HU000033455T2

(19) **HU**

(11) Lajstromszám: **E 033 455**

(13) **T2**

MAGYARORSZÁG
Szellemi Tulajdon Nemzeti Hivatala

EURÓPAI SZABADALOM
SZÖVEGÉNEK FORDÍTÁSA

(21) Magyar ügyszám: **E 12 152662**
(22) A bejelentés napja: **2007. 12. 21.**

(96) Az európai bejelentés bejelentési száma:
EP 20070152662
(97) Az európai bejelentés közzétételi adatai:
EP 2479267 A1 **2012. 07. 25.**
(97) Az európai szabadalom megadásának meghirdetési adatai:
EP 2479267 B1 **2016. 12. 21.**

(51) Int. Cl.:

C12N 9/32	*(2006.01)*
C12N 1/00	*(2006.01)*
C12N 1/20	*(2006.01)*
C12Q 1/68	*(2006.01)*
C12N 15/86	*(2006.01)*
C12P 19/04	*(2006.01)*
C12P 21/06	*(2006.01)*
C12P 21/08	*(2006.01)*
C07H 21/04	*(2006.01)*
C12N 15/00	*(2006.01)*

(30) Elsőbbségi adatok:

877068 P	**2006. 12. 21.**	**US**
892823 P	**2007. 03. 02.**	**US**

(72) Feltaláló(k):
SLUPSKA, Malgorzata, San Diego, CA 92126 (US)
ZHANG, Yan, Apex, NC 27502 (US)
Hazlewood, Geoff, Berkshire, Berkshire RG14 2RS (GB)
CHANG, Cathy, San Marcos, CA 92069 (US)
LUGINBUHL, Peter, San Diego, CA 92130 (US)
BURKE, Ellen, San Diego, CA 92106 (US)
CAYOUETTE, Michelle, San Diego, CA 92103 (US)
Gunavardena, Uvini, San Diego, CA 92130 (US)
GHASSEMIAN, Majid, Carlsbad, CA 92008 (US)
SILVERSTONE, Aron, Durham, NC 27705 (US)

(73) Jogosult(ak):
BASF Enzymes LLC, San Diego, CA 92121 (US)
Syngenta Participations AG, 4058 Basel (CH)

(74) Képviselő:
SBGK Szabadalmi Ügyvivői Iroda, Budapest

(54) **Amilázok és glükoamilázok, az ezeket kódoló nukleinsavak, valamint eljárások ezek előállítására és alkalmazására**

Az európai szabadalom ellen, megadásának az Európai Szabadalmi Közlönyben való meghirdetésétől számított kilenc hónapon belül, felszólalást lehet benyújtani az Európai Szabadalmi Hivatalnál. (Európai Szabadalmi Egyezmény 99. cikk(1))

A fordítást a szabadalmas az 1995. évi XXXIII. törvény 84/H. §-a szerint nyújtotta be. A fordítás tartalmi helyességét a Szellemi Tulajdon Nemzeti Hivatala nem vizsgálta.

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 2 479 267 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
21.12.2016 Bulletin 2016/51

(21) Application number: **12152662.8**

(22) Date of filing: **21.12.2007**

(51) Int Cl.:
C12N 9/32 *(2006.01)* ***C12P 21/06*** *(2006.01)*
C12N 15/00 *(2006.01)* ***C12Q 1/68*** *(2006.01)*
C12N 1/20 *(2006.01)* ***C12N 1/00*** *(2006.01)*
C12N 15/86 *(2006.01)* ***C12P 19/04*** *(2006.01)*
C12P 21/08 *(2006.01)* ***C07H 21/04*** *(2006.01)*

(54) **Amylases and glucoamylases, nucleic acids encoding them and methods for making and using them**

Amylasen und Glucoamylasen, dafür kodierende Nukleinsäuren sowie Verfahren zu ihrer Herstellung und Verwendung

Amylases et glucoamylases, acides nucléiques les codant, et leurs procédés de fabrication

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU LV MC MT NL PL PT RO SE SI SK TR

(30) Priority: **21.12.2006 US 877068 P**
02.03.2007 US 892823 P

(43) Date of publication of application:
25.07.2012 Bulletin 2012/30

(60) Divisional application:
16180480.2 / 3 101 128

(62) Document number(s) of the earlier application(s) in accordance with Art. 76 EPC:
07869797.6 / 2 069 490

(73) Proprietors:
- **BASF Enzymes LLC**
  San Diego, CA 92121 (US)
- **Syngenta Participations AG**
  4058 Basel (CH)

(72) Inventors:
- **Slupska, Malgorzata**
  San Diego, CA 92126 (US)
- **Zhang, Yan**
  Apex, NC 27502 (US)
- **Hazlewood, Geoff**
  Berkshire, Berkshire RG14 2RS (GB)
- **Chang, Cathy**
  San Marcos, CA 92069 (US)
- **Luginbuhl, Peter**
  San Diego, CA 92130 (US)
- **Burke, Ellen**
  San Diego, CA 92106 (US)
- **Cayouette, Michelle**
  San Diego, CA 92103 (US)
- **Gunavardena, Uvini**
  San Diego, CA 92130 (US)
- **Ghassemian, Majid**
  Carlsbad, CA 92008 (US)
- **Silverstone, Aron**
  Durham, NC 27705 (US)

(74) Representative: **Lasar, Andrea Gisela**
Maiwald Patentanwalts GmbH
Elisenhof
Elisenstraße 3
80335 München (DE)

(56) References cited:
WO-A2-03/012071

- **DATABASE UniProt [Online] 17 October 2006 (2006-10-17), "RecName: Full=Glucoamylase; EC=3.2.1.3; AltName: Full=1,4-alpha-D-glucan glucohydrolase; AltName: Full=Glucan 1,4-alpha-glucosidase;", XP002675122, retrieved from EBI accession no. UNIPROT:Q0CPK9 Database accession no. Q0CPK9 -& DATABASE ENA [Online] 10 September 2006 (2006-09-10), "Aspergillus terreus NIH2624 glucoamylase precursor", XP002675123, retrieved from EBI Database accession no. EAU34822**

Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

EP 2 479 267 B1

Description

TECHNICAL FIELD

[0001] This invention relates to molecular and cellular biology and biochemistry. In one aspect, the invention is directed to polypeptides having glucoamylase activity, polynucleotides encoding the polypeptides, and methods for making and using these polynucleotides and polypeptides. In one aspect, the polypeptides of the invention can be used as the exo-acting glucoamylases, e.g., to catalyze the hydrolysis of polysaccharides comprising glucose monomers, such as starch (a polymer of glucose monomers joined by 1,4-alpha or 1,6- alpha linkages), into sugars. In one aspect, the invention is directed to polypeptides having thermostable glucoamylase activity, including 1,4-alpha-D-glucan glucohydrolase activity. In one aspect, the polypeptides of the invention can be used as glucoamylases to catalyze the hydrolysis of polysaccharides such as starch into sugars, such as glucose. The invention is also directed to nucleic acid constructs, vectors, and host cells comprising the nucleic acid sequences of the invention as well as recombinant methods for producing the polypeptides of the invention. The invention is also directed to the use of glucoamylases of the invention in polysaccharide (e.g., starch) conversion processes, including production of high fructose corn syrup (HFCS), ethanol, dextrose, and dextrose syrups.

BACKGROUND

[0002] Starch is a complex carbohydrate often found in the human diet. The structure of starch is glucose polymers linked by α-1,4 and α-1,6 glucosidic bonds. Commercially, glucoamylases are used to further hydrolyze cornstarch, which has already been partially hydrolyzed with an alpha-amylase. The most widely utilized glucoamylase is produced from the fungus *Aspergillus niger;* one of the problems with the commercial use of this enzyme is its relatively low thermostability.

[0003] In general, starch to fructose processing consists of four steps: liquefaction of granular starch, saccharification of the liquefied starch into dextrose, purification, and isomerization to fructose. The object of a starch liquefaction process is to convert a concentrated suspension of starch polymer granules into a solution of soluble shorter chain length dextrins of low viscosity. This step is essential for convenient handling with standard equipment and for efficient conversion to glucose or other sugars. To liquefy granular starch, it is necessary to gelatinize the granules by raising the temperature of the granular starch to over about 72°C. The heating process instantaneously disrupts the insoluble starch granules to produce a water soluble starch solution. The solubilized starch solution is then liquefied by amylase. A starch granule is composed of: 69-74% amylopectin, 26-31% amylose, 11-14% water, 0.2-0.4% protein, 0.5-0.9% lipid, 0.05-0.1% ash, 0.02-0.03% phosphorus, 0.1% pentosan. Approximately 70% of a granule is amorphous and 30% is crystalline.

[0004] Staling of baked products (such as bread) has been recognized as a problem which becomes more serious as more time lies between the moment of preparation of the bread product and the moment of consumption. The term staling is used to describe changes undesirable to the consumer in the properties of the bread product after leaving the oven, such as an increase of the firmness of the crumb, a decrease of the elasticity of the crumb, and changes in the crust, which becomes tough and leathery. The firmness of the bread crumb increases further during storage up to a level, which is considered as negative. The increase in crumb firmness, which is considered as the most important aspect of staling, is recognized by the consumer a long time before the bread product has otherwise become unsuitable for consumption.

[0005] There is a need in the industry for new amylases, e.g., acid amylases, useful for various uses including commercial cornstarch liquefaction processes or improved manufacturing having new or improved performance characteristics over the industry standard enzymes, e.g., from *Bacillus licheniformis.* There is also an industry drive to identify amylases and glucoamylases capable of efficiently hydrolyzing granular starch (e.g. raw granular starch) at low temperatures without the need for a high temperature starch gelatinization step; the enzymes of the invention, e.g. glucoamylases, can be utilized to fulfill this need.

[0006] There is also a need for new amylases having utility in automatic dish wash (ADW) products and laundry detergent. In ADW products, the amylase will function at pH 10-11 and at 45-60°C in the presence of calcium chelators and oxidative conditions. For laundry, activity at pH 9-10 and 40°C in the appropriate detergent matrix will be required. Amylases are also useful in textile desizing, brewing processes, starch modification in the paper and pulp industry and other processes described in the art.

[0007] Database entry Q0CPK9 shows the amino acid sequence of a glucoamylase from *Aspergillus terreus* and database entry EAU34822 shows the corresponding nucleic acid sequence.

[0008] WO 03/012071 A2 describes the identification of different types of enzymes, including glucoamylases, from *Aspergillus fumigatus.*

SUMMARY

[0009] The invention provides isolated, synthetic or recombinant nucleic acids comprising a nucleic acid sequence having at least 98%, 99%, or more, or complete (100%) sequence identity to a nucleic acid of the invention, e.g., an exemplary nucleic acid of the invention. In one aspect, the nucleic acid encodes at least one polypeptide having glucoamylase activity, and the sequence identities are determined by analysis with a sequence comparison algorithm or by a visual inspection. In another aspect, the disclosure provides nucleic acids for use as probes, inhibitory molecules (e.g., antisense, iRNAs, such as siRNA, microRNA or miRNAs), transcriptional or translational regulation, and the like.

[0010] The nucleic acids of the invention include isolated, synthetic or recombinant nucleic acids comprising a nucleic acid sequence as set forth in SEQ ID NO:47.

[0011] Exemplary nucleic acids of the invention also include isolated, synthetic or recombinant nucleic acids encoding a polypeptide of the invention, e.g., an exemplary polypeptide having a sequence as set forth in SEQ ID NO:48.

[0012] In one aspect, the polypeptide of the invention has an exo-acting glucoamylase activity. In one embodiment, the polypeptides of the invention can catalyze the hydrolysis of polysaccharides and/or oligosaccharides comprising glucose monomers, such as starch (a polymer of glucose monomers joined by 1,4-alpha or 1,6- alpha linkages).

[0013] Glucoamylases of the invention can be used commercially in the initial stages (liquefaction) of polysaccharide, oligosaccharide or starch processing; in wet corn milling; in alcohol production; as cleaning agents in detergent matrices; in the textile industry for starch desizing; in baking applications; in the beverage industry; in oilfields in drilling processes; in inking of recycled paper and in animal feed. Glucoamylases of the invention can be used in textile desizing, brewing processes, polysaccharide, oligosaccharide or starch modification in the paper and pulp industry and other processes. For example, the invention provides methods for liquefaction saccharification as illustrated in Figure 5 using polypeptides of the disclosure.

[0014] Glucoamylases of the invention can be used to catalyze the hydrolysis of polysaccharides, e.g., starches, into sugars; or to hydrolyze internal α-1,4-glucosidic linkages in starch to produce smaller molecular weight malto-dextrins. Because the breakdown of polysaccharides and/or oligosaccharides, e.g., starches, is important in the digestive system and in commercial preparation processes, glucoamylases of the invention are used in foods and feeds and processes for preparing them, and in or as digestive aids. Glucoamylases of the invention can be used in the initial stages (liquefaction) of starch processing; in wet corn milling; in alcohol production; as cleaning agents in detergent matrices; in the textile industry for starch desizing; in baking applications; in the beverage industry; in oilfields in drilling processes; in inking of recycled paper; and in animal feed.

[0015] Enzymes of the invention can have an exo-acting glucoamylase activity and can be used to further hydrolyze cornstarch which has already been partially hydrolyzed with an alpha-amylase to produce glucose; and in one aspect of this process of the invention the glucose is converted to a mixture of glucose and fructose by a glucose isomerase enzyme. In another aspect of this process of the invention, this mixture is enriched with fructose to produce a high fructose corn syrup. In alternative aspects, polypeptides of the invention are used in any of step of polysaccharide, oligosaccharide or starch to fructose processing, e.g., including the four steps: liquefaction of granular starch, saccharification of the liquefied polysaccharide, oligosaccharide or starch into dextrose, purification, and isomerization to fructose. One aspect of the invention using at least one polypeptide of the invention comprises a polysaccharide, oligosaccharide or starch liquefaction process for converting a concentrated suspension of polysaccharide, oligosaccharide or starch polymer granules into a solution of soluble shorter chain length dextrins of low viscosity.

[0016] The invention also provides an enzymatic liquefaction process using at least one polypeptide of the invention comprising adjusting the pH of a granular polysaccharide, oligosaccharide or starch slurry to the pH optimum of an enzyme of the invention (e.g., a glucoamylase of the invention) to be utilized, e.g., between 6.0 and 6.5, or between about 5.5 and 7.0; calcium hydroxide, sodium hydroxide or sodium carbonate can be added for this purpose (the addition of calcium hydroxide has the advantage of also providing calcium ions which are known to stabilize the alpha-amylase against inactivation). In one aspect, upon addition of the amylase (e.g., an alpha-amylase) of the disclosure, the suspension is pumped through a steam jet to instantaneously raise the temperature to between 80°C to 115°C, and the starch is immediately gelatinized and, due to the presence of alpha-amylase, depolymerized through random hydrolysis of a (1-4) glycosidic bonds by the alpha-amylase to a fluid mass which is easily pumped.

[0017] In alternative aspects to this polysaccharide and/or oligosaccharide (e.g., starch) liquefaction process, a glucoamylase of the invention is added to the polysaccharide (e.g., starch) suspension, the suspension is held at a temperature of 80-100°C to partially hydrolyze the granules, e.g., starch granules, and the partially hydrolyzed polysaccharide / starch suspension is pumped through a jet at temperatures in excess of about 105°C to thoroughly gelatinize any remaining granular structure. After cooling the gelatinized polysaccharide / starch, a second addition of a glucoamylase of the invention can be made to further hydrolyze the polysaccharide / starch.

[0018] In one aspect, the disclosure also provides amylase-encoding and glucoamylase-encoding nucleic acids with a common novelty in that they are derived from mixed cultures, e.g., from environmental sources. The disclosure provides amylase-encoding and glucoamylase-encoding nucleic acids isolated from mixed cultures comprising a nucleic acid

sequence having at least about 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete (100%) sequence identity to an exemplary nucleic acid of the invention over a region of at least about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500, 1550 or more, residues, wherein the nucleic acid encodes at least one polypeptide having an amylase and/or glucoamylase activity, and the sequence identities are determined by analysis with a sequence comparison algorithm or by a visual inspection.

[0019] In one aspect, the disclosure provides amylase-encoding and glucoamylase-encoding nucleic acids isolated from mixed cultures, e.g., from environmental sources, comprising a nucleic acid of the invention, e.g., an exemplary nucleic acid of the invention, e.g., a sequence as set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:9, SEQ ID NO:11, etc., and subsequences thereof, e.g., at least about 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500 or more residues in length, or over the full length of a gene or transcript; or, a nucleic acid encoding a polypeptide of the invention.

[0020] In one aspect, the disclosure also provides amylase-encoding and glucoamylase-encoding nucleic acids with a common novelty in that they are derived from environmental sources (see Table 1 below, 2nd column for examples of sequences isolated from "unknown" or environmental sources), e.g., mixed environmental sources. In one aspect, the disclosure provides amylase-encoding and glucoamylase-encoding nucleic acids isolated from environmental sources, e.g., mixed environmental sources, comprising a nucleic acid sequence having at least about 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete (100%) sequence identity to an exemplary nucleic acid of the invention over a region of at least about 25, 30, 40, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500, 1550 or more, residues, wherein the nucleic acid encodes at least one polypeptide having an amylase and/or glucoamylase activity, and the sequence identities are determined by analysis with a sequence comparison algorithm or by a visual inspection.

[0021] In one aspect, the disclosure provides amylase-encoding and glucoamylase-encoding nucleic acids isolated from environmental sources, e.g., mixed environmental sources, comprising a nucleic acid of the disclosure, e.g., an exemplary nucleic acid sequence of the invention as set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:9, SEQ ID NO:11, etc., SEQ ID NO:583, SEQ ID NO:585, and subsequences thereof, e.g., at least about 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500 or more residues in length, or over the full length of a gene or transcript; or, a nucleic acid encoding a polypeptide of the invention.

[0022] In one aspect, the disclosure also provides glucoamylases and amylases, and amylase-encoding and glucoamylase-encoding nucleic acids, with a common novelty in that they are derived from archeal sources, including the archeal-derived amylases and/or glucoamylases of the disclosure.

[0023] In one aspect, the sequence comparison algorithm is a BLAST version 2.2.2 algorithm where a filtering setting is set to blastall -p blastp -d "nr pataa" -F F, and all other options are set to default.

[0024] Another aspect of the disclosure is an isolated, synthetic or recombinant nucleic acid including at least 10 consecutive bases of a nucleic acid sequence of the disclosure, sequences substantially identical thereto, and the sequences complementary thereto.

[0025] In one aspect, the amylase activity comprises α-amylase or a β-amylase activity, including the ability to hydrolyze internal alpha-1,4-glucosidic linkages in starch to produce smaller molecular weight malto-dextrins. In one aspect, the α-amylase activity includes hydrolyzing internal alpha-1,4-glucosidic linkages in starch at random. The glucoamylase and/or amylase activity can comprise an α-amylase activity, a β-amylase, activity, a 1,4-α-D-glucan glucohydrolase activity, an exoamylase activity, a glucan α-maltotetrahydrolase activity, a maltase activity, an isomaltase activity, a glucan 1, 4, α-glucosidase activity, an α-glucosidase activity, a sucrase activity or an agarase activity (e.g., a β-agarase activity).

[0026] The amylase activity can comprise hydrolyzing glucosidic bonds. In one aspect, the glucosidic bonds comprise an α-1,4-glucosidic bond. In another aspect, the glucosidic bonds comprise an α-1,6-glucosidic bond. In one aspect, the amylase activity comprises hydrolyzing glucosidic bonds in polysaccharides, e.g., starches, e.g., liquefied starch. The amylase activity can further comprise hydrolyzing glucosidic bonds into maltodextrins. In one aspect, the amylase activity comprises cleaving a maltose or a D-glucose unit from non-reducing end of the starch.

[0027] In one aspect, the isolated, synthetic or recombinant nucleic acid encodes a polypeptide having an amylase and/or glucoamylase activity which is thermostable. The polypeptide can retain an amylase activity under conditions comprising a temperature range of anywhere between about 0°C to about 37°C, or, between about 37°C to about 95°C or more, e.g., 98°C, 100°C or more; between about 55°C to about 85°C, between about 70°C to about 95°C, or, between

about 90°C to about 95°C. For example, the exemplary polypeptide having a sequence as set forth in SEQ ID NO:437 is thermostable, retaining 50% activity after 25 minutes at 100°C in the absence of added calcium.

[0028] In another aspect, the isolated, synthetic or recombinant nucleic acid encodes a polypeptide having an amylase and/or glucoamylase activity which is thermotolerant. The polypeptide can retain an amylase and/or glucoamylase activity after exposure to a temperature in the range from greater than 37°C to about 95°C or anywhere in the range from greater than 55°C to about 85°C. In one aspect, the polypeptide retains an amylase and/or glucoamylase activity after exposure to a temperature in the range from greater than 90°C to about 95°C at pH 4.5.

[0029] The disclosure provides isolated, synthetic or recombinant nucleic- acids comprising a sequence that hybridizes under stringent conditions to a nucleic acid of the disclosure, e.g., an exemplary nucleic acid of the disclosure, a nucleic acid comprising a sequence as set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:55, SEQ ID NO:57, SEQ ID NO:59, SEQ ID NO:61, SEQ ID NO:63, SEQ ID NO:65, SEQ ID NO:67, SEQ ID NO:69, SEQ ID NO:71, SEQ ID NO:73, SEQ ID NO:75, SEQ ID NO:77, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81 and/or SEQ ID NO:82, and/or fragments or subsequences thereof. In one aspect, the nucleic acid encodes a polypeptide having an amylase and/or glucoamylase activity. The nucleic acid can be at least about 25, 30, 40, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500 or more residues in length or the full length of the gene or transcript. In one aspect, the stringent conditions include a wash step comprising a wash in 0.2X SSC at a temperature of about 65°C for about 15 minutes. In one aspect, stringent conditions comprise hybridization under conditions comprising a buffer having 0.15M NaCl for 15 minutes at 72°C.

[0030] The disclosure provides a nucleic acid probe for identifying a nucleic acid encoding a polypeptide having an amylase and/or glucoamylase activity, wherein the probe comprises at least about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000 or more, consecutive bases of a sequence comprising a sequence of the invention, or fragments or subsequences thereof, wherein the probe identifies the nucleic acid by binding or hybridization. The probe can comprise an oligonucleotide comprising at least about 10 to 50, about 20 to 60, about 30 to 70, about 40 to 80, or about 60 to 100 consecutive bases of a sequence comprising a sequence of the disclosure, or fragments or subsequences thereof.

[0031] The disclosure provides a nucleic acid probe for identifying a nucleic acid encoding a polypeptide having an amylase and/or glucoamylase activity, wherein the probe comprises a nucleic acid comprising a sequence at least about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 90, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000 or more residues having at least about 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete (100%) sequence identity to a nucleic acid of the disclosure, wherein the sequence identities are determined by analysis with a sequence comparison algorithm or by visual inspection.

[0032] The probe can comprise an oligonucleotide comprising at least about 10 to 50, about 20 to 60, about 30 to 70, about 40 to 80, or about 60 to 100 consecutive bases of a nucleic acid sequence of the disclosure, or a subsequence thereof.

[0033] The disclosure provides an amplification primer sequence pair for amplifying a nucleic acid encoding a polypeptide having an amylase and/or glucoamylase activity, wherein the primer pair is capable of amplifying a nucleic acid comprising a sequence of the disclosure, or fragments or subsequences thereof. One or each member of the amplification primer sequence pair can comprise an oligonucleotide comprising at least about 10 to 50 consecutive bases of the sequence.

[0034] The disclosure provides methods of amplifying a nucleic acid encoding a polypeptide having an amylase and/or glucoamylase activity comprising amplification of a template nucleic acid with an amplification primer sequence pair capable of amplifying a nucleic acid sequence of the disclosure, or fragments or subsequences thereof.

[0035] The invention provides expression cassettes (including, e.g., vectors and cloning vehicles) comprising a nucleic acid of the invention or a subsequence thereof. In one aspect, the expression cassette can comprise the nucleic acid that is operably linked to a promoter. The promoter can be a viral, bacterial, mammalian or plant promoter. In one aspect, the plant promoter can be a potato, rice, corn, wheat, tobacco or barley promoter. The promoter can be a constitutive promoter. The constitutive promoter can comprise CaMV35S. In another aspect, the promoter can be an inducible promoter. In one aspect, the promoter can be a tissue-specific promoter or an environmentally regulated or a developmentally regulated promoter. Thus, the promoter can be, e.g., a seed-specific, a leaf-specific, a root-specific, a stem-specific or an abscission-induced promoter. In one aspect, the expression cassette can further comprise a plant or plant virus expression vector. In alternative embodiments, the plant promoter is a corn seed embryo-specific: globulin promoter (see, e.g., Belanger (1991) Molecular basis for allelic polymorphism of the maize Globulin-1 gene. Genetics 129:863-872);

or a corn seed endosperm-specific: γ-zein promoter (see, e.g., Lopes (1995) Identification of two opaque2 modifier loci in Quality Protein Maize. Mol. Gen. Genet. 247: 603-613); or a rice seed endosperm-specific: GTL1 promoter (see, e.g., Takaiwa (1991) Analysis of the 5' flanking region responsible for the endosperm-specific expression of a rice glutelin chimeric gene in transgenic tobacco. Plant Mol. Biol. 16:49-58).

[0036] The invention provides cloning vehicles comprising an expression cassette (e.g., a vector) of the invention or a nucleic acid of the invention. The cloning vehicle can be a viral vector, a plasmid, a phage, a phagemid, a cosmid, a fosmid, a bacteriophage or an artificial chromosome. The viral vector can comprise an adenovirus vector, a retroviral vector or an adeno-associated viral vector. The cloning vehicle can comprise a bacterial artificial chromosome (BAC), a plasmid, a bacteriophage P1-derived vector (PAC), a yeast artificial chromosome (YAC), or a mammalian artificial chromosome (MAC).

[0037] The invention provides transformed cells (or "host cells") comprising a nucleic acid of the invention or an expression cassette (e.g., a vector) of the invention, or a cloning vehicle of the invention. In one aspect, the transformed cell can be a bacterial cell, a mammalian cell, a fungal cell, a yeast cell, an insect cell or a plant cell. In one aspect, the plant cell can be from any plant, for example plants used for forage and/or feed for any animal, including ruminants, or as a source of feedstock to produce energy or fuel. Plants of particular interest may include crop plants and feedstock plants, for example, maize, alfalfa, sunflower, *Brassica,* soybean, cotton, safflower, peanut, sorghum, wheat, oat, rye, millet, barley, rice, conifers, grasses, e.g., switch grass and *Miscanthus,* legume crops, e.g., pea, bean and soybean, starchy tuber/roots, e.g., potato, sweet potato, cassava, taro, canna, sugar beet, sugar cane and the like.

[0038] The disclosure provides transgenic non-human animals comprising a nucleic acid of the disclosure or an expression cassette (e.g., a vector) of the disclosure. In one aspect, the animal is a mouse. The disclosure provides cells or cell lines isolated from these transgenic non-human animals.

[0039] The disclosure provides transgenic plants comprising a nucleic acid of the invention or an expression cassette (e.g., a vector) of the invention. The transgenic plant can be any plant, but in one embodiment the plant would be used for forage and/or feed for any animal or as a feedstock to produce energy or fuel, such as, maize, alfalfa, sunflower, *Brassica,* soybean, cotton, safflower, peanut, sorghum, wheat, oat, rye, millet, barley, rice, conifers, grasses, e.g., switch grass and *Miscanthus,* legume crops, e.g., pea, bean and soybean, starchy tuber/roots, e.g., potato, sweet potato, cassava, taro, canna, sugar beet, sugar cane and the like.

[0040] The disclosure provides transgenic seeds comprising a nucleic acid of the invention or an expression cassette (e.g., a vector) of the disclosure. The transgenic seed can from any plant, but in one embodiment the plant would be used for forage and/or feed for any animal or as a feedstock to produce energy or fuel, such as, maize, alfalfa, sunflower, *Brassica,* soybean, cotton, safflower, peanut, sorghum, wheat, oat, rye, millet, barley, rice, conifers, grasses, e.g., switch grass and *Miscanthus,* legume crops, e.g., pea, bean and soybean, starchy tuber/roots, e.g., potato, sweet potato, cassava, taro, canna, sugar beet, sugar cane and the like.

[0041] Any plant, plant part, plant tissue, plant seed or plant cell may be used for introduction of a nucleotide of the disclosure, either stably (e.g., as a transgenic plant, or cell or cell line derived therefrom) or transiently; thus the disclosure provides plants, plant parts, plant tissues, plant seeds and plant cells comprising a nucleic acid and/or polypeptide of the disclosure, wherein the plant can be (but is not limited to) corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus, B. rapa, B. juncea),* including those *Brassica* species useful as sources of seed oil, such as canola, alfalfa (*Medicago sativa),* rice (*Oryza sativa*), rye (*Secale cereale*), *sorghum (Sorghum bicolor, Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet *(Setaria italica),* finger *millet* (*Eleusine coracana*)), sunflower (*Helianthus annuus),* safflower (*Carthamus tinctorius*), wheat *(Triticum aestivum),* soybean *(Glycine max*), tobacco *(Nicotiana tabacum),* potato *(Solanum tuberosum),* peanuts *(Arachis hypogaea*), *cotton* (*Gossypium barbadense, Gossypium hirsutum*), sweet potato (*Ipomoea batatus),* cassava (*Manihot esculenta),* coffee (*Cofea* spp.), coconut *(Cocos nucifera),* pineapple *(Ananas comosus),* citrus trees *(Citrus* spp.), cocoa *(Theobroma cacao),* tea *(Camellia sinensis),* banana *(Musa* spp.), avocado (*Persea americana*), *fig (Ficus casica),* guava *(Psidium guajava),* mango (*Mangifera indica),* olive *(Olea europaea),* papaya *(Carica papaya),* cashew *(Anacardium occidentale),* macadamia (*Macadamia integrifolia*), almond *(Prunus amygdalus),* sugar beets (*Beta* sp., e.g., *Beta vulgaris),* sugarcane *(Saccharum* spp.), *Andropogoneae* (grasses), *Chenopodiaceae* (flowering plants), oats, barley, vegetables, ornamentals, and conifers; and vegetables, e.g., tomatoes *(Lycopersicon esculentum),* lettuce (e.g., *Lactuca sativa),* green beans *(Phaseolus vulgaris*), lima beans (*Phaseolus limensis),* peas (*Lathyrus* spp.), and members of the genus Cucumis such as *cucumber (C. sativus),* cantaloupe (*C. cantalupensis),* and musk melon (C. *melo*); and ornamentals, including *azalea* (*Rhododendron* spp.), hydrangea *(Macrophylla hydrangea),* hibiscus *(Hibiscus rosasanensis),* roses *(Rosa* spp.), tulips (*Tulipa* spp.), daffodils *(Narcissus* spp.), petunias *(Petunia hybrida*), carnation *(Dianthus caryophyllus),* poinsettia (*Euphorbia pulcherrima*), canna *(Cannaceae spp.)* and chrysanthemum; and conifers that may be used, including, for example, pines such as loblolly pine *(Pinus taeda),* slash pine *(Pinus elliotii),* ponderosa pine (*Pinus ponderosa*), lodgepole pine *(Pinus contorta),* and Monterey pine *(Pinus radiata),* Douglas-fir (*Pseudotsuga menziesii);* Western hemlock *(Tsuga canadensis*); Sitka *spruce (Picea glauca*); redwood *(Sequoia sempervirens*); true firs such as silver *fir (Abies amabilis)* and balsam fir *(Abies balsamea);* and cedars such as Western red cedar *(Thuja plicata)* and Alaska yellow-cedar (*Chamaecyparis nootkat-*

ensis); and leguminous plants, including, but not limited to, beans and peas, where in alternative aspects beans may include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, etc., and legumes can include, but are not limited to, *Arachis,* e.g., peanuts, Vicia, e.g., crown vetch, hairy vetch, adzuki bean, mung bean, and chickpea, Lupinus, e.g., lupine, trifolium, Phaseolus, e.g., common bean and lima bean, Pisum, e.g., field bean, Melilotus, e.g., clover, Medicago, e.g., alfalfa, Lotus, e.g., trefoil, lens, e.g., lentil, and false indigo; also including forage and turf grasses, such as alfalfa, switchgrass (*Panicum* virgatum), *Miscanthus,* orchard grass, tall fescue, perennial ryegrass, creeping bent grass, and redtop.

[0042] In alternative aspects, plants, plant parts, plant tissues, plant seeds and plant cells comprising a nucleic acid and/or polypeptide of the disclosure also include crop plants and plants used to produce energy or fuel (e.g., ethanol or other biofuels, e.g., bioethanols, biopropanols, biobutanols, or biodiesel), for example, corn, alfalfa, sunflower, Brassica, soybean, cotton, safflower, peanut, sorghum, wheat, oat, rye, millet, barley, rice, conifers, grasses, e.g., switch grass and *Miscanthus,* legume crops, e.g., pea, bean and soybean, starchy tuber/roots, e.g., potato, sweet potato, cassava, taro, canna, sugar cane and/or sugar beet and the like.

[0043] The invention provides an antisense oligonucleotide comprising a nucleic acid sequence complementary to or capable of hybridizing under stringent conditions to a nucleic acid of the invention. The disclosure provides methods of inhibiting the translation of an amylase message in a cell comprising administering to the cell or expressing in the cell an antisense oligonucleotide comprising a nucleic acid sequence complementary to or capable of hybridizing under stringent conditions to a nucleic acid of the disclosure.

[0044] The invention provides an isolated, synthetic or recombinant polypeptide comprising an amino acid sequence having at least 99%, or more, or complete (100%) sequence identity to an exemplary polypeptide or peptide of the invention. Exemplary polypeptide or peptide sequences of the invention include SEQ ID NO:48 and/or subsequences thereof and variants thereof, e.g., at least about 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500 or more residues in length, or over the full length of an enzyme. Exemplary polypeptide or peptide sequences of the invention include sequence encoded by the nucleic acid of the invention. Exemplary polypeptide or peptide sequences of the disclosure include polypeptides or peptides specifically bound by an antibody of the disclosure. In one aspect, a polypeptide of the disclosure has at least one amylase activity, e.g., an alpha amylase activity.

[0045] In alternative embodiments, the polypeptides of the invention lack a signal sequence (leader sequence) or a carbohydrate binding module. In alternative embodiments, the polypeptides of the invention further comprise one or more heterologous sequences, which can comprise a heterologous signal sequence (leader sequence), a heterologous catalytic domain (CD) (i.e., active site), or a heterologous carbohydrate binding module, or epitope, purification tag or label. In one aspect, the heterologous signal sequence, heterologous carbohydrate binding module or heterologous catalytic domain (CD) is derived from another amylase enzyme (an amylase other than an enzyme of this invention), or is derived from a non-amylase enzyme.

[0046] In alternative embodiments, the polypeptides of the invention can be used to generate antibodies which bind specifically to an exemplary polypeptide of the invention (SEQ ID NO:48).

[0047] In alternative embodiments, the polypeptides of the invention can be synthetic or in a peptidomimetic form.

[0048] Another aspect of the disclosure is an isolated, synthetic or recombinant polypeptide or peptide including at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 125, 150, 175, 200, 225, 250, 300, 350, 400, 450 or 500 or more consecutive amino acid residues of a polypeptide or peptide sequence of the disclosure, sequences substantially identical thereto, and the sequences complementary thereto.

[0049] In one aspect, the amylase activity of a polypeptide or peptide of the disclosure comprises an α-amylase activity, including the ability to hydrolyze internal alpha-1,4-glucosidic linkages in starch to produce smaller molecular weight malto-dextrins. In one aspect, the α-amylase activity includes hydrolyzing internal alpha-1,4-glucosidic linkages in starch at random. The amylase activity can comprise a glucoamylase activity, a 1,4-α-D-glucan glucohydrolase activity, an α-amylase activity, an exoamylase activity, or a β-amylase activity. The amylase activity can comprise hydrolyzing glucosidic bonds. In one aspect, the glucosidic bonds comprise an α-1,4-glucosidic bond. In another aspect, the glucosidic bonds comprise an α-1,6-glucosidic bond. In one aspect, the amylase activity comprises hydrolyzing glucosidic bonds in starch, e.g., liquefied starch. The amylase activity can further comprise hydrolyzing glucosidic bonds into maltodextrins. In one aspect, the amylase activity comprises cleaving a maltose or a D-glucose unit from non-reducing end of the starch.

[0050] In one aspect, the amylase activity of the invention comprises a glucoamylase activity, which can comprise catalysis of the hydrolysis of glucosidic bonds. The glucoamylase activity of the invention can comprise catalyzing the step-wise hydrolytic release of D-glucose from the non-reducing ends of starch or other related dextrins. The glucoamylase activity can comprise a 1,4-α-D-glucan glucohydralase activity. The glucoamylase activity can comprise catalysis of the hydrolysis of malto-dextrins resulting in the generation of free glucose. The glucoamylase activity can comprise an exoamylase activity. The glucoamylase activity can comprise an α-amylase or a β-amylase activity. The hydrolyzed glucosidic bonds can comprise α-1,4-glucosidic bonds or α-1,6-glucosidic bonds. The glucoamylase activity can comprise hydrolyzing glucosidic bonds in a starch. The glucoamylase activity can further comprise hydrolyzing glucosidic bonds

in the starch to produce maltodextrines. The glucoamylase activity can comprise cleaving a maltose or a D-glucose unit from non-reducing end of the starch.

[0051] In one aspect, the glucoamylase activity can be thermostable. The polypeptide can retain a glucoamylase activity under conditions comprising a temperature range of between about 37°C to about 95°C, between about 55°C to about 85°C, between about 70°C to about 95°C, or between about 90°C to about 95°C. In another aspect, the glucoamylase activity can be thermotolerant. The polypeptide can retain a glucoamylase activity after exposure to a temperature in the range from greater than 37°C to about 95°C, or in the range from greater than 55°C to about 85°C. In one aspect, the polypeptide can retain a glucoamylase activity after exposure to a temperature in the range from greater than 90°C to about 95°C at pH 4.5.

[0052] In one aspect, the glucoamylase activity is thermostable, e.g., wherein the polypeptide retains a glucoamylase activity under conditions comprising a temperature range from about -100°C to about -80°C, about -80°C to about -40°C, about -40°C to about -20°C, about -20°C to about 0°C, about 0°C to about 5°C, about 5°C to about 15°C, about 15°C to about 25°C, about 25°C to about 37°C, about 37°C to about 45°C, about 45°C to about 55°C, about 55°C to about 70°C, about 70°C to about 75°C, about 75°C to about 85°C, about 85°C to about 90°C, about 90°C to about 95°C, about 95°C to about 100°C, about 100°C to about 105°C, about 105°C to about 110°C, about 110°C to about 120°C, or 95°C, 96°C, 97°C, 98°C, 99°C, 100°C, 101°C, 102°C, 103°C, 104°C, 105°C, 106°C, 107°C, 108°C, 109°C, 110°C, 111°C, 112°C, 113°C, 114°C, 115°C or more. In some embodiments, the thermostable polypeptide retains activity, e.g., a glucoamylase activity, at a temperature in the ranges described above, at about pH 3.0, about pH 3.5, about pH 4.0, about pH 4.5, about pH 5.0, about pH 5.5, about pH 6.0, about pH 6.5, about pH 7.0, about pH 7.5, about pH 8.0, about pH 8.5, about pH 9.0, about pH 9.5, about pH 10.0, about pH 10.5, about pH 11.0, about pH 11.5, about pH 12.0 or more.

[0053] In one aspect, the glucoamylase, activity is thermotolerant, e.g., wherein the polypeptide retains a glucoamylase activity after exposure to a temperature in the range from about -100°C to about -80°C, about -80°C to about -40°C, about -40°C to about - 20°C, about -20°C to about 0°C, about 0°C to about 5°C, about 5°C to about 15°C, about 15°C to about 25°C, about 25°C to about 37°C, about 37°C to about 45°C, about 45°C to about 55°C, about 55°C to about 70°C, about 70°C to about 75°C, about 75°C to about 85°C, about 85°C to about 90°C, about 90°C to about 95°C, about 95°C to about 100°C, about 100°C to about 105°C, about 105°C to about 110°C, about 110°C to about 120°C, or 95°C, 96°C, 97°C, 98°C, 99°C, 100°C, 101°C, 102°C, 103°C, 104°C, 105°C, 106°C, 107°C, 108°C, 109°C, 110°C, 111°C, 112°C, 113°C, 114°C, 115°C or more. The thermotolerant polypeptides can retain activity, e.g. a glucoamylase activity, after exposure to a temperature in the range from about -100°C to about -80°C, about -80°C to about -40°C, about -40°C to about -20°C, about -20°C to about 0°C, about 0°C to about 5°C, about 5°C to about 15°C, about 15°C to about 25°C, about 25°C to about 37°C, about 37°C to about 45°C, about 45°C to about 55°C, about 55°C to about 70°C, about 70°C to about 75°C, about 75°C to about 85°C, about 85°C to about 90°C, about 90°C to about 95°C, about 95°C to about 100°C, about 100°C to about 105°C, about 105°C to about 110°C, about 110°C to about 120°C, or 95°C, 96°C, 97°C, 98°C, 99°C, 100°C, 101°C, 102°C, 103°C, 104°C, 105°C, 106°C, 107°C, 108°C, 109°C, 110°C, 111°C, 112°C, 113°C, 114°C, 115°C or more. In some embodiments, the thermotolerant polypeptides according to the invention retains activity, e.g. amylase activity and/or glucoamylase activity, after exposure to a temperature in the ranges described above, at about pH 3.0, about pH 3.5, about pH 4.0, about pH 4.5, about pH 5.0, about pH 5.5, about pH 6.0, about pH 6.5, about pH 7.0, about pH 7.5, about pH 8.0, about pH 8.5, about pH 9.0, about pH 9.5, about pH 10.0, about pH 10.5, about pH 11.0, about pH 11.5, about pH 12.0 or more.

[0054] In one aspect, the glucoamylase activity of polypeptides encoded by nucleic acids of the invention retain activity under acidic conditions comprising about pH 6.5, pH 6, pH 5.5, pH 5, pH 4.5, pH 4.0, pH 3.5, pH 3.0 or less (more acidic) pH, or, retain a glucoamylase activity after exposure to acidic conditions comprising about pH 6.5, pH 6, pH 5.5, pH 5, pH 4.5, pH 4.0, pH 3.5, pH 3.0 or less (more acidic) pH; or, retain activity under basic conditions comprising about pH 7, pH 7.5 pH 8.0, pH 8.5, pH 9, pH 9.5, pH 10, pH 10.5, pH 11, pH 11.5, pH 12, pH 12.5 or more (more basic) or, retain an amylase activity and/or glucoamylase activity after exposure to basic conditions comprising about pH 7, pH 7.5 pH 8.0, pH 8.5, pH 9, pH 9.5, pH 10, pH 10.5, pH 11, pH 11.5, pH 12, pH 12.5 or more (more basic). In one aspect, glucoamylase activity of polypeptides encoded by nucleic acids of the invention retain activity at a temperature of at least about 80°C, 81°C, 82°C, 83°C, 84°C, 85°C, 86°C, 87°C, 88°C, 89°C, 90°C, 91°C , 92°C , 93°C , 94°C, 95°C, 96°C, 97°C, 98°C, 99°C, 100°C, 101°C, 102°C, 103°C, 103.5°C, 104°C, 105°C, 107°C, 108°C, 109°C or 110°C, or more, and a basic pH of at least about pH 7.5 pH 8.0, pH 8.5, pH 9, pH 9.5, pH 10, pH 10.5, pH 11, pH 11.5, pH 12, pH 12.5 or more (more basic).

[0055] In one aspect, the isolated, synthetic or recombinant polypeptide can comprise the polypeptide of the invention that lacks a signal sequence. In one aspect, the isolated, synthetic or recombinant polypeptide can comprise the polypeptide of the invention comprising a heterologous signal sequence, such as a heterologous amylase or non-amylase signal sequence.

[0056] In one aspect, the disclosure provides a signal sequence comprising a peptide as set forth in Table 1. In one aspect, the disclosure provides a signal sequence consisting of a peptide as set forth in Table 1. In one aspect, the disclosure provides chimeric proteins comprising a first domain comprising a signal sequence of the invention and at

least a second domain. The protein can be a fusion protein. The second domain can comprise an enzyme. The enzyme can be any glucoamylase (e.g., a glucoamylase of the invention, or, another amylase or glucoamylase).

[0057] In one aspect, the enzymatic activity (e.g., glucoamylase activity) of an enzyme of this invention comprises a specific activity at about 37°C in the range from about 1 to about 1200 units per milligram of protein, or, about 100 to about 1000 units per milligram of protein. In another aspect, the enzymatic activity (e.g., glucoamylase activity) of an enzyme of this invention comprises a specific activity from about 100 to about 1000 units per milligram of protein, or, from about 500 to about 750 units per milligram of protein. Alternatively, the enzymatic activity (e.g., glucoamylase activity) of an enzyme of this invention comprises a specific activity at 37°C in the range from about 1 to about 750 units per milligram of protein, or, from about 500 to about 1200 units per milligram of protein. In one aspect, the enzymatic activity (e.g., glucoamylase activity) of an enzyme of this invention comprises a specific activity at 37°C in the range from about 1 to about 500 units per milligram of protein, or, from about 750 to about 1000 units per milligram of protein. In another aspect, the enzymatic activity (e.g., glucoamylase activity) of an enzyme of this invention comprises a specific activity at 37°C in the range from about 1 to about 250 units per milligram of protein. Alternatively, the enzymatic activity (e.g., glucoamylase activity) of an enzyme of this invention comprises a specific activity at 37°C in the range from about 1 to about 100 units per milligram of protein. In another aspect, the thermotolerance comprises retention of at least half of the specific activity of the enzymatic activity (e.g., glucoamylase activity) of an enzyme of this invention at 37°C after being heated to an elevated temperature, such as a temperature from about 0°C to about 20°C, about 20°C to about 37°C, about 37°C to about 50°C, about 50°C to about 70°C, about 70°C to about 75°C, about 75°C to about 80°C, about 80°C to about 85°C, about 85°C to about 90°C, about 90°C to about 95°C, about 95°C to about 100°C, about 100°C to about 110°C, or higher. Alternatively, the thermotolerance can comprise retention of specific activity at 37°C in the range from about 1 to about 1200 units per milligram of protein, or, from about 500 to about 1000 units per milligram of protein, after being heated to an elevated temperature. In another aspect, the thermotolerance can comprise retention of specific activity at 37°C in the range from about 1 to about 500 units per milligram of protein after being heated to an elevated temperature, as described above.

[0058] The invention provides isolated, synthetic or recombinant polypeptides of the invention, wherein the polypeptide comprises at least one glycosylation site. In one aspect, glycosylation can be an N-linked glycosylation. In one aspect, the polypeptide can be glycosylated after being expressed in a *P. pastoris* or a *S. pombe.* The disclosure also provides methods for adding glycosylation to a polypeptide, either post-translationally or chemically, to change the property of the polypeptides, e.g., its thermal stability, solubility, tendency to aggregate, and the like.

[0059] In one aspect, the polypeptide can retain the enzymatic activity (e.g., glucoamylase activity) of an enzyme of this invention under conditions comprising about pH 6.5, pH 6, pH 5.5, pH 5, pH 4.5, pH 4.0, pH 3.5, pH 3.0 or less (more acidic) pH. In another aspect, the polypeptide can retain the enzymatic activity (e.g., glucoamylase activity) of an enzyme of this invention under conditions comprising about pH 7, pH 7.5 pH 8.0, pH 8.5, pH 9, pH 9.5, pH 10, pH 10.5, pH 11.0, pH 11.5, pH 12, pH 12.5 or more (more basic) pH. In one aspect, the polypeptide can retain the enzymatic activity (e.g., glucoamylase activity) of an enzyme of this invention after exposure to conditions comprising about pH 6.5, pH 6, pH 5.5, pH 5, pH 4.5, pH 4.0, pH 3.5, pH 3.0 or less (more acidic) pH. In another aspect, the polypeptide can retain the enzymatic activity (e.g., glucoamylase activity) of an enzyme of this invention after exposure to conditions comprising about pH 7, pH 7.5 pH 8.0, pH 8.5, pH 9, pH 9.5, pH 10, pH 10.5, pH 11.0, pH 11.5, pH 12, pH 12.5 or more (more basic) pH.

[0060] The disclosure provides protein preparations comprising a polypeptide of the invention, wherein the protein preparation comprises a liquid, a solid or a gel.

[0061] The disclosure provides heterodimers comprising a polypeptide of the invention and a second domain. In one aspect, the second domain can be a polypeptide and the heterodimer can be a fusion protein. In one aspect, the second domain can be an epitope or a tag. In one aspect, the disclosure provides homodimers comprising a polypeptide of the invention.

[0062] The disclosure provides immobilized polypeptides having glucoamylase activity, wherein the polypeptide comprises a polypeptide of the invention, a polypeptide encoded by a nucleic acid of the invention, or a polypeptide comprising a polypeptide of the invention and a second domain. In one aspect, the polypeptide can be immobilized on a cell, a metal, a resin, a polymer, a ceramic, a glass, a microelectrode, a graphitic particle, a bead, a gel, a plate, an array or a capillary tube.

[0063] The disclosure provides arrays comprising an immobilized nucleic acid of the invention. The disclosure provides arrays comprising an antibody of the invention.

[0064] The disclosure provides isolated, synthetic or recombinant antibodies that specifically bind to a polypeptide of the invention or to a polypeptide encoded by a nucleic acid of the invention. The antibody can be a monoclonal or a polyclonal antibody. The disclosure provides hybridomas comprising an antibody, e.g., an antibody that specifically binds to a polypeptide of the invention or to a polypeptide encoded by a nucleic acid of the invention.

[0065] The disclosure provides food supplements for an animal comprising a polypeptide of the invention, e.g., a polypeptide encoded by the nucleic acid of the invention. In one aspect, the polypeptide in the food supplement can be

glycosylated. The invention provides edible enzyme delivery matrices comprising a polypeptide of the invention, e.g., a polypeptide encoded by the nucleic acid of the invention. In one aspect, the delivery matrix comprises a pellet. In one aspect, the polypeptide can be glycosylated. In one aspect, the amylase activity is thermotolerant. In another aspect, the amylase activity is thermostable.

[0066] The disclosure provides method of isolating or identifying a polypeptide having glucoamylase activity comprising the steps of: (a) providing an antibody of the disclosure; (b) providing a sample comprising polypeptides; and (c) contacting the sample of step (b) with the antibody of step (a) under conditions wherein the antibody can specifically bind to the polypeptide, thereby isolating or identifying a polypeptide having glucoamylase activity.

[0067] The disclosure provides methods of making an anti-amylase antibody comprising administering to a non-human animal a nucleic acid of the invention or a polypeptide of the invention or subsequences thereof in an amount sufficient to generate a humoral immune response, thereby making an anti-glycoamylase antibody. The disclosure provides methods of making an anti-glycoamylase immune comprising administering to a non-human animal a nucleic acid of the invention or a polypeptide of the invention or subsequences thereof in an amount sufficient to generate an immune response.

[0068] The invention provides methods of producing a recombinant polypeptide comprising the steps of: (a) providing a nucleic acid of the invention operably linked to a promoter; and (b) expressing the nucleic acid of step (a) under conditions that allow expression of the polypeptide, thereby producing a recombinant polypeptide. The method further comprises transforming a host cell with the nucleic acid of step (a) followed by expressing the nucleic acid of step (a), thereby producing a recombinant polypeptide in a transformed cell.

[0069] The disclosure provides methods for identifying a polypeptide having glucoamylase activity comprising the following steps: (a) providing a polypeptide of the invention; or a polypeptide encoded by a nucleic acid of the invention; (b) providing an amylase substrate; and (c) contacting the polypeptide or a fragment or variant thereof of step (a) with the substrate of step (b) and detecting a decrease in the amount of substrate or an increase in the amount of a reaction product, wherein a decrease in the amount of the substrate or an increase in the amount of the reaction product detects a polypeptide having glucoamylase activity. In one aspect, the substrate can be a polysaccharide, oligosaccharide or starch, e.g., a liquefied starch.

[0070] The disclosure provides methods for identifying a glucoamylase substrate comprising the following steps: (a) providing a polypeptide of the invention; or a polypeptide encoded by a nucleic acid of the invention; (b) providing a test substrate; and (c) contacting the polypeptide of step (a) with the test substrate of step (b) and detecting a decrease in the amount of substrate or an increase in the amount of reaction product, wherein a decrease in the amount of the substrate or an increase in the amount of a reaction product identifies the test substrate as an amylase or a glucoamylase substrate.

[0071] The disclosure provides methods of determining whether a test compound specifically binds to a polypeptide comprising the following steps: (a) expressing a nucleic acid or a vector comprising the nucleic acid under conditions permissive for translation of the nucleic acid to a polypeptide, wherein the nucleic acid comprises a nucleic acid of the disclosure, or, providing a polypeptide of the disclosure; (b) providing a test compound; (c) contacting the polypeptide with the test compound; and (d) determining whether the test compound of step (b) specifically binds to the polypeptide.

[0072] The disclosure provides methods for identifying a modulator of an amylase or a glucoamylase activity comprising the following steps: (a) providing a polypeptide of the disclosure or a polypeptide encoded by a nucleic acid of the disclosure; (b) providing a test compound; (c) contacting the polypeptide of step (a) with the test compound of step (b) and measuring an activity of the amylase or glucoamylase, wherein a change in the amylase or glucoamylase activity measured in the presence of the test compound compared to the activity in the absence of the test compound provides a determination that the test compound modulates the amylase or glucoamylase activity. In one aspect, the amylase or a glucoamylase activity can be measured by providing an amylase or glucoamylase substrate and detecting a decrease in the amount of the substrate or an increase in the amount of a reaction product, or, an increase in the amount of the substrate or a decrease in the amount of a reaction product. A decrease in the amount of the substrate or an increase in the amount of the reaction product with the test compound as compared to the amount of substrate or reaction product without the test compound identifies the test compound as an activator of amylase or glucoamylase activity. An increase in the amount of the substrate or a decrease in the amount of the reaction product with the test compound as compared to the amount of substrate or reaction product without the test compound identifies the test compound as an inhibitor of amylase or glucoamylase activity.

[0073] The disclosure provides computer systems comprising a processor and a data storage device wherein said data storage device has stored thereon a polypeptide sequence or a nucleic acid sequence of the disclosure (e.g., a polypeptide encoded by a nucleic acid of the disclosure). In one aspect, the computer system can further comprise a sequence comparison algorithm and a data storage device having at least one reference sequence stored thereon. In another aspect, the sequence comparison algorithm comprises a computer program that indicates polymorphisms. In one aspect, the computer system can further comprise an identifier that identifies one or more features in said sequence. The disclosure provides computer readable media having stored thereon a polypeptide sequence or a nucleic acid

sequence of the invention. The disclosure provides methods for identifying a feature in a sequence comprising the steps of: (a) reading the sequence using a computer program which identifies one or more features in a sequence, wherein the sequence comprises a polypeptide sequence or a nucleic acid sequence of the disclosure; and (b) identifying one or more features in the sequence with the computer program. The disclosure provides methods for comparing a first sequence to a second sequence comprising the steps of: (a) reading the first sequence and the second sequence through use of a computer program which compares sequences, wherein the first sequence comprises a polypeptide sequence or a nucleic acid sequence of the disclosure; and (b) determining differences between the first sequence and the second sequence with the computer program. The step of determining differences between the first sequence and the second sequence can further comprise the step of identifying polymorphisms. In one aspect, the method can further comprise an identifier that identifies one or more features in a sequence. In another aspect, the method can comprise reading the first sequence using a computer program and identifying one or more features in the sequence.

[0074] The disclosure provides methods for isolating or recovering a nucleic acid encoding a polypeptide having an amylase and/or glucoamylase activity from an environmental sample comprising the steps of: (a) providing an amplification primer sequence pair for amplifying a nucleic acid encoding a polypeptide having an amylase and/or glucoamylase activity, wherein the primer pair is capable of amplifying a nucleic acid of the disclosure; (b) isolating a nucleic acid from the environmental sample or treating the environmental sample such that nucleic acid in the sample is accessible for hybridization to the amplification primer pair; and, (c) combining the nucleic acid of step (b) with the amplification primer pair of step (a) and amplifying nucleic acid from the environmental sample, thereby isolating or recovering a nucleic acid encoding a polypeptide having an amylase and/or glucoamylase activity from an environmental sample. One or each member of the amplification primer sequence pair can comprise an oligonucleotide comprising at least about 10 to 50 consecutive bases of a sequence of the disclosure.

[0075] The disclosure provides methods for isolating or recovering a nucleic acid encoding a polypeptide having an amylase and/or glucoamylase activity from an environmental sample comprising the steps of: (a) providing a polynucleotide probe comprising a nucleic acid of the disclosure or a subsequence thereof; (b) isolating a nucleic acid from the environmental sample or treating the environmental sample such that nucleic acid in the sample is accessible for hybridization to a polynucleotide probe of step (a); (c) combining the isolated nucleic acid or the treated environmental sample of step (b) with the polynucleotide probe of step (a); and (d) isolating a nucleic acid that specifically hybridizes with the polynucleotide probe of step (a), thereby isolating or recovering a nucleic acid encoding a polypeptide having an amylase and/or glucoamylase activity from an environmental sample. The environmental sample can comprise a water sample, a liquid sample, a soil sample, an air sample or a biological sample. In one aspect, the biological sample can be derived from a bacterial cell, a protozoan cell, an insect cell, a yeast cell, a plant cell, a fungal cell or a mammalian cell.

[0076] The disclosure provides methods of generating a variant of a nucleic acid encoding a polypeptide having an amylase and/or glucoamylase activity comprising the steps of: (a) providing a template nucleic acid comprising a nucleic acid of the disclosure; and (b) modifying, deleting or adding one or more nucleotides in the template sequence, or a combination thereof, to generate a variant of the template nucleic acid. In one aspect, the method can further comprise expressing the variant nucleic acid to generate a variant amylase or glucoamylase polypeptide. The modifications, additions or deletions can be introduced by a method comprising error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, *in vivo* mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis, gene reassembly, gene site saturated mutagenesis (GSSM), synthetic ligation reassembly (SLR) or a combination thereof. In another aspect, the modifications, additions or deletions are introduced by a method comprising recombination, recursive sequence recombination, phosphothioate-modified DNA mutagenesis, uracil-containing template mutagenesis, gapped duplex mutagenesis, point mismatch repair mutagenesis, repair-deficient host strain mutagenesis, chemical mutagenesis, radiogenic mutagenesis, deletion mutagenesis, restriction-selection mutagenesis, restriction-purification mutagenesis, artificial gene synthesis, ensemble mutagenesis, chimeric nucleic acid multimer creation and a combination thereof.

[0077] In one aspect, the method can be iteratively repeated until an amylase or glucoamylase having an altered or different activity or an altered or different stability from that of a polypeptide encoded by the template nucleic acid is produced. In one aspect, the variant amylase or glucoamylase polypeptide is thermotolerant, and retains some activity after being exposed to an elevated temperature. In another aspect, the variant amylase or glucoamylase polypeptide has increased glycosylation as compared to the amylase or glucoamylase encoded by a template nucleic acid. Alternatively, the variant amylase or glucoamylase polypeptide has an amylase or glucoamylase activity under a high temperature, wherein the amylase or glucoamylase encoded by the template nucleic acid is not active under the high temperature. In one aspect, the method can be iteratively repeated until an amylase or glucoamylase coding sequence having an altered codon usage from that of the template nucleic acid is produced. In another aspect, the method can be iteratively repeated until an amylase or glucoamylase gene having higher or lower level of message expression or stability from that of the template nucleic acid is produced.

[0078] The disclosure provides methods for modifying codons in a nucleic acid encoding a polypeptide having an

amylase and/or glucoamylase activity to increase its expression in a host cell, the method comprising the following steps: (a) providing a nucleic acid of the disclosure encoding a polypeptide having an amylase and/or glucoamylase activity; and, (b) identifying a non-preferred or a less preferred codon in the nucleic acid of step (a) and replacing it with a preferred or neutrally used codon encoding the same amino acid as the replaced codon, wherein a preferred codon is a codon over-represented in coding sequences in genes in the host cell and a non-preferred or less preferred codon is a codon under-represented in coding sequences in genes in the host cell, thereby modifying the nucleic acid to increase its expression in a host cell.

[0079] The disclosure provides methods for modifying codons in a nucleic acid encoding a polypeptide having an amylase and/or glucoamylase activity; the method comprising the following steps: (a) providing a nucleic acid of the disclosure; and, (b) identifying a codon in the nucleic acid of step (a) and replacing it with a different codon encoding the same amino acid as the replaced codon, thereby modifying codons in a nucleic acid encoding an amylase or glucoamylase.

[0080] The disclosure provides methods for modifying codons in a nucleic acid encoding a polypeptide having an amylase and/or glucoamylase activity to increase its expression in a host cell, the method comprising the following steps: (a) providing a nucleic acid of the disclosure encoding an amylase or glucoamylase polypeptide; and, (b) identifying a non-preferred or a less preferred codon in the nucleic acid of step (a) and replacing it with a preferred or neutrally used codon encoding the same amino acid as the replaced codon, wherein a preferred codon is a codon over-represented in coding sequences in genes in the host cell and a non-preferred or less preferred codon is a codon under-represented in coding sequences in genes in the host cell, thereby modifying the nucleic acid to increase its expression in a host cell.

[0081] The disclosure provides methods for modifying a codon in a nucleic acid encoding a polypeptide having an amylase and/or glucoamylase activity to decrease its expression in a host cell, the method comprising the following steps: (a) providing a nucleic acid of the disclosure; and (b) identifying at least one preferred codon in the nucleic acid of step (a) and replacing it with a non-preferred or less preferred codon encoding the same amino acid as the replaced codon, wherein a preferred codon is a codon over-represented in coding sequences in genes in a host cell and a non-preferred or less preferred codon is a codon under-represented in coding sequences in genes in the host cell, thereby modifying the nucleic acid to decrease its expression in a host cell. In one aspect, the host cell can be a bacterial cell, a fungal cell, an insect cell, a yeast cell, a plant cell or a mammalian cell.

[0082] The disclosure provides methods for producing a library of nucleic acids encoding a plurality of modified amylase or glucoamylase active sites or substrate binding sites, wherein the modified active sites or substrate binding sites are derived from a first nucleic acid comprising a sequence encoding a first active site or a first substrate binding site the method comprising the following steps: (a) providing a first nucleic acid encoding a first active site or first substrate binding site, wherein the first nucleic acid sequence comprises a sequence that hybridizes under stringent conditions to a nucleic acid of the disclosure, and the nucleic acid encodes an amylase or glucoamylase active site or an amylase or glucoamylase substrate binding site; (b) providing a set of mutagenic oligonucleotides that encode naturally-occurring amino acid variants at a plurality of targeted codons in the first nucleic acid; and, (c) using the set of mutagenic oligonucleotides to generate a set of active site-encoding or substrate binding site-encoding variant nucleic acids encoding a range of amino acid variations at each amino acid codon that was mutagenized, thereby producing a library of nucleic acids encoding a plurality of modified amylase or glucoamylase active sites or substrate binding sites. In one aspect, the method comprises mutagenizing the first nucleic acid of step (a) by a method comprising an optimized directed evolution system, gene site-saturation mutagenesis (GSSM), synthetic ligation reassembly (SLR), error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, in vivo mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis, gene reassembly, gene site saturated mutagenesis (GSSM), synthetic ligation reassembly (SLR) and a combination thereof. In another aspect, the method comprises mutagenizing the first nucleic acid of step (a) or variants by a method comprising recombination, recursive sequence recombination, phosphothioate-modified DNA mutagenesis, uracil-containing template mutagenesis, gapped duplex mutagenesis, point mismatch repair mutagenesis, repair-deficient host strain mutagenesis, chemical mutagenesis, radiogenic mutagenesis, deletion mutagenesis, restriction-selection mutagenesis, restriction-purification mutagenesis, artificial gene synthesis, ensemble mutagenesis, chimeric nucleic acid multimer creation and a combination thereof.

[0083] The disclosure provides methods for making a small molecule comprising the following steps: (a) providing a plurality of biosynthetic enzymes capable of synthesizing or modifying a small molecule, wherein one of the enzymes comprises an amylase or glucoamylase enzyme encoded by a nucleic acid of the disclosure; (b) providing a substrate for at least one of the enzymes of step (a); and (c) reacting the substrate of step (b) with the enzymes under conditions that facilitate a plurality of biocatalytic reactions to generate a small molecule by a series of biocatalytic reactions. The disclosure provides methods for modifying a small molecule comprising the following steps: (a) providing an amylase or glucoamylase enzyme, wherein the enzyme comprises a polypeptide of the disclosure, or, a polypeptide encoded by a nucleic acid of the disclosure, or a subsequence thereof; (b) providing a small molecule; and (c) reacting the enzyme of step (a) with the small molecule of step (b) under conditions that facilitate an enzymatic reaction catalyzed by the

amylase or glucoamylase enzyme, thereby modifying a small molecule by an amylase or glucoamylase enzymatic reaction. In one aspect, the method can comprise a plurality of small molecule substrates for the enzyme of step (a), thereby generating a library of modified small molecules produced by at least one enzymatic reaction catalyzed by the amylase or glucoamylase enzyme. In one aspect, the method can comprise a plurality of additional enzymes under conditions that facilitate a plurality of biocatalytic reactions by the enzymes to form a library of modified small molecules produced by the plurality of enzymatic reactions. In another aspect, the method can further comprise the step of testing the library to determine if a particular modified small molecule which exhibits a desired activity is present within the library. The step of testing the library can further comprise the steps of systematically eliminating all but one of the biocatalytic reactions used to produce a portion of the plurality of the modified small molecules within the library by testing the portion of the modified small molecule for the presence or absence of the particular modified small molecule with a desired activity, and identifying at least one specific biocatalytic reaction that produces the particular modified small molecule of desired activity.

[0084] The disclosure provides methods for determining a functional fragment of an amylase or glucoamylase enzyme comprising the steps of: (a) providing an amylase or glucoamylase enzyme, wherein the enzyme comprises a polypeptide of the disclosure, or a polypeptide encoded by a nucleic acid of the disclosure, or a subsequence thereof; and (b) deleting a plurality of amino acid residues from the sequence of step (a) and testing the remaining subsequence for an amylase or glucoamylase activity, thereby determining a functional fragment of an amylase or glucoamylase enzyme. In one aspect, the amylase or glucoamylase activity is measured by providing an amylase or glucoamylase substrate and detecting a decrease in the amount of the substrate or an increase in the amount of a reaction product.

[0085] The disclosure provides methods for whole cell engineering of new or modified phenotypes by using real-time metabolic flux analysis, the method comprising the following steps: (a) making a modified cell by modifying the genetic composition of a cell, wherein the genetic composition is modified by addition to the cell of a nucleic acid of the disclosure; (b) culturing the modified cell to generate a plurality of modified cells; (c) measuring at least one metabolic parameter of the cell by monitoring the cell culture of step (b) in real time; and, (d) analyzing the data of step (c) to determine if the measured parameter differs from a comparable measurement in an unmodified cell under similar conditions, thereby identifying an engineered phenotype in the cell using real-time metabolic flux analysis. In one aspect, the genetic composition of the cell can be modified by a method comprising deletion of a sequence or modification of a sequence in the cell, or, knocking out the expression of a gene. In one aspect, the method can further comprise selecting a cell comprising a newly engineered phenotype. In another aspect, the method can comprise culturing the selected cell, thereby generating a new cell strain comprising a newly engineered phenotype.

[0086] The invention provides methods for hydrolyzing a polysaccharide, oligosaccharide or starch, comprising the following steps: (a) providing a polypeptide having glucoamylase activity, wherein the polypeptide comprises a polypeptide of the invention; (b) providing a composition comprising a polysaccharide, oligosaccharide or starch; and (c) contacting the polypeptide of step (a) with the composition of step (b) under conditions wherein the polypeptide hydrolyzes the polysaccharide, oligosaccharide or starch. In one aspect, the composition comprising polysaccharide, oligosaccharide or starch, that comprises an α-1,4-glucosidic bond or an α-1,6-glucosidic bond.

[0087] The invention provides methods for liquefying or removing a polysaccharide, oligosaccharide or starch, from a composition comprising the following steps: (a) providing a polypeptide having glucoamylase activity, wherein the polypeptide comprises a polypeptide of the invention; (b) providing a composition comprising a polysaccharide, oligosaccharide or starch; and (c) contacting the polypeptide of step (a) with the composition of step (b) under conditions wherein the polypeptide removes or liquefies the polysaccharide, oligosaccharide or starch.

[0088] The disclosure provides methods of increasing thermotolerance or thermostability of an amylase polypeptide, the method comprising glycosylating an amylase or glucoamylase polypeptide, wherein the polypeptide comprises at least thirty contiguous amino acids of a polypeptide of the disclosure; or a polypeptide encoded by a nucleic acid sequence of the disclosure, thereby increasing the thermotolerance or thermostability of the amylase or glucoamylase polypeptide. In one aspect, the amylase or glucoamylase specific activity can be thermostable or thermotolerant at a temperature in the range from greater than about 37°C to about 95°C.

[0089] The disclosure provides methods for overexpressing a recombinant amylase or glucoamylase polypeptide in a cell comprising expressing a vector comprising a nucleic acid comprising a nucleic acid of the disclosure or a nucleic acid sequence of the disclosure, wherein the sequence identities are determined by analysis with a sequence comparison algorithm or by visual inspection, wherein overexpression is effected by use of a high activity promoter, a dicistronic vector or by gene amplification of the vector.

[0090] The invention provides detergent compositions comprising a polypeptide of the invention or a polypeptide encoded by a nucleic acid of the invention, wherein the polypeptide comprises glucoamylase activity. In one aspect, the glucoamylase can be a nonsurface-active glucoamylase. In another aspect, the glucoamylase can be a surface-active glucoamylase.

[0091] The invention provides methods for washing an object comprising the following steps: (a) providing a composition comprising a polypeptide having glucoamylase activity, wherein the polypeptide comprises: a polypeptide of the invention

or a polypeptide encoded by a nucleic acid of the invention; (b) providing an object; and (c) contacting the polypeptide of step (a) and the object of step (b) under conditions wherein the composition can wash the object.

[0092] The invention provides methods for hydrolyzing a polysaccharide, oligosaccharide or starch, e.g., in a feed or a food prior to consumption by an animal, comprising the following steps: (a) obtaining a composition, e.g., a feed material, comprising a polysaccharide, oligosaccharide or starch, wherein the polypeptide comprises: a polypeptide of the invention or a polypeptide encoded by a nucleic acid of the invention; and (b) adding the polypeptide of step (a) to the composition, e.g., the feed or food material, in an amount sufficient for a sufficient time period to cause hydrolysis of the polysaccharide, oligosaccharide or starch, thereby hydrolyzing the polysaccharide, oligosaccharide or starch. In one aspect, the food or feed comprises rice, corn, barley, wheat, legumes, or potato.

[0093] For example, in one embodiment, the disclosure provide compositions comprising a combination of an amylase and a glucoamylase (where one or both of these enzymes is an enzyme of this disclosure) for the hydrolysis of a polysaccharide, oligosaccharide or starch, e.g., a rice, corn, barley, wheat, legumes, or potato starch. In one aspect, enzyme loading for a combination of an amylase and a glucoamylase comprises a 1:10 amylase:glucoamylase ratio; wherein in one embodiment a total enzyme load is between 0.015% - 0.0255% enzyme necessary to completely hydrolyze 33% of a flour, e.g., a corn flour. Alternative exemplary ranges for loading are around 0.01% (w/w) for hydrolysis of 23% purified corn starch and between 0.015%- 0.2% (w/w) for hydrolysis of corn starch in 33% corn flour.

[0094] The invention provides methods for textile desizing comprising the following steps: (a) providing a polypeptide having glucoamylase activity, wherein the polypeptide comprises a polypeptide of the invention or a polypeptide encoded by a nucleic acid of the invention; (b) providing a fabric; and (c) contacting the polypeptide of step (a) and the fabric of step (b) under conditions wherein the glucoamylase can desize the fabric.

[0095] The invention provides methods for deinking of paper or fibers comprising the following steps: (a) providing a polypeptide having glucoamylase activity, wherein the polypeptide comprises a polypeptide of the invention; (b) providing a composition comprising paper or fiber; and (c) contacting the polypeptide of step (a) and the composition of step (b) under conditions wherein the polypeptide can deink the paper or fiber.

[0096] The invention provides methods for treatment of lignocellulosic fibers comprising the following steps: (a) providing a polypeptide having glucoamylase activity, wherein the polypeptide comprises a polypeptide of the invention; (b) providing a lignocellulosic fiber; and (c) contacting the polypeptide of step (a) and the fiber of step (b) under conditions wherein the polypeptide can treat the fiber thereby improving the fiber properties.

[0097] The invention provides methods for producing a high-maltose or a high-glucose syrup comprising the following steps: (a) providing a polypeptide glucoamylase activity, wherein the polypeptide comprises an enzyme of the invention; (b) providing a composition comprising a polysaccharide, oligosaccharide or starch; and (c) contacting the polypeptide of step (a) and the fabric of step (b) under conditions wherein the polypeptide of step (a) can liquefy the composition of step (b) thereby producing a soluble polysaccharide, oligosaccharide or starch, hydrolysate and saccharify the soluble polysaccharide, oligosaccharide or starch, hydrolysate thereby producing the syrup. In one aspect, the starch can be from rice, corn, barley, wheat, legumes, potato, or sweet potato.

[0098] The invention provides methods for improving the flow of the polysaccharide-comprising, e.g., a starch-containing, production fluids comprising the following steps: (a) providing a polypeptide having glucoamylase activity, wherein the polypeptide comprises a polypeptide of the invention; (b) providing production fluid; and (c) contacting the polypeptide of step (a) and the production fluid of step (b) under conditions wherein the glucoamylase can hydrolyze the polysaccharide, oligosaccharide or starch, in the production fluid thereby improving its flow by decreasing its density. In one aspect, the production fluid can be from a subterranean formation.

[0099] The invention provides anti-staling compositions comprising a polypeptide of the invention or a polypeptide encoded by a nucleic acid of the invention. The disclosure provides methods for preventing staling of the baked products comprising the following steps: (a) providing a polypeptide having glucoamylase activity, wherein the polypeptide comprises a polypeptide of the invention; (b) providing a composition containing a polysaccharide, oligosaccharide or starch, used for baking; (c) combining the polypeptide of step (a) with the composition of the step (b) under conditions wherein the polypeptide can hydrolyze the polysaccharide, oligosaccharide or starch, in the composition used for baking thereby preventing staling of the baked product. In one aspect, the baked product can be bread.

[0100] The invention provides methods for using a glucoamylase in brewing or alcohol production comprising the following steps: (a) providing a polypeptide having glucoamylase activity, wherein the polypeptide comprises a polypeptide of the invention; (b) providing a composition containing a polysaccharide, oligosaccharide or starch, and used for brewing or in alcohol production; (c) combining the polypeptide of step (a) with the composition of the step (b) under conditions wherein the polypeptide can hydrolyze the polysaccharide, oligosaccharide or starch, in the composition used for brewing or in alcohol production. In one aspect, the composition containing a polysaccharide, oligosaccharide or starch, can be beer.

[0101] The disclosure provides methods of making a transgenic plant comprising the following steps: (a) introducing a heterologous nucleic acid sequence into the cell, wherein the heterologous nucleic sequence comprises a nucleic acid sequence of the disclosure, thereby producing a transformed plant cell; and (b) producing a transgenic plant from the

transformed cell. In one aspect, the step (a) can further comprise introducing the heterologous nucleic acid sequence by electroporation or microinjection of plant cell protoplasts. In another aspect, the step (a) can further comprise introducing the heterologous nucleic acid sequence directly to plant tissue by DNA particle bombardment. Alternatively, the step (a) can further comprise introducing the heterologous nucleic acid sequence into the plant cell DNA using an Agrobacterium tumefaciens host. In one aspect, the plant cell can be a potato, corn, rice, wheat, tobacco, or barley cell.

[0102] The disclosure provides methods of expressing a heterologous nucleic acid sequence in a plant cell comprising the following steps: (a) transforming the plant cell with a heterologous nucleic acid sequence operably linked to a promoter, wherein the heterologous nucleic sequence comprises a nucleic acid of the disclosure; (b) growing the plant under conditions wherein the heterologous nucleic acids sequence is expressed in the plant cell.

[0103] The disclosure also provides a process for preparing a dough or a baked product prepared from the dough which comprises adding an amylase or glucoamylase of the disclosure to the dough in an amount which is effective to retard the staling of the bread. The disclosure also provides a dough comprising said amylase or glucoamylase and a premix comprising flour together with said amylase or glucoamylase. Finally, the disclosure provides an enzymatic baking additive, which contains said amylase or glucoamylase. The use of the glucoamylase in accordance with the present invention provides an improved anti-staling effect as measured by, e.g. less crumb firming, retained crumb elasticity, improved slice-ability (e.g. fewer crumbs, non-gummy crumb), improved palatability or flavor.

[0104] The enzyme-comprising compositions of the invention (e.g., comprising polypeptides, nucleic acids of this invention) can be formulated in a variety of forms, e.g., as liquids, gels, pills, tablets, sprays, powders, food, feed pellets or encapsulated forms, including nanoencapsulated forms. Any of these embodiments can be designed or further formulated as delayed release or "controlled release" compositions.

[0105] The invention provides delayed release ("controlled release") compositions comprising a desired ingredient coated by a latex polymer (or equivalent) coating. In one aspect, the desired ingredient comprises an enzyme, e.g., an enzyme of the invention. In one aspect, a coated composition comprises a drug or pharmaceutical. In one aspect, the desired ingredient comprises a small molecule, a drug, a polysaccharide, a lipid, a nucleic acid, a vitamin, an antibiotics or an insecticide. In one aspect, the desired ingredient comprises a pellet or a matrix, e.g., a pellet or a matrix comprising an edible material (e.g., as an animal food or feed or supplement or medicament). The disclosure also provides methods for the "controlled release" or "delayed release" of a composition, wherein the composition is coated by a latex polymer (or equivalent) coating.

[0106] In one aspect, the latex polymer coating comprises a latex paint, or equivalent. The latex polymer coating can comprise a (meth)acrylate, a vinyl acetate, a styrene, an ethylene, a vinyl chloride, a butadiene, a vinylidene chloride, a vinyl versatate, a vinyl propionate, a t-butyl acrylate, an acrylonitrile, a neoprene, a maleate, a fumarate, equivalents thereof, combinations thereof and/or derivatives thereof.

[0107] The disclosure provides methods for the delayed release or controlled release of a composition comprising: (i) (a) providing a composition, and, providing a latex polymer coating; and (b) coating the composition with the latex polymer coating; (ii) the method of (i), wherein the composition comprises a drug or pharmaceutical; or (iii) the method of (i) or (ii), wherein the composition comprises or consists of the polypeptide of the disclosure, or a polypeptide encoded by the nucleic acid of the disclosure.

[0108] The invention provides oil well drilling fluids comprising the polypeptides of the invention. The invention provides methods for changing the viscosity of a composition comprising: (i) (a) providing a composition and a polypeptide of the invention, and, providing a composition; and (b) treating the composition with the polypeptide of the invention; or (ii) the method of (i), wherein the composition comprises a soil or a drilling mud.

[0109] The disclosure provides methods for aiding in the carrying away of drilling mud comprising: (a) providing a composition and a polypeptide of the disclosure, and a drilling mud; and (b) treating the drilling mud with a composition comprising a polypeptide of the disclosure.

[0110] The invention provides bio-bleaching solutions comprising a polypeptide of the invention. The invention provides methods for bio-bleaching a composition comprising: (i) (a) providing a composition and a polypeptide of the invention; and (b) treating the composition with a polypeptide of the invention; or (ii) the method of (i), wherein the composition is a paper or a pulp product.

[0111] The invention provides methods for making a fuel comprising: (i) (a) providing a polypeptide of the invention; (b) providing a composition comprising a polysaccharide, oligosaccharide or starch; and (c) contacting the polypeptide of (a) with the composition of (b) under conditions wherein the polypeptide hydrolyzes the polysaccharide, oligosaccharide or starch; or (ii) the method of (i) wherein the polypeptide is a thermostable enzyme; (iii) the method of (i) wherein the fuel is ethanol-based.

[0112] The invention provides disinfectants comprising a polypeptide of the invention.

[0113] The invention provides biodefense or bio-detoxifying agents comprising a polypeptide of the invention.

[0114] The invention provides dairy products comprising a polypeptide of the invention.

[0115] The invention provides methods for processing a biomass material comprising lignocellulose comprising (i) (a) providing a composition comprising a polypeptide of the invention, and, providing a biomass material; and (b) contacting

the composition comprising a polypeptide of the invention, with the biomass material; (ii) the method of (i), wherein the biomass material comprises or is derived from an agricultural crop, or is a byproduct of a food or a feed production, or is a lignocellulosic waste product, or is a plant residue or a waste paper or waste paper product; (iii) the method of (i) or (ii), wherein the polypeptide has activity comprising a glucoamylase activity; (iv) the method of any of (ii), wherein the plant residue comprise stems, leaves, hulls, husks, cobs, wood, wood chips, wood pulp and sawdust, or, the paper waste comprises discarded or used photocopy paper, computer printer paper, notebook paper, notepad paper, typewriter paper, newspapers, magazines, cardboard and paper-based packaging materials; or (v) the method of (i) to (iv), wherein the processing of the biomass material generates a bioethanol, biopropanol, biobutanol, or a biodiesel; (vi) the method of (i) to (v), wherein the biomass material comprises a lignocellulose.

[0116] The invention provides biomass materials comprising a polypeptide of the invention.

[0117] The invention provides methods for making bioethanol, biopropanol, biobutanol, or a biodiesel comprising: (i) (a) providing a polypeptide of the invention, and, providing a composition comprising a polysaccharide or oligosaccharide; and (b) contacting the composition comprising a polysaccharide or oligosaccharide with a polypeptide of the invention; (ii) the method of (i), wherein the composition comprising a polysaccharide or oligosaccharide comprises a plant, plant product or plant derivative; (iii) the method of (ii), wherein the plant or plant product comprises cane sugar plants or plant products, beets or sugarbeets, wheat, corn, soybeans, potato, rice or barley; (iv) the method of any of (i) to (iii), wherein the polypeptide has activity comprising a glucoamylase activity; or (v) the method of any of (i) to (iv), wherein the polysaccharide or oligosaccharide comprises a fermentable sugar.

[0118] The invention provides methods for making a fuel comprising (i) (a) providing a polypeptide of the invention, and, providing a composition comprising a fermentable sugar; and (b) contacting the composition comprising a fermentable sugar with a polypeptide of the invention; (ii) the method of (i), wherein the composition comprising a fermentable sugar comprises a plant, plant product or plant derivative; (iii) the method of (ii), wherein the plant or plant product comprises cane sugar plants or plant products, beets or sugarbeets, wheat, corn, soybeans, potato, rice or barley; (iv) the method of any of (i) to (iii), wherein the polypeptide has activity comprising a glucoamylase activity; or (v) the method of any of (i) to (iv), wherein the fuel comprises a bioethanol or a gasoline-ethanol mix, or comprises a bioethanol, biopropanol, biobutanol, or a biodiesel.

[0119] The disclosure provides fuels comprising (a) a polypeptide of the disclosure; (b) the fuel of (a), wherein the polypeptide has activity comprising amylase, glucoamylase, glucosidase, e.g. alpha-glucosidase or beta-glucosidase activity; (c) the fuel of (a) or (b), wherein the fuel is derived from a plant material, or the fuel is derived from a potato, soybean (rapeseed), barley, rye, corn, oats, wheat, beet or sugar cane; or (d) the fuel of any of (a) to (c), wherein the fuel comprises a bioethanol or a gasoline-ethanol mix, or comprises a bioethanol, biopropanol, biobutanol, or a biodiesel.

[0120] The invention provides methods for producing a sugar (e.g., a monosaccharide), the method comprising: (i) (a) providing at least one polypeptide having a glucoamylase activity; (b) providing a composition comprising a polysaccharide or an oligosaccharide; and (c) contacting the composition of step (b) with the polypeptide of step (a), thereby generating sugars; (ii) the method of (i), wherein the composition comprising a polysaccharide or an oligosaccharide comprises a starch; (iii) the method of any of (i) or (ii), wherein the polysaccharide, oligosaccharide and/or sugar comprises or is a fermentable sugar; (iv) the method of any of (i), (ii), or (iii), further comprising fermenting the sugar to produce an alcohol; or (v) the method of (iv), wherein the alcohol is an ethanol, propanol or butanol.

[0121] The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

[0122]

Figure 1 is a block diagram of a computer system.

Figure 2 is a flow diagram illustrating one aspect of a process for comparing a new nucleotide or protein sequence with a database of sequences in order to determine the homology levels between the new sequence and the sequences in the database.

Figure 3 is a flow diagram illustrating one aspect of a process in a computer for determining whether two sequences are homologous.

Figure 4 is a flow diagram illustrating one aspect of an identifier process 300 for detecting the presence of a feature in a sequence.

Figure 5 illustrates an exemplary method of the invention for liquefaction saccharification, as discussed in detail, below.

Figure 6 illustrates an exemplary corn wet milling process of the invention using at least one enzyme of the invention, as discussed in detail, below.

Figure 7, Figure 8 and Figure 9 illustrate alternative exemplary polysaccharide, e.g., starch, processing methods (e.g., industrial processes), as discussed in detail, below.

Figure 10 and Figure 11 illustrate the influence of pH in the range of between about pH 3.5 to 6.0 on the hydrolysis of starch by seven (7) glucoamylases and two (2) amylases of this disclosure, as discussed in detail, below.

Figure 12 illustrates how enzymes of the invention can be used in ethanol production from corn by dry milling, including their use in both "conventional processes" and "simultaneous liquefaction saccharification and fermentation processes", as discussed in detail, below.

Figure 13, Figure 14, Figure 15, Figure 16, Figure 17, Figure 18, Figure 19 and Figure 20, illustrate rate comparisons of exemplary enzymes of the disclosure in different storage buffers, as discussed in detail in Example 1, below.

Figure 21 illustrates an SDS PAGE showing the results of proteolysis of exemplary enzyme SEQ ID NO:52 by pepsin; and Figure 22 illustrates the characterization of the peptides generated in this pepsin digest of SEQ ID NO:52; and Figure 23A illustrates the small peptide isolation scheme used for the peptides generated by the proteolysis of SEQ ID NO:52 by pepsin; Figure 23B illustrates an SDS PAGE of the results of the small peptide isolation scheme; Figure 23C illustrates the LC/MS profile of the C18 RP eluted fraction; Figure 23D illustrates the sequence of the peptides identified by the LC MS/MS analysis; Figure 23E and Figure 23F illustrate the "Asn-Xaa-Ser/Thr" sequins (motifs) in the sequence output (highlighted in blue); asparagines predicted to be N-glycosylated are highlighted in red, as discussed in detail in Example 22, below.

Figure 24 illustrates Table 1, which summarizes data comparing initial rates of granular corn starch and soluble starch (dextrin) hydrolysis by exemplary enzymes of the disclosure and a benchmark enzyme *A. niger* glucoamylase, as discussed in detail in Example 23, below.

Figure 25 illustrates the effect of temperature on the activity of the exemplary glucoamylase SEQ ID NO:20 and the *A. niger* "benchmark" enzyme with soluble starch (dextrin) as a substrate, as discussed in detail in Example 23, below.

Figure 26 illustrates tables summarizing the efficiency of exemplary enzyme purification protocols on selected enzymes of this disclosure, and corresponding activity data on, inter alia, raw starch and soluble starch comparing purified and unpurified enzyme, as discussed in detail in Example 18, below.

Figure 27 illustrates the theoretical basis of the CERALPHA™ α-amylase assay procedure, as discussed in detail in Example 27, below.

Figure 28A shows data demonstrating the effect of temperature on the activity of e.g., an exemplary glucoamylase of the disclosure with granular starch as a substrate, as discussed in detail in Example 29, below.

Figure 28B shows data demonstrating the effect of temperature on the activity of e.g., an exemplary glucoamylase with soluble starch (dextrin) as a substrate, as discussed in detail in Example 29, below.

Figure 29A shows data demonstrating the effect of pH on the activity of e.g., an exemplary glucoamylase of the disclosure with granular starch as a substrate, as discussed in detail in Example 29, below.

Figure 29B shows data demonstrating the effect of pH on the activity of e.g., an exemplary glucoamylase with soluble starch (dextrin) as a substrate, as discussed in detail in Example 29, below.

Figure 30 shows data demonstrating the effect of temperature on starch hydrolysis by the characterized α-amylases, as discussed in detail in Example 29, below.

Figure 31A shows data demonstrating the effect pH on the activities of exemplary α-Amylases and/or glucoamylases with Granular Starch as substrate, as discussed in detail in Example 29, below.

Figure 31B shows data demonstrating the effect pH on the activities of exemplary α-Amylases and/or glucoamylases with Soluble Starch as substrate, as discussed in detail in Example 29, below.

[0123] Like reference symbols in the various drawings indicate like elements.

DETAILED DESCRIPTION

[0124] The disclosure provides amylase enzymes, e.g., alpha amylases, polynucleotides encoding the enzymes, methods of making and using these polynucleotides and polypeptides. The invention is directed to novel polypeptides having glucoamylase activity, nucleic acids encoding them. The polypeptides of the invention can be used in a variety of diagnostic, therapeutic, and industrial contexts. The polypeptides of the invention can be used as, e.g., an additive for a detergent, for processing foods and for chemical synthesis utilizing a reverse reaction. Additionally, the polypeptides of the invention can be used in fabric treatment, alcohol production, and as additives to food or animal feed.

[0125] In one aspect, the glucoamylase of the invention is active at a high and/or at a low temperature, or, over a wide range of temperature. For example, they can be active in the temperatures ranging between 20°C to 90°C, between 30°C to 80°C, or between 40°C to 70°C. In alternative aspects, the glucoamylase of the invention can have activity in acidic pHs as low as pH 5.5, pH 5.0, pH 4.5, pH 4.0, and pH 3.5. In alternative aspects, the glucoamylase of the invention can have activity in alkaline pHs as high as pH 8, pH 9.5, pH 10, pH 10.5, and pH 11. In one aspect, the glucoamylase of the invention is active in the temperature range of between about 40°C to about 70°C under conditions of low water

activity (low water content). For example, the invention provides glucoamylases, with the ability to hydrolyze a polysaccharide, oligosaccharide or starch, e.g., a granular starch (including raw granular starch), at low temperatures, e.g., in the range of about 30°C to 40°C; at low pH, e.g., in the range of about pH 3.5 to pH 6.0; and, at low temperatures and low pH, e.g., in the range of about 30°C to 40°C and at low pH in the range of about pH 3.5 to pH 6.0.

[0126] The disclosure also provides methods for further modifying the exemplary amylases and/or glucoamylases of the disclosure to generate proteins with desirable properties. For example, amylases generated by the methods of the disclosure can have altered enzymatic activity, thermal stability, pH/activity profile, pH/stability profile (such as increased stability at low, e.g. pH<6 or pH<5, or high, e.g. pH>9, pH values), stability towards oxidation, Ca^{2+} dependency, specific activity and the like. The disclosure provides for altering any property of interest. For instance, the alteration may result in a variant which, as compared to a parent enzyme, has altered enzymatic activity, or, pH or temperature activity profiles.

Generating and Manipulating Nucleic Acids

[0127] In one aspect, the invention provides isolated, synthetic or recombinant nucleic acids comprising a nucleic acid sequence having at least 98%, 99%, or more, or complete (100%) sequence identity to the nucleic acid of the invention. In one aspect, the nucleic acid encodes at least one polypeptide having glucoamylase activity.

[0128] "Synthetic" nucleic acids (including oligonucleotides), polypeptides or proteins of the invention include those prepared by any chemical synthesis, e.g., as described, below.

[0129] The phrases "nucleic acid" or "nucleic acid sequence" includes oligonucleotides, nucleotides, polynucleotides, or to a fragment of any of these, to DNA or RNA (e.g., mRNA, rRNA, tRNA) of genomic, recombinant or synthetic origin which may be single-stranded or double-stranded and may represent a sense or antisense strand, to peptide nucleic acid (PNA), or to any DNA-like or RNA-like material, natural or synthetic in origin, including, e.g., iRNA such as miRNA or siRNA, ribonucleoproteins (e.g., iRNPs). The term encompasses nucleic acids, i.e., oligonucleotides, containing known analogues of natural nucleotides. The term also encompasses nucleic-acid-like structures with synthetic backbones, see e.g., Mata (1997) Toxicol. Appl. Pharmacol. 144:189-197; Strauss-Soukup (1997) Biochemistry 36:8692-8698; Samstag (1996) Antisense Nucleic Acid Drug Dev 6:153-156.

[0130] The invention provides "recombinant" and synthetic nucleic acids, which can include nucleic acids adjacent to a "backbone" nucleic acid to which it is not adjacent in its natural environment. In one aspect, nucleic acids represent 5% or more of the number ofnucleic acid inserts in a population of nucleic acid "backbone molecules." "Backbone molecules" according to the invention include nucleic acids such as expression vectors, self-replicating nucleic acids, viruses, integrating nucleic acids, and other vectors or nucleic acids used to maintain or manipulate a nucleic acid insert of interest. In one aspect, the enriched nucleic acids represent 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more of the number of nucleic acid inserts in the population of recombinant backbone molecules. "Recombinant" polypeptides or proteins refer to polypeptides or proteins produced by recombinant DNA techniques; e.g., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide or protein.

[0131] "Oligonucleotide" includes either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands, which may be chemically synthesized (i.e., as synthetic nucleic acids). In alternative embodiments, synthetic nucleic acids and oligonucleotides of the invention have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. In alternative embodiments, a synthetic oligonucleotide can ligate to a fragment that has not been dephosphorylated.

[0132] The term "gene" includes a nucleic acid sequence comprising a segment of DNA involved in producing a transcription product (e.g., a message), which in turn is translated to produce a polypeptide chain, or regulates gene transcription, reproduction or stability. Genes can include regions preceding and following the coding region, such as leader and trailer, promoters and enhancers, as well as, where applicable, intervening sequences (introns) between individual coding segments (exons).

[0133] The invention provides isolated and recombinant nucleic acids, including expression cassettes such as expression vectors encoding the polypeptides of the invention. The disclosure provides probes comprising or consisting of nucleic acids of the disclosure. The disclosure also includes methods for discovering new amylase sequences using the nucleic acids of the disclosure. The disclosure also includes methods for inhibiting the expression of amylase genes, transcripts and polypeptides using the nucleic acids of the disclosure. Also provided are methods for modifying the nucleic acids of the disclosure by, e.g., synthetic ligation reassembly, optimized directed evolution system and/or gene site saturation mutagenesis (GSSM).

[0134] The nucleic acids of the invention can be made, isolated and/or manipulated by, e.g., cloning and expression of cDNA libraries, amplification of message or genomic DNA by PCR, and the like. In practicing the methods of the disclosure, homologous genes can be modified by manipulating a template nucleic acid, as described herein. The invention can be practiced in conjunction with any method or protocol or device known in the art, which are well described

in the scientific and patent literature.

General Techniques

[0135] The nucleic acids used to practice this invention, whether RNA, iRNA, antisense nucleic acid, cDNA, genomic DNA, vectors, viruses or hybrids thereof, may be isolated from a variety of sources, genetically engineered, amplified, and/or expressed/ generated recombinantly. Recombinant polypeptides generated from these nucleic acids can be individually isolated or cloned and tested for a desired activity. Any recombinant expression system can be used, including bacterial, mammalian, yeast, insect or plant cell expression systems.

[0136] The disclosure provides methods for optimizing amylase enzymes and enzyme-encoding nucleic acid sequences, e.g., making "variant sequences", comprising use of sequences of the invention using, e.g., "saturation mutagenesis" or "GSSM" (includes a method that uses degenerate oligonucleotide primers to introduce point mutations into a polynucleotide, as described in detail, below); "optimized directed evolution system" or "optimized directed evolution" (includes a method for reassembling fragments of related nucleic acid sequences, e.g., related genes, and explained in detail, below) and/or "synthetic ligation reassembly" or "SLR" (includes a method of ligating oligonucleotide fragments in a non-stochastic fashion, and explained in detail, below). "Variant" includes polynucleotides or polypeptides of the disclosure modified at one or more base pairs, codons, introns, exons, or amino acid residues (respectively) yet still retain the biological activity of an amylase and/or glucoamylase of the invention. Variants can be produced by any number of means included methods such as, for example, error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, in vivo mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis, gene reassembly, GSSM and any combination thereof. Techniques for producing variant amylase having activity at a pH or temperature, for example, that is different from an exemplary enzyme of this disclosure, or a wild-type amylase, are provided herein.

[0137] Nucleic acids of the invention can be completely or partially synthetic, and in alternative aspects, they can be synthesized *in vitro* by well-known chemical synthesis techniques, as described in, e.g., Adams (1983) J. Am. Chem. Soc. 105:661; Belousov (1997) Nucleic Acids Res. 25:3440-3444; Frenkel (1995) Free Radic. Biol. Med. 19:373-380; Blommers (1994) Biochemistry 33:7886-7896; Narang (1979) Meth. Enzymol. 68:90; Brown (1979) Meth. Enzymol. 68:109; Beaucage (1981) Tetra. Lett. 22:1859; U.S. Patent No. 4,458,066.

[0138] Techniques for the manipulation of nucleic acids, such as, e.g., subcloning, labeling probes (e.g., random-primer labeling using Klenow polymerase, nick translation, amplification), sequencing, hybridization and the like are well described in the scientific and patent literature, see, e.g., Sambrook, ed., MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc., New York (1997); LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y. (1993).

[0139] Another useful means of obtaining and manipulating nucleic acids used to practice the methods of the invention is to clone from genomic samples, and, if desired, screen and re-clone inserts isolated or amplified from, e.g., genomic clones or cDNA clones. Sources of nucleic acid used in the methods of the invention include genomic or cDNA libraries contained in, e.g., mammalian artificial chromosomes (MACs), see, e.g., U.S. Patent Nos. 5,721,118; 6,025,155; human artificial chromosomes, see, e.g., Rosenfeld (1997) Nat. Genet. 15:333-335; yeast artificial chromosomes (YAC); bacterial artificial chromosomes (BAC); P1 artificial chromosomes, see, e.g., Woon (1998) Genomics 50:306-316; P1-derived vectors (PACs), see, e.g., Kern (1997) Biotechniques 23:120-124; cosmids, recombinant viruses, phages or plasmids.

[0140] In one aspect, a nucleic acid encoding a polypeptide of the disclosure is assembled in appropriate phase with a leader sequence capable of directing secretion of the translated polypeptide or fragment thereof.

[0141] The disclosure provides fusion proteins and nucleic acids encoding them. A polypeptide of the disclosure can be fused to a heterologous peptide or polypeptide, such as N-terminal identification peptides which impart desired characteristics, such as increased stability or simplified purification. Peptides and polypeptides of the disclosure can also be synthesized and expressed as fusion proteins with one or more additional domains linked thereto for, e.g., producing a more immunogenic peptide, to more readily isolate a recombinantly synthesized peptide, to identify and isolate antibodies and antibody-expressing B cells, and the like. Detection and purification facilitating domains include, e.g., metal chelating peptides such as polyhistidine tracts and histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle WA). The inclusion of a cleavable linker sequences such as Factor Xa or enterokinase (Invitrogen, San Diego CA) between a purification domain and the motif-comprising peptide or polypeptide to facilitate purification. For example, an expression vector can include an epitope-encoding nucleic acid sequence linked to six histidine residues followed by a thioredoxin and an enterokinase cleavage site (see e.g., Williams (1995) Biochemistry 34:1787-1797; Dobeli (1998) Protein Expr. Purif. 12:404-414). The histidine residues facilitate detection and purification while the enterokinase cleavage site provides a means for purifying the

epitope from the remainder of the fusion protein. Technology pertaining to vectors encoding fusion proteins and application of fusion proteins are well described in the scientific and patent literature, see e.g., Kroll (1993) DNA Cell. Biol., 12:441-53.

Transcriptional and translational control sequences

[0142] The invention provides nucleic acid (e.g., DNA) sequences of the invention operatively linked to expression (e.g., transcriptional or translational) control sequence(s), e.g., promoters or enhancers, to direct or modulate RNA synthesis/ expression. Promoters that are used to practice this invention include all sequences capable of driving transcription of a coding sequence in a cell, e.g., a plant cell. Thus, promoters used in the constructs of the invention include *cis*-acting transcriptional control elements and regulatory sequences that are involved in regulating or modulating the timing and/or rate of transcription of a gene. For example, a promoter used to practice this invention can be a *cis*-acting transcriptional control element, including an enhancer, a promoter, a transcription terminator, an origin of replication, a chromosomal integration sequence, 5' and 3' untranslated regions, or an intronic sequence, which are involved in transcriptional regulation. These cis-acting sequences typically interact with proteins or other biomolecules to carry out (turn on/off, regulate, modulate, etc.) transcription. "Constitutive" promoters used to practice this invention can be those that drive expression continuously under most environmental conditions and states of development or cell differentiation. Promoters used to practice this invention can be "inducible" or "regulatable" promoters direct expression of the nucleic acid of the invention under the influence of environmental conditions or developmental conditions. Examples of environmental conditions that may affect transcription by inducible promoters include anaerobic conditions, elevated temperature, drought, or the presence of light.

[0143] In one embodiment, a promoter sequence can be "operably linked to" a coding sequence of the invention, e.g., when RNA polymerase which initiates transcription at the promoter will transcribe the coding sequence into mRNA.

[0144] Promoters used to practice this invention include "tissue-specific" promoters, which are transcriptional control elements that are only active in particular cells or tissues or organs, e.g., in plants or animals. Tissue-specific regulation may be achieved by certain intrinsic factors which ensure that genes encoding proteins specific to a given tissue are expressed. Such factors are known to exist in mammals and plants so as to allow for specific tissues to develop.

[0145] The expression control sequence(s) used to practice this invention can be in an expression vector. Exemplary bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda PR, PL and trp. Exemplary eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein I. Promoters suitable for expressing a polypeptide in bacteria include the *E. coli* lac or trp promoters, the lacI promoter, the lacZ promoter, the T3 promoter, the T7 promoter, the gpt promoter, the lambda PR promoter, the lambda PL promoter, promoters from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), and the acid phosphatase promoter. Eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, heat shock promoters, the early and late SV40 promoter, LTRs from retroviruses, and the mouse metallothionein-I promoter. Other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses may also be used.

Tissue-Specific Plant Promoters

[0146] The invention provides expression cassettes that can be expressed in a tissue-specific manner, e.g., that can express a glucoamylase of the invention in a tissue-specific manner. The invention also provides plants or seeds that express a glucoamylase of the invention in a tissue-specific manner. The tissue-specificity can be seed specific, stem specific, leaf specific, root specific, fruit specific and the like.

[0147] In one aspect, a constitutive promoter such as the CaMV 35S promoter can be used for expression in specific parts of the plant or seed or throughout the plant. For example, for overexpression, a plant promoter fragment can be employed which will direct expression of a nucleic acid in some or all tissues of a plant, e.g., a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens,* and other transcription initiation regions from various plant genes known to those of skill. Such genes include, e.g., *ACT11* from *Arabidopsis* (Huang (1996) Plant Mol. BioL 33:125-139); *Cat3* from *Arabidopsis* (GenBank No. U43147, Zhong (1996) Mol. Gen. Genet. 251:196-203); the gene encoding stearoyl-acyl carrier protein desaturase from *Brassica napus* (Genbank No. X74782, Solocombe (1994) Plant Physiol. 104:1167-1176); *GPc1* from maize (GenBank No. X15596; Martinez (1989) J. Mol. Biol 208:551-565); the *Gpc2* from maize (GenBank No. U45855, Manjunath (1997) Plant Mol. Biol. 33:97-112); plant promoters described in U.S. Patent Nos. 4,962,028; 5,633,440.

[0148] The invention uses tissue-specific or constitutive promoters derived from viruses which can include, e.g., the tobamovirus subgenomic promoter (Kumagai (1995) Proc. Natl. Acad. Sci. USA 92:1679-1683; the rice tungro bacilliform virus (RTBV), which replicates only in phloem cells in infected rice plants, with its promoter which drives strong phloem-

specific reporter gene expression; the cassava vein mosaic virus (CVMV) promoter, with highest activity in vascular elements, in leaf mesophyll cells, and in root tips (Verdaguer (1996) Plant Mol. Biol. 31:1129-1139).

[0149] Alternatively, the plant promoter may direct expression of the nucleic acid in a specific tissue, organ or cell type (*i.e.* tissue-specific promoters) or may be otherwise under more precise environmental or developmental control or under the control of an inducible promoter. Examples of environmental conditions that may affect transcription include anaerobic conditions, elevated temperature, the presence of light, or sprayed with chemicals/hormones. For example, the invention incorporates the drought-inducible promoter of maize (Busk (1997) supra); the cold, drought, and high salt inducible promoter from potato (Kirch (1997) Plant Mol. Biol. 33:897 909).

[0150] Tissue-specific promoters can promote transcription only within a certain time frame of developmental stage within that tissue. See, e.g., Blazquez (1998) Plant Cell 10:791-800, characterizing the *Arabidopsis* LEAFY gene promoter. See also Cardon (1997) Plant J 12:367-77, describing the transcription factor SPL3, which recognizes a conserved sequence motif in the promoter region of the *A. thaliana* floral meristem identity gene AP1; and Mandel (1995) Plant Molecular Biology, Vol. 29, pp 995-1004, describing the meristem promoter eIF4. Tissue specific promoters which are active throughout the life cycle of a particular tissue can be used. In one aspect, the nucleic acids of the invention are operably linked to a promoter active primarily only in cotton fiber cells. In one aspect, the nucleic acids of the invention are operably linked to a promoter active primarily during the stages of cotton fiber cell elongation, e.g., as described by Rinehart (1996) supra. The nucleic acids can be operably linked to the Fb12A gene promoter to be preferentially expressed in cotton fiber cells (Ibid) . See also, John (1997) Proc. Natl. Acad. Sci. USA 89:5769-5773; John, et al., U.S. Patent Nos. 5,608,148 and 5,602,321, describing cotton fiber-specific promoters and methods for the construction of transgenic cotton plants. Root-specific promoters may also be used to express the nucleic acids of the invention. Examples of root-specific promoters include the promoter from the alcohol dehydrogenase gene (DeLisle (1990) Int. Rev. Cytol. 123:39-60). Other promoters that can be used to express the nucleic acids of the invention include, e.g., ovule-specific, embryo-specific, endosperm-specific, integument-specific, seed coat-specific promoters, or some combination thereof; a leaf-specific promoter (see, e.g., Busk (1997) Plant J. 11:1285 1295, describing a leaf-specific promoter in maize); the ORF13 promoter from *Agrobacterium rhizogenes* (which exhibits high activity in roots, see, e.g., Hansen (1997) supra); a maize pollen specific promoter (see, e.g., Guerrero (1990) Mol. Gen. Genet. 224:161 168); a tomato promoter active during fruit ripening, senescence and abscission of leaves and, to a lesser extent, of flowers can be used (see, e.g., Blume (1997) Plant J. 12:731 746); a pistil-specific promoter from the potato SK2 gene (see, e.g., Ficker (1997) Plant Mol. Biol. 35:425 431); the Blec4 gene from pea, which is active in epidermal tissue of vegetative and floral shoot apices of transgenic alfalfa making it a useful tool to target the expression of foreign genes to the epidermal layer of actively growing shoots or fibers; the ovule-specific BEL1 gene (see, e.g., Reiser (1995) Cell 83:735-742, GenBank No. U39944); and/or, the promoter in Klee, U.S. Patent No. 5,589,583, describing a plant promoter region is capable of conferring high levels of transcription in meristematic tissue and/or rapidly dividing cells.

[0151] Alternatively, plant promoters which are inducible upon exposure to plant hormones, such as auxins, are used to express the nucleic acids of the invention. For example, the invention can use the auxin-response elements E1 promoter fragment (AuxREs) in the soybean (*Glycine max* L.) (Liu (1997) Plant Physiol. 115:397-407); the auxin-responsive *Arabidopsis* GST6 promoter (also responsive to salicylic acid and hydrogen peroxide) (Chen (1996) Plant J. 10: 955-966); the auxin-inducible parC promoter from tobacco (Sakai (1996) 37:906-913); a plant biotin response element (Streit (1997) Mol. Plant Microbe Interact. 10:933-937); and, the promoter responsive to the stress hormone abscisic acid (Sheen (1996) Science 274:1900-1902).

[0152] The nucleic acids of the invention can also be operably linked to plant promoters which are inducible upon exposure to chemicals reagents which can be applied to the plant, such as herbicides or antibiotics. For example, the maize In2-2 promoter, activated by benzenesulfonamide herbicide safeners, can be used (De Veylder (1997) Plant Cell Physiol. 38:568-577); application of different herbicide safeners induces distinct gene expression patterns, including expression in the root, hydathodes, and the shoot apical meristem. Coding sequence can be under the control of, *e.g.,* a tetracycline-inducible promoter, *e.g.,* as described with transgenic tobacco plants containing the *Avena sativa* L. (oat) arginine decarboxylase gene (Masgrau (1997) Plant J. 11:465-473); or, a salicylic acid-responsive element (Stange (1997) Plant J. 11:1315-1324). Using chemically- (e.g., hormone- or pesticide-) induced promoters, *i.e.,* promoter responsive to a chemical which can be applied to the transgenic plant in the field, expression of a polypeptide of the invention can be induced at a particular stage of development of the plant. Thus, the invention also provides for transgenic plants containing an inducible gene encoding for polypeptides of the invention whose host range is limited to target plant species, such as corn, rice, barley, wheat, potato, sugar cane, sugar beet, or other crops, inducible at any stage of development of the crop.

[0153] One of skill will recognize that a tissue-specific plant promoter may drive expression of operably linked sequences in tissues other than the target tissue. Thus, a tissue-specific promoter is one that drives expression preferentially in the target tissue or cell type, but may also lead to some expression in other tissues as well.

[0154] The nucleic acids of the invention can also be operably linked to plant promoters which are inducible upon exposure to chemicals reagents. These reagents include, e.g., herbicides, synthetic auxins, or antibiotics which can be

applied, e.g., sprayed, onto transgenic plants. Inducible expression of amylase-producing nucleic acids of the disclosure will allow the grower to select plants with the optimal starch / sugar ratio. The development of plant parts can thus controlled. In this way the invention provides the means to facilitate the harvesting of plants and plant parts. For example, in various embodiments, the maize In2-2 promoter, activated by benzenesulfonamide herbicide safeners, is used (De Veylder (1997) Plant Cell Physiol. 38:568-577); application of different herbicide safeners induces distinct gene expression patterns, including expression in the root, hydathodes, and the shoot apical meristem. Coding sequences of the invention are also under the control of a tetracycline-inducible promoter, e.g., as described with transgenic tobacco plants containing the *Avena sativa* L. (oat) arginine decarboxylase gene (Masgrau (1997) Plant J. 11:465-473); or, a salicylic acid-responsive element (Stange (1997) Plant J. 11:1315-1324).

[0155] If proper polypeptide expression is desired, a polyadenylation region at the 3'-end of the coding region should be included. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from genes in the *Agrobacterial* T-DNA.

[0156] The invention provides "expression cassettes" comprising any sequence of the invention "operably linked" to a transcriptional regulator; e.g., wherein "operably linked" as used herein refers to a functional relationship between two or more nucleic acid (e.g., DNA) segments. Typically, it refers to the functional relationship of transcriptional regulatory sequence to a transcribed sequence. For example, a promoter is operably linked to a coding sequence, such as a nucleic acid of the invention, if it stimulates or modulates the transcription of the coding sequence in an appropriate host cell or other expression system. Generally, promoter transcriptional regulatory sequences that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, i.e., they are *cis*-acting. However, some transcriptional regulatory sequences, such as enhancers, need not be physically contiguous or located in close proximity to the coding sequences whose transcription they enhance.

Modification of Coding Sequences and Adjacent Sequences

[0157] The transgenic expression in plants of genes derived from heterologous sources may involve the modification of those genes to achieve and optimize their expression in plants. In particular, bacterial ORFs which encode separate enzymes but which are encoded by the same transcript in the native microbe are best expressed in plants on separate transcripts. To achieve this, each microbial ORF is isolated individually and cloned within a cassette which provides a plant promoter sequence at the 5' end of the ORF and a plant transcriptional terminator at the 3' end of the ORF. The isolated ORF sequence preferably includes the initiating ATG codon and the terminating STOP codon but may include additional sequence beyond the initiating ATG and the STOP codon. In addition, the ORF may be truncated, but still retain the required activity; for particularly long ORFs, truncated versions which retain activity may be preferable for expression in transgenic organisms. By "plant promoter" and "plant transcriptional terminator" it is intended to mean promoters and transcriptional terminators which operate within plant cells. This includes promoters and transcription terminators which may be derived from non-plant sources such as viruses (an example is the Cauliflower Mosaic Virus).

[0158] In some cases, modification to the ORF coding sequences and adjacent sequence is not required. It is sufficient to isolate a fragment containing the ORF of interest and to insert it downstream of a plant promoter. For example, Gaffney et. al. (Science 261: 754-756 (1993)) have expressed the *Pseudomonas nahG* gene in transgenic plants under the control of the CaMV 35S promoter and the CaMV *tml* terminator successfully without modification of the coding sequence and with nucleotides of the *Pseudomonas* gene upstream of the ATG still attached, and nucleotides downstream of the STOP codon still attached to the *nahG* ORF. Preferably as little adjacent microbial sequence should be left attached upstream of the ATG and downstream of the STOP codon. In practice, such construction may depend on the availability of restriction sites.

[0159] In other cases, the expression of genes derived from microbial sources may provide problems in expression. These problems have been well characterized in the art and are particularly common with genes derived from certain sources, such as *Bacillus.* These problems may apply to the nucleotide sequence of this invention and the modification of these genes can be undertaken using techniques now well known in the art. The following problems may be encountered:

Codon Usage

[0160] The preferred codon usage in plants differs from the preferred codon usage in certain microorganisms. Comparison of the usage of codons within a cloned microbial ORF to usage in plant genes (and in particular genes from the target plant) will enable an identification of the codons within the ORF which should preferably be changed. Typically plant evolution has tended towards a strong preference of the nucleotides C and G in the third base position of monocotyledons, whereas dicotyledons often use the nucleotides A or T at this position. By modifying a gene to incorporate preferred codon usage for a particular target transgenic species, many of the problems described below for GC/AT content and illegitimate splicing will be overcome.

GC/AT Content

[0161] Plant genes typically have a GC content of more than 35%. ORF sequences which are rich in A and T nucleotides can cause several problems in plants. Firstly, motifs of ATTTA are believed to cause destabilization of messages and are found at the 3' end of many short-lived mRNAs. Secondly, the occurrence of polyadenylation signals such as AATAAA at inappropriate positions within the message is believed to cause premature truncation of transcription. In addition, monocotyledons may recognize AT-rich sequences as splice sites (see below).

Sequences Adjacent to the Initiating Methionine

[0162] Plants differ from microorganisms in that their messages do not possess a defined ribosome binding site. Rather, it is believed that ribosomes attach to the 5' end of the message and scan for the first available ATG at which to start translation. Nevertheless, it is believed that there is a preference for certain nucleotides adjacent to the ATG and that expression of microbial genes can be enhanced by the inclusion of a eukaryotic consensus translation initiator at the ATG. Clontech (1993/1994 catalog, page 210) have suggested one sequence as a consensus translation initiator for the expression of the *E. coli uidA* gene in plants. Further, Joshi, N.A.R. 15: 6643-6653 (1987), has compared many plant sequences adjacent to the ATG and suggests another consensus sequence. In situations where difficulties are encountered in the expression of microbial ORFs in plants, inclusion of one of these sequences at the initiating ATG may improve translation. In such cases the last three nucleotides of the consensus may not be appropriate for inclusion in the modified sequence due to their modification of the second AA residue. Preferred sequences adjacent to the initiating methionine may differ between different plant species. A survey of 14 maize genes located in the GenBank database provided the following results:

Position Before the Initiating ATG in 14 Maize Genes:

	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1
C3	8	4	6	2	5	6	0	10	7
T3	0	3	4	3	2	1	1	1	0
A2	3	1	4	3	2	3	7	2	3
G6	3	6	0	6	5	4	6	1	5

[0163] This analysis can be done for the desired plant species into which the nucleotide sequence is being incorporated, and the sequence adjacent to the ATG modified to incorporate the preferred nucleotides.

Removal of Illegitimate Splice Sites

[0164] Genes cloned from non-plant sources and not optimized for expression in plants may also contain motifs which may be recognized in plants as 5' or 3' splice sites, and be cleaved, thus generating truncated or deleted messages. These sites can be removed using the techniques well known in the art.

[0165] Techniques for the modification of coding sequences and adjacent sequences are well known in the art. In cases where the initial expression of a microbial ORF is low and it is deemed appropriate to make alterations to the sequence as described above, then the construction of synthetic genes can be accomplished according to methods well known in the art. These are, for example, described in the published patent disclosures EP 0 385 962 (to Monsanto), EP 0 359 472 (to Lubrizol) and WO 93/07278 (to Ciba-Geigy). In most cases it is preferable to assay the expression of gene constructions using transient assay protocols (which are well known in the art) prior to their transfer to transgenic plants.

Plant Promoters

[0166] The compositions of the disclosure may contain nucleic acid sequences for transformation and expression in a plant of interest. The nucleic acid sequences may be present in DNA constructs or expression cassettes. In alternative embodiments, "expression cassette" as used herein means a nucleic acid molecule capable of directing expression of a particular, nucleotide sequence in an appropriate host cell, comprising a promoter operatively linked to the nucleotide sequence of interest, which is operatively linked to termination signals. It also typically comprises sequences required for proper translation of the nucleotide sequence. The coding region usually codes for a protein of interest but may also code for a functional RNA of interest, for example antisense RNA or a nontranslated RNA, in the sense or antisense direction. The expression cassette comprising the nucleotide sequence of interest may be chimeric, meaning that at

least one of its components is heterologous with respect to at least one of its other components. The expression cassette may also be one that is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. In alternative embodiments, the expression cassette is heterologous with respect to the host, i.e., the particular DNA sequence of the expression cassette does not occur naturally in the host cell and must have been introduced into the host cell or an ancestor of the host cell by a transformation event. The expression of the nucleotide sequence in the expression cassette may be under the control of a constitutive promoter or of an inducible promoter that initiates transcription only when the host cell is exposed to some particular external stimulus. In alternative embodiments, the promoter can also be specific to a particular tissue or organ or stage of development.

[0167] The present disclosure encompasses the transformation of plants with expression cassettes capable of expressing polynucleotides. In alternative embodiments, the expression cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region (i.e., a promoter) and a polynucleotide of interest. The expression cassette may optionally comprise a transcriptional and translational termination region (i.e. termination region) functional in plants. In some embodiments, the expression cassette comprises a selectable marker gene to allow for selection for stable transformants. Expression constructs of the invention may also comprise a leader sequence and/or a sequence allowing for inducible expression of the polynucleotide of interest. *See,* Guo et. al. (2003) Plant J. 34:383-92 and Chen et. al. (2003) Plant J. 36:731-40 for examples of sequences allowing for inducible expression.

[0168] In alternative embodiments, the regulatory sequences of the expression construct are operably linked to the polynucleotide of interest. By "operably linked" is intended a functional linkage between a promoter and a second sequence wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. In alternative embodiments, operably linked means that the nucleotide sequences being linked are contiguous.

[0169] Any promoter capable of driving expression in the plant of interest may be used in the practice of the invention. The promoter may be native or analogous or foreign or heterologous to the plant host. In alternative embodiments, the terms "heterologous" and "exogenous" when used herein to refer to a nucleic acid sequence (e.g. a DNA or RNA sequence) or a gene, refer to a sequence that originates from a source foreign to the particular host cell or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that is endogenous to the particular host cell but has been modified. The terms also include non-naturally occurring multiple copies of a naturally occurring DNA sequence. Thus, the terms refer to a DNA segment that is foreign or heterologous to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is not ordinarily found. Exogenous DNA segments are expressed to yield exogenous polypeptides.

[0170] In alternative embodiments, a "homologous" nucleic acid (e.g. DNA) sequence is a nucleic acid (e.g. DNA or RNA) sequence naturally associated with a host cell into which it is introduced.

[0171] The choice of promoters to be included can depend upon several factors, including, but not limited to, efficiency, selectability, inducibility, desired expression level, and cell- or tissue-preferential expression. It is a routine matter for one of skill in the art to modulate the expression of a sequence by appropriately selecting and positioning promoters and other regulatory regions relative to that sequence.

[0172] In alternative embodiments, suitable promoters initiate transcription only, or predominantly, in certain cell types. Thus, as used herein a cell type- or tissue-preferential promoter is one that drives expression preferentially in the target tissue, but may also lead to some expression in other cell types or tissues as well. Methods for identifying and characterizing promoter regions in plant genomic DNA include, for example, those described in the following references: Jordano, et. al., Plant Cell, 1:855-866 (1989); Bustos, et. al., Plant Cell, 1:839-854 (1989); Green, et. al., EMBO J. 7, 4035-4044 (1988); Meier, et. al., Plant Cell, 3, 309-316 (1991); and Zhang, et. al., Plant Physiology 110: 1069-1079 (1996).

[0173] In alternative embodiments, tissue preferred regulated genes and/or promoters which have been reported in plants can be used. Reported tissue preferred genes that can be used in alternative embodiments include the genes encoding the seed storage proteins (such as napin, cruciferin, beta-conglycinin, and phaseolin, prolamines, glutelins, globulins, and zeins) zeins or oil body proteins (such as oleosin), or genes involved in fatty acid biosynthesis (including acyl carrier protein, stearoyl-ACP desaturase, and fatty acid desaturases (fad 2-1)), and other genes expressed during embryo development (such as Bce4, see, for example, EP 255378 and Kridl et. al., (1991) Seed Science Research, 1:209). Examples of tissue-specific promoters that can be used to practice this invention, which have been described, include the lectin (Vodkin, Prog. Clin. Biol. Res., 138;87 (1983); Lindstrom et. al., (1990) Der. Genet., 11:160), corn alcohol dehydrogenase 1 (Dennis et. al., Nucleic Acids Res., 12:3983 (1984)), corn light harvesting complex (see, e.g., Simpson, (1986) Science, 233:34; Bansal (1992) Proc. Natl. Acad. Sci. USA 89:3654), corn heat shock protein (see, e.g., Odell et. al., (1985) Nature, 313:810; pea small subunit RuBP carboxylase (see, e.g., Poulsen et. al., (1986) Mol. Gen. Genet., 205:193-200; Cashmore et. al., (1983) Gen. Eng. of Plants, Plenum Press, New York, 29-38); Ti plasmid mannopine synthase (see, e.g., Langridge et. al., (1989) Proc. Natl. Acad. Sci. USA, 86:3219-3223), Ti plasmid nopaline synthase (Langridge et. al., (1989) Proc. Natl. Acad. Sci. USA, 86:3219-3223), petunia chalcone isomerase (see, e.g., vanTunen (1988) EMBO J. 7:1257); bean glycine rich protein 1 (see, e.g., Keller (1989) Genes Dev. 3:1639) ; truncated CaMV 35s (see, e.g., Odell (1985) Nature 313:810) ; potato patatin (see, e.g., Wenzler (1989) Plant Mol. Biol. 13:347;

root cell (see, e.g., Yamamoto (1990) Nucleic Acids Res. 18:7449) ; maize zein (see, e.g., Reina (1990) Nucleic Acids Res. 18:6425; Lopes et. al. (1995) Mol. Gen. Genet. 247: 603-613; Kriz (1987) Mol. Gen. Genet. 207:90; Wandelt (1989) Nucleic Acids Res., 17:2354; Langridge (1983) Cell, 34:1015; Reina (1990) Nucleic Acids Res., 18:7449), ADP-gpp promoter (see, e.g., U.S. Patent No. 7,102,057); globulin-1 (see, e.g., Belanger (1991) Genetics 129:863); α-globulin (Sunilkumar, et. al. (2002), Transgenic Res. 11 :347-359); α-tubulin; cab (see, e.g., Sullivan (1989) Mol. Gen. Genet., 215:431); PEPCase (see e.g., Hudspeth & Grula, (1989) Plant Molec. Biol., 12:579-589); R gene complex-associated promoters (Chandler et. al., (1989) Plant Cell, 1:1175); pea vicilin promoter (Czako et. al., (1992) Mol. Gen. Genet., 235:33; U.S. Pat. No. 5,625,136); GTL1 promoter (Takaiwa et. al. (1991) Plant Mol. Biol. 16 (1), 49-58); chalcone synthase promoters (Franken et. al., (1991) EMBO J., 10:2605); and/or a GY1 promoter (Sims & Goldburg (1989) Nuc. Acid Res. 17(11) 4368) and the like.

[0174] In alternative embodiments, a class of fruit-preferred promoters expressed at or during antithesis through fruit development, at least until the beginning of ripening, is used, e.g., as discussed in U.S. 4,943,674. The promoter for polygalacturonase gene is active in fruit ripening. A polygalacturonase gene also can be used, e.g., as described in U.S. Patent No. 4,535,060, U.S. Patent No. 4,769,061, U.S. Patent No. 4,801,590, and U.S. Patent No. 5,107,065.

[0175] Other examples of tissue-preferred promoters that can be used include those that direct expression in leaf cells following damage to the leaf (for example, from chewing insects), in tubers (for example, patatin gene promoter), and in fiber cells (an example of a developmentally-regulated fiber cell protein is E6 (John & Crow (1992) PNAS 89:5769-5773). The E6 gene is most active in fiber, although low levels of transcripts are found in leaf, ovule and flower.

[0176] In alternative embodiments, promoters active in photosynthetic tissue can be used in order to drive transcription in green tissues such as leaves and stems; these promoters are suitable when they drive expression only or predominantly in such tissues. In alternative embodiments, the promoter may confer expression constitutively throughout the plant, or differentially with respect to the green tissues, or differentially with respect to the developmental stage of the green tissue in which expression occurs, or in response to external stimuli.

[0177] Examples of promoters that can be used to practice this invention include the ribulose-1,5-bisphosphate carboxylase (RbcS) promoters such as the RbcS promoter from eastern larch *(Larix laricina),* the pine cab6 promoter (Yamamoto et. al. (1994) Plant Cell Physiol. 35:773-778), the Cab-1 gene promoter from wheat (Fejes et. al. (1990) Plant Mol. Biol. 15:921-932), the CAB-1 promoter from spinach (Lubberstedt et. al. (1994) Plant Physiol. 104:997-1006), the cab1R promoter from rice (Luan et. al. (1992) Plant Cell 4:971-981), the pyruvate orthophosphate dikinase (PPDK) promoter from corn (Matsuoka et. al. (1993) Proc. Natl. Acad. Sci USA 90:9586-9590), the tobacco Lhcb1*2 promoter (Cerdan et. al. (1997) Plant Mol. Biol. 33:245-255), the Arabidopsis thaliana SUC2 sucrose-H+ symporter promoter (Truernit et. al. (1995) Planta 196:564-570), and thylakoid membrane protein promoters from spinach (psaD, psaF, psaE, PC, FNR, atpC, atpD, cab, rbcS. Other promoters that drive transcription in stems, leafs and green tissue are described in U.S. Patent Publication No. 2007/0006346.

[0178] The tissue specificity of some "tissue preferred" promoters may not be absolute; in alternative embodiments reporter genes such as Gus or green fluorescent protein, cyan fluorescent protein, yellow fluorescent protein or red fluorescent protein are used. In alternative embodiments tissue preferred expression can be achieved with "leaky" expression by a combination of different tissue-preferred promoters. Other tissue preferred promoters can be used, and they can be isolated by one skilled in the art (see U.S. 5,589,379).

[0179] In one aspect, plant promoters which are inducible upon exposure to plant hormones, such as auxins, are used to express the nucleic acids of the invention. For example, the invention can use the auxin-response elements E1 promoter fragment (AuxREs) in the soybean *(Glycine max* L.) (Liu (1997) Plant Physiol. 115:397-407); the auxin-responsive *Arabidopsis* GST6 promoter (also responsive to salicylic acid and hydrogen peroxide) (Chen (1996) Plant J. 10: 955-966); the auxin-inducible parC promoter from tobacco (Sakai (1996) 37:906-913); a plant biotin response element (Streit (1997) Mol. Plant Microbe Interact. 10:933-937); and, the promoter responsive to the stress hormone abscisic acid (Sheen (1996) Science 274:1900-1902).

[0180] The nucleic acids of the invention can also be operably linked to plant promoters which are inducible upon exposure to chemicals reagents which can be applied to the plant, such as herbicides or antibiotic. For example, gene expression systems that are activated in the presence of a chemical ligand, including ethanol, such as can be found in WO 96/27673; WO 93/01294; WO 94/03619; WO 02/061102. The maize In2-2 promoter, activated by benzenesulfonamide herbicide safeners, can be used (De Veylder (1997) Plant Cell Physiol. 38:568-577); application of different herbicide safeners induces distinct gene expression patterns, including expression in the root, hydathodes, and the shoot apical meristem. Coding sequence can be under the control of, *e.g.,* a tetracycline-inducible promoter, e.g., as described with transgenic tobacco plants containing the *Avena sativa* L. (oat) arginine decarboxylase gene (Masgrau (1997) Plant J. 11:465-473); estrogen, such as, the ecdysone receptor (WO 01/52620) or, a salicylic acid-responsive element (Stange (1997) Plant J. 11:1315-1324). Using chemically- (e.g., hormone- or pesticide-) induced promoters, *i.e.*, promoter responsive to a chemical which can be applied to the transgenic plant in the field, expression of a polypeptide of the invention can be induced at a particular stage of development of the plant.

[0181] Examples of some constitutive promoters which can be used to practice this invention, and which have been

described, include rice actin 1 (Wang et. al. (1992) Mol. Cell. Biol., 12:3399; U.S. Patent No. 5,641,876); other actin isoforms (McElroy et. al. (1990) Plant Cell 2: 163-171 and McElroy et. al. (1991) Mol. Gen. Genet. 231: 150-160); CaMV 35S (Odell et. al. (1985) Nature, 313:810); CaMV 19S (Lawton et. al. (1987) Plant Mol. Biol. 9:315-324; U.S. Patent No. 5,639,949); nos (Ebert et. al. (1987) PNAS USA 84:5745-5749); Adh (Walker et. al. (1987) PNAS USA 84:6624-6628), sucrose synthase (Yang & Russell (1990) PNAS USA 87:4144-4148); and the ubiquitin promoters (*e.g.* sunflower - Binet et. al. (1991) Plant Science 79: 87-94; maize - Christensen et. al. (1989) Plant Molec. Biol. 12: 619-632; and *Arabidopsis* - Callis et. al., J. Biol. Chem. (1990) 265:12486-12493; and Norris et. al., Plant Mol. BioL (1993) 21:895-906.

[0182] A variety of transcriptional terminators can be used in expression cassettes to practice this invention. These transcriptional terminators are responsible for the termination of transcription beyond the transgene and correct mRNA polyadenylation. The termination region may be native with the transcriptional initiation region, may be native with the operably linked DNA sequence of interest, may be native with the plant host, or may be derived from another source (i.e., foreign or heterologous to the promoter, the DNA sequence of interest, the plant host, or any combination thereof). Appropriate transcriptional terminators are those that are known to function in plants and include the CAMV 35S terminator, the tml terminator, the nopaline synthase terminator and the pea rbcs E9 terminator. These can be used in both monocotyledons and dicotyledons. In addition, a gene's native transcription terminator may be used.

[0183] Numerous sequences have been found to enhance gene expression from within the transcriptional unit and these sequences can be used in conjunction with the genes of this invention to increase their expression in transgenic plants. For example, various intron sequences have been shown to enhance expression, particularly in monocotyledonous cells. For example, the introns of the maize Adhl gene have been found to significantly enhance the expression of the wild-type gene under its cognate promoter when introduced into maize cells.

[0184] In alternative embodiments, non-translated leader sequences derived from viruses can be used to enhance expression, and these are particularly effective in dicotyledonous cells. In alternative embodiments, leader sequences from Tobacco Mosaic Virus (TMV, the "W-sequence"), Maize Chlorotic Mottle Virus (MCMV), and Alfalfa Mosaic Virus (AMV) are used, and these have been shown to be effective in enhancing expression (e.g. Gallie et. al. Nucl. Acids Res. 15: 8693-8711 (1987); Skuzeski et. al. Plant Molec. Biol. 15: 65-79 (1990)).

Targeting of the Gene Product Within the Cell

[0185] In alternative embodiments, various mechanisms for targeting gene products are used; and these are known to exist in plants, and the sequences controlling the functioning of these mechanisms have been characterized in some detail. Sequences have been characterized which cause the targeting of gene products to other cell compartments. Amino terminal sequences can be responsible for targeting a protein of interest to any cell compartment, such as, a vacuole, mitochondrion, peroxisome, protein bodies, endoplasmic reticulum, chloroplast, starch granule, amyloplast, apoplast or cell wall of a plant (*e.g.* Unger et. al. Plant Molec. Biol. 13: 411-418 (1989); Rogers et. al. (1985) Proc. Natl. Acad. Sci. USA 82: 6512-651; U.S. Patent No. 7,102,057; WO 2005/096704). In alternative embodiments, various signal sequence are used, e.g., the signal sequence may be an N-terminal signal sequence from *waxy*, an N-terminal signal sequence from γ-zein, a starch binding domain, a C-terminal starch binding domain, a chloroplast targeting sequence, which imports the mature protein to the chloroplast (Comai et. al. (1988) J. Biol. Chem. 263: 15104-15109; van den Broeck, et. al. (1985) Nature 313: 358-363; U.S. Patent No. 5,639,949) or a secretion signal sequence from aleurone cells (Koehler & Ho, Plant Cell 2: 769-783 (1990)). In alternative embodiments, amino terminal sequences are used in conjunction with carboxy terminal sequences; these are responsible for vacuolar targeting of gene products (Shinshi et. al. (1990) Plant Molec. Biol. 14: 357-368).

[0186] The signal sequence selected can include the known cleavage site, and the fusion constructed should take into account any amino acids after the cleavage site(s), which are required for cleavage. In some cases this requirement may be fulfilled by the addition of a small number of amino acids between the cleavage site and the transgene ATG or, alternatively, replacement of some amino acids within the transgene sequence. These construction techniques are well known in the art and are equally applicable to any cellular compartment.

[0187] The above-described mechanisms for cellular targeting can be utilized not only in conjunction with their cognate promoters, but also in conjunction with heterologous promoters so as to effect a specific cell-targeting goal under the transcriptional regulation of a promoter that has an expression pattern different to that of the promoter from which the targeting signal derives.

Expression vectors and cloning vehicles

[0188] The invention provides expression vectors and cloning vehicles comprising nucleic acids of the invention, e.g., a sequence encoding the glucoamylase of the invention. Expression vectors and cloning vehicles of the invention can comprise viral particles, baculovirus, phage, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes, viral DNA (e.g., vaccinia, adenovirus, foul pox virus, pseudorabies and derivatives of SV40), P1-based artificial chro-

mosomes, yeast plasmids, yeast artificial chromosomes, and any other vectors specific for specific hosts of interest (such as bacillus, *Aspergillus* and yeast). Vectors of the invention can include chromosomal, non-chromosomal and synthetic DNA sequences. Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. Exemplary vectors are include: bacterial: pQE vectors (Qiagen), pBLUESCRIPT™ (pBluescript) plasmids, pNH vectors, (lambda-ZAP vectors (Stratagene); ptrc99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene), pSVK3, pBPV, pMSG, pSVLSV40 (Pharmacia). However, any other plasmid or other vector may be used so long as they are replicable and viable in the host. Low copy number or high copy number vectors may be employed with the present invention. "Plasmids" can be commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. Equivalent plasmids to those described herein are known in the art and will be apparent to the ordinarily skilled artisan.

[0189] The invention provides "expression cassettes" comprising any sequence of the invention "operably linked" to a transcriptional regulator; the term "expression cassette" as used herein can refer to a nucleotide sequence which is capable of affecting expression of a structural gene (e.g., a protein coding sequence, such as a glucoamylase of the invention) in a host compatible with such sequences. Expression cassettes include at least a promoter operably linked with the polypeptide coding sequence; and, optionally, with other sequences, e.g., transcription termination signals. Additional factors necessary or helpful in effecting expression may also be used, e.g., enhancers. Thus, expression cassettes also include plasmids, expression vectors, recombinant viruses, any form of recombinant "naked DNA" vector, and the like.

[0190] A "vector" comprises a nucleic acid which can infect, transfect, transiently or permanently transduce a cell. It will be recognized that a vector can be a naked nucleic acid, or a nucleic acid complexed with protein or lipid. The vector optionally comprises viral or bacterial nucleic acids and/or proteins, and/or membranes (e.g., a cell membrane, a viral lipid envelope, etc.). Vectors used to practice this invention include, but are not limited to replicons (e.g., RNA replicons, bacteriophages) to which fragments of DNA may be attached and become replicated. Vectors used to practice this invention include, but are not limited to RNA, autonomous self-replicating circular or linear DNA or RNA (e.g., plasmids, viruses, and the like, see, e.g., U.S. Patent No. 5,217,879), and include both the expression and non-expression plasmids. Where a recombinant microorganism or cell culture is described as hosting an "expression vector" this includes both extra-chromosomal circular and linear DNA and DNA that has been incorporated into the host chromosome(s). Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous structure, or is incorporated within the host's genome.

[0191] The expression vector can comprise a promoter, a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. Mammalian expression vectors can comprise an origin of replication, any necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking non-transcribed sequences. In some aspects, DNA sequences derived from the SV40 splice and polyadenylation sites may be used to provide the required non-transcribed genetic elements.

[0192] In one aspect, the expression vectors contain one or more selectable marker genes to permit selection of host cells containing the vector. Such selectable markers include genes encoding dihydrofolate reductase or genes conferring neomycin resistance for eukaryotic cell culture, genes conferring tetracycline or ampicillin resistance in *E. coli,* and the *S. cerevisiae* TRP1 gene. Promoter regions can be selected from any desired gene using chloramphenicol transferase (CAT) vectors or other vectors with selectable markers.

[0193] Vectors for expressing the polypeptide or fragment thereof in eukaryotic cells can also contain enhancers to increase expression levels. Enhancers are cis-acting elements of DNA, usually from about 10 to about 300 bp in length that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin bp 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and the adenovirus enhancers.

[0194] A nucleic acid sequence can be inserted into a vector by a variety of procedures. In general, the sequence is ligated to the desired position in the vector following digestion of the insert and the vector with appropriate restriction endonucleases. Alternatively, blunt ends in both the insert and the vector may be ligated. A variety of cloning techniques are known in the art, e.g., as described in Ausubel and Sambrook. Such procedures and others are deemed to be within the scope of those skilled in the art.

[0195] The vector can be in the form of a plasmid, a viral particle, or a phage. Other vectors include chromosomal, non-chromosomal and synthetic DNA sequences, derivatives of SV40; bacterial plasmids, phage DNA, baculovirus, yeast plasmids, vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. A variety of cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by, e.g., Sambrook.

[0196] Particular bacterial vectors which can be used include the commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017), pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden), GEM1 (Promega Biotec, Madison, WI, USA) pQE70, pQE60, pQE-9 (Qiagen), pD10, psiX174 pBluescript II

KS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene), ptrc99a, pKK223-3, pKK233-3, DR540, pRIT5 (Pharmacia), pKK232-8 and pCM7. Particular eukaryotic vectors include pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, and pSVL (Pharmacia). However, any other vector may be used as long as it is replicable and viable in the host cell.

[0197] The nucleic acids of the invention can be expressed in expression cassettes, vectors or viruses and transiently or stably expressed in plant cells and seeds. One exemplary transient expression system uses episomal expression systems, e.g., cauliflower mosaic virus (CaMV) viral RNA generated in the nucleus by transcription of an episomal minichromosome containing supercoiled DNA, see, e.g., Covey (1990) Proc. Natl. Acad. Sci. USA 87:1633-1637. Alternatively, coding sequences, i.e., all or sub-fragments of sequences of the invention can be inserted into a plant host cell genome becoming an integral part of the host chromosomal DNA. Sense or antisense transcripts can be expressed in this manner. A vector comprising the sequences (e.g., promoters or coding regions) from nucleic acids of the invention can comprise a marker gene that confers a selectable phenotype on a plant cell or a seed. For example, the marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulfuron or Basta.

[0198] Expression vectors capable of expressing nucleic acids and proteins in plants are well known in the art, and can include, *e.g.*, vectors from *Agrobacterium* spp., potato virus X (see, *e.g.*, Angell (1997) EMBO J. 16:3675-3684), tobacco mosaic virus (see, *e.g.*, Casper (1996) Gene 173:69-73), tomato bushy stunt virus (see, *e.g.*, Hillman (1989) Virology 169:42-50), tobacco etch virus (see, *e.g.*, Dolja (1997) Virology 234:243-252), bean golden mosaic virus (see, *e.g.*, Morinaga (1993) Microbiol Immunol. 37:471-476), cauliflower mosaic virus (see, *e.g.*, Cecchini (1997) Mol. Plant Microbe Interact. 10:1094-1101), maize Ac/Ds transposable element (see, *e.g.*, Rubin (1997) Mol. Cell. Biol. 17:6294-6302; Kunze (1996) Curr. Top. Microbiol. Immunol. 204:161-194), and the maize suppressor-mutator (Spm) transposable element (see, *e.g.*, Schlappi (1996) Plant Mol. Biol. 32:717-725); and derivatives thereof.

[0199] In one aspect, the expression vector can have two replication systems to allow it to be maintained in two organisms, for example in mammalian or insect cells for expression and in a prokaryotic host for cloning and amplification. Furthermore, for integrating expression vectors, the expression vector can contain at least one sequence homologous to the host cell genome. It can contain two homologous sequences which flank the expression construct. The integrating vector can be directed to a specific locus in the host cell by selecting the appropriate homologous sequence for inclusion in the vector. Constructs for integrating vectors are well known in the art.

[0200] Expression vectors of the invention may also include a selectable marker gene to allow for the selection of bacterial strains that have been transformed, e.g., genes which render the bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin, neomycin and tetracycline. Selectable markers can also include biosynthetic genes, such as those in the histidine, tryptophan and leucine biosynthetic pathways.

Host cells and transformed cells

[0201] The invention also provides a transformed cell comprising a nucleic acid sequence of the invention, e.g., a sequence encoding a glucoamylase of the invention, or a vector, expression cassette, or cloning vehicle of the invention. The host cell may be any of the host cells familiar to those skilled in the art, including prokaryotic cells, eukaryotic cells, such as bacterial cells, fungal cells, yeast cells, mammalian cells, insect cells, or plant cells. Exemplary bacterial cells include any species within the genera *Escherichia*, *Bacillus, Streptomyces, Salmonella*, *Pseudomonas* and *Staphylococcus,* including, e.g., *Escherichia coli*, *Lactococcus lactis, Bacillus subtilis, Bacillus cereus, Salmonella typhimurium*, *Pseudomonas fluorescens*. Exemplary yeast cells include any species of *Pichia, Saccharomyces*, *Schizosaccharomyces,* or *Schwanniomyces*, including *Pichia pastoris, Saccharomyces cerevisiae,* or *Schizosaccharomyces pombe.* Exemplary insect cells include any species of *Spodoptera* or *Drosophila,* including *Drosophila S2* and *Spodoptera Sf9.* Exemplary animal cells include CHO, COS or Bowes melanoma or any mouse or human cell line. The selection of an appropriate host is within the abilities of those skilled in the art. Techniques for transforming a wide variety of higher plant species are well known and described in the technical and scientific literature. See, e.g., Weising (1988) Ann. Rev. Genet. 22:421-477; U.S. Patent No. 5,750,870.

[0202] The vector, expression cassette, or cloning vehicle can be introduced into the host cells using any of a variety of techniques, including transformation, transfection, transduction, viral infection, gene guns, or Ti-mediated gene transfer. Particular methods include calcium phosphate transfection, DEAE-Dextran mediated transfection, lipofection, or electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

[0203] In one aspect, the nucleic acids or vectors of the invention are introduced into the cells for screening, thus, the nucleic acids enter the cells in a manner suitable for subsequent expression of the nucleic acid. The method of introduction is largely dictated by the targeted cell type. Exemplary methods include $CaPO_4$ precipitation, liposome fusion, lipofection (e.g., LIPOFECTIN™), electroporation, viral infection, etc. The candidate nucleic acids may stably integrate into the genome of the host cell (for example, with retroviral introduction) or may exist either transiently or stably in the cytoplasm (i.e. through the use of traditional plasmids, utilizing standard regulatory sequences, selection markers, etc.). As many pharmaceutically important screens require human or model mammalian cell targets, retroviral vectors capable of trans-

fecting such targets are preferred.

[0204] Where appropriate, the engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes of the invention. Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter may be induced by appropriate means (e.g., temperature shift or chemical induction) and the cells may be cultured for an additional period to allow them to produce the desired polypeptide or fragment thereof.

[0205] Cells can be harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract is retained for further purification. Microbial cells employed for expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents. Such methods are well known to those skilled in the art. The expressed polypeptide or fragment thereof can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the polypeptide. If desired, high performance liquid chromatography (HPLC) can be employed for final purification steps.

[0206] Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts and other cell lines capable of expressing proteins from a compatible vector, such as the C127, 3T3, CHO, HeLa and BHK cell lines.

[0207] The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Depending upon the host employed in a recombinant production procedure, the polypeptides produced by host cells containing the vector may be glycosylated or may be non-glycosylated. Polypeptides of the invention may or may not also include an initial methionine amino acid residue.

[0208] Cell-free translation systems can also be employed to produce a polypeptide of the invention. Cell-free translation systems can use mRNAs transcribed from a DNA construct comprising a promoter operably linked to a nucleic acid encoding the polypeptide or fragment thereof. In some aspects, the DNA construct may be linearized prior to conducting an in vitro transcription reaction. The transcribed mRNA is then incubated with an appropriate cell-free translation extract, such as a rabbit reticulocyte extract, to produce the desired polypeptide or fragment thereof.

[0209] The expression vectors can contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli.*

Amplification of Nucleic Acids

[0210] In practicing the invention, nucleic acids of the invention and nucleic acids encoding the polypeptides of the invention can be reproduced by amplification. Amplification can also be used to clone or modify the nucleic acids of the invention. Thus, the disclosure provides amplification primer sequence pairs for amplifying nucleic acids of the invention. One of skill in the art can design amplification primer sequence pairs for any part of or the full length of these sequences.

[0211] Amplification reactions can also be used to quantify the amount of nucleic acid in a sample (such as the amount of message in a cell sample), label the nucleic acid (e.g., to apply it to an array or a blot), detect the nucleic acid, or quantify the amount of a specific nucleic acid in a sample. In one aspect, message isolated from a cell or a cDNA library is amplified.

[0212] The skilled artisan can select and design suitable oligonucleotide amplification primers. Amplification methods are also well known in the art, and include, e.g., polymerase chain reaction, PCR (see, e.g., PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS, ed. Innis, Academic Press, N.Y. (1990) and PCR STRATEGIES (1995), ed. Innis, Academic Press, Inc., N.Y., ligase chain reaction (LCR) (see, e.g., Wu (1989) Genomics 4:560; Landegren (1988) Science 241:1077; Barringer (1990) Gene 89:117); transcription amplification (see, e.g., Kwoh (1989) Proc. Natl. Acad. Sci. USA 86:1173); and, self-sustained sequence replication (see, e.g., Guatelli (1990) Proc. Natl. Acad. Sci. USA 87:1874); Q Beta replicase amplification (see, e.g., Smith (1997) J. Clin. Microbiol. 35:1477-1491), automated Q-beta replicase amplification assay (see, e.g., Burg (1996) Mol. Cell. Probes 10:257-271) and other RNA polymerase mediated techniques (e.g., NASBA, Cangene, Mississauga, Ontario); see also Berger (1987) Methods Enzymol. 152:307-316; Sambrook; Ausubel; U.S. Patent Nos. 4,683,195 and 4,683,202; Sooknanan (1995) Biotechnology 13:563-564.

Determining the degree of sequence identity

[0213] The invention provides nucleic acids encoding a polypeptide having glucoamylase activity comprising sequences having at least 99%, or more, or complete (100%) sequence identity to an exemplary nucleic acid of the invention. The invention provides polypeptides having glucoamylase activity comprising sequences having at least 99%, or more, or complete (100%) sequence identity to an exemplary polypeptide of the invention. The extent of sequence identity

(homology) may be determined using any computer program and associated parameters, including those described herein, such as BLAST 2.2.2. or FASTA version 3.0t78, with the default parameters.

[0214] The phrase "substantially identical" in the context of two nucleic acids can refer to two or more sequences that have, e.g., at least 98%, 99%, or more nucleotide (sequence) identity, when compared and aligned for maximum correspondence, as measured using one any known sequence comparison algorithm, as discussed in detail below, or by visual inspection. In alternative aspects, the disclosure provides nucleic acid and polypeptide sequences having substantial identity to an exemplary sequence of the disclosure over a region of at least about 10, 20, 30, 40, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000 or more residues, or a region ranging from between about 50 residues to the full length of the nucleic acid or polypeptide. Nucleic acid sequences of the invention can be substantially identical over the entire length of a polypeptide coding region.

[0215] Homologous sequences also include RNA sequences in which uridines replace the thymines in the nucleic acid sequences. The homologous sequences may be obtained using any of the procedures described herein or may result from the correction of a sequencing error. It will be appreciated that the nucleic acid sequences as set forth herein can be represented in the traditional single character format (see, e.g., Stryer, Lubert. Biochemistry, 3rd Ed., W. H Freeman & Co., New York) or in any other format which records the identity of the nucleotides in a sequence.

[0216] Various sequence comparison programs identified herein are used in this aspect of the invention. Protein and/or nucleic acid sequence identities (homologies) may be evaluated using any of the variety of sequence comparison algorithms and programs known in the art. Such algorithms and programs include, but are not limited to, TBLASTN, BLASTP, FASTA, TFASTA, and CLUSTALW (Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85(8):2444-2448, 1988; Altschul et al., J. Mol. Biol. 215(3):403-410, 1990; Thompson et al., Nucleic Acids Res. 22(2):4673-4680, 1994; Higgins et al., Methods Enzymol. 266:383-402, 1996; Altschul et al., J. Mol. Biol. 215(3):403-410, 1990; Altschul et al., Nature Genetics 3:266-272, 1993).

[0217] Homology or sequence identity can be measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of homology to various deletions, substitutions and other modifications. The terms "homology" and "identity" in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same when compared and aligned for maximum correspondence over a comparison window or designated region as measured using any number of sequence comparison algorithms or by manual alignment and visual inspection. For sequence comparison, one sequence can act as a reference sequence, e.g., a sequence of the invention, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0218] A "comparison window", as used herein, includes reference to a segment of any one of the numbers of contiguous residues. For example, contiguous residues ranging anywhere from 20 to the full length of an exemplary polypeptide or nucleic acid sequence of the invention are compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. If the reference sequence has the requisite sequence identity to an exemplary nucleic acid sequence of the invention, e.g., 98%, 99%, or more sequence identity to a sequence of the invention, that sequence is within the scope of the invention.

[0219] Methods of alignment of sequence for comparison are well known in the art. In alternative aspects, optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482, 1981, by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443, 1970, by the search for similarity method of person & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetic Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection. Other algorithms for determining homology or identity include, for example, in addition to a BLAST program (Basic Local Alignment Search Tool at the National Center for Biological Information), ALIGN, AMAS (Analysis of Multiply Aligned Sequences), AMPS (Protein Multiple Sequence Alignment), ASSET (Aligned Segment Statistical Evaluation Tool), BANDS, BESTSCOR, BIOSCAN (Biological Sequence Comparative Analysis Node), BLIMPS (BLocks IMProved Searcher), FASTA, Intervals & Points, BMB, CLUSTAL V, CLUSTAL W, CONSENSUS, LCONSENSUS, WCONSENSUS, Smith-Waterman algorithm, DARWIN, Las Vegas algorithm, FNAT (Forced Nucleotide Alignment Tool), Framealign, Framesearch, DYNAMIC, FILTER, FSAP (Fristensky Sequence Analysis Package), GAP (Global Alignment Program), GENAL, GIBBS, GenQuest, ISSC (Sensitive Sequence Comparison), LALIGN (Local Sequence Alignment), LCP (Local Content Program), MACAW (Multiple Alignment Construction & Analysis Workbench), MAP (Multiple Alignment Program), MBLKP, MBLKN, PIMA (Pattern-Induced Multi-sequence Alignment), SAGA (Sequence Alignment by Genetic Algorithm) and WHAT-IF. Such alignment programs can also be used to screen genome databases to identify polynucleotide sequences having sub-

stantially identical sequences. A number of genome databases are available, for example, a substantial portion of the human genome is available as part of the Human Genome Sequencing Project (Gibbs, 1995). Several genomes have been sequenced, e.g., M. genitalium (Fraser et al., 1995), M. jannaschii (Bult et al., 1996), H. influenzae (Fleischmann et al., 1995), E. coli (Blattner et al., 1997), and yeast (S. cerevisiae) (Mewes et al., 1997), and D. melanogaster (Adams et al., 2000). Significant progress has also been made in sequencing the genomes of model organism, such as mouse, *C. elegans*, and *Arabadopsis* sp. Databases containing genomic information annotated with some functional information are maintained by different organization, and are accessible via the internet.

[0220] BLAST, BLAST 2.0 and BLAST 2.2.2 algorithms also can be used. They are described, e.g., in Altschul (1977) Nuc. Acids Res. 25:3389-3402; Altschul (1990) J. Mol. Biol. 215:403-410. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul (1990) supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectations (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff(1989) Proc. Natl. Acad. Sci. USA 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N= -4, and a comparison of both strands. The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873). One measure of similarity provided by BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a references sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001. In one aspect, protein and nucleic acid sequence homologies are evaluated using the Basic Local Alignment Search Tool ("BLAST"). For example, five specific BLAST programs can be used to perform the following task: (1) BLASTP and BLAST3 compare an amino acid query sequence against a protein sequence database; (2) BLASTN compares a nucleotide query sequence against a nucleotide sequence database; (3) BLASTX compares the six-frame conceptual translation products of a query nucleotide sequence (both strands) against a protein sequence database; (4) TBLASTN compares a query protein sequence against a nucleotide sequence database translated in all six reading frames (both strands); and, (5) TBLASTX compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database. The BLAST programs identify homologous sequences by identifying similar segments, which are referred to herein as "high-scoring segment pairs," between a query amino or nucleic acid sequence and a test sequence which is preferably obtained from a protein or nucleic acid sequence database. High-scoring segment pairs are preferably identified (i.e., aligned) by means of a scoring matrix, many of which are known in the art. Preferably, the scoring matrix used is the BLOSUM62 matrix (Gonnet et al., Science 256:1443-1445, 1992; Henikoff and Henikoff, Proteins 17:49-61, 1993). Less preferably, the PAM or PAM250 matrices may also be used (see, e.g., Schwartz and Dayhoff, eds., 1978, Matrices for Detecting Distance Relationships: Atlas of Protein Sequence and Structure, Washington: National Biomedical Research Foundation).

[0221] In one aspect of the disclosure, to determine if a nucleic acid has the requisite sequence identity to be within the scope of the invention, the NCBI BLAST 2.2.2 programs is used, default options to blastp. There are about 38 setting options in the BLAST 2.2.2 program. In this exemplary aspect of the disclosure, all default values are used except for the default filtering setting (i.e., all parameters set to default except filtering which is set to OFF); in its place a "-F F" setting is used, which disables filtering. Use of default filtering often results in Karlin-Altschul violations due to short length of sequence.

[0222] The default values used in this exemplary aspect of the disclosure, and to determine the values in Figure 3, as discussed above, include:

"Filter for low complexity: ON
Word Size: 3
Matrix: Blosum62
Gap Costs: Existence: 11

Extension:1"

[0223] Other default settings can be: filter for low complexity OFF, word size of 3 for protein, BLOSUM62 matrix, gap existence penalty of -11 and a gap extension penalty of -1. An exemplary NCBI BLAST 2.2.2 program setting has the "-W" option default to 0. This means that, if not set, the word size defaults to 3 for proteins and 11 for nucleotides.

Computer systems and computer program products

[0224] To determine and identify sequence identities, structural homologies, motifs and the like in silico, the sequence of the invention can be stored, recorded, and manipulated on any medium which can be read and accessed by a computer. Accordingly, the disclosure provides computers, computer systems, computer readable mediums, computer programs products and the like recorded or stored thereon the nucleic acid and polypeptide sequences of the invention. As used herein, the words "recorded" and "stored" refer to a process for storing information on a computer medium. A skilled artisan can readily adopt any known methods for recording information on a computer readable medium to generate manufactures comprising one or more of the nucleic acid and/or polypeptide sequences of the invention.

[0225] The disclosure also provides computers and processors comprising computer program products comprising sequences of the invention; and as used herein, the terms "computer," "computer program" and "processor" are used in their broadest general contexts and incorporate all such devices, as described in detail, below. A "coding sequence of" or a "sequence encodes" a particular polypeptide or protein, is a nucleic acid sequence which is transcribed and translated into a polypeptide or protein when placed under the control of appropriate regulatory sequences.

[0226] Another aspect of the disclosure is a computer readable medium having recorded thereon at least one nucleic acid and/or polypeptide sequence of the invention. Computer readable media include magnetically readable media, optically readable media, electronically readable media and magnetic/optical media. For example, the computer readable media may be a hard disk, a floppy disk, a magnetic tape, CD-ROM, Digital Versatile Disk (DVD), Random Access Memory (RAM), or Read Only Memory (ROM) as well as other types of other media known to those skilled in the art.

[0227] Aspects of the disclosure include systems (e.g., internet based systems), particularly computer systems, which store and manipulate the sequences and sequence information described herein. One example of a computer system 100 is illustrated in block diagram form in Figure 1. As used herein, "a computer system" refers to the hardware components, software components, and data storage components used to analyze a nucleotide or polypeptide sequence of the invention. The computer system 100 can include a processor for processing, accessing and manipulating the sequence data. The processor 105 can be any well-known type of central processing unit, such as, for example, the Pentium III from Intel Corporation, or similar processor from Sun, Motorola, Compaq, AMD or International Business Machines. The computer system 100 is a general purpose system that comprises the processor 105 and one or more internal data storage components 110 for storing data, and one or more data retrieving devices for retrieving the data stored on the data storage components. A skilled artisan can readily appreciate that any one of the currently available computer systems are suitable.

[0228] In one aspect, the computer system 100 includes a processor 105 connected to a bus which is connected to a main memory 115 (preferably implemented as RAM) and one or more internal data storage devices 110, such as a hard drive and/or other computer readable media having data recorded thereon. The computer system 100 can further include one or more data retrieving device 118 for reading the data stored on the internal data storage devices 110. The data retrieving device 118 may represent, for example, a floppy disk drive, a compact disk drive, a magnetic tape drive, or a modem capable of connection to a remote data storage system (e.g., via the internet) etc. In some embodiments, the internal data storage device 110 is a removable computer readable medium such as a floppy disk, a compact disk, a magnetic tape, etc. containing control logic and/or data recorded thereon. The computer system 100 may advantageously include or be programmed by appropriate software for reading the control logic and/or the data from the data storage component once inserted in the data retrieving device. The computer system 100 includes a display 120 which is used to display output to a computer user. It should also be noted that the computer system 100 can be linked to other computer systems 125a-c in a network or wide area network to provide centralized access to the computer system 100. Software for accessing and processing the nucleotide or amino acid sequences of the invention can reside in main memory 115 during execution. In some aspects, the computer system 100 may further comprise a sequence comparison algorithm for comparing a nucleic acid sequence of the invention. The algorithm and sequence(s) can be stored on a computer readable medium. A "sequence comparison algorithm" refers to one or more programs which are implemented (locally or remotely) on the computer system 100 to compare a nucleotide sequence with other nucleotide sequences and/or compounds stored within a data storage means. For example, the sequence comparison algorithm may compare the nucleotide sequences of the invention stored on a computer readable medium to reference sequences stored on a computer readable medium to identify homologies or structural motifs.

[0229] The parameters used with the above algorithms may be adapted depending on the sequence length and degree of homology studied. In some aspects, the parameters may be the default parameters used by the algorithms in the

absence of instructions from the user. Figure 2 is a flow diagram illustrating one aspect of a process 200 for comparing a new nucleotide or protein sequence with a database of sequences in order to determine the homology levels between the new sequence and the sequences in the database. The database of sequences can be a private database stored within the computer system 100, or a public database such as GENBANK that is available through the Internet. The process 200 begins at a start state 201 and then moves to a state 202 wherein the new sequence to be compared is stored to a memory in a computer system 100. As discussed above, the memory could be any type of memory, including RAM or an internal storage device. The process 200 then moves to a state 204 wherein a database of sequences is opened for analysis and comparison. The process 200 then moves to a state 206 wherein the first sequence stored in the database is read into a memory on the computer. A comparison is then performed at a state 210 to determine if the first sequence is the same as the second sequence. It is important to note that this step is not limited to performing an exact comparison between the new sequence and the first sequence in the database. Well-known methods are known to those of skill in the art for comparing two nucleotide or protein sequences, even if they are not identical. For example, gaps can be introduced into one sequence in order to raise the homology level between the two tested sequences. The parameters that control whether gaps or other features are introduced into a sequence during comparison are normally entered by the user of the computer system. Once a comparison of the two sequences has been performed at the state 210, a determination is made at a decision state 210 whether the two sequences are the same. Of course, the term "same" is not limited to sequences that are absolutely identical. Sequences that are within the homology parameters entered by the user will be marked as "same" in the process 200. If a determination is made that the two sequences are the same, the process 200 moves to a state 214 wherein the name of the sequence from the database is displayed to the user. This state notifies the user that the sequence with the displayed name fulfills the homology constraints that were entered. Once the name of the stored sequence is displayed to the user, the process 200 moves to a decision state 218 wherein a determination is made whether more sequences exist in the database. If no more sequences exist in the database, then the process 200 terminates at an end state 220. However, if more sequences do exist in the database, then the process 200 moves to a state 224 wherein a pointer is moved to the next sequence in the database so that it can be compared to the new sequence. In this manner, the new sequence is aligned and compared with every sequence in the database. It should be noted that if a determination had been made at the decision state 212 that the sequences were not homologous, then the process 200 would move immediately to the decision state 218 in order to determine if any other sequences were available in the database for comparison. Accordingly, one aspect of the disclosure is a computer system comprising a processor, a data storage device having stored thereon a nucleic acid sequence of the invention and a sequence comparer for conducting the comparison. The sequence comparer may indicate a homology level between the sequences compared or identify structural motifs, or it may identify structural motifs in sequences which are compared to these nucleic acid codes and polypeptide codes. Figure 3 is a flow diagram illustrating one embodiment of a process 250 in a computer for determining whether two sequences are homologous. The process 250 begins at a start state 252 and then moves to a state 254 wherein a first sequence to be compared is stored to a memory. The second sequence to be compared is then stored to a memory at a state 256. The process 250 then moves to a state 260 wherein the first character in the first sequence is read and then to a state 262 wherein the first character of the second sequence is read. It should be understood that if the sequence is a nucleotide sequence, then the character would normally be either A, T, C, G or U. If the sequence is a protein sequence, then it can be a single letter amino acid code so that the first and sequence sequences can be easily compared. A determination is then made at a decision state 264 whether the two characters are the same. If they are the same, then the process 250 moves to a state 268 wherein the next characters in the first and second sequences are read. A determination is then made whether the next characters are the same. If they are, then the process 250 continues this loop until two characters are not the same. If a determination is made that the next two characters are not the same, the process 250 moves to a decision state 274 to determine whether there are any more characters either sequence to read. If there are not any more characters to read, then the process 250 moves to a state 276 wherein the level of homology between the first and second sequences is displayed to the user. The level of homology is determined by calculating the proportion of characters between the sequences that were the same out of the total number of sequences in the first sequence. Thus, if every character in a first 100 nucleotide sequence aligned with an every character in a second sequence, the homology level would be 100%.

[0230] Alternatively, the computer program can compare a reference sequence to a sequence of the invention to determine whether the sequences differ at one or more positions. The program can record the length and identity of inserted, deleted or substituted nucleotides or amino acid residues with respect to the sequence of either the reference or the invention. The computer program may be a program which determines whether a reference sequence contains a single nucleotide polymorphism (SNP) with respect to a sequence of the invention, or, whether a sequence of the invention comprises a SNP of a known sequence. Thus, in some aspects, the computer program is a program which identifies SNPs. The method may be implemented by the computer systems described above and the method illustrated in Figure 3. The method can be performed by reading a sequence of the invention and the reference sequences through the use of the computer program and identifying differences with the computer program.

[0231] In other aspects the computer based system comprises an identifier for identifying features within a nucleic

acid or polypeptide of the invention. An "identifier" refers to one or more programs which identifies certain features within a nucleic acid sequence. For example, an identifier may comprise a program which identifies an open reading frame (ORF) in a nucleic acid sequence. Figure 4 is a flow diagram illustrating one aspect of an identifier process 300 for detecting the presence of a feature in a sequence. The process 300 begins at a start state 302 and then moves to a state 304 wherein a first sequence that is to be checked for features is stored to a memory 115 in the computer system 100. The process 300 then moves to a state 306 wherein a database of sequence features is opened. Such a database would include a list of each feature's attributes along with the name of the feature. For example, a feature name could be "Initiation Codon" and the attribute would be "ATG". Another example would be the feature name "TAATAA Box" and the feature attribute would be "TAATAA". An example of such a database is produced by the University of Wisconsin Genetics Computer Group. Alternatively, the features may be structural polypeptide motifs such as alpha helices, beta sheets, or functional polypeptide motifs such as enzymatic active sites, helix-turn-helix motifs or other motifs known to those skilled in the art. Once the database of features is opened at the state 306, the process 300 moves to a state 308 wherein the first feature is read from the database. A comparison of the attribute of the first feature with the first sequence is then made at a state 310. A determination is then made at a decision state 316 whether the attribute of the feature was found in the first sequence. If the attribute was found, then the process 300 moves to a state 318 wherein the name of the found feature is displayed to the user. The process 300 then moves to a decision state 320 wherein a determination is made whether move features exist in the database. If no more features do exist, then the process 300 terminates at an end state 324. However, if more features do exist in the database, then the process 300 reads the next sequence feature at a state 326 and loops back to the state 310 wherein the attribute of the next feature is compared against the first sequence. If the feature attribute is not found in the first sequence at the decision state 316, the process 300 moves directly to the decision state 320 in order to determine if any more features exist in the database. Thus, in one aspect, the disclosure provides a computer program that identifies open reading frames (ORFs).

[0232] A polypeptide or nucleic acid sequence of the invention can be stored and manipulated in a variety of data processor programs in a variety of formats. For example, a sequence can be stored as text in a word processing file, such as MicrosoftWORD or WORDPERFECT or as an ASCII file in a variety of database programs familiar to those of skill in the art, such as DB2™, SYBASE™, or ORACLE™. In addition, many computer programs and databases may be used as sequence comparison algorithms, identifiers, or sources of reference nucleotide sequences or polypeptide sequences to be compared to a nucleic acid sequence of the invention. The programs and databases used to practice the disclosure include, but are not limited to: MACPATTERN™ (EMBL), DiscoveryBase (Molecular Applications Group), GENEMINE™ (Molecular Applications Group), LOOK™ (Molecular Applications Group), MACLOOK™ (Molecular Applications Group), BLAST and BLAST2 (NCBI), BLASTN and BLASTX (Altschul et al, J. Mol. Biol. 215: 403, 1990), FASTA (Pearson and Lipman, Proc. Natl. Acad. Sci. USA, 85: 2444, 1988), FASTDB™ (Brutlag et al. Comp. App. Biosci. 6:237-245, 1990), Catalyst (Molecular Simulations Inc.), CATALYST™ /SHAPE™ (Molecular Simulations Inc.), CERIUS2.DB ACCESS™ (Molecular Simulations Inc.), HYPOGEN™ (Molecular Simulations Inc.), INSIGHT II™, (Molecular Simulations Inc.), DISCOVER™ (Molecular Simulations Inc.), CHARMM™ (CHARMm™) (Molecular Simulations Inc.), Felix (Molecular Simulations Inc.), DELPHI™, (Molecular Simulations Inc.), QUANTEMM™, (Molecular Simulations Inc.), HOMOLOGY™ (Molecular Simulations Inc.), MODELER™ (Molecular Simulations Inc.), ISIS™ (Molecular Simulations Inc.), QUANTA™ /Protein Design (Molecular Simulations Inc.), WEBLAB™ (Molecular Simulations Inc.), WEBLAB DIVERSITY EXPLORER™ (Molecular Simulations Inc.), GENE EXPLORER™ (Molecular Simulations Inc.), SEQFOLD™ (Molecular Simulations Inc.), the MDL Available Chemicals Directory database, the MDL Drug Data Report data base, the Comprehensive Medicinal Chemistry database, Derwent's World Drug Index database, the BioByteMasterFile database, the Genbank database, and the Genseqn database. Many other programs and data bases would be apparent to one of skill in the art given the present disclosure.

[0233] Motifs which may be detected using the above programs include sequences encoding leucine zippers, helix-turn-helix motifs, glycosylation sites, ubiquitination sites, alpha helices, and beta sheets, signal sequences encoding signal peptides which direct the secretion of the encoded proteins, sequences implicated in transcription regulation such as homeoboxes, acidic stretches, enzymatic active sites, substrate binding sites, and enzymatic cleavage sites.

Hybridization of nucleic acids

[0234] The disclosure provides isolated, synthetic or recombinant nucleic acids that hybridize under stringent conditions to an exemplary sequence of the invention, or a nucleic acid that encodes a polypeptide of the invention. The stringent conditions can be highly stringent conditions, medium stringent conditions, low stringent conditions, including the high and reduced stringency conditions described herein. In one aspect, it is the stringency of the wash conditions that set forth the conditions which determine whether a nucleic acid is within the scope of the invention, as discussed below.

[0235] "Hybridization" protocols include processes by which a nucleic acid strand joins with a complementary strand through base pairing. Hybridization reactions can be sensitive and selective so that a particular sequence of interest can be identified even in samples in which it is present at low concentrations. Stringent conditions can be defined by,

for example, the concentrations of salt or formamide in the prehybridization and hybridization solutions, or by the hybridization temperature, and are well known in the art. For example, stringency can be increased by reducing the concentration of salt, increasing the concentration of formamide, or raising the hybridization temperature, altering the time of hybridization, as described in detail, below. In alternative aspects, nucleic acids of the disclosure are defined by their ability to hybridize under various stringency conditions (e.g., high, medium, and low), as set forth herein.

[0236] In alternative embodiments, nucleic acids of the disclosure as defined by their ability to hybridize under stringent conditions can be between about five residues and the full length of nucleic acid of the disclosure; e.g., they can be at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 55, 60, 65, 70, 75, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, or more, residues in length. Nucleic acids shorter than full length are also included. These nucleic acids can be useful as, e.g., hybridization probes, labeling probes, PCR oligonucleotide probes, iRNA, antisense or sequences encoding antibody binding peptides (epitopes), motifs, active sites and the like.

[0237] In one aspect, nucleic acids of the disclosure are defined by their ability to hybridize under high stringency comprises conditions of about 50% formamide at about 37°C to 42°C. In one aspect, nucleic acids of the disclosure are defined by their ability to hybridize under reduced stringency comprising conditions in about 35% to 25% formamide at about 30°C to 35°C.

[0238] Alternatively, nucleic acids of the disclosure are defined by their ability to hybridize under high stringency comprising conditions at 42°C in 50% formamide, 5X SSPE, 0.3% SDS, and a repetitive sequence blocking nucleic acid, such as cot-1 or salmon sperm DNA (e.g., 200 ug/ml sheared and denatured salmon sperm DNA). In one aspect, nucleic acids of the disclosure are defined by their ability to hybridize under reduced stringency conditions comprising 35% formamide at a reduced temperature of 35°C.

[0239] Following hybridization, the filter may be washed with 6X SSC, 0.5% SDS at 50°C. These conditions are considered to be "moderate" conditions above 25% formamide and "low" conditions below 25% formamide. A specific example of "moderate" hybridization conditions is when the above hybridization is conducted at 30% formamide. A specific example of "low stringency" hybridization conditions is when the above hybridization is conducted at 10% formamide.

[0240] The temperature range corresponding to a particular level of stringency can be further narrowed by calculating the purine to pyrimidine ratio of the nucleic acid of interest and adjusting the temperature accordingly. Nucleic acids of the disclosure are also defined by their ability to hybridize under high, medium, and low stringency conditions as set forth in Ausubel and Sambrook. Variations on the above ranges and conditions are well known in the art. Hybridization conditions are discussed further, below.

[0241] The above procedure may be modified to identify nucleic acids having decreasing levels of homology to the probe sequence. For example, to obtain nucleic acids of decreasing homology to the detectable probe, less stringent conditions may be used. For example, the hybridization temperature may be decreased in increments of 5°C from 68°C to 42°C in a hybridization buffer having a Na^+ concentration of approximately 1 M. Following hybridization, the filter may be washed with 2X SSC, 0.5% SDS at the temperature of hybridization. These conditions are considered to be "moderate" conditions above 50°C and "low" conditions below 50°C. A specific example of "moderate" hybridization conditions is when the above hybridization is conducted at 55°C. A specific example of "low stringency" hybridization conditions is when the above hybridization is conducted at 45°C.

[0242] Alternatively, the hybridization may be carried out in buffers, such as 6X SSC, containing formamide at a temperature of 42°C. In this case, the concentration of formamide in the hybridization buffer may be reduced in 5% increments from 50% to 0% to identify clones having decreasing levels of homology to the probe. Following hybridization, the filter may be washed with 6X SSC, 0.5% SDS at 50°C. These conditions are considered to be "moderate" conditions above 25% formamide and "low" conditions below 25% formamide. A specific example of "moderate" hybridization conditions is when the above hybridization is conducted at 30% formamide. A specific example of "low stringency" hybridization conditions is when the above hybridization is conducted at 10% formamide.

[0243] However, the selection of a hybridization format is not critical - it is the stringency of the wash conditions that set forth the conditions which determine whether a nucleic acid is within the scope of the disclosure. Wash conditions used to identify nucleic acids within the scope of the disclosure include, e.g.: a salt concentration of about 0.02 molar at pH 7 and a temperature of at least about 50°C or about 55°C to about 60°C; or, a salt concentration of about 0.15 M NaCl at 72°C for about 15 minutes; or, a salt concentration of about 0.2X SSC at a temperature of at least about 50°C or about 55°C to about 60°C for about 15 to about 20 minutes; or, the hybridization complex is washed twice with a solution with a salt concentration of about 2X SSC containing 0.1% SDS at room temperature for 15 minutes and then washed twice by 0.1X SSC containing 0.1% SDS at 68°C for 15 minutes; or, equivalent conditions. See Sambrook, Tijssen and Ausubel for a description of SSC buffer and equivalent conditions.

[0244] These methods may be used to isolate nucleic acids of the disclosure.

Oligonucleotides probes and methods for using them

[0245] The disclosure also provides nucleic acid probes that can be used, e.g., for identifying nucleic acids encoding a polypeptide with an amylase activity or fragments thereof or for identifying amylase genes. In one aspect, the probe comprises at least 10 consecutive bases of a nucleic acid of the disclosure. Alternatively, a probe of the disclosure can be at least about 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 110, 120, 130, 150 or about 10 to 50, about 20 to 60 about 30 to 70, consecutive bases of a sequence as set forth in a nucleic acid of the disclosure. The probes identify a nucleic acid by binding and/or hybridization. The probes can be used in arrays of the disclosure, see discussion below, including, e.g., capillary arrays. The probes of the disclosure can also be used to isolate other nucleic acids or polypeptides.

[0246] The probes of the disclosure can be used to determine whether a biological sample, such as a soil sample, contains an organism having a nucleic acid sequence of the invention or an organism from which the nucleic acid was obtained. In such procedures, a biological sample potentially harboring the organism from which the nucleic acid was isolated is obtained and nucleic acids are obtained from the sample. The nucleic acids are contacted with the probe under conditions which permit the probe to specifically hybridize to any complementary sequences present in the sample. Where necessary, conditions which permit the probe to specifically hybridize to complementary sequences may be determined by placing the probe in contact with complementary sequences from samples known to contain the complementary sequence, as well as control sequences which do not contain the complementary sequence. Hybridization conditions, such as the salt concentration of the hybridization buffer, the formamide concentration of the hybridization buffer, or the hybridization temperature, may be varied to identify conditions which allow the probe to hybridize specifically to complementary nucleic acids (see discussion on specific hybridization conditions).

[0247] If the sample contains the organism from which the nucleic acid was isolated, specific hybridization of the probe is then detected. Hybridization may be detected by labeling the probe with a detectable agent such as a radioactive isotope, a fluorescent dye or an enzyme capable of catalyzing the formation of a detectable product. Many methods for using the labeled probes to detect the presence of complementary nucleic acids in a sample are familiar to those skilled in the art. These include Southern Blots, Northern Blots, colony hybridization procedures, and dot blots. Protocols for each of these procedures are provided in Ausubel and Sambrook.

[0248] Alternatively, more than one probe (at least one of which is capable of specifically hybridizing to any complementary sequences which are present in the nucleic acid sample), may be used in an amplification reaction to determine whether the sample contains an organism containing a nucleic acid sequence of the disclosure (e.g., an organism from which the nucleic acid was isolated). In one aspect, the probes comprise oligonucleotides. In one aspect, the amplification reaction may comprise a PCR reaction. PCR protocols are described in Ausubel and Sambrook (see discussion on amplification reactions). In such procedures, the nucleic acids in the sample are contacted with the probes, the amplification reaction is performed, and any resulting amplification product is detected. The amplification product may be detected by performing gel electrophoresis on the reaction products and staining the gel with an intercalator such as ethidium bromide. Alternatively, one or more of the probes may be labeled with a radioactive isotope and the presence of a radioactive amplification product may be detected by autoradiography after gel electrophoresis.

[0249] Probes derived from sequences near the 3' or 5' ends of a nucleic acid sequence of the invention can also be used in chromosome walking procedures to identify clones containing additional, e.g., genomic sequences. Such methods allow the isolation of genes which encode additional proteins of interest from the host organism.

[0250] In one aspect, nucleic acid sequences of the disclosure are used as probes to identify and isolate related nucleic acids. In some aspects, the so-identified related nucleic acids may be cDNAs or genomic DNAs from organisms other than the one from which the nucleic acid of the invention was first isolated. In such procedures, a nucleic acid sample is contacted with the probe under conditions which permit the probe to specifically hybridize to related sequences. Hybridization of the probe to nucleic acids from the related organism is then detected using any of the methods described above.

[0251] In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency can vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (e.g., GC v. AT content), and nucleic acid type (e.g., RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter. Hybridization can be carried out under conditions of low stringency, moderate stringency or high stringency. As an example of nucleic acid hybridization, a polymer membrane containing immobilized denatured nucleic acids is first prehybridized for 30 minutes at 45°C in a solution consisting of 0.9 M NaCl, 50 mM NaH_2PO4, pH 7.0, 5.0 mM Na_2EDTA, 0.5% SDS, 10X Denhardt's, and 0.5 mg/ml polyriboadenylic acid. Approximately 2 X 10^7 cpm (specific activity 4-9 X 10^8 cpm/ug) of ^{32}P end-labeled oligonucleotide probe can then added to the solution. After 12-16 hours of incubation, the membrane is washed for 30 minutes at room temperature (RT) in 1X SET (150 mM NaCl, 20 mM Tris hydrochloride, pH 7.8, 1 mM Na_2EDTA) containing 0.5% SDS, followed by a 30 minute wash in fresh 1X SET at Tm-10°C for the oligonucleotide probe. The membrane is then exposed to auto-

radiographic film for detection of hybridization signals.

[0252] By varying the stringency of the hybridization conditions used to identify nucleic acids, such as cDNAs or genomic DNAs, which hybridize to the detectable probe, nucleic acids having different levels of homology to the probe can be identified and isolated. Stringency may be varied by conducting the hybridization at varying temperatures below the melting temperatures of the probes. The melting temperature, Tm, is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly complementary probe. Very stringent conditions are selected to be equal to or about 5°C lower than the Tm for a particular probe. The melting temperature of the probe may be calculated using the following exemplary formulas. For probes between 14 and " 70 nucleotides in length the melting temperature (Tm) is calculated using the formula: Tm=81.5+16.6(log [Na+])+0.41(fraction G+C)-(600/N) where N is the length of the probe. If the hybridization is carried out in a solution containing formamide, the melting temperature may be calculated using the equation: Tm=81.5+16.6 (log [Na+])+0.41(fraction G+C)-(0.63% formamide)-(600/N) where N is the length of the probe. Prehybridization may be carried out in 6X SSC, 5X Denhardt's reagent, 0.5% SDS, 100 μg/ml denatured fragmented salmon sperm DNA or 6X SSC, 5X Denhardt's reagent, 0.5% SDS, 100 μg/ml denatured fragmented salmon sperm DNA, 50% formamide. Formulas for SSC and Denhardt's and other solutions are listed, e.g., in Sambrook.

[0253] Hybridization is conducted by adding the detectable probe to the prehybridization solutions listed above. Where the probe comprises double stranded DNA, it is denatured before addition to the hybridization solution. The filter is contacted with the hybridization solution for a sufficient period of time to allow the probe to hybridize to cDNAs or genomic DNAs containing sequences complementary thereto or homologous thereto. For probes over 200 nucleotides in length, the hybridization may be carried out at 15-25°C below the Tm. For shorter probes, such as oligonucleotide probes, the hybridization may be conducted at 5-10°C below the Tm. In one aspect, hybridizations in 6X SSC are conducted at approximately 68°C. In one aspect, hybridizations in 50% formamide containing solutions are conducted at approximately 42°C. All of the foregoing hybridizations would be considered to be under conditions of high stringency.

[0254] Following hybridization, the filter is washed to remove any non-specifically bound detectable probe. The stringency used to wash the filters can also be varied depending on the nature of the nucleic acids being hybridized, the length of the nucleic acids being hybridized, the degree of complementarity, the nucleotide sequence composition (e.g., GC v. AT content), and the nucleic acid type (e.g., RNA v. DNA). Examples of progressively higher stringency condition washes are as follows: 2X SSC, 0.1% SDS at room temperature for 15 minutes (low stringency); 0.1X SSC, 0.5% SDS at room temperature for 30 minutes to 1 hour (moderate stringency); 0.1X SSC, 0.5% SDS for 15 to 30 minutes at between the hybridization temperature and 68°C (high stringency); and 0.15M NaCl for 15 minutes at 72°C (very high stringency). A final low stringency wash can be conducted in 0.1X SSC at room temperature. The examples above are merely illustrative of one set of conditions that can be used to wash filters. One of skill in the art would know that there are numerous recipes for different stringency washes.

[0255] Nucleic acids which have hybridized to the probe can be identified by autoradiography or other conventional techniques. The above procedure may be modified to identify nucleic acids having decreasing levels of homology to the probe sequence. For example, to obtain nucleic acids of decreasing homology to the detectable probe, less stringent conditions may be used. For example, the hybridization temperature may be decreased in increments of 5°C from 68°C to 42°C in a hybridization buffer having a Na+ concentration of approximately 1M. Following hybridization, the filter may be washed with 2X SSC, 0.5% SDS at the temperature of hybridization. These conditions are considered to be "moderate" conditions above 50°C and "low" conditions below 50°C. An example of "moderate" hybridization conditions is when the above hybridization is conducted at 55°C. An example of "low stringency" hybridization conditions is when the above hybridization is conducted at 45°C.

[0256] Alternatively, the hybridization protocols may be carried out in buffers, such as 6X SSC, containing formamide at a temperature of 42°C. In this case, the concentration of formamide in the hybridization buffer may be reduced in 5% increments from 50% to 0% to identify clones having decreasing levels of homology to the probe. Following hybridization, the filter may be washed with 6X SSC, 0.5% SDS at 50°C. These conditions are considered to be "moderate" conditions above 25% formamide and "low" conditions below 25% formamide. A specific example of "moderate" hybridization conditions is when the above hybridization is conducted at 30% formamide. A specific example of "low stringency" hybridization conditions is when the above hybridization is conducted at 10% formamide.

[0257] These probes and methods of the disclosure can be used to isolate nucleic acids having a sequence with at least 98% or 99% sequence identity ("homology") to an exemplary nucleic acid sequence of the invention. Homology may be measured using an alignment algorithm, as discussed herein. For example, the homologous polynucleotides may have a coding sequence which is a naturally occurring allelic variant of one of the coding sequences described herein. Such allelic variants may have a substitution, deletion or addition of one or more nucleotides when compared to a nucleic acid of the invention.

[0258] Additionally, the probes and methods of the disclosure can be used to isolate nucleic acids which encode polypeptides having at least about 99%, sequence identity (homology) to a polypeptide of the invention.

Inhibiting Expression of Amylase

[0259] The disclosure provides nucleic acids complementary to (e.g., antisense sequences to) the nucleic acid sequences of the invention, e.g., nucleic acids comprising antisense, siRNA, miRNA, ribozymes. Antisense sequences are capable of inhibiting the transport, splicing or transcription of amylase-encoding and glucoamylase-encoding genes. The inhibition can be effected through the targeting of genomic DNA or messenger RNA. The transcription or function of targeted nucleic acid can be inhibited, for example, by hybridization and/or cleavage. One particularly useful set of inhibitors includes oligonucleotides which are able to either bind amylase gene or message, in either case preventing or inhibiting the production or function of amylase. The association can be through sequence specific hybridization. Another useful class of inhibitors includes oligonucleotides which cause inactivation or cleavage of amylase message. The oligonucleotide can have enzyme activity which causes such cleavage, such as ribozymes. The oligonucleotide can be chemically modified or conjugated to an enzyme or composition capable of cleaving the complementary nucleic acid. A pool of many different such oligonucleotides can be screened for those with the desired activity.

[0260] Methods and compositions for inhibition of expression of amylase, glucoamylase, glucosidase and other polysaccharide hydrolyzing enzymes can have a variety of industrial applications. For example, inhibition of glucosidase expression can slow or prevent spoilage. Spoilage can occur when polysaccharides, lipids or polypeptides, e.g., structural polysaccharides, are enzymatically degraded. This can lead to the deterioration, or rot, of fruits and vegetables. Compositions that inhibit the expression and/or activity of glucosidases, e.g., antibodies, antisense oligonucleotides, ribozymes and RNAi, can be used to slow or prevent spoilage. Thus, in one aspect, the disclosure provides methods and compositions comprising application onto a plant or plant product (e.g., a fruit, seed, root, leaf, etc.) antibodies, antisense oligonucleotides, ribozymes and RNAi of the disclosure, e.g., to slow or prevent spoilage, or for another purpose. These compositions also can be expressed by the plant (e.g., a transgenic plant) or another organism (e.g., a bacterium or other microorganism transformed with a glucosidase gene of the disclosure).

Antisense Oligonucleotides

[0261] The disclosure provides antisense oligonucleotides capable of binding amylase message which can inhibit proteolytic activity by targeting mRNA. Strategies for designing antisense oligonucleotides are well described in the scientific and patent literature, and the skilled artisan can design such amylase oligonucleotides using the novel reagents of the disclosure. For example, gene walking/ RNA mapping protocols to screen for effective antisense oligonucleotides are well known in the art, see, e.g., Ho (2000) Methods Enzymol. 314:168-183, describing an RNA mapping assay, which is based on standard molecular techniques to provide an easy and reliable method for potent antisense sequence selection. See also Smith (2000) Eur. J. Pharm. Sci. 11:191-198.

[0262] Naturally occurring nucleic acids are used as antisense oligonucleotides. The antisense oligonucleotides can be of any length; for example, in alternative aspects, the antisense oligonucleotides are between about 5 to 100, about 10 to 80, about 15 to 60, about 18 to 40. The optimal length can be determined by routine screening. The antisense oligonucleotides can be present at any concentration. The optimal concentration can be determined by routine screening. A wide variety of synthetic, non-naturally occurring nucleotide and nucleic acid analogues are known which can address this potential problem. For example, peptide nucleic acids (PNAs) containing non-ionic backbones, such as N-(2-aminoethyl) glycine units can be used. Antisense oligonucleotides having phosphorothioate linkages can also be used, as described in WO 97/03211; WO 96/39154; Mata (1997) Toxicol Appl Pharmacol 144:189-197; Antisense Therapeutics, ed. Agrawal (Humana Press, Totowa, N.J., 1996). Antisense oligonucleotides having synthetic DNA backbone analogues provided by the disclosure can also include phosphoro-dithioate, methylphosphonate, phosphoramidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene(methylimino), 3'-N-carbamate, and morpholino carbamate nucleic acids, as described above.

[0263] Combinatorial chemistry methodology can be used to create vast numbers of oligonucleotides that can be rapidly screened for specific oligonucleotides that have appropriate binding affinities and specificities toward any target, such as the sense and antisense amylase sequences of the disclosure (see, e.g., Gold (1995) J. of Biol. Chem. 270:13581-13584).

Inhibitory Ribozymes

[0264] The disclosure provides ribozymes capable of binding amylase message. These ribozymes can inhibit amylase activity by, e.g., targeting mRNA. Strategies for designing ribozymes and selecting the amylase-specific antisense sequence for targeting are well described in the scientific and patent literature, and the skilled artisan can design such ribozymes using the novel reagents of the disclosure. Ribozymes act by binding to a target RNA through the target RNA binding portion of a ribozyme which is held in close proximity to an enzymatic portion of the RNA that cleaves the target RNA. Thus, the ribozyme recognizes and binds a target RNA through complementary base-pairing, and once bound to

the correct site, acts enzymatically to cleave and inactivate the target RNA. Cleavage of a target RNA in such a manner will destroy its ability to direct synthesis of an encoded protein if the cleavage occurs in the coding sequence. After a ribozyme has bound and cleaved its RNA target, it can be released from that RNA to bind and cleave new targets repeatedly.

[0265] In some circumstances, the enzymatic nature of a ribozyme can be advantageous over other technologies, such as antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its transcription, translation or association with another molecule) as the effective concentration of ribozyme necessary to effect a therapeutic treatment can be lower than that of an antisense oligonucleotide. This potential advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, a ribozyme is typically a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding, but also on the mechanism by which the molecule inhibits the expression of the RNA to which it binds. That is, the inhibition is caused by cleavage of the RNA target and so specificity is defined as the ratio of the rate of cleavage of the targeted RNA over the rate of cleavage of non-targeted RNA. This cleavage mechanism is dependent upon factors additional to those involved in base pairing. Thus, the specificity of action of a ribozyme can be greater than that of antisense oligonucleotide binding the same RNA site.

[0266] The ribozyme of the disclosure, e.g., an enzymatic ribozyme RNA molecule, can be formed in a hammerhead motif, a hairpin motif, as a hepatitis delta virus motif, a group I intron motif and/or an RNaseP-like RNA in association with an RNA guide sequence. Examples of hammerhead motifs are described by, e.g., Rossi (1992) Aids Research and Human Retroviruses 8:183; hairpin motifs by Hampel (1989) Biochemistry 28:4929, and Hampel (1990) Nuc. Acids Res. 18:299; the hepatitis delta virus motif by Perrotta (1992) Biochemistry 31:16; the RNaseP motif by Guerrier-Takada (1983) Cell 35:849; and the group I intron by Cech U.S. Pat. No. 4,987,071. Those skilled in the art will recognize that a ribozyme of the disclosure, e.g., an enzymatic RNA molecule of this disclosure, can have a specific substrate binding site complementary to one or more of the target gene RNA regions. A ribozyme of the disclosure can have a nucleotide sequence within or surrounding that substrate binding site which imparts an RNA cleaving activity to the molecule.

RNA interference (RNAi)

[0267] In one aspect, the disclosure provides an RNA inhibitory molecule, a so-called "RNAi" molecule, comprising an amylase enzyme sequence of the disclosure (which includes both sense and antisense strands). The RNAi molecule can comprise a double-stranded RNA (dsRNA) molecule, e.g., siRNA and/or miRNA. The RNAi molecule comprises a double-stranded RNA (dsRNA) molecule. The RNAi can inhibit expression of an amylase gene. In one aspect, the RNAi, e.g., siRNA and/or miRNA, is about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more duplex nucleotides in length. RNAi can enter a cell and cause the degradation of a single-stranded RNA (ssRNA) of similar or identical sequences, including endogenous mRNAs. When a cell is exposed to double-stranded RNA (dsRNA), mRNA from the homologous gene is selectively degraded by a process called RNA interference (RNAi). A possible basic mechanism behind RNAi is the breaking of a double-stranded RNA (dsRNA) matching a specific gene sequence into short pieces called short interfering RNA, which trigger the degradation of mRNA that matches its sequence. In one aspect, the RNAi's of the disclosure are used in gene-silencing therapeutics, see, e.g., Shuey (2002) Drug Discov. Today 7:1040-1046. In one aspect, the disclosure provides methods to selectively degrade RNA using the RNAi's of the disclosure. The process may be practiced *in vitro, ex vivo* or *in vivo.* In one aspect, the RNAi molecules of the disclosure can be used to generate a loss-of-function mutation in a cell, an organ or an animal. Methods for making and using RNAi molecules, e.g., siRNA and/or miRNA, for selectively degrade RNA are well known in the art, see, e.g., U.S. Patent No. 6,506,559; 6,511,824; 6,515,109; 6,489,127.

Modification of Nucleic Acids

[0268] The disclosure provides methods of generating variants of the nucleic acids of the disclosure, e.g., those encoding an amylase. These methods can be repeated or used in various combinations to generate amylases having an altered or different activity or an altered or different stability from that of an amylase encoded by the template nucleic acid. These methods also can be repeated or used in various combinations, e.g., to generate variations in gene/ message expression, message translation or message stability. In another aspect, the genetic composition of a cell is altered by, e.g., modification of a homologous gene ex vivo, followed by its reinsertion into the cell.

[0269] A nucleic acid of the disclosure can be altered by any means. For example, random or stochastic methods, or, non-stochastic, or "directed evolution," methods, see, e.g., U.S. Patent No. 6,361,974. Methods for random mutation of genes are well known in the art, see, e.g., U.S. Patent No. 5,830,696. For example, mutagens can be used to randomly mutate a gene. Mutagens include, e.g., ultraviolet light or gamma irradiation, or a chemical mutagen, e.g., mitomycin, nitrous acid, photoactivated psoralens, alone or in combination, to induce DNA breaks amenable to repair by recombination. Other chemical mutagens include, for example, sodium bisulfite, nitrous acid, hydroxylamine, hydrazine or formic

acid. Other mutagens are analogues of nucleotide precursors, e.g., nitrosoguanidine, 5-bromouracil, 2-aminopurine, or acridine. These agents can be added to a PCR reaction in place of the nucleotide precursor thereby mutating the sequence. Intercalating agents such as proflavine, acriflavine, quinacrine and the like can also be used.

[0270] Any technique in molecular biology can be used, e.g., random PCR mutagenesis, see, e.g., Rice (1992) Proc. Natl. Acad. Sci. USA 89:5467-5471; or, combinatorial multiple cassette mutagenesis, see, e.g., Crameri (1995) Biotechniques 18:194-196. Alternatively, nucleic acids, e.g., genes, can be reassembled after random, or "stochastic," fragmentation, see, e.g., U.S. Patent Nos. 6,291,242; 6,287,862; 6,287,861; 5,955,358; 5,830,721; 5,824,514; 5,811,238; 5,605,793. In alternative aspects, modifications, additions or deletions are introduced by error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, in vivo mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis, gene reassembly, gene site saturated mutagenesis (GSSM), synthetic ligation reassembly (SLR), recombination, recursive sequence recombination, phosphothioate-modified DNA mutagenesis, uracil-containing template mutagenesis, gapped duplex mutagenesis, point mismatch repair mutagenesis, repair-deficient host strain mutagenesis, chemical mutagenesis, radiogenic mutagenesis, deletion mutagenesis, restriction-selection mutagenesis, restriction-purification mutagenesis, artificial gene synthesis, ensemble mutagenesis, chimeric nucleic acid multimer creation, and/or a combination of these and other methods.

[0271] The following publications describe a variety of recursive recombination procedures and/or methods which can be incorporated into the methods of the disclosure: Stemmer (1999) "Molecular breeding of viruses for targeting and other clinical properties" Tumor Targeting 4:1-4; Ness (1999) Nature Biotechnology 17:893-896; Chang (1999) "Evolution of a cytokine using DNA family shuffling" Nature Biotechnology 17:793-797; Minshull (1999) "Protein evolution by molecular breeding" Current Opinion in Chemical Biology 3:284-290; Christians (1999) "Directed evolution of thymidine kinase for AZT phosphorylation using DNA family shuffling" Nature Biotechnology 17:259-264; Crameri (1998) "DNA shuffling of a family of genes from diverse species accelerates directed evolution" Nature 391:288-291; Crameri (1997) "Molecular evolution of an arsenate detoxification pathway by DNA shuffling," Nature Biotechnology 15:436-438; Zhang (1997) "Directed evolution of an effective fucosidase from a galactosidase by DNA shuffling and screening" Proc. Natl. Acad. Sci. USA 94:4504-4509; Patten et al. (1997) "Applications of DNA Shuffling to Pharmaceuticals and Vaccines" Current Opinion in Biotechnology 8:724-733; Crameri et al. (1996) "Construction and evolution of antibody-phage libraries by DNA shuffling" Nature Medicine 2:100-103; Gates et al. (1996) "Affinity selective isolation of ligands from peptide libraries through display on a lac repressor 'headpiece dimer'" Journal of Molecular Biology 255:373-386; Stemmer (1996) "Sexual PCR and Assembly PCR" In: The Encyclopedia of Molecular Biology. VCH Publishers, New York. pp.447-457; Crameri and Stemmer (1995) "Combinatorial multiple cassette mutagenesis creates all the permutations of mutant and wildtype cassettes" BioTechniques 18:194-195; Stemmer et al. (1995) "Single-step assembly of a gene and entire plasmid form large numbers of oligodeoxyribonucleotides" Gene, 164:49-53; Stemmer (1995) "The Evolution of Molecular Computation" Science 270: 1510; Stemmer (1995) "Searching Sequence Space" Bio/Technology 13:549-553; Stemmer (1994) "Rapid evolution of a protein in vitro by DNA shuffling" Nature 370:389-391; and Stemmer (1994) "DNA shuffling by random fragmentation and reassembly: In vitro recombination for molecular evolution." Proc. Natl. Acad. Sci. USA 91:10747-10751.

[0272] Mutational methods of generating diversity include, for example, site-directed mutagenesis (Ling et al. (1997) "Approaches to DNA mutagenesis: an overview" Anal Biochem. 254(2): 157-178; Dale et al. (1996) "Oligonucleotide-directed random mutagenesis using the phosphorothioate method" Methods Mol. Biol. 57:369-374; Smith (1985) "In vitro mutagenesis" Ann. Rev. Genet. 19:423-462; Botstein & Shortle (1985) "Strategies and applications of in vitro mutagenesis" Science 229:1193-1201; Carter (1986) "Site-directed mutagenesis" Biochem. J. 237:1-7; and Kunkel (1987) "The efficiency of oligonucleotide directed mutagenesis" in Nucleic Acids & Molecular Biology (Eckstein, F. and Lilley, D. M. J. eds., Springer Verlag, Berlin)); mutagenesis using uracil containing templates (Kunkel (1985) "Rapid and efficient site-specific mutagenesis without phenotypic selection" Proc. Natl. Acad. Sci. USA 82:488-492; Kunkel et al. (1987) "Rapid and efficient site-specific mutagenesis without phenotypic selection" Methods in Enzymol. 154, 367-382; and Bass et al. (1988) "Mutant Trp repressors with new DNA-binding specificities" Science 242:240-245); oligonucleotide-directed mutagenesis (Methods in Enzymol. 100: 468-500 (1983); Methods in Enzymol. 154: 329-350 (1987); Zoller & Smith (1982) "Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any DNA fragment" Nucleic Acids Res. 10:6487-6500; Zoller & Smith (1983) "Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors" Methods in Enzymol. 100:468-500; and Zoller & Smith (1987) Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template" Methods in Enzymol. 154:329-350); phosphorothioate-modified DNA mutagenesis (Taylor et al. (1985) "The use of phosphorothioate-modified DNA in restriction enzyme reactions to prepare nicked DNA" Nucl. Acids Res. 13: 8749-8764; Taylor et al. (1985) "The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA" Nucl. Acids Res. 13: 8765-8787 (1985); Nakamaye (1986) "Inhibition of restriction endonuclease Nci I cleavage by phosphorothioate groups and its application to oligonucleotide-directed mutagenesis" Nucl. Acids Res. 14: 9679-9698; Sayers et al. (1988) "Y-T Exonucleases in phosphorothioate-based oli-

gonucleotide-directed mutagenesis" Nucl. Acids Res. 16:791-802; and Sayers et al. (1988) "Strand specific cleavage of phosphorothioate-containing DNA by reaction with restriction endonucleases in the presence of ethidium bromide" Nucl. Acids Res. 16: 803-814); mutagenesis using gapped duplex DNA (Kramer et al. (1984) "The gapped duplex DNA approach to oligonucleotide-directed mutation construction" Nucl. Acids Res. 12: 9441-9456; Kramer & Fritz (1987) Methods in Enzymol. "Oligonucleotide-directed construction of mutations via gapped duplex DNA" 154:350-367; Kramer et al. (1988) "Improved enzymatic in vitro reactions in the gapped duplex DNA approach to oligonucleotide-directed construction of mutations" Nucl. Acids Res. 16: 7207; and Fritz et al. (1988) "Oligonucleotide-directed construction of mutations: a gapped duplex DNA procedure without enzymatic reactions in vitro" Nucl. Acids Res. 16: 6987-6999).

[0273] Additional protocols that can be used include point mismatch repair (Kramer (1984) "Point Mismatch Repair" Cell 38:879-887), mutagenesis using repair-deficient host strains (Carter et al. (1985) "Improved oligonucleotide site-directed mutagenesis using M13 vectors" Nucl. Acids Res. 13: 4431-4443; and Carter (1987) "Improved oligonucleotide-directed mutagenesis using M13 vectors" Methods in Enzymol. 154: 382-403), deletion mutagenesis (Eghtedarzadeh (1986) "Use of oligonucleotides to generate large deletions" Nucl. Acids Res. 14: 5115), restriction-selection and restriction-selection and restriction-purification (Wells et al. (1986) "Importance of hydrogen-bond formation in stabilizing the transition state of subtilisin" Phil. Trans. R. Soc. Lond. A 317: 415-423), mutagenesis by total gene synthesis (Nambiar et al. (1984) "Total synthesis and cloning of a gene coding for the ribonuclease S protein" Science 223: 1299-1301; Sakamar and Khorana (1988) "Total synthesis and expression of a gene for the a-subunit of bovine rod outer segment guanine nucleotide-binding protein (transducin)" Nucl. Acids Res. 14: 6361-6372; Wells et al. (1985) "Cassette mutagenesis: an efficient method for generation of multiple mutations at defined sites" Gene 34:315-323; and Grundstrom et al. (1985) "Oligonucleotide-directed mutagenesis by microscale 'shot-gun' gene synthesis" Nucl. Acids Res. 13: 3305-3316), double-strand break repair (Mandecki (1986); Arnold (1993) "Protein engineering for unusual environments" Current Opinion in Biotechnology 4:450-455. "Oligonucleotide-directed double-strand break repair in plasmids of Escherichia coli: a method for site-specific mutagenesis" Proc. Natl. Acad. Sci. USA, 83:7177-7181). Additional details on many of the above methods can be found in Methods in Enzymology Volume 154, which also describes useful controls for trouble-shooting problems with various mutagenesis methods.

[0274] Protocols that can be used are described, e.g., in U.S. Patent Nos. 5,605,793 to Stemmer (Feb. 25, 1997), "Methods for In Vitro Recombination;" U.S. Pat. No. 5,811,238 to Stemmer et al. (Sep. 22, 1998) "Methods for Generating Polynucleotides having Desired Characteristics by Iterative Selection and Recombination;" U.S. Pat. No. 5,830,721 to Stemmer et al. (Nov. 3, 1998), "DNA Mutagenesis by Random Fragmentation and Reassembly;" U.S. Pat. No. 5,834,252 to Stemmer, et al. (Nov. 10, 1998) "End-Complementary Polymerase Reaction;" U.S. Pat. No. 5,837,458 to Minshull, et al. (Nov. 17, 1998), "Methods and Compositions for Cellular and Metabolic Engineering;" WO 95/22625, Stemmer and Crameri, "Mutagenesis by Random Fragmentation and Reassembly;" WO 96/33207 by Stemmer and Lipschutz "End Complementary Polymerase Chain Reaction;" WO 97/20078 by Stemmer and Crameri "Methods for Generating Polynucleotides having Desired Characteristics by Iterative Selection and Recombination;" WO 97/35966 by Minshull and Stemmer, "Methods and Compositions for Cellular and Metabolic Engineering;" WO 99/41402 by Punnonen et al. "Targeting of Genetic Vaccine Vectors;" WO 99/41383 by Punnonen et al. "Antigen Library Immunization;" WO 99/41369 by Punnonen et al. "Genetic Vaccine Vector Engineering;" WO 99/41368 by Punnonen et al. "Optimization of Immunomodulatory Properties of Genetic Vaccines;" EP 752008 by Stemmer and Crameri, "DNA Mutagenesis by Random Fragmentation and Reassembly;" EP 0932670 by Stemmer "Evolving Cellular DNA Uptake by Recursive Sequence Recombination;" WO 99/23107 by Stemmer et al., "Modification of Virus Tropism and Host Range by Viral Genome Shuffling;" WO 99/21979 by Apt et al., "Human Papillomavirus Vectors;" WO 98/31837 by del Cardayre et al. "Evolution of Whole Cells and Organisms by Recursive Sequence Recombination;" WO 98/27230 by Patten and Stemmer, "Methods and Compositions for Polypeptide Engineering;" WO 98/27230 by Stemmer et al., "Methods for Optimization of Gene Therapy by Recursive Sequence Shuffling and Selection," WO 00/00632, "Methods for Generating Highly Diverse Libraries," WO 00/09679, "Methods for Obtaining in Vitro Recombined Polynucleotide Sequence Banks and Resulting Sequences," WO 98/42832 by Arnold et al., "Recombination of Polynucleotide Sequences Using Random or Defined Primers," WO 99/29902 by Arnold et al., "Method for Creating Polynucleotide and Polypeptide Sequences," WO 98/41653 by Vind, "An in Vitro Method for Construction of a DNA Library," WO 98/41622 by Borchert et al., "Method for Constructing a Library Using DNA Shuffling," and WO 98/42727 by Pati and Zarling, "Sequence Alterations using Homologous Recombination."

[0275] Protocols that can be used (providing details regarding various diversity generating methods) are described, e.g., in U.S. Patent application serial no. (USSN) 09/407,800, "SHUFFLING OF CODON ALTERED GENES" by Patten et al. filed Sep. 28, 1999; "EVOLUTION OF WHOLE CELLS AND ORGANISMS BY RECURSIVE SEQUENCE RECOMBINATION" by del Cardayre et al., United States Patent No. 6,379,964; "OLIGONUCLEOTIDE MEDIATED NUCLEIC ACID RECOMBINATION" by Crameri et al., United States Patent Nos. 6,319,714; 6,368,861; 6,376,246; 6,423,542; 6,426,224 and PCT/US00/01203; "USE OF CODON-VARIED OLIGONUCLEOTIDE SYNTHESIS FOR SYNTHETIC SHUFFLING" by Welch et al., United States Patent No. 6,436,675; "METHODS FOR MAKING CHARACTER

STRINGS, POLYNUCLEOTIDES & POLYPEPTIDES HAVING DESIRED CHARACTERISTICS" by Selifonov et al., filed Jan. 18, 2000, (PCT/US00/01202) and, e.g. "METHODS FOR MAKING CHARACTER STRINGS, POLYNUCLEOTIDES & POLYPEPTIDES HAVING DESIRED CHARACTERISTICS" by Selifonov et al., filed Jul. 18, 2000 (U.S. Ser. No. 09/618,579); "METHODS OF POPULATING DATA STRUCTURES FOR USE IN EVOLUTIONARY SIMULATIONS" by Selifonov and Stemmer, filed Jan. 18, 2000 (PCT/US00/01138); and "SINGLE-STRANDED NUCLEIC ACID TEMPLATE-MEDIATED RECOMBINATION AND NUCLEIC ACID FRAGMENT ISOLATION" by Affholter, filed Sep. 6, 2000 (U.S. Ser. No. 09/656,549); and United States Patent Nos. 6,177,263; 6,153,410.

[0276] Non-stochastic, or "directed evolution," methods include, e.g., gene site saturation mutagenesis (GSSM), synthetic ligation reassembly (SLR), or a combination thereof are used to modify the nucleic acids of the disclosure to generate amylases with new or altered properties (e.g., activity under highly acidic or alkaline conditions, high temperatures, and the like). Polypeptides encoded by the modified nucleic acids can be screened for an activity before testing for proteolytic or other activity. Any testing modality or protocol can be used, e.g., using a capillary array platform. See, e.g., U.S. Patent Nos. 6,361,974; 6,280,926; 5,939,250.

Saturation mutagenesis, or, GSSM

[0277] The disclosure also provides methods for making enzyme using Gene Site Saturation mutagenesis, or, GSSM, as described herein, and also in U.S. Patent Nos. 6,171,820 and 6,579,258.

[0278] In one aspect, codon primers containing a degenerate N,N,G/T sequence are used to introduce point mutations into a polynucleotide, e.g., an amylase or an antibody of the disclosure, so as to generate a set of progeny polypeptides in which a full range of single amino acid substitutions is represented at each amino acid position, e.g., an amino acid residue in an enzyme active site or ligand binding site targeted to be modified. These oligonucleotides can comprise a contiguous first homologous sequence, a degenerate N,N,G/T sequence, and, optionally, a second homologous sequence. The downstream progeny translational products from the use of such oligonucleotides include all possible amino acid changes at each amino acid site along the polypeptide, because the degeneracy of the N,N,G/T sequence includes codons for all 20 amino acids. In one aspect, one such degenerate oligonucleotide (comprised of, e.g., one degenerate N,N,G/T cassette) is used for subjecting each original codon in a parental polynucleotide template to a full range of codon substitutions. In another aspect, at least two degenerate cassettes are used - either in the same oligonucleotide or not, for subjecting at least two original codons in a parental polynucleotide template to a full range of codon substitutions. For example, more than one N,N,G/T sequence can be contained in one oligonucleotide to introduce amino acid mutations at more than one site. This plurality ofN,N,G/T sequences can be directly contiguous, or separated by one or more additional nucleotide sequence(s). In another aspect, oligonucleotides serviceable for introducing additions and deletions can be used either alone or in combination with the codons containing an N,N,G/T sequence, to introduce any combination or permutation of amino acid additions, deletions, and/or substitutions.

[0279] In one aspect, simultaneous mutagenesis of two or more contiguous amino acid positions is done using an oligonucleotide that contains contiguous N,N,G/T triplets, i.e. a degenerate (N,N,G/T)n sequence. In another aspect, degenerate cassettes having less degeneracy than the N,N,G/T sequence are used. For example, it may be desirable in some instances to use (e.g. in an oligonucleotide) a degenerate triplet sequence comprised of only one N, where said N can be in the first second or third position of the triplet. Any other bases including any combinations and permutations thereof can be used in the remaining two positions of the triplet. Alternatively, it may be desirable in some instances to use (e.g. in an oligo) a degenerate N,N,N triplet sequence.

[0280] In one aspect, use of degenerate triplets (e.g., N,N,G/T triplets) allows for systematic and easy generation of a full range of possible natural amino acids (for a total of 20 amino acids) into each and every amino acid position in a polypeptide (in alternative aspects, the methods also include generation of less than all possible substitutions per amino acid residue, or codon, position). For example, for a 100 amino acid polypeptide, 2000 distinct species (i.e. 20 possible amino acids per position X 100 amino acid positions) can be generated. Through the use of an oligonucleotide or set of oligonucleotides containing a degenerate N,N,G/T triplet, 32 individual sequences can code for all 20 possible natural amino acids. Thus, in a reaction vessel in which a parental polynucleotide sequence is subjected to saturation mutagenesis using at least one such oligonucleotide, there are generated 32 distinct progeny polynucleotides encoding 20 distinct polypeptides. In contrast, the use of a non-degenerate oligonucleotide in site-directed mutagenesis leads to only one progeny polypeptide product per reaction vessel. Nondegenerate oligonucleotides can optionally be used in combination with degenerate primers disclosed; for example, nondegenerate oligonucleotides can be used to generate specific point mutations in a working polynucleotide. This provides one means to generate specific silent point mutations, point mutations leading to corresponding amino acid changes, and point mutations that cause the generation of stop codons and the corresponding expression of polypeptide fragments.

[0281] In one aspect, each saturation mutagenesis reaction vessel contains polynucleotides encoding at least 20 progeny polypeptide (e.g., amylases) molecules such that all 20 natural amino acids are represented at the one specific amino acid position corresponding to the codon position mutagenized in the parental polynucleotide (other aspects use

less than all 20 natural combinations). The 32-fold degenerate progeny polypeptides generated from each saturation mutagenesis reaction vessel can be subjected to clonal amplification (e.g. cloned into a suitable host, e.g., E. coli host, using, e.g., an expression vector) and subjected to expression screening. When an individual progeny polypeptide is identified by screening to display a favorable change in property (when compared to the parental polypeptide, such as increased proteolytic activity under alkaline or acidic conditions), it can be sequenced to identify the correspondingly favorable amino acid substitution contained therein.

[0282] In one aspect, upon mutagenizing each and every amino acid position in a parental polypeptide using saturation mutagenesis as disclosed herein, favorable amino acid changes may be identified at more than one amino acid position. One or more new progeny molecules can be generated that contain a combination of all or part of these favorable amino acid substitutions. For example, if 2 specific favorable amino acid changes are identified in each of 3 amino acid positions in a polypeptide, the permutations include 3 possibilities at each position (no change from the original amino acid, and each of two favorable changes) and 3 positions. Thus, there are 3 x 3 x 3 or 27 total possibilities, including 7 that were previously examined - 6 single point mutations (i.e. 2 at each of three positions) and no change at any position.

[0283] In another aspect, site-saturation mutagenesis can be used together with another stochastic or non-stochastic means to vary sequence, e.g., synthetic ligation reassembly (see below), shuffling, chimerization, recombination and other mutagenizing processes and mutagenizing agents. This disclosure provides for the use of any mutagenizing process(es), including saturation mutagenesis, in an iterative manner.

Synthetic Ligation Reassembly (SLR)

[0284] The disclosure provides a non-stochastic gene modification system termed "synthetic ligation reassembly," or simply "SLR," a "directed evolution process," to generate polypeptides, e.g., amylases or antibodies of the invention, with new or altered properties. SLR is a method of ligating oligonucleotide fragments together non-stochastically. This method differs from stochastic oligonucleotide shuffling in that the nucleic acid building blocks are not shuffled, concatenated or chimerized randomly, but rather are assembled non-stochastically. See, e.g., U.S. Patent Nos. 6,773,900; 6,740,506; 6,713,282; 6,635,449; 6,605,449; 6,537,776.

[0285] In one aspect, SLR comprises the following steps: (a) providing a template polynucleotide, wherein the template polynucleotide comprises sequence encoding a homologous gene; (b) providing a plurality of building block polynucleotides, wherein the building block polynucleotides are designed to cross-over reassemble with the template polynucleotide at a predetermined sequence, and a building block polynucleotide comprises a sequence that is a variant of the homologous gene and a sequence homologous to the template polynucleotide flanking the variant sequence; (c) combining a building block polynucleotide with a template polynucleotide such that the building block polynucleotide cross-over reassembles with the template polynucleotide to generate polynucleotides comprising homologous gene sequence variations.

[0286] SLR does not depend on the presence of high levels of homology between polynucleotides to be rearranged. Thus, this method can be used to non-stochastically generate libraries (or sets) of progeny molecules comprised of over 10^{100} different chimeras. SLR can be used to generate libraries comprised of over 10^{1000} different progeny chimeras. Thus, aspects of the present disclosure include non-stochastic methods of producing a set of finalized chimeric nucleic acid molecule shaving an overall assembly order that is chosen by design. This method includes the steps of generating by design a plurality of specific nucleic acid building blocks having serviceable mutually compatible ligatable ends, and assembling these nucleic acid building blocks, such that a designed overall assembly order is achieved.

[0287] The mutually compatible ligatable ends of the nucleic acid building blocks to be assembled are considered to be "serviceable" for this type of ordered assembly if they enable the building blocks to be coupled in predetermined orders. Thus, the overall assembly order in which the nucleic acid building blocks can be coupled is specified by the design of the ligatable ends. If more than one assembly step is to be used, then the overall assembly order in which the nucleic acid building blocks can be coupled is also specified by the sequential order of the assembly step(s). In one aspect, the annealed building pieces are treated with an enzyme, such as a ligase (e.g. T4 DNA ligase), to achieve covalent bonding of the building pieces.

[0288] In one aspect, the design of the oligonucleotide building blocks is obtained by analyzing a set of progenitor nucleic acid sequence templates that serve as a basis for producing a progeny set of finalized chimeric polynucleotides. These parental oligonucleotide templates thus serve as a source of sequence information that aids in the design of the nucleic acid building blocks that are to be mutagenized, e.g., chimerized or shuffled. In one aspect of this method, the sequences of a plurality of parental nucleic acid templates are aligned in order to select one or more demarcation points. The demarcation points can be located at an area of homology, and are comprised of one or more nucleotides. These demarcation points are preferably shared by at least two of the progenitor templates. The demarcation points can thereby be used to delineate the boundaries of oligonucleotide building blocks to be generated in order to rearrange the parental polynucleotides. The demarcation points identified and selected in the progenitor molecules serve as potential chimerization points in the assembly of the final chimeric progeny molecules. A demarcation point can be an area of homology

(comprised of at least one homologous nucleotide base) shared by at least two parental polynucleotide sequences. Alternatively, a demarcation point can be an area of homology that is shared by at least half of the parental polynucleotide sequences, or, it can be an area of homology that is shared by at least two thirds of the parental polynucleotide sequences. Even more preferably a serviceable demarcation points is an area of homology that is shared by at least three fourths of the parental polynucleotide sequences, or, it can be shared by at almost all of the parental polynucleotide sequences. In one aspect, a demarcation point is an area of homology that is shared by all of the parental polynucleotide sequences.

[0289] In one aspect, a ligation reassembly process is performed exhaustively in order to generate an exhaustive library of progeny chimeric polynucleotides. In other words, all possible ordered combinations of the nucleic acid building blocks are represented in the set of finalized chimeric nucleic acid molecules. At the same time, in another aspect, the assembly order (i.e. the order of assembly of each building block in the 5' to 3 sequence of each finalized chimeric nucleic acid) in each combination is by design (or non-stochastic) as described above. Because of the non-stochastic nature of this invention, the possibility of unwanted side products is greatly reduced.

[0290] In another aspect, the ligation reassembly method is performed systematically. For example, the method is performed in order to generate a systematically compartmentalized library of progeny molecules, with compartments that can be screened systematically, e.g. one by one. In other words, through the selective and judicious use of specific nucleic acid building blocks, coupled with the selective and judicious use of sequentially stepped assembly reactions, a design can be achieved where specific sets of progeny products are made in each of several reaction vessels. This allows a systematic examination and screening procedure to be performed. Thus, these methods allow a potentially very large number of progeny molecules to be examined systematically in smaller groups. Because of its ability to perform chimerizations in a manner that is highly flexible yet exhaustive and systematic as well, particularly when there is a low level of homology among the progenitor molecules, these methods provide for the generation of a library (or set) comprised of a large number of progeny molecules. Because of the non-stochastic nature of the instant ligation reassembly, the progeny molecules generated preferably comprise a library of finalized chimeric nucleic acid molecules having an overall assembly order that is chosen by design. The saturation mutagenesis and optimized directed evolution methods also can be used to generate different progeny molecular species. It is appreciated that the disclosure provides freedom of choice and control regarding the selection of demarcation points, the size and number of the nucleic acid building blocks, and the size and design of the couplings. It is appreciated, furthermore, that the requirement for intermolecular homology is highly relaxed for the operability of this disclosure. In fact, demarcation points can even be chosen in areas of little or no intermolecular homology. For example, because of codon wobble, i.e. the degeneracy of codons, nucleotide substitutions can be introduced into nucleic acid building blocks without altering the amino acid originally encoded in the corresponding progenitor template.

[0291] Alternatively, a codon can be altered such that the coding for an originally amino acid is altered. This disclosure provides that such substitutions can be introduced into the nucleic acid building block in order to increase the incidence of intermolecular homologous demarcation points and thus to allow an increased number of couplings to be achieved among the building blocks, which in turn allows a greater number of progeny chimeric molecules to be generated.

[0292] In another aspect, the synthetic nature of the step in which the building blocks are generated allows the design and introduction of nucleotides (e.g., one or more nucleotides, which may be, for example, codons or introns or regulatory sequences) that can later be optionally removed in an in vitro process (e.g. by mutagenesis) or in an in vivo process (e.g. by utilizing the gene splicing ability of a host organism). It is appreciated that in many instances the introduction of these nucleotides may also be desirable for many other reasons in addition to the potential benefit of creating a serviceable demarcation point.

[0293] In one aspect, a nucleic acid building block is used to introduce an intron. Thus, functional introns are introduced into a man-made gene manufactured according to the methods described herein. The artificially introduced intron(s) can be functional in a host cells for gene splicing much in the way that naturally-occurring introns serve functionally in gene splicing.

Optimized Directed Evolution System

[0294] The disclosure provides a non-stochastic gene modification system termed "optimized directed evolution system" to generate polypeptides, e.g., amylases or antibodies of the disclosure, with new or altered properties. Optimized directed evolution is directed to the use of repeated cycles of reductive reassortment, recombination and selection that allow for the directed molecular evolution of nucleic acids through recombination. Optimized directed evolution allows generation of a large population of evolved chimeric sequences, wherein the generated population is significantly enriched for sequences that have a predetermined number of crossover events.

[0295] A crossover event is a point in a chimeric sequence where a shift in sequence occurs from one parental variant to another parental variant. Such a point is normally at the juncture of where oligonucleotides from two parents are ligated together to form a single sequence. This method allows calculation of the correct concentrations of oligonucleotide sequences so that the final chimeric population of sequences is enriched for the chosen number of crossover events.

This provides more control over choosing chimeric variants having a predetermined number of crossover events.

[0296] In addition, this method provides a convenient means for exploring a tremendous amount of the possible protein variant space in comparison to other systems. Previously, if one generated, for example, 10^{13} chimeric molecules during a reaction, it would be extremely difficult to test such a high number of chimeric variants for a particular activity. Moreover, a significant portion of the progeny population would have a very high number of crossover events which resulted in proteins that were less likely to have increased levels of a particular activity. By using these methods, the population of chimerics molecules can be enriched for those variants that have a particular number of crossover events. Thus, although one can still generate 10^{13} chimeric molecules during a reaction, each of the molecules chosen for further analysis most likely has, for example, only three crossover events. Because the resulting progeny population can be skewed to have a predetermined number of crossover events, the boundaries on the functional variety between the chimeric molecules is reduced. This provides a more manageable number of variables when calculating which oligonucleotide from the original parental polynucleotides might be responsible for affecting a particular trait.

[0297] One method for creating a chimeric progeny polynucleotide sequence is to create oligonucleotides corresponding to fragments or portions of each parental sequence. Each oligonucleotide preferably includes a unique region of overlap so that mixing the oligonucleotides together results in a new variant that has each oligonucleotide fragment assembled in the correct order. Alternatively protocols for practicing these methods of the invention can be found in U.S. Patent Nos. 6,773,900; 6,740,506; 6,713,282; 6,635,449; 6,605,449; 6,537,776; 6,361,974.

[0298] The number of oligonucleotides generated for each parental variant bears a relationship to the total number of resulting crossovers in the chimeric molecule that is ultimately created. For example, three parental nucleotide sequence variants might be provided to undergo a ligation reaction in order to find a chimeric variant having, for example, greater activity at high temperature. As one example, a set of 50 oligonucleotide sequences can be generated corresponding to each portions of each parental variant. Accordingly, during the ligation reassembly process there could be up to 50 crossover events within each of the chimeric sequences. The probability that each of the generated chimeric polynucleotides will contain oligonucleotides from each parental variant in alternating order is very low. If each oligonucleotide fragment is present in the ligation reaction in the same molar quantity it is likely that in some positions oligonucleotides from the same parental polynucleotide will ligate next to one another and thus not result in a crossover event. If the concentration of each oligonucleotide from each parent is kept constant during any ligation step in this example, there is a 1/3 chance (assuming 3 parents) that an oligonucleotide from the same parental variant will ligate within the chimeric sequence and produce no crossover.

[0299] Accordingly, a probability density function (PDF) can be determined to predict the population of crossover events that are likely to occur during each step in a ligation reaction given a set number of parental variants, a number of oligonucleotides corresponding to each variant, and the concentrations of each variant during each step in the ligation reaction. The statistics and mathematics behind determining the PDF is described below. By utilizing these methods, one can calculate such a probability density function, and thus enrich the chimeric progeny population for a predetermined number of crossover events resulting from a particular ligation reaction. Moreover, a target number of crossover events can be predetermined, and the system then programmed to calculate the starting quantities of each parental oligonucleotide during each step in the ligation reaction to result in a probability density function that centers on the predetermined number of crossover events. These methods are directed to the use of repeated cycles of reductive reassortment, recombination and selection that allow for the directed molecular evolution of a nucleic acid encoding a polypeptide through recombination. This system allows generation of a large population of evolved chimeric sequences, wherein the generated population is significantly enriched for sequences that have a predetermined number of crossover events. A crossover event is a point in a chimeric sequence where a shift in sequence occurs from one parental variant to another parental variant. Such a point is normally at the juncture of where oligonucleotides from two parents are ligated together to form a single sequence. The method allows calculation of the correct concentrations of oligonucleotide sequences so that the final chimeric population of sequences is enriched for the chosen number of crossover events. This provides more control over choosing chimeric variants having a predetermined number of crossover events.

[0300] In addition, these methods provide a convenient means for exploring a tremendous amount of the possible protein variant space in comparison to other systems. By using the methods described herein, the population of chimerics molecules can be enriched for those variants that have a particular number of crossover events. Thus, although one can still generate 10^{13} chimeric molecules during a reaction, each of the molecules chosen for further analysis most likely has, for example, only three crossover events. Because the resulting progeny population can be skewed to have a predetermined number of crossover events, the boundaries on the functional variety between the chimeric molecules is reduced. This provides a more manageable number of variables when calculating which oligonucleotide from the original parental polynucleotides might be responsible for affecting a particular trait.

[0301] In one aspect, the method creates a chimeric progeny polynucleotide sequence by creating oligonucleotides corresponding to fragments or portions of each parental sequence. Each oligonucleotide preferably includes a unique region of overlap so that mixing the oligonucleotides together results in a new variant that has each oligonucleotide fragment assembled in the correct order. See also U.S. Patent Nos. 6,773,900; 6,740,506; 6,713,282; 6,635,449;

6,605,449; 6,537,776; 6,361,974.

Determining Crossover Events

[0302] Aspects of the disclosure include a system and software that receive a desired crossover probability density function (PDF), the number of parent genes to be reassembled, and the number of fragments in the reassembly as inputs. The output of this program is a "fragment PDF" that can be used to determine a recipe for producing reassembled genes, and the estimated crossover PDF of those genes. The processing described herein is preferably performed in MATLAB™ (The Mathworks, Natick, Massachusetts) a programming language and development environment for technical computing.

Iterative Processes

[0303] In practicing the disclosure, these processes can be iteratively repeated. For example, a nucleic acid (or, the nucleic acid) responsible for an altered or new amylase phenotype is identified, re-isolated, again modified, re-tested for activity. This process can be iteratively repeated until a desired phenotype is engineered. For example, an entire biochemical anabolic or catabolic pathway can be engineered into a cell, including, e.g., starch hydrolysis activity.

[0304] Similarly, if it is determined that a particular oligonucleotide has no affect at all on the desired trait (e.g., a new amylase phenotype), it can be removed as a variable by synthesizing larger parental oligonucleotides that include the sequence to be removed. Since incorporating the sequence within a larger sequence prevents any crossover events, there will no longer be any variation ofthis sequence in the progeny polynucleotides. This iterative practice of determining which oligonucleotides are most related to the desired trait, and which are unrelated, allows more efficient exploration all of the possible protein variants that might be provide a particular trait or activity.

In vivo shuffling

[0305] *In vivo* shuffling of molecules can be used in methods of the disclosure that provide variants of polypeptides of the disclosure, e.g., antibodies, amylases, and the like. *In vivo* shuffling can be performed utilizing the natural property of cells to recombine multimers. While recombination *in vivo* has provided the major natural route to molecular diversity, genetic recombination remains a relatively complex process that involves 1) the recognition of homologies; 2) strand cleavage, strand invasion, and metabolic steps leading to the production of recombinant chiasma; and finally 3) the resolution of chiasma into discrete recombined molecules. The formation of the chiasma requires the recognition of homologous sequences.

[0306] In one aspect, the disclosure provides a method for producing a hybrid polynucleotide from at least a first polynucleotide (e.g., a glucoamylase of the invention) and a second polynucleotide (e.g., an enzyme, such as a glucoamylase of the invention or any other amylase, or, a tag or an epitope). The disclosure can be used to produce a hybrid polynucleotide by introducing at least a first polynucleotide and a second polynucleotide which share at least one region of partial sequence homology into a suitable host cell. The regions of partial sequence homology promote processes which result in sequence reorganization producing a hybrid polynucleotide. The term "hybrid polynucleotide", as used herein, is any nucleotide sequence which results from the method of the present disclosure and contains sequence from at least two original polynucleotide sequences. Such hybrid polynucleotides can result from intermolecular recombination events which promote sequence integration between DNA molecules. In addition, such hybrid polynucleotides can result from intramolecular reductive reassortment processes which utilize repeated sequences to alter a nucleotide sequence within a DNA molecule.

Producing sequence variants

[0307] The disclosure also provides additional methods for making sequence variants of the nucleic acid (e.g., amylase) sequences of the disclosure. The disclosure also provides additional methods for isolating amylases using the nucleic acids and polypeptides of the disclosure. In one aspect, the disclosure provides for variants of an amylase coding sequence (e.g., a gene, cDNA or message) of the disclosure, which can be altered by any means, including, e.g., random or stochastic methods, or, non-stochastic, or "directed evolution," methods, as described above.

[0308] The isolated variants may be naturally occurring. Variants can also be created *in vitro.* Variants may be created using genetic engineering techniques such as site directed mutagenesis, random chemical mutagenesis, Exonuclease III deletion procedures, and standard cloning techniques. Alternatively, such variants, fragments, analogs, or derivatives may be created using chemical synthesis or modification procedures. Other methods of making variants are also familiar to those skilled in the art. These include procedures in which nucleic acid sequences obtained from natural isolates are modified to generate nucleic acids which encode polypeptides having characteristics which enhance their value in

industrial or laboratory applications. In such procedures, a large number of variant sequences having one or more nucleotide differences with respect to the sequence obtained from the natural isolate are generated and characterized. These nucleotide differences can result in amino acid changes with respect to the polypeptides encoded by the nucleic acids from the natural isolates.

[0309] For example, variants may be created using error prone PCR. In error prone PCR, PCR is performed under conditions where the copying fidelity of the DNA polymerase is low, such that a high rate of point mutations is obtained along the entire length of the PCR product. Error prone PCR is described, e.g., in Leung, D.W., et al., Technique, 1:11-15, 1989) and Caldwell, R. C. & Joyce G.F., PCR Methods Applic., 2:28-33, 1992. Briefly, in such procedures, nucleic acids to be mutagenized are mixed with PCR primers, reaction buffer, $MgCl_2$, $MnCl_2$, Taq polymerase and an appropriate concentration of dNTPs for achieving a high rate of point mutation along the entire length of the PCR product. For example, the reaction may be performed using 20 fmoles of nucleic acid to be mutagenized, 30 pmole of each PCR primer, a reaction buffer comprising 50mM KCl, 10mM Tris HCl (pH 8.3) and 0.01% gelatin, 7mM MgCl2, 0.5mM $MnCl_2$, 5 units of Taq polymerase, 0.2mM dGTP, 0.2mM dATP, 1mM dCTP, and 1mM dTTP. PCR may be performed for 30 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min. However, it will be appreciated that these parameters may be varied as appropriate. The mutagenized nucleic acids are cloned into an appropriate vector and the activities of the polypeptides encoded by the mutagenized nucleic acids is evaluated.

[0310] Variants may also be created using oligonucleotide directed mutagenesis to generate site-specific mutations in any cloned DNA of interest. Oligonucleotide mutagenesis is described, e.g., in Reidhaar-Olson (1988) Science 241:53-57. Briefly, in such procedures a plurality of double stranded oligonucleotides bearing one or more mutations to be introduced into the cloned DNA are synthesized and inserted into the cloned DNA to be mutagenized. Clones containing the mutagenized DNA are recovered and the activities of the polypeptides they encode are assessed.

[0311] Another method for generating variants is assembly PCR. Assembly PCR involves the assembly of a PCR product from a mixture of small DNA fragments. A large number of different PCR reactions occur in parallel in the same vial, with the products of one reaction priming the products of another reaction. Assembly PCR is described in, e.g., U.S. Patent No. 5,965,408.

[0312] Still another method of generating variants is sexual PCR mutagenesis. In sexual PCR mutagenesis, forced homologous recombination occurs between DNA molecules of different but highly related DNA sequence in vitro, as a result of random fragmentation of the DNA molecule based on sequence homology, followed by fixation of the crossover by primer extension in a PCR reaction. Sexual PCR mutagenesis is described, e.g., in Stemmer (1994) Proc. Natl. Acad. Sci. USA 91:10747-10751. Briefly, in such procedures a plurality of nucleic acids to be recombined are digested with DNase to generate fragments having an average size of 50-200 nucleotides. Fragments of the desired average size are purified and resuspended in a PCR mixture. PCR is conducted under conditions which facilitate recombination between the nucleic acid fragments. For example, PCR may be performed by resuspending the purified fragments at a concentration of 10-30ng/:l in a solution of 0.2mM of each dNTP, 2.2mM $MgCl_2$, 50mM KCL, 10mM Tris HCl, pH 9.0, and 0.1% Triton X-100. 2.5 units of Taq polymerase per 100:1 of reaction mixture is added and PCR is performed using the following regime: 94°C for 60 seconds, 94°C for 30 seconds, 50-55°C for 30 seconds, 72°C for 30 seconds (30-45 times) and 72°C for 5 minutes. However, it will be appreciated that these parameters may be varied as appropriate. In some aspects, oligonucleotides may be included in the PCR reactions. In other aspects, the Klenow fragment of DNA polymerase I may be used in a first set of PCR reactions and Taq polymerase may be used in a subsequent set of PCR reactions. Recombinant sequences are isolated and the activities of the polypeptides they encode are assessed.

[0313] Variants may also be created by *in vivo* mutagenesis. In some aspects, random mutations in a sequence of interest are generated by propagating the sequence of interest in a bacterial strain, such as an *E. coli* strain, which carries mutations in one or more of the DNA repair pathways. Such "mutator" strains have a higher random mutation rate than that of a wild-type parent. Propagating the DNA in one of these strains will eventually generate random mutations within the DNA. Mutator strains suitable for use for in vivo mutagenesis are described, e.g., in PCT Publication No. WO 91/16427.

[0314] Variants may also be generated using cassette mutagenesis. In cassette mutagenesis a small region of a double stranded DNA molecule is replaced with a synthetic oligonucleotide "cassette" that differs from the native sequence. The oligonucleotide often contains completely and/or partially randomized native sequence.

[0315] Recursive ensemble mutagenesis may also be used to generate variants. Recursive ensemble mutagenesis is an algorithm for protein engineering (protein mutagenesis) developed to produce diverse populations of phenotypically related mutants whose members differ in amino acid sequence. This method uses a feedback mechanism to control successive rounds of combinatorial cassette mutagenesis. Recursive ensemble mutagenesis is described, e.g., in Arkin (1992) Proc. Natl. Acad. Sci. USA 89:7811-7815.

[0316] In some aspects, variants are created using exponential ensemble mutagenesis. Exponential ensemble mutagenesis is a process for generating combinatorial libraries with a high percentage of unique and functional mutants, wherein small groups of residues are randomized in parallel to identify, at each altered position, amino acids which lead to functional proteins. Exponential ensemble mutagenesis is described, e.g., in Delegrave (1993) Biotechnology Res.

11:1548-1552. Random and site-directed mutagenesis are described, e.g., in Arnold (1993) Current Opinion in Biotechnology 4:450-455.

[0317] In some aspects, the variants are created using shuffling procedures wherein portions of a plurality of nucleic acids which encode distinct polypeptides are fused together to create chimeric nucleic acid sequences which encode chimeric polypeptides as described in, e.g., U.S. Patent Nos. 5,965,408; 5,939,250 (see also discussion, above).

[0318] The disclosure also provides variants of polypeptides of the disclosure (e.g., amylases) comprising sequences in which one or more of the amino acid residues (e.g., of an exemplary polypeptide of the invention) are substituted with a conserved or non-conserved amino acid residue (e.g., a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code. Conservative substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Thus, polypeptides of the disclosure include those with conservative substitutions of sequences of the invention, e.g., the exemplary polypeptides of the invention, including but not limited to the following replacements: replacements of an aliphatic amino acid such as Alanine, Valine, Leucine and Isoleucine with another aliphatic amino acid; replacement of a Serine with a Threonine or vice versa; replacement of an acidic residue such as Aspartic acid and Glutamic acid with another acidic residue; replacement of a residue bearing an amide group, such as Asparagine and Glutamine, with another residue bearing an amide group; exchange of a basic residue such as Lysine and Arginine with another basic residue; and replacement of an aromatic residue such as Phenylalanine, Tyrosine with another aromatic residue. Other variants are those in which one or more of the amino acid residues of the polypeptides of the invention includes a substituent group. A conservative amino acid substitution can also comprise substituting one amino acid for another of the same class (e.g., substitution of one hydrophobic amino acid, such as isoleucine, valine, leucine, or methionine, for another, or substitution of one polar amino acid for another, such as substitution of arginine for lysine, glutamic acid for aspartic acid or glutamine for asparagine). One or more amino acids can be deleted, for example, from an amylase, resulting in modification of the structure of the polypeptide, without significantly altering its biological activity. For example, amino- or carboxyl-terminal amino acids that are not required for amylase activity can be removed.

[0319] Other variants are those in which the polypeptide is associated with another compound, such as a compound to increase the half-life of the polypeptide, for example, polyethylene glycol.

[0320] Additional variants are those in which additional amino acids are fused to the polypeptide, such as a leader sequence, a secretory sequence, a proprotein sequence or a sequence which facilitates purification, enrichment, or stabilization of the polypeptide.

[0321] In some aspects, the variants, fragments, derivatives and analogs of the polypeptides of the disclosure retain the same biological function or activity as the exemplary polypeptides, e.g., amylase activity, as described herein. In other aspects, the variant, fragment, derivative, or analog includes a proprotein, such that the variant, fragment, derivative, or analog can be activated by cleavage of the proprotein portion to produce an active polypeptide.

Optimizing codons to achieve high levels of protein expression in host cells

[0322] The disclosure provides methods for modifying amylase-encoding and glucoamylase-encoding nucleic acids to modify codon usage, and codon optimized amylase-encoding and glucoamylase-encoding nucleic acids, including SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81 and SEQ ID NO:82; these exemplary amylase-encoding and glucoamylase-encoding nucleic acids were generated as discussed in detail in Example 28, below, from SEQ ID NO:51, SEQ ID NO:3, SEQ ID NO:47 and SEQ ID NO:25, respectively:

Enzyme	Wild-type SEQ ID NO:	Codon-optimized SEQ ID NO:
Amylase	51	79
Amylase	3	80
Glucoamylase	47	81
Glucoamylase	25	82

[0323] In one aspect, the disclosure provides methods for modifying codons in a nucleic acid encoding an amylase to increase or decrease its expression in a host cell. The disclosure also provides nucleic acids encoding an amylase modified to increase its expression in a host cell, amylase so modified, and methods of making the modified amylases. The method comprises identifying a "non-preferred" or a "less preferred" codon in amylase-encoding and glucoamylase-encoding nucleic acid and replacing one or more of these non-preferred or less preferred codons with a "preferred codon" encoding the same amino acid as the replaced codon and at least one non-preferred or less preferred codon in the nucleic acid has been replaced by a preferred codon encoding the same amino acid. A preferred codon is a codon over-

represented in coding sequences in genes in the host cell and a non-preferred or less preferred codon is a codon under-represented in coding sequences in genes in the host cell.

[0324] Host cells for expressing the nucleic acids, expression cassettes and vectors of the invention include bacteria, yeast, fungi, plant cells, insect cells and mammalian cells. Thus, the disclosure provides methods for optimizing codon usage in all of these cells, codon-altered nucleic acids and polypeptides made by the codon-altered nucleic acids. Exemplary host cells include gram negative bacteria, such as any species from the genus *Escherichia* or *Pseudomonas, e.g., Escherichia coli* and *Pseudomonas fluorescens*); or gram positive bacteria, such as any species from the genus *Bacillus, Streptomyces, Lactococcus, Lactobacillus,* e.g., *Bacillus cereus, Lactobacillus gasseri, Lactococcus lactis, Lactococcus cremoris*, *Bacillus subtilis.* Exemplary host cells also include eukaryotic organisms, e.g., various yeast, such as *Schizosaccharomyces* sp., *Aspergillus* sp., *Hansenula* sp., *Kluyveromyces* sp., *Pichia* sp. *and Saccharomyces* sp., including, e.g., *Saccharomyces cerevisiae, Schizosaccharomyces pombe*, *Pichia pastoris,* and *Kluyveromyces lactis*, *Hansenula polymorpha, Aspergillus niger,* and mammalian cells and cell lines and insect cells and cell lines. Thus, the disclosure also includes nucleic acids and polypeptides optimized for expression in these organisms and species.

[0325] For example, the codons of a nucleic acid encoding an amylase isolated from a bacterial cell are modified such that the nucleic acid is optimally expressed in a bacterial cell different from the bacteria from which the amylase was derived, a yeast, a fungi, a plant cell, an insect cell or a mammalian cell. Methods for optimizing codons are well known in the art, see, e.g., U.S. Patent No. 5,795,737; Baca (2000) Int. J. Parasitol. 30:113-118; Hale (1998) Protein Expr. Purif. 12:185-188; Narum (2001) Infect. Immun. 69:7250-7253. See also Narum (2001) Infect. Immun. 69:7250-7253, describing optimizing codons in mouse systems; Outchkourov (2002) Protein Expr. Purif. 24:18-24, describing optimizing codons in yeast; Feng (2000) Biochemistry 39:15399-15409, describing optimizing codons in *E. coli*; Humphreys (2000) Protein Expr. Purif. 20:252-264, describing optimizing codon usage that affects secretion in *E. coli.*

Transgenic non-human animals

[0326] The disclosure provides transgenic non-human animals comprising a nucleic acid, a polypeptide (e.g., an amylase), an expression cassette or vector or a transfected or transformed cell of the invention. The disclosure also provides methods of making and using these transgenic non-human animals.

[0327] The transgenic non-human animals can be, e.g., goats, rabbits, sheep, pigs, cows, rats and mice, comprising the nucleic acids of the disclosure. These animals can be used, e.g., as *in vivo* models to study amylase activity, or, as models to screen for agents that change the amylase activity *in vivo.* The coding sequences for the polypeptides to be expressed in the transgenic non-human animals can be designed to be constitutive, or, under the control of tissue-specific, developmental-specific or inducible transcriptional regulatory factors. Transgenic non-human animals can be designed and generated using any method known in the art; see, e.g., U.S. Patent Nos. 6,211,428; 6,187,992; 6,156,952; 6,118,044; 6,111,166; 6,107,541; 5,959,171; 5,922,854; 5,892,070; 5,880,327; 5,891,698; 5,639,940; 5,573,933; 5,387,742; 5,087,571, describing making and using transformed cells and eggs and transgenic mice, rats, rabbits, sheep, pigs and cows. See also, e.g., Pollock (1999) J. Immunol. Methods 231:147-157, describing the production of recombinant proteins in the milk of transgenic dairy animals; Baguisi (1999) Nat. Biotechnol. 17:456-461, demonstrating the production of transgenic goats. U.S. Patent No. 6,211,428, describes making and using transgenic non-human mammals which express in their brains a nucleic acid construct comprising a DNA sequence. U.S. Patent No. 5,387,742, describes injecting cloned recombinant or synthetic DNA sequences into fertilized mouse eggs, implanting the injected eggs in pseudo-pregnant females, and growing to term transgenic mice whose cells express proteins related to the pathology of Alzheimer's disease. U.S. Patent No. 6,187,992, describes making and using a transgenic mouse whose genome comprises a disruption of the gene encoding amyloid precursor protein (APP).

[0328] "Knockout animals" can also be used. For example, in one aspect, the transgenic or modified animals of the disclosure comprise a "knockout animal," e.g., a "knockout mouse," engineered not to express an endogenous gene, which is replaced with a gene expressing an amylase and/or a glucoamylase of the invention, or, a fusion protein comprising an amylase and/or a glucoamylase of the invention.

Transgenic Plants and Seeds

[0329] The disclosure provides transgenic plants and seeds comprising a nucleic acid, a polypeptide, an expression cassette or vector or a transfected or transformed cell of the invention. The disclosure also provides plant products, e.g., oils, seeds, leaves, extracts and the like, comprising a nucleic acid and/or a polypeptide of the invention. The transgenic plant can be dicotyledonous (a dicot) or monocotyledonous (a monocot). The disclosure provides transgenic plants with a modified taste, solids content and/or texture, wherein that modification is generated by expressing at least one enzyme of the invention either constitutively or selectively in the transgenic plant (or seed, or fruit, etc.), as described, e.g., in U.S. Pat. Application No. 20060195940.

[0330] The disclosure also provides methods of making and using these transgenic plants and seeds. The transgenic

plant or plant cell expressing a polypeptide of the present invention may be constructed in accordance with any method known in the art. See, for example, U.S. Pat. No. 6,309,872.

[0331] Nucleic acids and expression constructs of the invention can be introduced into a plant cell by any means. The term "introducing" in the context of a polynucleotide, for example, a nucleotide construct of interest, is intended to mean presenting to the plant the polynucleotide in such a manner that the polynucleotide gains access to the interior of a cell of the plant. Where more than one polynucleotide is to be introduced, these polynucleotides can be assembled as part of a single nucleotide construct, or as separate nucleotide constructs, and can be located on the same or different transformation vectors. Accordingly, these polynucleotides can be introduced into the host cell of interest in a single transformation event, in separate transformation events, or, for example, in plants, as part of a breeding protocol. The methods of the disclosure do not depend on a particular method for introducing one or more polynucleotides into a plant, only that the polynucleotide(s) gains access to the interior of at least one cell of the plant. Methods for introducing polynucleotides into plants are known in the art including, but not limited to, transient transformation methods, stable transformation methods, and virus-mediated methods.

[0332] "Transient transformation" in the context of a polynucleotide is intended to mean that a polynucleotide is introduced into the plant and does not integrate into the genome of the plant.

[0333] By "stably introducing" or "stably introduced" in the context of a polynucleotide introduced into a plant is intended the introduced polynucleotide is stably incorporated into the plant genome, and thus the plant is stably transformed with the polynucleotide.

[0334] In alternative embodiments, "stable transformation" or "stably transformed" is intended to mean that a polynucleotide, for example, a nucleotide construct described herein, introduced into a plant integrates into the genome of the plant and is capable of being inherited by the progeny thereof, more particularly, by the progeny of multiple successive generations. Introduction into the genome of a desired plant can be such that the enzyme is regulated by endogenous transcriptional or translational control elements. Transformation techniques for both monocotyledons and dicotyledons are well known in the art.

[0335] The nucleic acids of the invention can be used to confer desired traits on essentially any plant. Nucleic acids of the disclosure can be used to manipulate metabolic pathways of a plant in order to optimize or alter host's expression of glucanase, mannanase, or xylanase. They can change amylase, glucoamylase, glucanase, mannanase, or xylanase activity in a plant. Alternatively, a glucoamylase, of the invention can be used in production of a transgenic plant to produce a compound not naturally produced by that plant. This can lower production costs or create a novel product. In one embodiment, the enzyme of the invention may be expressed in such a way that the enzyme will not come in contact with it's substrate until desired. For example, an enzyme of the invention may be targeted and retained in the endoplasmic reticulum of a plant cell. Retention of the enzyme, in the endoplasmic reticulum of the cell, will prevent the enzyme from coming in contact with its substrate. The enzyme and substrate may then be brought into contact through any means able to disrupt the subcellular architecture, such as, grinding, milling, heating, and the like. See, WO 98/11235, WO 2003/18766, and WO 2005/096704.

[0336] Selectable marker genes can be added to the gene construct in order to identify plant cells or tissues that have successfully integrated the transgene. This may be necessary because achieving incorporation and expression of genes in plant cells is a rare event, occurring in just a few percent of the targeted tissues or cells. Selectable marker genes encode proteins that provide resistance to agents that are normally toxic to plants, such as antibiotics or herbicides. Only plant cells that have integrated the selectable marker gene will survive when grown on a medium containing the appropriate antibiotic or herbicide. Selection markers used routinely in transformation include the nptII gene, which confers resistance to kanamycin and related antibiotics (Messing & Vierra. Gene 19: 259-268 (1982); Bevan et. al., Nature 304:184-187 (1983)), the bar gene, which confers resistance to the herbicide phosphinothricin (White et. al., Nucl. Acids Res 18: 1062 (1990), Spencer et. al. Theor. Appl. Genet 79: 625-631 (1990)), the hph gene, which confers resistance to the antibiotic hygromycin (Blochinger & Diggelmann, Mol Cell Biol 4: 2929-2931), the dhfr gene, which confers resistance to methatrexate (Bourouis et. al., EMBO J. 2(7): 1099-1104 (1983)), the EPSPS gene, which confers resistance to glyphosate (U.S. Pat. Nos. 4,940,935 and 5,188,642),

[0337] Alternatively, transgenic plant material can be identified through a positive selection system, such as, the system utilizing the mannose-6-phosphate isomerase gene, which provides the ability to metabolize mannose (U.S. Pat. Nos. 5,767,378 and 5,994,629).

[0338] In one aspect, making transgenic plants or seeds comprises incorporating sequences of the invention and, optionally, marker genes into a target expression construct (e.g., a plasmid), along with positioning of the promoter and the terminator sequences. This can involve transferring the modified gene into the plant through a suitable method. One or more of the sequences of the invention may be combined with sequences that confer resistance to insect, disease, drought, increase yield, improve nutritional quality of the grain, improve ethanol yield and the like.

[0339] For example, a construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts, or the constructs can be introduced directly to plant tissue using ballistic methods, such as DNA particle bombardment. For example, see, e.g., Christou (1997) Plant Mol.

Biol. 35:197-203; Pawlowski (1996) Mol. Biotechnol. 6:17-30; Klein (1987) Nature 327:70-73; Takumi (1997) Genes Genet. Syst. 72:63-69, discussing use of particle bombardment to introduce transgenes into wheat; and Adam (1997) supra, for use of particle bombardment to introduce YACs into plant cells. For example, Rinehart (1997) supra, used particle bombardment to generate transgenic cotton plants. Apparatus for accelerating particles is described U.S. Pat. No. 5,015,580; and, the commercially available BioRad (Biolistics) PDS-2000 particle acceleration instrument; see also, John, U.S. Patent No. 5,608,148; and Ellis, U.S. Patent No. 5, 681,730, describing particle-mediated transformation of gymnosperms.

[0340] In one aspect, protoplasts can be immobilized and injected with a nucleic acid, e.g., an expression construct. Although plant regeneration from protoplasts is not easy with cereals, plant regeneration is possible in legumes using somatic embryogenesis from protoplast derived callus. Organized tissues can be transformed with naked DNA using gene gun technique, where DNA is coated on tungsten microprojectiles, shot 1/100th the size of cells, which carry the DNA deep into cells and organelles. Transformed tissue is then induced to regenerate, usually by somatic embryogenesis. This technique has been successful in several cereal species including maize and rice.

[0341] Nucleic acids, e.g., expression constructs, can also be introduced in to plant cells using recombinant viruses. Plant cells can be transformed using viral vectors, such as, e.g., tobacco mosaic virus derived vectors (Rouwendal (1997) Plant Mol. Biol. 33:989-999), see Porta (1996) "Use of viral replicons for the expression of genes in plants," Mol. Biotechnol. 5:209-221.

[0342] Alternatively, nucleic acids, e.g., an expression construct, can be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria. *Agrobacterium tumefaciens*-mediated transformation techniques, including disarming and use of binary vectors, are well described in the scientific literature. See, e.g., Horsch (1984) Science 233:496-498; Fraley (1983) Proc. Natl. Acad. Sci. USA 80:4803 (1983); Gene Transfer to Plants, Potrykus, ed. (Springerlag, Berlin 1995). The DNA in an A. *tumefaciens* cell is contained in the bacterial chromosome as well as in another structure known as a Ti

[0343] (tumor-inducing) plasmid. The Ti plasmid contains a stretch of DNA termed T-DNA (~20 kb long) that is transferred to the plant cell in the infection process and a series of vir (virulence) genes that direct the infection process. *A. tumefaciens* can only infect a plant through wounds: when a plant root or stem is wounded it gives off certain chemical signals, in response to which, the vir genes of *A. tumefaciens* become activated and direct a series of events necessary for the transfer of the T-DNA from the Ti plasmid to the plant's chromosome. The T-DNA then enters the plant cell through the wound. One speculation is that the T-DNA waits until the plant DNA is being replicated or transcribed, then inserts itself into the exposed plant DNA. In order to use *A. tumefaciens* as a transgene vector, the tumor-inducing section of T-DNA have to be removed, while retaining the T-DNA border regions and the vir genes. The transgene is then inserted between the T-DNA border regions, where it is transferred to the plant cell and becomes integrated into the plant's chromosomes.

[0344] The disclosure provides for the transformation of monocotyledonous plants using the nucleic acids of the invention, including important cereals, see Hiei (1997) Plant Mol. Biol. 35:205-218. See also, e.g., Horsch, Science (1984) 233:496; Fraley (1983) Proc. Natl. Acad. Sci USA 80:4803; Thykjaer (1997) supra; Park (1996) Plant Mol. Biol. 32:1135-1148, discussing T-DNA integration into genomic DNA. See also D'Halluin, U.S. Patent No. 5,712,135, describing a process for the stable integration of a DNA comprising a gene that is functional in a cell of a cereal, or other monocotyledonous plant.

[0345] In one aspect, the third step can involve selection and regeneration of whole plants capable of transmitting the incorporated target gene to the next generation. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker that has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans et al., Protoplasts Isolation and Culture, Handbook of Plant Cell Culture, pp. 124-176, MacMillilan Publishing Company, New York, 1983; and Binding, Regeneration of Plants, Plant Protoplasts, pp. 21-73, CRC Press, Boca Raton, 1985. Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee (1987) Ann. Rev. of Plant Phys. 38:467-486. To obtain whole plants from transgenic tissues such as immature embryos, they can be grown under controlled environmental conditions in a series of media containing nutrients and hormones, a process known as tissue culture. Once whole plants are generated and produce seed, evaluation of the progeny begins.

[0346] After the expression cassette is stably incorporated in transgenic plants, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed. See, for example, Welsh J. R., Fundamentals of Plant Genetics and Breeding, John Wiley & Sons, NY (1981); Crop Breeding, Wood D. R. (Ed.) American Society of Agronomy Madison, Wis. (1983); Mayo O., The Theory of Plant Breeding, Second Edition, Clarendon Press, Oxford (1987); Singh, D. P., Breeding for Resistance to Diseases and Insect Pests, Springer-Verlag, NY (1986); and Wricke and Weber, Quantitative Genetics and Selection Plant Breeding, Walter de Gruyter and Co., Berlin (1986).

[0347] Since transgenic expression of the nucleic acids of the invention leads to phenotypic changes, plants comprising the recombinant nucleic acids of the invention can be sexually crossed with a second plant to obtain a final product. Thus, the seed of the disclosure can be derived from a cross between two transgenic plants of the disclosure, or a cross between a plant of the disclosure and another plant. The desired effects (e.g., expression of the polypeptides of the invention to produce a plant in which flowering behavior is altered) can be enhanced when both parental plants express the polypeptides (e.g., a glucanase, mannanase, or xylanase) of the disclosure. The desired effects can be passed to future plant generations by standard propagation means.

[0348] Any plant may be used for introduction of the nucleotide of interest, including, but not limited to, corn or maize (*Zea ways*), Brassica sp. (e.g., *B. napus, B. rapa*, *B. juncea*), particularly those Brassica species useful as sources of seed oil, such as canola, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), *sorghum* (*Sorghum bicolor, Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger *millet* (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), *cotton* (*Gossypium barbadense, Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (Cofea spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), *fig* (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

[0349] Vegetables may include tomatoes (*Lycopersicon esculentacm*), lettuce (e.g., *Lactuca sativa),* green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus Cucumis such as *cucumber (C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals may include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), canna (*Cannaceae spp.*) and chrysanthemum. Conifers that may be employed, including, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*), Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka *spruce (Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver *fir (Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). Leguminous plants may include, but are not limited to, beans and peas. Beans may include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, etc. Legumes may include, but are not limited to, Arachis, e.g., peanuts, Vicia, e.g., crown vetch, hairy vetch, adzuki bean, mung bean, and chickpea, Lupinus, e.g., lupine, trifolium, Phaseolus, e.g., common bean and lima bean, Pisum, e.g., field bean, Melilotus, e.g., clover, Medicago, e.g., alfalfa, Lotus, e.g., trefoil, lens, e.g., lentil, and false indigo. Forage and turf grasses may include alfalfa, switchgrass (*Panicum* virgatum), *Miscanthus*, orchard grass, tall fescue, perennial ryegrass, creeping bent grass, and redtop.

[0350] Plants of particular interest may include crop plants and plants used to produce energy or fuel, for example, maize, alfalfa, sunflower, Brassica, soybean, cotton, safflower, peanut, sorghum, wheat, oat, rye, millet, barley, rice, conifers, grasses, e.g., switch grass and *Miscanthus*, legume crops, e.g., pea, bean and soybean, starchy tuber/roots, e.g., potato, sweet potato, cassava, taro, canna, sugar beet, sugar cane and the like.

[0351] In alternative embodiments, the nucleic acids of the invention are expressed in plants which contain fiber cells, including, e.g., cotton, silk cotton tree (Kapok, Ceiba pentandra), desert willow, creosote bush, winterfat, balsa, ramie, kenaf, hemp, roselle, jute, sisal abaca and flax. In alternative embodiments, the transgenic plants of the disclosure can be members of the genus *Gossypium,* including members of any *Gossypium* species, such as *G. arboreum;. G. herbaceum*, *G. barbadense,* and *G.*

hirsutum.

[0352] The disclosure also provides transgenic plants to be used for producing large amounts of the polypeptides (e.g., a glucanase, mannanase, or xylanase or antibody) of the disclosure. For example, see Palmgren (1997) Trends Genet. 13:348; Chong (1997) Transgenic Res. 6:289-296 (producing human milk protein beta-casein in transgenic potato plants using an auxin-inducible, bidirectional mannopine synthase (mas1',2') promoter with *Agrobacterium tumefaciens*-mediated leaf disc transformation methods).

[0353] Using known procedures, one of skill can screen for plants of the disclosure by detecting the increase or decrease of transgene mRNA or protein in transgenic plants. Means for detecting and quantitation of mRNAs or proteins are well known in the art.

Polypeptides and peptides

[0354] In one aspect, the invention provides isolated, synthetic or recombinant polypeptides having glucoamylase activity having a sequence identity (e.g., at least 99%, or more, or complete (100%) sequence identity) to the sequence of the invention, SEQ ID NO:48,

[0355] In one embodiment, the polypeptides of the invention can catalyze the hydrolysis of polysaccharides comprising glucose monomers, such as starch (a polymer of glucose monomers joined by 1,4-alpha or 1,6- alpha linkages). In one aspect, the polypeptide has a glucoamylase activity. Glucoamylases of the invention include polypeptides having a glucoamylase activity. An amylase activity of the invention includes polypeptides having glucoamylase activity, such as the ability to hydrolyze glucose polymers linked by α-1,4-and α-1,6-glucosidic bonds. In one aspect, the polypeptides of the invention have glucoamylase activity, hydrolyzing internal α-1,4-glucosidic linkages to yield smaller molecular weight malto-dextrins. An amylase activity of the invention also includes glucan 1,4-α-glucosidase activity; or, 1,4-α-D-glucan glucohydrolase, commonly called glucoamylase but also called amyloglucosidase and γ-amylase that, in one aspect, releases β-D-glucose from 1,4-α-, 1,6-α- and 1,3-α-linked glucans.

[0356] In one embodiment, the polypeptides of the invention can be used to generate an antibody that specifically binds to (is specific for) a polypeptide of the invention, e.g., an exemplary enzyme of the invention).

[0357] In one aspect, the glucoamylase activity of a polypeptide of the invention comprises catalysis of the hydrolysis of glucosidic bonds. The glucoamylase activity can comprise catalyzing the step-wise hydrolytic release of D-glucose from the non-reducing ends of starch or other related dextrins. The glucoamylase activity can comprise a 1,4-α-D-glucan glucohydralase activity. The glucoamylase activity can comprise catalysis of the hydrolysis of malto-dextrins resulting in the generation of free glucose. The glucoamylase activity can comprise an exoamylase activity. The glucoamylase activity can comprise an α-amylase or a β-amylase activity. The hydrolyzed glucosidic bonds can comprise α-1,4-glucosidic bonds or α-1,6-glucosidic bonds. The glucoamylase activity can comprise hydrolyzing glucosidic bonds in a polysaccharide, oligosaccharide or starch. The glucoamylase activity can further comprise hydrolyzing glucosidic bonds in the starch to produce maltodextrines. The glucoamylase activity can comprise cleaving a maltose or a D-glucose unit from non-reducing end of the polysaccharide, oligosaccharide or starch.

[0358] In one aspect, the disclosure provides alpha-amylases (α-amylases) that are endo-acting enzymes that can hydrolyze starch, a polymer of glucose monomers joined by 1,4-alpha or 1,6- alpha linkages, to short maltodextrins. In one aspect, the invention provides glucoamylases that are exo-acting hydro lases that can release beta-D-glucose from the non-reducing ends of starch and related saccharides. Glucoamylases of this invention can be used commercially to liquefy and saccharify starch during ethanol production using processes such as the dry milling process. In one aspect of a process of the invention, the dry milling process, ground whole corn (the mash) is subjected to an elevated temperature (to promote gelatinization of starch) and hydrolyzed by one or more thermostable amylases, including at least one enzyme of the disclosure, resulting in polysaccharide, e.g., starch, liquefaction. In one aspect, the hydrolyzed polysaccharide, e.g., starch, is further digested by a glucoamylase of the invention, which can be added after the mash temperature cools down; and in one aspect, the glucose released from the polysaccharide, e.g., starch, is fermented to ethanol by yeast added at the conclusion of the polysaccharide, e.g., starch, hydrolysis process, or during saccharification of the polysaccharide, e.g., starch,.

[0359] In one aspect, glucoamylases) of the invention hydrolyze internal polysaccharide bonds, e.g., α-1,4- and 1,6-glucosidic bonds in a polysaccharide, oligosaccharide or starch, to produce smaller molecular weight maltodextrines. In one aspect, this hydrolysis is largely at random. Thus, the invention provides methods for producing smaller molecular weight maltodextrines. Glucosidases of the invention can be used in laboratory and industrial settings to hydrolyze a polysaccharide, oligosaccharide or starch, or any maltodextrine-comprising compound for a variety of purposes. These glucosidases can be used alone to provide specific hydrolysis or can be combined with other glucosidases to provide a "cocktail" with a broad spectrum of activity. Exemplary uses include the removal or partial or complete hydrolysis of a polysaccharide, oligosaccharide or starch, or any maltodextrine-comprising compound from biological, food, animal feed, pharmaceutical or industrial samples.

[0360] For example, the glucoamylase of the invention can be formulated in laundry detergents to aid in the removal of polysaccharide-comprising, e.g., starch-containing, stains. Glucosidases of the disclosure can be used as cleaning agents in detergent matrices (see industrial applications below). The glucosidases of the disclosure can be used in the initial stages (liquefaction) of polysaccharide, e.g., starch, processing, in wet corn milling, in alcohol production, in the textile industry for starch desizing, in baking applications, in the beverage industry, in oilfields in drilling processes; in inking of recycled paper; and in animal feed.

[0361] Glucosidases of the disclosure (e.g., glucoamylases) can have a glucosidase activity under various conditions, e.g., extremes in pH and/or temperature, oxidizing agents, and the like. The disclosure provides methods leading to alternative glucosidase preparations with different catalytic efficiencies and stabilities, e.g., towards temperature, oxidizing agents and changing wash conditions. In one aspect, glucosidase variants can be produced using techniques of site-directed mutagenesis and/or random mutagenesis. In one aspect, directed evolution can be used to produce a great

variety of glucosidase variants with alternative specificities and stability.

[0362] The proteins of the invention are also useful as research reagents to identify glucoamylase modulators, e.g., activators or inhibitors of glucoamylase activity. Briefly, test samples (compounds, broths, extracts, and the like) are added to glucoamylase assays to determine their ability to inhibit substrate cleavage. Inhibitors identified in this way can be used in industry and research to reduce or prevent undesired proteolysis. As with glucoamylase, inhibitors can be combined to increase the spectrum of activity.

[0363] A glucoamylase activity of the invention also includes hydrolyzing a polysaccharide, oligosaccharide or starch, at high temperatures, low temperatures, alkaline pHs and at acidic pHs. For example, in one aspect, the invention provides polypeptides, and nucleic acids encoding them, having a glucoamylase activity which is thermostable. The polypeptide can retain a glucoamylase activity under conditions comprising a temperature range of between about 37°C to about 95°C; between about 55°C to about 85°C, between about 70°C to about 95°C, or, between about 90°C to about 95°C. In another aspect, a polypeptide of the invention can have a glucoamylase activity which is thermotolerant. The polypeptide can retain a glucoamylase activity after exposure to a temperature in the range from greater than 37°C to about 95°C or anywhere in the range from greater than 55°C to about 85°C.

[0364] The invention provides "amino acids" or "amino acid sequences of the invention", including an oligopeptide, peptide, polypeptide, or protein sequence, or to a fragment, portion, or subunit of any of these, and to naturally occurring, recombinant or synthetic molecules. The terms "polypeptide" and "protein" include amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres, or non-peptide bonds (synthetic bonds, synthetic polypeptides) and may contain modified amino acids other than the 20 gene-encoded amino acids. The term "polypeptide" also includes peptides and polypeptide fragments, motifs and the like. The term also includes glycosylated polypeptides. The peptides and polypeptides of the invention also include all "mimetic" and "peptidomimetic" forms, as described in further detail, below.

[0365] The term "isolated" includes a material removed from its original environment, e.g., the natural environment if it is naturally occurring. For example, a naturally occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment. As used herein, an isolated material or composition can also be a "purified" composition, i.e., it does not require absolute purity; rather, it is intended as a relative definition. Individual nucleic acids obtained from a library can be conventionally purified to electrophoretic homogeneity. In alternative aspects, the invention provides nucleic acids which have been purified from genomic DNA or from other sequences in a library or other environment by at least one, two, three, four, five or more orders of magnitude.

[0366] The disclosure also provides "amylase variants" and "glucoamylase variants" which can comprise an amino acid sequence which is derived from the amino acid sequence of a "precursor amylase", e.g., in one aspect, an exemplary sequence of the disclosure (e.g., SEQ ID NO:2, SEQ ID NO:4, etc., or any polypeptide of this disclosure). The precursor glycoamylase or amylase also can include naturally-occurring glucoamylases or amylases and recombinant amylases. The amino acid sequence of the glucoamylase or amylase variant can be "derived" from the precursor glucoamylase or amylase amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification can be of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor amylase rather than manipulation of the precursor amylase enzyme per se. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein, as well as methods known to those skilled in the art.

[0367] Activities of exemplary sequences of the disclosure are listed in the table ("Table 1") immediately below. To aid in reading the table, for example, in the first row, SEQ ID NO:1, 2, represent the exemplary polypeptide of the disclosure having a sequence as set forth in SEQ ID NO:2, encoded by, e.g., SEQ ID NO:1; and this exemplary sequence was initially isolated from *Cochliobolus heterostrophus,* ATCC 48331; no signal sequence is predicted (but under certain cell in vivo conditions, the sequence may have a signal sequence); the polypeptide has "glycosidase" activity, which can be more specifically designated "amylase" enzyme activity; and the corresponding "EC" number for amylase enzymes (an EC number is the number assigned to a type of enzyme according to a scheme of standardized enzyme nomenclature developed by the Enzyme Commission of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, or IUBMB); "CMB20" designating "carbohydrate binding domain"; and the last column indicates a "genetic source", or the source of the exemplary sequence as determined by homology analysis of the 18/16S RNA of the cell from which it was initially isolated. In the second row, SEQ ID NO:11, 12, represent the exemplary polypeptide of the disclosure having a sequence as set forth in SEQ ID NO:12, encoded by, e.g., SEQ ID NO:11; and this exemplary sequence was initially isolated from an unknown source; the "SS site" designates the amino terminal resides that are the signal sequence, and for SEQ ID NO:12 it is the amino terminal 21 amino acid residues that make up the signal sequence (or, MFNQVLYGLAATALWQGQVVA, i.e., residues 1 to 21 of SEQ ID NO:12); and the polypeptide has "glycosidase" activity, which can be more specifically designated as a "glycoamylase" enzyme activity; this enzyme

having an EC number of 3.2.1.3; with "carbohydrate binding domain"; and a genetic match of the 18/16S RNA of the cell from which it was initially isolated is *Fusarium equiseti.*

Table 1

SEQ ID NO:	Source	SS site	Signal Sequence	General Activity	An Exemplary Specific Activity	EC Number	CBM20	*Source (as determined by 18/16S RNA)*
1,2	Cochliobolus heterostrophus ATCC 48331			Glycosidase	amylase	3.2.1.1	CBM20	*Cochliobolus heterostrophus*
11,12	Unknown	AA 1-21	MFNQVLYGLAATALWQGQVVA	Glycosidase	glucoamylase	3.2.1.3	CBM20	*Fusariumequiseti 100%*
13, 14	Fusarium verticillioides GZ3639	AA 1-21	MFTQILYGLTALSALQGQVTA	Glycosidase	glucoamylase	3.2.1.3	CBM20	*Fusarium verticillioides* GZ3639
15, 16	Cochliobolus heterostrophus ATCC 48332	AA 1-20	MLSKILLPVVALAASANAHG	Glycosidase	glucoamylase		CBM20	*Cochliobolus heterostrophus*
17, 18	Fusarium verticillioides GZ3639			Glycosidase	glucoamylase	3.2.1.3	CBM20	*Fusarium verticillioides* GZ3639
19, 20	Unknown	AA 1-22	MLTLNVLTALLAPIVLSSALPA	Glycosidase	glucoamylase	3.2.1.3	no	*Penicillium chrysogenum* 100%
21, 22	Unknown	AA 1-18	MVLARLAWLAGLVSTAVA	Glycosidase	amylase	3.2.1.1	no	*Penicillium expansum* 99%
23, 24	Unknown	AA 1-20	MKLSHTLTALLLPLICTVSA	Glycosidase	amylase	3.2.1.1	no	*Penicillium chrysogenum* 100%
25, 26	Unknown	AA 1-21	MTISRLSSVLFALALGQSALA	Glycosidase	glucoamylase	3.2.1.3	CBM20	*Penicillium verruculosum 100%*
27, 28	Unknown	AA 1-20	MYILSSAFLLGSLALQSVLG	Glycosidase	glucoamylase	3.2.1.3	CBM20	*Fusarium merismoides 99%*
29, 30	Unknown	AA 1-21	MLFSSLLRALSASLLAGAVQG	Glycosidase	glucoamylase	3.2.1.3	CBM20	*Phoma herbarum 99*
3, 4	Cochliobolus heterostrophus ATCC 48331	AA 1-20	MLLLNIFTTLFFYITCIVSA	Glycosidase	amylase	3.2.1.1	CBM20	*Cochliobolus heterostrophus*
31, 32	Unknown	AA1-18	MVLARLAWLAGLVSTAIA	Glycosidase	amylase	3.2.1.1	no	*Penicillium chrysogenum 100%*
33, 34	Unknown	AA1-18	MVGFNILTLALLAPAALS	Glycosidase	glucoamylase	3.2.1.3	no	*Penicillium herquei 99% Fusarium*

(continued)

SEQ ID NO:	Source	SS site	Signal Sequence	General Activity	An Exemplary Specific Activity	EC Number	CBM20	*Source (as determined by 18/16S RNA)*
35, 36	Unknown	AA 1-20	MAPRFWTTLCALTLGSAALA	Glycosidase	glucoamylase	3.2.1.3	CBM20	*oxysporum 100%*
37, 38	Unknown	AA 1-19	MAPRFWIALWALTFGQAIA	Glycosidase	glucoamylase	3.2.1.3	CBM20	*Cordyceps ophioglossoide s* 99%
39, 40	Unknown	AA1-20	MAPRFWTALWALTLGHAWA	Glycosidase	glucoamylase	3.2.1.3	CBM20	*Penicillium chrysogenum 100%*
41, 42	Unknown			Glycosidase	glucoamylase	3.2.1.3	no	*Cucurbitaria berberidis 98%*
43, 44	Cochliobolus heterostrophus ATCC 48332	AA 1-23	MTHTSFVQASTVLSSLLALTAGQ	Glycosidase	α-glucosidase			*Cochliobolus heterostrophus*
45, 46	Unknown	AA 1-19	MKLLQLAALVASLSPFTNA	Glycosidase	amylase	3.2.1.1	no	*Fusarium equiseti 100%*
47, 48	Unknown	AA 1-20	MTRILTLALHGLALVQSVVG	Glycosidase	glucoamylase	3.2.1.3	CBM20	*Aspergillus versicolor 99*
49, 50	Aspergillus terreus	AA 1-18	MSFFLSCLYLSLCGSALA	Glycosidase	amylase	3.2.1.1	no	*Aspergillus terreus*
5,6	Cochliobolus heterostrophus ATCC 48331			Glycosidase	α-glucosidase			*Cochliobolus heterostrophus*
51, 52	Aspergillus terreus	AA 1-20	MKWTFSLLLLLSVFGQATHA	Glycosidase	amylase	3.2.1.1	CBM20	*Aspergillus terreus*
53, 54	Aspergillus terreus	AA 1-20	MKLSRALTVFLLHLTSTALA	Glycosidase	amylase	3.2.1.1	no	*Aspergillus terreus*
55, 56	Unknown	AA 1-27	MLKQFTKRLITLTSLLALVLVAPLASA	Glycosidase		3.2.1.1		
57, 58	Unknown			Glycosidase		3.2.1.10		
59, 60	Unknown			Glycosidase		3.2.1.10		
61, 62	Unknown	AA 1-20	MVAGFGLYGAALLTPMAAQA	Glycosidase		3.2.1.1		
63, 64	Unknown			Glycosidase		3.2.1.20		
65, 66	Unknown	AA 1-25	MKLKYLALVLLAVASIGLLSTPVGA	Glycosidase		3.2.1.1		
67, 68	Unknown	AA 1-23	MKKNTISALVAGMVLGFASNAMA	Glycosidase		3.2.1.1		
69, 70	Unknown	AA1-44	MNRPGTGASGRPQSRSATSWQSRNGGWLLASLLAVCFATAPVRA	Glycosidase		3.2.1.1		

(continued)

	Cochliobolus heterostrophus ATCC 48331		Glycosidase	α-glucosidase				*Cochliobolus heterostrophus*
5,6	Cochliobolus heterostrophus ATCC 48331		Glycosidase	glucoamylase	3.2.1.3	no		*Cochliobolus heterostrophus*
7, 8	Unknown		Glycosidase		3.2.1.20			
71, 72	Thermomyces lanuginosus ATCC 200065		Glycosidase		3.2.1.3			
73, 74	Unknown	AA 1-24	Glycosidase	amylase	3.2.1.1			*Penicillium expansum* 99%
75, 76	Unknown	AA 1-22	Glycosidase	glucoamylase	3.2.1.3	no		
9, 10			MLTLNVLTALLAPGVLSSALPA					

[0368] Polypeptides of the invention can also be shorter than the full length of exemplary polypeptides. In alternative aspects, the invention provides polypeptides (peptides, fragments) ranging in size between about 5 and the full length of a polypeptide, e.g., an enzyme; exemplary sizes being of about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, or more residues, e.g., contiguous residues of an exemplary glucoamylase of the invention. Peptides of the invention can be useful as, e.g., labeling probes, antigens, toleragens, motifs, amylase active sites.

[0369] Polypeptides and peptides of the invention can be isolated from natural sources, be synthetic, or be recombinantly generated polypeptides. Peptides and proteins can be recombinantly expressed *in vitro* or *in vivo.* The peptides and polypeptides of the invention can be made and isolated using any method known in the art. Polypeptide and peptides of the invention can also be synthesized, whole or in part, using chemical methods well known in the art. See e.g., Caruthers (1980) Nucleic Acids Res. Symp. Ser. 215-223; Horn (1980) Nucleic Acids Res. Symp. Ser. 225-232; Banga, A.K., Therapeutic Peptides and Proteins, Formulation, Processing and Delivery Systems (1995) Technomic Publishing Co., Lancaster, PA. For example, peptide synthesis can be performed using various solid-phase techniques (see e.g., Roberge (1995) Science 269:202; Merrifield (1997) Methods Enzymol. 289:3-13) and automated synthesis may be achieved, e.g., using the ABI 431 A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer.

[0370] The peptides and polypeptides of the invention can also be glycosylated. The glycosylation can be added post-translationally either chemically or by cellular biosynthetic mechanisms, wherein the later incorporates the use of known glycosylation motifs, which can be native to the sequence or can be added as a peptide or added in the nucleic acid coding sequence. The glycosylation can be O-linked or N-linked. Glycosylation can be added to any polypeptide of the invention to generate an enzyme that is more thermotolerant or thermostable than the "parent" enzyme (to which the glycosylation was added). The glycosylation can be added by either chemical or by cellular biosynthetic mechanisms.

[0371] The disclosure provides amylases having a broad range of specific activity over a broad range of temperatures, e.g., at about 37°C in the range from about 10 to 10,000, or, 100 to about 1000 units per milligram of protein. Glucoamylases of the invention can have activity at temperatures as high as 120°C. In alternative aspects, the amylase used in these methods is active at these temperatures, e.g., active at temperatures in a range of between about 80°C to about 115°C, between about 100°C to about 110°C, and from about 105°C to about 108°C. However, glucoamylases of the invention can also have activity at low temperatures, e.g., as low as 4°C to 5°C.

[0372] The Tm of an enzyme of the invention can be shifted (for example, can be shifted between about 10°C to 90°C) by heat activation. For example, the Tm of SEQ ID NO:336/337 can be shifted about 17°C to 87°C by heat activation: for example, 80°C preincubation for 5 minutes.

[0373] The peptides and polypeptides of the disclosure, as defined above, include all "mimetic" and "peptidomimetic" forms. The terms "mimetic" and "peptidomimetic" refer to a synthetic chemical compound which has substantially the same structural and/or functional characteristics of the polypeptides of the invention. The mimetic can be either entirely composed of synthetic, non-natural analogues of amino acids, or, is a chimeric molecule of partly natural peptide amino acids and partly non-natural analogs of amino acids. The mimetic can also incorporate any amount of natural amino acid conservative substitutions as long as such substitutions also do not substantially alter the mimetic's structure and/or activity. As with polypeptides of the disclosure which are conservative variants, routine experimentation will determine whether a mimetic has a structure and/or function that is not substantially altered.

[0374] Polypeptide mimetic compositions of the disclosure can contain any combination of non-natural structural components (e.g., they can be completely or partially synthetic, or "mimetic"). In alternative aspect, mimetic compositions of the disclosure include one or all of the following three structural groups: a) residue linkage groups other than the natural amide bond ("peptide bond") linkages; b) non-natural residues in place of naturally occurring amino acid residues; or c) residues which induce secondary structural mimicry, i.e., to induce or stabilize a secondary structure, e.g., a beta turn, gamma turn, beta sheet, alpha helix conformation, and the like. For example, a polypeptide of the disclosure can be characterized as a mimetic when all or some of its residues are joined by chemical means other than natural peptide bonds. Individual peptidomimetic residues can be joined by peptide bonds, other chemical bonds or coupling means, such as, e.g., glutaraldehyde, N-hydroxysuccinimide esters, bifunctional maleimides, N,N'-dicyclohexylcarbodiimide (DCC) or N,N'-diisopropylcarbodiimide (DIC). Linking groups that can be an alternative to the traditional amide bond ("peptide bond") linkages include, e.g., ketomethylene (e.g., -C(=O)-CH_2- for -C(=O)-NH-), aminomethylene (CH_2-NH), ethylene, olefin (CH=CH), ether (CH_2-O), thioether (CH_2-S), tetrazole (CN_4-), thiazole, retroamide, thioamide, or ester (see, e.g., Spatola (1983) in Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Vol. 7, pp 267-357, "Peptide Backbone Modifications," Marcell Dekker, NY).

[0375] A polypeptide of the disclosure can also be characterized as a mimetic by containing all or some non-natural residues in place of naturally occurring amino acid residues. Non-natural residues are well described in the scientific and patent literature; a few exemplary non-natural compositions useful as mimetics of natural amino acid residues and guidelines are described below. Mimetics of aromatic amino acids can be generated by replacing by, e.g., D- or L-naphylalanine; D- or L- phenylglycine; D- or L-2 thieneylalanine; D- or L-1, -2, 3-, or 4- pyreneylalanine; D- or L-3

thieneylalanine; D-or L-(2-pyridinyl)-alanine; D- or L-(3-pyridinyl)-alanine; D- or L-(2-pyrazinyl)-alanine; D- or L-(4-isopropyl)-phenylglycine; D-(trifluoromethyl)-phenylglycine; D-(trifluoromethyl)-phenylalanine; D-p-fluoro-phenylalanine; D- or L-p-biphenylphenylalanine; D- or L-p-methoxy-biphenylphenylalanine; D- or L-2-indole(alkyl)alanines; and, D- or L-alkylainines, where alkyl can be substituted or unsubstituted methyl, ethyl, propyl, hexyl, butyl, pentyl, isopropyl, isobutyl, sec-isotyl, iso-pentyl, or a non-acidic amino acids. Aromatic rings of a non-natural amino acid include, e.g., thiazolyl, thiophenyl, pyrazolyl, benzimidazolyl, naphthyl, furanyl, pyrrolyl, and pyridyl aromatic rings.

[0376] Mimetics of acidic amino acids can be generated by substitution by, e.g., non-carboxylate amino acids while maintaining a negative charge; (phosphono)alanine; sulfated threonine. Carboxyl side groups (e.g., aspartyl or glutamyl) can also be selectively modified by reaction with carbodiimides (R'-N-C-N-R') such as, e.g., 1-cyclohexyl-3(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3(4-azonia- 4,4-dimetholpentyl) carbodiimide. Aspartyl or glutamyl can also be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions. Mimetics of basic amino acids can be generated by substitution with, e.g., (in addition to lysine and arginine) the amino acids ornithine, citrulline, or (guanidino)-acetic acid, or (guanidino)alkyl-acetic acid, where alkyl is defined above. Nitrile derivative (e.g., containing the CN-moiety in place of COOH) can be substituted for asparagine or glutamine. Asparaginyl and glutaminyl residues can be deaminated to the corresponding aspartyl or glutamyl residues. Arginine residue mimetics can be generated by reacting arginyl with, e.g., one or more conventional reagents, including, e.g., phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, or ninhydrin, preferably under alkaline conditions. Tyrosine residue mimetics can be generated by reacting tyrosyl with, e.g., aromatic diazonium compounds or tetranitromethane. N-acetylimidizol and tetranitromethane can be used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Cysteine residue mimetics can be generated by reacting cysteinyl residues with, e.g., alpha-haloacetates such as 2-chloroacetic acid or chloroacetamide and corresponding amines; to give carboxymethyl or carboxyamidomethyl derivatives. Cysteine residue mimetics can also be generated by reacting cysteinyl residues with, e.g., bromo-trifluoroacetone, alpha-bromo-beta-(5-imidozoyl) propionic acid; chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide; methyl 2-pyridyl disulfide; p-chloromercuribenzoate; 2-chloromercuri-4 nitrophenol; or, chloro-7-nitrobenzo-oxa-1,3-diazole. Lysine mimetics can be generated (and amino terminal residues can be altered) by reacting lysinyl with, e.g., succinic or other carboxylic acid anhydrides. Lysine and other alpha-amino-containing residue mimetics can also be generated by reaction with imidoesters, such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitro-benzenesulfonic acid, O-methylisourea, 2,4, pentanedione, and transamidase-catalyzed reactions with glyoxylate. Mimetics of methionine can be generated by reaction with, e.g., methionine sulfoxide. Mimetics of proline include, e.g., pipecolic acid, thiazolidine carboxylic acid, 3- or 4- hydroxy proline, dehydroproline, 3- or 4-methylproline, or 3,3,-dimethylproline. Histidine residue mimetics can be generated by reacting histidyl with, e.g., diethylprocarbonate or para-bromophenacyl bromide. Other mimetics include, e.g., those generated by hydroxylation of proline and lysine; phosphorylation of the hydroxyl groups of seryl or threonyl residues; methylation of the alpha-amino groups of lysine, arginine and histidine; acetylation of the N-terminal amine; methylation of main chain amide residues or substitution with N-methyl amino acids; or amidation of C-terminal carboxyl groups.

[0377] A residue, e.g., an amino acid, of a polypeptide of the disclosure can also be replaced by an amino acid (or peptidomimetic residue) of the opposite chirality. Thus, any amino acid naturally occurring in the L-configuration (which can also be referred to as the R or S, depending upon the structure of the chemical entity) can be replaced with the amino acid of the same chemical structural type or a peptidomimetic, but of the opposite chirality, referred to as the D-amino acid, but also can be referred to as the R- or S- form.

[0378] The disclosure also provides methods for modifying the polypeptides of the disclosure by either natural processes, such as post-translational processing (e.g., phosphorylation, acylation, etc), or by chemical modification techniques, and the resulting modified polypeptides. Modifications can occur anywhere in the polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also a given polypeptide may have many types of modifications. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of a phosphatidylinositol, cross-linking cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristolyation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, and transfer-RNA mediated addition of amino acids to protein such as arginylation. See, e.g., Creighton, T.E., Proteins - Structure and Molecular Properties 2nd Ed., W.H. Freeman and Company, New York (1993); Posttranslational Covalent Modification of Proteins, B.C. Johnson, Ed., Academic Press, New York, pp. 1-12 (1983).

[0379] Solid-phase chemical peptide synthesis methods can also be used to synthesize polypeptides or fragments of the disclosure. Such method have been known in the art since the early 1960's (Merrifield, R. B., J. Am. Chem. Soc., 85:2149-2154, 1963) (See also Stewart, J. M. and Young, J. D., Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical

Co., Rockford, Ill., pp. 11-12)) and have recently been employed in commercially available laboratory peptide design and synthesis kits (Cambridge Research Biochemicals). Such commercially available laboratory kits have'generally utilized the teachings of H. M. Geysen et al, Proc. Natl. Acad. Sci., USA, 81:3998 (1984) and provide for synthesizing peptides upon the tips of a multitude of "rods" or "pins" all of which are connected to a single plate. When such a system is utilized, a plate of rods or pins is inverted and inserted into a second plate of corresponding wells or reservoirs, which contain solutions for attaching or anchoring an appropriate amino acid to the pin's or rod's tips. By repeating such a process step, i.e., inverting and inserting the rod's and pin's tips into appropriate solutions, amino acids are built into desired peptides. In addition, a number of available FMOC peptide synthesis systems are available. For example, assembly of a polypeptide or fragment can be carried out on a solid support using an Applied Biosystems, Inc. Model 431A™ automated peptide synthesizer. Such equipment provides ready access to the peptides of the invention, either by direct synthesis or by synthesis of a series of fragments that can be coupled using other known techniques.

[0380] The invention provides novel glucoamylases, including the exemplary enzymes of the invention, nucleic acids encoding them, and methods for using them. In one aspect, the polypeptides of the invention have a glucoamylase activity, as described herein, including, e.g., the ability to hydrolyze polysaccharides, oligosaccharides and/or starches, into sugars. In one aspect, the polypeptides of the invention have a glucoamylase activity. In alternative aspects, the glucoamylases of the invention have activities that have been modified from those of the exemplary glucoamylases described herein.

[0381] The invention includes glucoamylases of the invention with and without signal sequences (including signal sequences of the disclosure, see e.g., Table 1, or other signal sequences). The invention also includes polypeptides (e.g., fusion proteins) comprising a signal sequence of the disclosure, see, e.g., Table 1. The polypeptide comprising a signal sequence of the disclosure can be an amylase and/or a glucoamylase of the disclosure or another amylase or another enzyme or other polypeptide.

[0382] The disclosure includes immobilized amylases, glucoamylases, anti-glucoamylase, anti-amylase antibodies and fragments thereof. The disclosure provides methods for inhibiting amylase and/or glucoamylase activity, e.g., using dominant negative mutants or anti-amylase or anti-glucoamylase antibodies of the invention. The disclosure relates to heterocomplexes, e.g., fusion proteins, heterodimers, etc., comprising the amylases and/or glucoamylases of the disclosure.

[0383] In one aspect, glucoamylases of the invention hydrolyze internal polysaccharide or oligosaccharide bonds, e.g., α-1,4- and 1,6-glucosidic bonds in starch to produce smaller molecular weight maltodextrines. In one aspect, this hydrolysis is largely at random. Thus, the disclosure provides methods for producing smaller molecular weight maltodextrines.

[0384] Glucoamylases of the invention can be used in laboratory and industrial settings to hydrolyze polysaccharide, oligosaccharide or starch or any maltodextrine-comprising compound for a variety of purposes. These glucoamylases can be used alone to provide specific hydrolysis or can be combined with other amylases to provide a "cocktail" with a broad spectrum of activity. Exemplary uses include the removal or partial or complete hydrolysis of polysaccharide, oligosaccharide or starch or any maltodextrine-comprising compound from biological, food, animal feed, pharmaceutical or industrial samples.

[0385] For example, the amylases of the present disclosure can be formulated in laundry detergents to aid in the removal of polysaccharide-comprising, e.g., starch-containing, stains. In one aspect, the invention provides detergents comprising glucoamylases of the invention, including glucoamylases active under alkaline conditions, and methods of making and using them. These detergent compositions include laundry and dishwashing (e.g., autodishwashing) solutions and application. Glucoamylases of the invention can be used as cleaning agents in any detergent matrices (see industrial applications below). The glucoamylases of the present invention can be used in the initial stages (liquefaction) of polysaccharide, e.g., starch, processing, in wet corn milling, in alcohol production, in the textile industry for polysaccharide, e.g., starch, desizing, in baking applications, in the beverage industry, in oilfields in drilling processes; in inking of recycled paper; and in animal feed.

[0386] Glucoamylases of the invention can have an amylase activity under various conditions, e.g., extremes in pH and/or temperature, oxidizing agents, and the like. The disclosure provides methods leading to alternative amylase preparations with different catalytic efficiencies and stabilities, e.g., towards temperature, oxidizing agents and changing wash conditions. In one aspect, amylase variants can be produced using techniques of site-directed mutagenesis and/or random mutagenesis. In one aspect, directed evolution can be used to produce a great variety of amylase variants with alternative specificities and stability.

[0387] The disclosure also provides methods of discovering new amylases and/or glucoamylases using the nucleic acids, polypeptides and antibodies of the invention. In one aspect, lambda phage libraries are screened for expression-based discovery of amylases. In one aspect, the invention uses lambda phage libraries in screening to allow detection of toxic clones; improved access to substrate; reduced need for engineering a host, by-passing the potential for any bias resulting from mass excision of the library; and, faster growth at low clone densities. Screening of lambda phage libraries can be in liquid phase or in solid phase. In one aspect, the invention provides screening in liquid phase. This gives a

greater flexibility in assay conditions; additional substrate flexibility; higher sensitivity for weak clones; and ease of automation over solid phase screening.

[0388] The disclosure provides screening methods using the proteins and nucleic acids of the invention and robotic automation to enable the execution of many thousands of biocatalytic reactions and screening assays in a short period of time, e.g., per day, as well as ensuring a high level of accuracy and reproducibility (see discussion of arrays, below). As a result, a library of derivative compounds can be produced in a matter of weeks. For further teachings on modification of molecules, including small molecules, see PCT/US94/09174.

[0389] The present invention includes glucoamylase enzymes which are non-naturally occurring carbonyl hydrolase variants (e.g., glucoamylase variants) having a different proteolytic activity, stability, substrate specificity, pH profile and/or performance characteristic as compared to the precursor carbonyl hydrolase from which the amino acid sequence of the variant is derived. Specifically, such variants have an amino acid sequence not found in nature, which is derived by substitution of a plurality of amino acid residues of a precursor glucoamylase with different amino acids. The precursor glucoamylase may be naturally-occurring or recombinant. The useful glucoamylase variants encompass the substitution of any of the naturally occurring L-amino acids at the designated amino acid residue positions.

Amylase and glucoamylase Signal Sequences

[0390] The disclosure provides signal sequences consisting of or comprising a peptide having a sequence comprising residues 1 to 12, 1 to 13, 1 to 14, 1 to 15, 1 to 16, 1 to 17, 1 to 18, 1 to 19, 1 to 20, 1 to 21, 1 to 22, 1 to 23, 1 to 24, 1 to 25, 1 to 26, 1 to 27, 1 to 28, 1 to 28, 1 to 30 or 1 to 31, 1 to 32, 1 to 33, 1 to 34, 1 to 35, 1 to 36, 1 to 37, 1 to 38, or 1 to 39, or longer, of a polypeptide of the invention. For example, the disclosure provides amylase (e.g., alpha amylase) or glucoamylase signal sequences and nucleic acids encoding these signal sequences, e.g., exemplary peptides of the invention having sequences as set forth in the Table above.

[0391] The amylase and/or glucoamylase signal sequences of the disclosure can be isolated peptides, or, sequences joined to another amylase and/or glucoamylase or a non-amylase or non-glucoamylase polypeptide, e.g., as a fusion protein. In one aspect, the disclosure provides polypeptides comprising amylase and/or glucoamylase signal sequences of the disclosure. In one aspect, polypeptides comprising amylase and/or glucoamylase signal sequences of the disclosure comprise sequences heterologous to a glucoamylase of the invention (e.g., a fusion protein comprising an amylase signal sequence of the disclosure and sequences from another amylase or a non-amylase protein). In one aspect, the disclosure provides glucoamylases of the invention with heterologous signal sequences, e.g., sequences with a yeast signal sequence. For example, a glucoamylase of the invention comprising a heterologous signal sequence in a vectors, e.g., a pPIC series vector (Invitrogen, Carlsbad, CA).

[0392] In one aspect, the signal sequences of the disclosure are identified following identification of novel amylase and/or glucoamylase polypeptides. The pathways by which proteins are sorted and transported to their proper cellular location are often referred to as protein targeting pathways. One of the most important elements in all of these targeting systems is a short amino acid sequence at the amino terminus of a newly synthesized polypeptide called the signal sequence. This signal sequence directs a protein to its appropriate location in the cell and is removed during transport or when the protein reaches its final destination. Most lysosomal, membrane, or secreted proteins have an amino-terminal signal sequence that marks them for translocation into the lumen of the endoplasmic reticulum. More than 100 signal sequences for proteins in this group have been determined. The signal sequences can vary in length from between about 13 to 36, or anywhere from between about 10 to 40, amino acid residues. Various methods of recognition of signal sequences are known to those of skill in the art. For example, in one aspect, novel amylase signal peptides are identified by a method referred to as SIGNALP™. SignalP™ uses a combined neural network which recognizes both signal peptides and their cleavage sites; see, e.g., Nielsen (1997) "Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites", Protein Engineering, vol. 10, no. 1, p. 1-6.

[0393] It should be understood that in some aspects glucoamylases of the invention may not have signal sequences. In one aspect, the invention provides the glucoamylases of the invention lacking all or part of a signal sequence, e.g. the signal sequences of the invention (see Table 1). In one aspect, the disclosure provides a nucleic acid sequence encoding a signal sequence from one amylase operably linked to a nucleic acid sequence of a different amylase or, optionally, a signal sequence from a non-amylase protein may be desired. Table 1 shows exemplary signal sequences of the disclosure.

Amylases and glycoamylase prepro and signal sequences and catalytic domains

[0394] In addition to signal sequences (e.g., signal peptides (SPs)), as discussed above, the disclosure provides prepro domains and catalytic domains (CDs). The SPs, prepro domains and/or CDs of the invention can be isolated, synthetic or recombinant peptides or can be part of a fusion protein, e.g., as a heterologous domain in a chimeric protein. The disclosure provides nucleic acids encoding these catalytic domains (CDs) (e.g., "active sites"), prepro domains and

signal sequences (SPs, e.g., a peptide having a sequence comprising/ consisting of amino terminal residues of a polypeptide of the invention).

[0395] The amylase and/or glucoamylase signal sequences (SPs), catalytic domains (CDs) and/or prepro sequences of the disclosure can be isolated peptides, or, sequences joined to another amylase or a non-amylase or a non-glucoamylase polypeptide, e.g., as a fusion (chimeric) protein. In one aspect, polypeptides comprising amylase signal sequences SPs and/or prepro of the disclosure comprise sequences heterologous to glucoamylases of the invention (e.g., a fusion protein comprising an SP and/or prepro of the disclosure and sequences from another amylase and/or glucoamylase, or a non-amylase or a non-glucoamylase protein). In one aspect, the invention provides glucoamylases of the invention with heterologous CDs, SPs and/or prepro sequences, e.g., sequences with a yeast signal sequence. A glucoamylase of the invention can comprise a heterologous CD, SP and/or prepro in a vector, e.g., a pPIC series vector (Invitrogen, Carlsbad, CA).

[0396] In one aspect, SPs, CDs, and/or prepro sequences of the disclosure are identified following identification of novel amylase polypeptides. The pathways by which proteins are sorted and transported to their proper cellular location are often referred to as protein targeting pathways. One of the most important elements in all of these targeting systems is a short amino acid sequence at the amino terminus of a newly synthesized polypeptide called the signal sequence. This signal sequence directs a protein to its appropriate location in the cell and is removed during transport or when the protein reaches its final destination. Most lysosomal, membrane, or secreted proteins have an amino-terminal signal sequence that marks them for translocation into the lumen of the endoplasmic reticulum. The signal sequences can vary in length from 13 to 45 or more amino acid residues. Various methods of recognition of signal sequences are known to those of skill in the art. For example, in one aspect, novel hydrolase signal peptides are identified by a method referred to as SignalP. SignalP uses a combined neural network which recognizes both signal peptides and their cleavage sites. (Nielsen, et al., "Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites." Protein Engineering, vol. 10, no. 1, p. 1-6 (1997).

[0397] In some aspects, a glucoamylase of the invention may not have SPs and/or prepro sequences, and/or catalytic domains (CDs). In one aspect, the invention provides glucoamylases lacking all or part of an SP, a CD and/or a prepro domain. In one aspect, the invention provides a nucleic acid sequence encoding a signal sequence (SP), a CD and/or prepro from one glucoamylase operably linked to a nucleic acid sequence of a different amylase and/or glucoamylase, or, optionally, a signal sequence (SPs), a CD and/or prepro domain from a non-amylase or non-glucoamylase protein may be desired.

[0398] The disclosure also provides isolated, synthetic or recombinant polypeptides comprising signal sequences (SPs), prepro domain and/or catalytic domains (CDs) of the disclosure and heterologous sequences. The heterologous sequences are sequences not naturally associated (e.g., to an amylase) with an SP, prepro domain and/or CD. The sequence to which the SP, prepro domain and/or CD are not naturally associated can be on the SP's, prepro domain and/or CD's amino terminal end, carboxy terminal end, and/or on both ends of the SP and/or CD. In one aspect, the disclosure provides an isolated, synthetic or recombinant polypeptide comprising (or consisting of) a polypeptide comprising a signal sequence (SP), prepro domain and/or catalytic domain (CD) of the disclosure with the proviso that it is not associated with any sequence to which it is naturally associated (e.g., amylase and/or a glucoamylase sequence). Similarly in one aspect, the disclosure provides isolated, synthetic or recombinant nucleic acids encoding these polypeptides. Thus, in one aspect, the isolated, synthetic or recombinant nucleic acid of the disclosure comprises coding sequence for a signal sequence (SP), prepro domain and/or catalytic domain (CD) of the disclosure and a heterologous sequence (i.e., a sequence not naturally associated with the a signal sequence (SP), prepro domain and/or catalytic domain (CD) of the disclosure). The heterologous sequence can be on the 3' terminal end, 5' terminal end, and/or on both ends of the SP, prepro domain and/or CD coding sequence.

[0399] The polypeptides of the invention include glucoamylases in an active or inactive form. For example, the polypeptides of the invention include proproteins before "maturation" or processing of prepro sequences, e.g., by a proprotein-processing enzyme, such as a proprotein convertase to generate an "active" mature protein. The polypeptides of the invention include glucoamylases inactive for other reasons, e.g., before "activation" by a post-translational processing event, e.g., an endo- or exo-peptidase or proteinase action, a phosphorylation event, an amidation, a glycosylation or a sulfation, a dimerization event, and the like. Methods for identifying "prepro" domain sequences, CDs, and signal sequences are well known in the art, see, e.g., Van de Ven (1993) Crit. Rev. Oncog. 4(2):115-136. For example, to identify a prepro sequence, the protein is purified from the extracellular space and the N-terminal protein sequence is determined and compared to the unprocessed form.

[0400] The polypeptides of the invention include all active forms, including active subsequences, e.g., catalytic domains (CDs) or active sites, of an enzyme of the invention. In one aspect, the invention provides catalytic domains or active sites as set forth below. In one aspect, the invention provides a peptide or polypeptide comprising or consisting of an active site domain as predicted through use of a database such as Pfam (which is a large collection of multiple sequence alignments and hidden Markov models covering many common protein families, The Pfam protein families database, A. Bateman, E. Birney, L. Cerruti, R. Durbin, L. Etwiller, S.R. Eddy, S. Griffiths-Jones, K.L. Howe, M. Marshall, and E.L.L.

Sonnhammer, Nucleic Acids Research, 30(1):276-280, 2002) or equivalent.

Hybrid amylases and glucoamylases, and peptide libraries

[0401] In one aspect, the disclosure provides hybrid amylases and/or a glucoamylases and fusion proteins, including peptide libraries, comprising sequences of the invention. The peptide libraries of the disclosure can be used to isolate peptide modulators (e.g., activators or inhibitors) of targets, such as amylase and/or a glucoamylase substrates, receptors, enzymes. The peptide libraries of the disclosure can be used to identify formal binding partners of targets, such as ligands, e.g., cytokines, hormones and the like.

[0402] In one aspect, the fusion proteins of the disclosure (e.g., the peptide moiety) are conformationally stabilized (relative to linear peptides) to allow a higher binding affinity for targets. The disclosure provides fusions of amylases and/or glucoamylases of the disclosure and other peptides, including known and random peptides. They can be fused in such a manner that the structure of the amylases and/or a glucoamylases is not significantly perturbed and the peptide is metabolically or structurally conformationally stabilized. This allows the creation of a peptide library that is easily monitored both for its presence within cells and its quantity.

[0403] Amino acid sequence variants of the disclosure can be characterized by a predetermined nature of the variation, a feature that sets them apart from a naturally occurring form, e.g., an allelic or interspecies variation of an amylase and/or a glucoamylase sequence. In one aspect, the variants of the disclosure exhibit the same qualitative biological activity as the naturally occurring analogue. Alternatively, the variants can be selected for having modified characteristics. In one aspect, while the site or region for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed amylase and/or a glucoamylase variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, as discussed herein for example, M13 primer mutagenesis and PCR mutagenesis. Screening of the mutants can be done using assays of proteolytic activities. In alternative aspects, amino acid substitutions can be single residues; insertions can be on the order of from about 1 to 20 amino acids, although considerably larger insertions can be done. Deletions can range from about 1 to about 20, 30, 40, 50, 60, 70 residues or more. To obtain a final derivative with the optimal properties, substitutions, deletions, insertions or any combination thereof may be used. Generally, these changes are done on a few amino acids to minimize the alteration of the molecule. However, larger changes may be tolerated in certain circumstances.

[0404] The invention provides glucoamylases where the structure of the polypeptide backbone, the secondary or the tertiary structure, e.g., an alpha-helical or beta-sheet structure, has been modified. In one aspect, the charge or hydrophobicity has been modified. In one aspect, the bulk of a side chain has been modified. Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative. For example, substitutions can be made which more significantly affect: the structure of the polypeptide backbone in the area of the alteration, for example a alpha-helical or a beta-sheet structure; a charge or a hydrophobic site of the molecule, which can be at an active site; or a side chain. The invention provides substitutions in polypeptide of the invention where (a) a hydrophilic residues, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g. lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g. glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g. glycine. The variants can exhibit the same qualitative biological activity (i.e. a glucoamylase activity) although variants can be selected to modify the characteristics of the glucoamylases as needed.

[0405] In one aspect, glucoamylases of the invention comprise epitopes or purification tags, signal sequences or other fusion sequences, etc. In one aspect, the glucoamylases of the invention can be fused to a random peptide to form a fusion polypeptide. By "fused" or "operably linked" herein is meant that the random peptide and the glucoamylase are linked together, in such a manner as to minimize the disruption to the stability of the glucoamylase structure, e.g., it retains glucoamylase activity. The fusion polypeptide (or fusion polynucleotide encoding the fusion polypeptide) can comprise further components as well, including multiple peptides at multiple loops.

[0406] In one aspect, the peptides and nucleic acids encoding them are randomized, either fully randomized or they are biased in their randomization, e.g. in nucleotide/residue frequency generally or per position. "Randomized" means that each nucleic acid and peptide consists of essentially random nucleotides and amino acids, respectively. In one aspect, the nucleic acids which give rise to the peptides can be chemically synthesized, and thus may incorporate any nucleotide at any position. Thus, when the nucleic acids are expressed to form peptides, any amino acid residue may be incorporated at any position. The synthetic process can be designed to generate randomized nucleic acids, to allow the formation of all or most of the possible combinations over the length of the nucleic acid, thus forming a library of randomized nucleic acids. The library can provide a sufficiently structurally diverse population of randomized expression products to affect a probabilistically sufficient range of cellular responses to provide one or more cells exhibiting a desired

response. Thus, the disclosure provides an interaction library large enough so that at least one of its members will have a structure that gives it affinity for some molecule, protein, or other factor.

Screening Methodologies and "On-line" Monitoring Devices

[0407] In practicing the methods of the disclosure, a variety of apparatus and methodologies can be used to in conjunction with the polypeptides and nucleic acids of the invention, e.g., to screen polypeptides for glucoamylase activity, to screen compounds as potential modulators, e.g., activators or inhibitors, of glucoamylase activity, for antibodies that bind to a polypeptide of the invention, for nucleic acids that hybridize to a nucleic acid of the invention, to screen for cells expressing a polypeptide of the invention and the like.

Capillary Arrays

[0408] Capillary arrays, such as the GIGAMATRIX™, Diversa Corporation, San Diego, CA, can be used to in the methods of the disclosure. Nucleic acids or polypeptides of the invention can be immobilized to or applied to an array, including capillary arrays. Arrays can be used to screen for or monitor libraries of compositions (e.g., small molecules, antibodies, nucleic acids, etc.) for their ability to bind to or modulate the activity of a nucleic acid or a polypeptide of the invention. Capillary arrays provide another system for holding and screening samples. For example, a sample screening apparatus can include a plurality of capillaries formed into an array of adjacent capillaries, wherein each capillary comprises at least one wall defining a lumen for retaining a sample. The apparatus can further include interstitial material disposed between adjacent capillaries in the array, and one or more reference indicia formed within of the interstitial material. A capillary for screening a sample, wherein the capillary is adapted for being bound in an array of capillaries, can include a first wall defining a lumen for retaining the sample, and a second wall formed of a filtering material, for filtering excitation energy provided to the lumen to excite the sample.

[0409] A polypeptide or nucleic acid, e.g., a ligand, can be introduced into a first component into at least a portion of a capillary of a capillary array. Each capillary of the capillary array can comprise at least one wall defining a lumen for retaining the first component. An air bubble can be introduced into the capillary behind the first component. A second component can be introduced into the capillary, wherein the second component is separated from the first component by the air bubble. A sample of interest can be introduced as a first liquid labeled with a detectable particle into a capillary of a capillary array, wherein each capillary of the capillary array comprises at least one wall defining a lumen for retaining the first liquid and the detectable particle, and wherein the at least one wall is coated with a binding material for binding the detectable particle to the at least one wall. The method can further include removing the first liquid from the capillary tube, wherein the bound detectable particle is maintained within the capillary, and introducing a second liquid into the capillary tube. The capillary array can include a plurality of individual capillaries comprising at least one outer wall defining a lumen. The outer wall of the capillary can be one or more walls fused together. Similarly, the wall can define a lumen that is cylindrical, square, hexagonal or any other geometric shape so long as the walls form a lumen for retention of a liquid or sample. The capillaries of the capillary array can be held together in close proximity to form a planar structure. The capillaries can be bound together, by being fused (e.g., where the capillaries are made of glass), glued, bonded, or clamped side-by-side. The capillary array can be formed of any number of individual capillaries, for example, a range from 100 to 4,000,000 capillaries. A capillary array can form a micro titer plate having about 100,000 or more individual capillaries bound together.

Arrays, or "Biochips"

[0410] Nucleic acids or polypeptides of the invention can be immobilized to or applied to an array, e.g., an "array" or "microarray" or "biochip" or "chip", which in one embodiment comprise a plurality of target elements, each target element comprising a defined amount of one or more polypeptides (including antibodies) or nucleic acids immobilized onto a defined area of a substrate surface, where at least one of the "target elements" is a polypeptide (e.g., an enzyme or antibody) of the invention, or a nucleic acid of the invention.

[0411] Arrays of the disclosure can be used to screen for or monitor libraries of compositions (e.g., small molecules, antibodies, nucleic acids, etc.) for their ability to bind to or modulate the activity of a nucleic acid or a polypeptide of the invention. For example, in one aspect of the disclosure, a monitored parameter is transcript expression of an amylase and/or a glucoamylase gene. One or more, or, all the transcripts of a cell can be measured by hybridization of a sample comprising transcripts of the cell, or, nucleic acids representative of or complementary to transcripts of a cell, by hybridization to immobilized nucleic acids on an array, or "biochip." By using an "array" of nucleic acids on a microchip, some or all of the transcripts of a cell can be simultaneously quantified. Alternatively, arrays comprising genomic nucleic acid can also be used to determine the genotype of a newly engineered strain made by the methods of the disclosure. Polypeptide arrays" can also be used to simultaneously quantify a plurality of proteins. The present disclosure can be

practiced with any known "array," also referred to as a "microarray" or "nucleic acid array" or "polypeptide array" or "antibody array" or "biochip," or variation thereof. Arrays are generically a plurality of "spots" or "target elements," each target element comprising a defined amount of one or more biological molecules, e.g., oligonucleotides, immobilized onto a defined area of a substrate surface for specific binding to a sample molecule, e.g., mRNA transcripts.

[0412] In practicing the methods of the disclosure, any known array and/or method of making and using arrays can be incorporated in whole or in part, or variations thereof, as described, for example, in U.S. Patent Nos. 6,277,628; 6,277,489; 6,261,776; 6,258,606; 6,054,270; 6,048,695; 6,045,996; 6,022,963; 6,013,440; 5,965,452; 5,959,098; 5,856,174; 5,830,645; 5,770,456; 5,632,957; 5,556,752; 5,143,854; 5,807,522; 5,800,992; 5,744,305; 5,700,637; 5,556,752; 5,434,049; see also, e.g., WO 99/51773; WO 99/09217; WO 97/46313; WO 96/17958; see also, e.g., Johnston (1998) Curr. Biol. 8:R171-R174; Schummer (1997) Biotechniques 23:1087-1092; Kern (1997) Biotechniques 23:120-124; Solinas-Toldo (1997) Genes, Chromosomes & Cancer 20:399-407; Bowtell (1999) Nature Genetics Supp. 21:25-32. See also published U.S. patent applications Nos. 20010018642; 20010019827; 20010016322; 20010014449; 20010014448; 20010012537; 20010008765.

Antibodies and Antibody-based screening methods

[0413] The disclosure provides isolated, synthetic or recombinant antibodies that specifically bind to an amylase and/or a glucoamylase of the disclosure. These antibodies can be used to isolate, identify or quantify the amylases and/or glucoamylases of the disclosure or related polypeptides. These antibodies can be used to isolate other polypeptides within the scope the invention or other related glucoamylases. The antibodies can be designed to bind to an active site of an amylase and/or a glucoamylase. Thus, the disclosure provides methods of inhibiting amylases and/or a glucoamylases using antibodies of the disclosure.

[0414] The disclosure provides antibodies that comprise a peptide or polypeptide derived from, modeled after or substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, capable of specifically binding an antigen or epitope, see, e.g. Fundamental Immunology, Third Edition, W.E. Paul, ed., Raven Press, N.Y. (1993); Wilson (1994) J. Immunol. Methods 175:267-273; Yarmush (1992) J. Biochem. Biophys. Methods 25:85-97. The term antibody includes antigen-binding portions, i.e., "antigen binding sites," (e.g., fragments, subsequences, complementarity determining regions (CDRs)) that retain capacity to bind antigen, including (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Single chain antibodies are also included by reference in the term "antibody."

[0415] The antibodies can be used in immunoprecipitation, staining, immunoaffinity columns, and the like. If desired, nucleic acid sequences encoding for specific antigens can be generated by immunization followed by isolation of polypeptide or nucleic acid, amplification or cloning and immobilization of polypeptide onto an array of the disclosure. Alternatively, the methods of the disclosure can be used to modify the structure of an antibody produced by a cell to be modified, e.g., an antibody's affinity can be increased or decreased. Furthermore, the ability to make or modify antibodies can be a phenotype engineered into a cell by the methods of the invention.

[0416] Methods of immunization, producing and isolating antibodies (polyclonal and monoclonal) are known to those of skill in the art and described in the scientific and patent literature, see, e.g., Coligan, CURRENT PROTOCOLS IN IMMUNOLOGY, Wiley/Greene, NY (1991); Stites (eds.) BASIC AND CLINICAL IMMUNOLOGY (7th ed.) Lange Medical Publications, Los Altos, CA ("Stites"); Goding, MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE (2d ed.) Academic Press, New York, NY (1986); Kohler (1975) Nature 256:495; Harlow (1988) ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publications, New York. Antibodies also can be generated in vitro, e.g., using recombinant antibody binding site expressing phage display libraries, in addition to the traditional in vivo methods using animals. See, e.g., Hoogenboom (1997) Trends Biotechnol. 15:62-70; Katz (1997) Annu. Rev. Biophys. Biomol. Struct. 26:27-45.

[0417] Polypeptides or peptides can be used to generate antibodies which bind specifically to the polypeptides, e.g., the glucoamylases of the invention. The resulting antibodies may be used in immunoaffinity chromatography procedures to isolate or purify the polypeptide or to determine whether the polypeptide is present in a biological sample. In such procedures, a protein preparation, such as an extract, or a biological sample is contacted with an antibody capable of specifically binding to one of the polypeptides of the invention.

[0418] In immunoaffinity procedures, the antibody is attached to a solid support, such as a bead or other column matrix. The protein preparation is placed in contact with the antibody under conditions in which the antibody specifically binds to one of the polypeptides of the invention. After a wash to remove non-specifically bound proteins, the specifically bound polypeptides are eluted.

[0419] The ability of proteins in a biological sample to bind to the antibody may be determined using any of a variety of procedures familiar to those skilled in the art. For example, binding may be determined by labeling the antibody with

a detectable label such as a fluorescent agent, an enzymatic label, or a radioisotope. Alternatively, binding of the antibody to the sample may be detected using a secondary antibody having such a detectable label thereon. Particular assays include ELISA assays, sandwich assays, radioimmunoassays, and Western Blots.

[0420] Polyclonal antibodies generated against the polypeptides of the invention can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to a non-human animal. The antibody so obtained will then bind the polypeptide itself. In this manner, even a sequence encoding only a fragment of the polypeptide can be used to generate antibodies which may bind to the whole native polypeptide. Such antibodies can then be used to isolate the polypeptide from cells expressing that polypeptide.

[0421] For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique, the trioma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique (see, e.g., Cole (1985) in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

[0422] Techniques described for the production of single chain antibodies (see, e.g., U.S. Patent No. 4,946,778) can be adapted to produce single chain antibodies to the polypeptides of the invention. Alternatively, transgenic mice may be used to express humanized antibodies to these polypeptides or fragments thereof.

[0423] Antibodies generated against the polypeptides of the invention may be used in screening for similar polypeptides (e.g., a glucoamylase) from other organisms and samples. In such techniques, polypeptides from the organism are contacted with the antibody and those polypeptides which specifically bind the antibody are detected. Any of the procedures described above may be used to detect antibody binding.

Kits

[0424] The disclosure provides kits comprising the compositions, e.g., nucleic acids, expression cassettes, vectors, cells, transgenic seeds or plants or plant parts, polypeptides (e.g., amylases and/or a glucoamylase) and/or antibodies of the disclosure. The kits also can contain instructional material teaching the methodologies and industrial uses of the invention, as described herein.

Measuring Metabolic Parameters

[0425] The methods of the disclosure provide whole cell evolution, or whole cell engineering, of a cell to develop a new cell strain having a new phenotype, e.g., a new or modified amylase and/or a glucoamylase activity, by modifying the genetic composition of the cell. The genetic composition can be modified by addition to the cell of a nucleic acid of the invention. To detect the new phenotype, at least one metabolic parameter of a modified cell is monitored in the cell in a "real time" or "on-line" time frame. In one aspect, a plurality of cells, such as a cell culture, is monitored in "real time" or "on-line." In one aspect, a plurality of metabolic parameters is monitored in "real time" or "on-line." Metabolic parameters can be monitored using the glucoamylases of the invention.

[0426] Metabolic flux analysis (MFA) is based on a known biochemistry framework. A linearly independent metabolic matrix is constructed based on the law of mass conservation and on the pseudo-steady state hypothesis (PSSH) on the intracellular metabolites. In practicing the methods of the disclosure, metabolic networks are established, including the:

- identity of all pathway substrates, products and intermediary metabolites
- identity of all the chemical reactions interconverting the pathway metabolites, the stoichiometry of the pathway reactions,
- identity of all the enzymes catalyzing the reactions, the enzyme reaction kinetics,
- the regulatory interactions between pathway components, e.g. allosteric interactions, enzyme-enzyme interactions etc,
- intracellular compartmentalization of enzymes or any other supramolecular organization of the enzymes, and,
- the presence of any concentration gradients of metabolites, enzymes or effector molecules or diffusion barriers to their movement.

[0427] Once the metabolic network for a given strain is built, mathematic presentation by matrix notion can be introduced to estimate the intracellular metabolic fluxes if the on-line metabolome data is available. Metabolic phenotype relies on the changes of the whole metabolic network within a cell. Metabolic phenotype relies on the change of pathway utilization with respect to environmental conditions, genetic regulation, developmental state and the genotype, etc. In one aspect of the methods of the disclosure, after the on-line MFA calculation, the dynamic behavior of the cells, their phenotype and other properties are analyzed by investigating the pathway utilization. For example, if the glucose supply is increased and the oxygen decreased during the yeast fermentation, the utilization of respiratory pathways will be reduced and/or stopped, and the utilization of the fermentative pathways will dominate. Control of physiological state of cell cultures will

become possible after the pathway analysis. The methods of the disclosure can help determine how to manipulate the fermentation by determining how to change the substrate supply, temperature, use of inducers, etc. to control the physiological state of cells to move along desirable direction. In practicing the methods of the disclosure, the MFA results can also be compared with transcriptome and proteome data to design experiments and protocols for metabolic engineering or gene shuffling, etc.

[0428] In practicing the methods of the disclosure, any modified or new phenotype can be conferred and detected, including new or improved characteristics in the cell. Any aspect of metabolism or growth can be monitored.

Monitoring expression of an mRNA transcript

[0429] In one aspect of the disclosure, the engineered phenotype comprises increasing or decreasing the expression of an mRNA transcript (e.g., an amylase and/or a glucoamylase message) or generating new (e.g., amylase and/or a glucoamylase) transcripts in a cell. This increased or decreased expression can be traced by testing for the presence of a glucoamylase of the invention or by glucoamylase activity assays. mRNA transcripts, or messages, also can be detected and quantified by any method known in the art, including, e.g., Northern blots, quantitative amplification reactions, hybridization to arrays, and the like. Quantitative amplification reactions include, e.g., quantitative PCR, including, e.g., quantitative reverse transcription polymerase chain reaction, or RT-PCR; quantitative real time RT-PCR, or "real-time kinetic RT-PCR" (see, e.g., Kreuzer (2001) Br. J. Haematol. 114:313-318; Xia (2001) Transplantation 72:907-914).

[0430] In one aspect of the disclosure, the engineered phenotype is generated by knocking out expression of a homologous gene. The gene's coding sequence or one or more transcriptional control elements can be knocked out, e.g., promoters or enhancers. Thus, the expression of a transcript can be completely ablated or only decreased.

[0431] In one aspect of the disclosure, the engineered phenotype comprises increasing the expression of a homologous gene. This can be effected by knocking out of a negative control element, including a transcriptional regulatory element acting in cis- or trans- , or, mutagenizing a positive control element. One or more, or, all the transcripts of a cell can be measured by hybridization of a sample comprising transcripts of the cell, or, nucleic acids representative of or complementary to transcripts of a cell, by hybridization to immobilized nucleic acids on an array.

Monitoring expression of a polypeptides, peptides and amino acids

[0432] In one aspect of the disclosure, the engineered phenotype comprises increasing or decreasing the expression of a polypeptide (e.g., an amylase and/or a glucoamylase) or generating new polypeptides in a cell. This increased or decreased expression can be traced by determining the amount of amylase and/or a glucoamylase present or by amylase and/or a glucoamylase activity assays. Polypeptides, peptides and amino acids also can be detected and quantified by any method known in the art, including, e.g., nuclear magnetic resonance (NMR), spectrophotometry, radiography (protein radio labeling), electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, various immunological methods, e.g. immunoprecipitation, immunodiffusion, immuno-electrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immuno-fluorescent assays, gel electrophoresis (e.g., SDS-PAGE), staining with antibodies, fluorescent activated cell sorter (FACS), pyrolysis mass spectrometry, Fourier-Transform Infrared Spectrometry, Raman spectrometry, GC-MS, and LC-Electrospray and cap-LC-tandem-electrospray mass spectrometries, and the like. Novel bioactivities can also be screened using methods, or variations thereof, described in U.S. Patent No. 6,057,103. Furthermore, as discussed below in detail, one or more, or, all the polypeptides of a cell can be measured using a protein array.

Industrial Applications

[0433] The invention provides many industrial uses and medical applications for the glucoamylase of the invention. For example, the invention provides enzymes and methods for liquefying polysaccharide, e.g., starch. Many amylases and/or glucoamylases and glucosidases used in processes for converting liquefied polysaccharide, e.g., starch, to glucose are unable to hydrolyze alpha (1,6) linkages, and this deficiency leaves approximately 5% of the sugar as pannose and isomaltose. However, in one aspect, enzymes of the invention can convert polysaccharide, e.g., starch, to glucose to maximize glucose production, including converting liquefied polysaccharide, e.g., starch, to glucose. In one aspect, the invention provides enzymes and methods for hydrolyzing 1,4-alpha and/or 1,6- alpha linkages (e.g., hydrolyzing starches) and hydrolyzing pannose and isomaltase. The glucosidases of the disclosure can be used in a variety of industrial processes, including biomass conversion to fuels (e.g., biofuels, such as bioethanol, biopropanol, biobutanol, or a biodiesel) and the like), e.g., including their use in the initial stages (liquefaction) of polysaccharide, e.g., starch, processing, in wet corn milling, in alcohol production, in the textile industry for polysaccharide, e.g., starch, desizing, in baking applications, in the beverage industry, in oilfields in drilling processes; in inking of recycled paper; and in animal feed. Thus, the invention also provides a fuel, e.g., a biofuel (such as a bioethanol, biopropanol, biobutanol,

or a biodiesel), comprising a polypeptide of the invention.

Detergent Compositions

[0434] The invention provides detergent compositions comprising one or more polypeptides of the invention, for example, glucoamylases of the invention, and methods of using these compositions. For methods of making and using detergent compositions, see, e.g., U.S. Patent No. 6,413,928; 6,399,561; 6,365,561; 6,380,147. The detergent compositions can be a one and two part aqueous composition, a non-aqueous liquid composition, a cast solid, a granular form, a particulate form, a compressed tablet, a gel and/or a paste and a slurry form. The invention also provides methods capable of a rapid removal of gross food soils, films of food residue and other minor food compositions using these detergent compositions. Glucoamylases of the invention can facilitate the removal of polysaccharide-comprising, e.g., starchy, stains by means of catalytic hydrolysis of a polysaccharide and/or oligosaccharide, e.g., starch. Glucoamylases of the invention can be used in dishwashing detergents in textile laundering detergents.

[0435] The actual active enzyme content depends upon the method of manufacture of a detergent composition and is not critical, assuming the detergent solution has the desired enzymatic activity. In one aspect, the amount of glucoamylase present in the final solution ranges from about 0.001 mg to 0.5 mg per gram of the detergent composition. The particular enzyme chosen for use in the process and products of this invention depends upon the conditions of final utility, including the physical product form, use pH, use temperature, and soil types to be degraded or altered. The enzyme can be chosen to provide optimum activity and stability for any given set of utility conditions. In one aspect, the polypeptides of the present invention are active in the pH ranges of from about 4 to about 12 and in the temperature range of from about 20°C to about 95°C. The detergents of the invention can comprise cationic, semi-polar nonionic or zwitterionic surfactants; or, mixtures thereof.

[0436] Glucoamylases of the present invention can be formulated into powdered and liquid detergents having pH between 4.0 and 12.0 at levels of about 0.01 to about 5% (preferably 0.1% to 0.5%) by weight. These detergent compositions can also include other enzymes such as known proteases, cellulases, lipases or endoglycosidases, as well as builders and stabilizers. The addition of glucoamylases of the invention to conventional cleaning compositions does not create any special use limitation. In other words, any temperature and pH suitable for the detergent is also suitable for the present compositions as long as the pH is within the above range, and the temperature is below the described enzyme's denaturing temperature. In addition, the polypeptides of the invention can be used in a cleaning composition without detergents, again either alone or in combination with builders and stabilizers.

[0437] The present invention provides cleaning compositions including detergent compositions for cleaning hard surfaces, detergent compositions for cleaning fabrics, dishwashing compositions, oral cleaning compositions, denture cleaning compositions, and contact lens cleaning solutions.

[0438] In one aspect, the invention provides a method for washing an object comprising contacting the object with a polypeptide of the invention under conditions sufficient for washing. In one aspect, a polypeptide of the invention (e.g., an alkaline-active glucoamylase) is used in a detergent, i.e., as a detergent additive. The detergent composition of the invention may, for example, be formulated as a hand or machine laundry detergent composition comprising a polypeptide of the invention. Detergent compositions of the invention include laundry and dishwashing (e.g., autodishwashing) solutions and application. A laundry additive suitable for pre-treatment of stained fabrics can comprise a polypeptide of the invention. A fabric softener composition can comprise a polypeptide of the invention. Alternatively, a polypeptide of the invention can be formulated as a detergent composition for use in general household hard surface cleaning operations. In alternative aspects, detergent additives and detergent compositions of the invention may comprise one or more other enzymes such as a protease, a lipase, a cutinase, an amylase, another glucoamylase, a carbohydrase, a cellulase, a pectinase, a mannanase, an arabinase, a galactanase, a xylanase, an oxidase, e.g., a lactase, and/or a peroxidase. The properties of the enzyme(s) of the invention are chosen to be compatible with the selected detergent (i.e. pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, etc.) and the enzyme(s) is present in effective amounts. In one aspect, glucoamylase enzymes of the invention are used to remove malodorous materials from fabrics. Various detergent compositions and methods for making them that can be used in practicing the invention are described in, e.g., U.S. Patent Nos. 6,333,301; 6,329,333; 6,326,341; 6,297,038; 6,309,871; 6,204,232; 6,197,070; 5,856,164.

Treating fabrics

[0439] The invention provides methods of treating fabrics using one or more polypeptides of the invention. The polypeptides of the invention can be used in any fabric-treating method, which are well known in the art, see, e.g., U.S. Patent No. 6,077,316. For example, in one aspect, the feel and appearance of a fabric is improved by a method comprising contacting the fabric with a glucoamylase of the invention in a solution. In one aspect, the fabric is treated with the solution under pressure.

[0440] In one aspect, the enzymes of the invention are applied during or after the weaving of textiles, or during the

desizing stage, or one or more additional fabric processing steps. During the weaving of textiles, the threads are exposed to considerable mechanical strain. Prior to weaving on mechanical looms, warp yarns are often coated with sizing starch or starch derivatives in order to increase their tensile strength and to prevent breaking. The enzymes of the invention can be applied to remove these sizing starch or starch derivatives. After the textiles have been woven, a fabric can proceed to a desizing stage. This can be followed by one or more additional fabric processing steps. Desizing is the act of removing size from textiles. After weaving, the size coating must be removed before further processing the fabric in order to ensure a homogeneous and wash-proof result. The invention provides a method of desizing comprising enzymatic hydrolysis of the size by the action of an enzyme of the invention.

[0441] The enzymes of the invention can be used to desize fabrics, including cotton-containing fabrics, as detergent additives, e.g., in aqueous compositions. The invention provides methods for producing a stonewashed look on indigo-dyed denim fabric and garments. For the manufacture of clothes, the fabric can be cut and sewn into clothes or garments, which are afterwards finished. In particular, for the manufacture of denim jeans, different enzymatic finishing methods have been developed. The finishing of denim garment normally is initiated with an enzymatic desizing step, during which garments are subjected to the action of amylolytic enzymes in order to provide softness to the fabric and make the cotton more accessible to the subsequent enzymatic finishing steps. The invention provides methods of finishing denim garments (e.g., a "bio-stoning process"), enzymatic desizing and providing softness to fabrics using the glucoamylases of the invention. The invention provides methods for quickly softening denim garments in a desizing and/or finishing process.

Foods and food processing: liquification of polysaccharides, e.g., starch

[0442] The enzymes of the invention have numerous applications in food processing industry. The glucoamylases of the invention are used in starch to fructose processing. In alternative aspect, processes of the invention comprise polysaccharide, e.g., starch, to fructose processing comprising four steps: liquefaction of granular starch, saccharification of the liquefied polysaccharide, e.g., starch, into dextrose, purification, and isomerization to fructose; and one, several or all of these steps can comprise use of one or more enzymes of the invention. Enzymes of the invention can be used in ethanol production from a biomass, e.g., a corn or a grass, by wet or by dry milling; for example, Figure 12 illustrates how enzymes of the invention can be used in ethanol production from corn by dry milling, including their use in both "conventional processes" and "simultaneous liquefaction saccharification and fermentation processes"

[0443] The invention provides methods of polysaccharide, e.g., starch, liquefaction using the enzymes of the invention. Concentrated suspensions of starch polymer granules are converted into a solution of soluble shorter chain length dextrins of low viscosity. This step is useful for convenient handling with standard equipment and for efficient conversion to glucose or 10^3 other sugars. In one aspect, the granular starch is liquefied by gelatinizing the granules by raising the temperature of the granular starch to over about 72°C. The heating process instantaneously disrupts the insoluble starch granules to produce a water soluble starch solution. The solubilized starch solution can then be liquefied by a glucoamylase of the invention. Thus, the invention provides enzymatic starch liquefaction processes using a glucoamylase of the invention.

[0444] Figure 7, Figure 8 and Figure 9 illustrate alternative exemplary starch processes, including starch liquefaction processes, of the invention using at least one enzyme of the invention. For example, Figure 7 illustrates an exemplary starch liquefaction process of the invention comprising treating a starch slurry (e.g., having about 30% to 35% solids) with steam for primary liquefaction (e.g., at about 105°C for about 5 minutes), input into a flash tank, followed by secondary liquefaction (e.g., at about 90°C to 95°C for about 90 minutes), each or one of these steps involving use of an enzyme of the invention. Figure 8 illustrates another exemplary starch liquefaction process of the invention comprising treating a starch slurry at about between pH 4 to pH 5, e.g., pH 4.5, adjusting the pH, calcium addition, liquefaction at about pH 5 to pH 6, e.g., pH 5.4, at about 95°C using a glucoamylase of the invention, followed by another pH and temperature adjustment for saccharification at about between pH 4 to pH 5, e.g., pH 4.5, at a temperature of between about 60°C to 65°C using a glucoamylase of the invention. Figure 9 illustrates another exemplary starch process of the invention comprising treating a starch slurry at about between pH 4 to pH 5, e.g., pH 4.5, (optional adjusting pH, calcium addition), combined liquefaction-saccharification using a glucoamylase of the invention at about between pH 4 to pH 5, e.g., pH 4.5, at a temperature of greater than about 90°C, or, greater than about 95°C, followed by another pH and temperature adjustment for saccharification at about between pH 4 to pH 5, e.g., pH 4.5, at a temperature of between about 60°C to 65°C using a glucoamylase of the invention. In one aspect, the combined liquefaction-saccharification of the invention is a "one-pot" process. In one aspect, the entire process is a "one-pot" process. Any one of these processes, and any one of these steps, can also comprise, or can further comprise, another enzyme of the disclosure (e.g., a glucosidase such as an α-1,6-glucosidase, a maltase, etc.), or another enzyme such as a pullulanase or an isomerase.

[0445] An exemplary enzymatic liquefaction process involves adjusting the pH of a granular starch slurry to between 6.0 and 6.5 and the addition of calcium hydroxide, sodium hydroxide or sodium carbonate. In one aspect, calcium hydroxide is added. This provides calcium ions to stabilize the glucoamylase of the invention against inactivation. In one aspect, upon addition of amylase, the suspension is pumped through a steam jet to instantaneously raise the temperature

to between 80°-115°C. In one aspect, the starch is immediately gelatinized and, due to the presence of amylase, depolymerized through random hydrolysis of α-1,4-glycosidic bonds by amylase to a fluid mass. The fluid mass can be easily pumped.

[0446] The invention provides various enzymatic polysaccharide, oligosaccharide and/or starch liquefaction processes using a glucoamylase of the invention. In one aspect of the liquefaction process, an amylase is added to the polysaccharide, oligosaccharide and/or starch suspension and the suspension is held at a temperature of between about 80°-100°C to partially hydrolyze the polysaccharide, oligosaccharide and/or starch granules. In one aspect, the partially hydrolyzed polysaccharide, oligosaccharide and/or starch suspension is pumped through a jet at temperatures in excess of about 105°C to thoroughly gelatinize any remaining granular structure. In one aspect, after cooling the gelatinized starch, a second addition of glucoamylase is made to further hydrolyze the polysaccharide, oligosaccharide and/or starch.

[0447] The invention provides enzymes and processes for hydrolyzing liquid (liquefied) and granular polysaccharide, oligosaccharide and/or starch. Such starch can be derived from any source, e.g., corn, wheat, milo, sorghum, rye or bulgher. The invention applies to any grain starch source which is useful in liquefaction, e.g., any other grain or vegetable source known to produce starch suitable for liquefaction. The methods of the invention comprise liquefying starch from any natural material, such as rice, germinated rice, corn, barley, milo, wheat, legumes and sweet potato. The liquefying process can substantially hydrolyze the starch to produce a syrup. The temperature range of the liquefaction can be any liquefaction temperature which is known to be effective in liquefying starch. For example, the temperature of the polysaccharide, oligosaccharide and/or starch can be between about 80°C to about 115°C, between about 100°C to about 110°C, and from about 105°C to about 108°C. In alternative aspects, the amylase and/or glucoamylase used in these methods is active at these temperatures, e.g., active at temperatures in a range of between about 80°C to about 115°C, between about 100°C to about 110°C, and from about 105°C to about 108°C.

[0448] The invention provides methods for liquefaction saccharification as illustrated in Figure 5. In one aspect, glucoamylases of the invention are used in the illustrated liquefaction step (some current industrial methods use *B. licheniformis* α-amylase). In one aspect, the process takes place at about pH 6.0 at a temperature anywhere in the range of between about 95°C to 105°C, for a length of time anywhere between about 0.5 and 5 hours, e.g., 60, 90 or 120 minutes. In one aspect, in a corn steep process, prior to liquefaction cellulases, proteases and/or protein thioreductases are added.

[0449] In one aspect of a liquefaction process of the invention, a glucoamylase of the invention that has activity at about pH 4.5 (or, anywhere between about pH 5 and pH 5), that may or may not be Ca^{2+} dependent is added. Eliminating the addition of salts in the front end of the process eliminates the need to remove them at the back end of the process. In one aspect of a liquefaction process, an amylase that is more active is used. This can allow one to decrease the amount of enzyme needed. In one aspect, liquefaction and saccharification are done in the same pot, as a "one-pot process," for example, under conditions comprising about 90°C to 95°C (or, anywhere between about 80°C to 105°C), as about a 3 hour process (or, as a process lasting between about 1 and 5 hours). In this aspect, the enzyme load can be cut in half again.

[0450] In one aspect of a saccharification process of the invention, a glucoamylase of the invention is used. In one aspect, glucoamylases of the invention are used in a saccharification step (in addition to or in place of an *A. niger* glucoamylase). In one aspect, the process takes place at about pH 4.5, in a temperature range of between about 60°C to 62°C (or, anywhere in the range of between about 50°C to 72°C, or, between about 40°C to 80°C) as a process lasting between about 12 and 96 or more hours. In one aspect of a saccharification process of the invention, a glucoamylase of the invention is used to give a higher level of dextrose in the syrup. In one aspect, other enzymes are added, e.g., pullulanases to increase the amount of glucose.

[0451] In one aspect, one, some or all of the enzymes used in processes of the invention (including the enzymes of the invention) are immobilized, e.g., immobilized on any surface, e.g., a flat surface or an enzyme column, e.g., immobilized on an array, a bead, fiber, pore, capillary and the like. In one aspect, by being immobilized, they can be reused.

[0452] In one aspect, the invention provides "enzyme cocktails" using at least one enzyme of the invention. In one aspect, "enzyme cocktails" are used in the processes of the invention, e.g., including the liquefaction saccharification methods as illustrated in Figure 5. For example, in one aspect, cell wall degrading enzymes (CWDE) are used, e.g., for textile, pulp and paper, and laundry processes of the invention, including, e.g., combinations of cellulases, hemicellulases, xylanase, galactomannanases, gluco-mannanases, arabinofuranosidases, and others. In one aspect, "enzyme cocktails" used in the processes of the invention for bio-bleaching (e.g., pulp and paper, laundry processes), include combinations of laccases, peroxidases, oxidases and the like. In one aspect, cell wall degrading enzymes are combined with bio-bleaching enzymes and enzymes of the invention to degrade plant cell walls to release color agents.

[0453] One exemplary enzyme cocktail of the invention comprising at least one glucoamylase of the invention can hydrolyze approximately great than 95% of the starch in milled corn into fermentable sugars in no more than 60 hours at about 30 to 40°C and at about pH 3.5 to pH 5.5 in the presence of yeast. In one aspect, the total quantity of enzyme protein required is not greater than 50 grams/ton of corn (0.005%w/w).

[0454] Enzymes of the invention can be used to treat dairy products; and the invention provides dairy products comprising a polypeptide of the invention, wherein optionally the dairy product comprises a milk, an ice cream, a cheese or

a yogurt.

[0455] Enzymes of the invention can be used in simultaneous liquefaction saccharification and fermentation (SLSF) processes; and advantages to using enzymes of this invention can include:

- Lower energy costs due to elimination of high temperature step;
- Improved yield due to more rapid fermentation;
- Reduced risk of bacterial contamination;
- yeast produces ethanol earlier than in conventional process;
- Reduced storage and handling of bulk yeast.

[0456] Properties of enzymes of the invention used in these processes of the invention can include: raw polysaccharide, oligosaccharide and/or starch hydrolyzing activity; temperature and pH activity profiles compatible with process conditions; high activity on non-gelatinized polysaccharide, oligosaccharide and/or starch; and/or active on 'resistant' fraction of raw starch.

[0457] In alternative aspects, enzymes of the invention have glucoamylase activity and can hydrolyze (partially or completely) polysaccharide, oligosaccharide and/or starch granules having complex multi-level structures, including amorphous and crystalline regions and/or branched and linear chains, e.g., can hydrolyze (partially or completely) raw starch, including: RS I - physically inaccessible starch; RS II - resistant starch granules as in raw potato and banana; RS III - retrograded as in cooked potato.

Processes to produce high MW dextrose syrups

[0458] The invention provides processes to produce high MW dextrose syrups using enzymes of the invention, including methods for producing oligosaccharides having a MW tightly groups at about 20,000 MW. In one aspect, glucoamylases of the invention can be used to liquefy a polysaccharide-comprising, oligosaccharide-comprising and/or starch starch-comprising composition, e.g., a corn starch, to produce an oligosaccharide pattern that is tightly grouped at about 20,000 MW (*Bacillus* amylases will produce syrups containing much higher MW fragments, and high MW oligosaccharides are not fully converted to glucose by glucoamylases, e.g., *Aspergillus* glucoamylases, during saccharification).

[0459] In one aspect, using the glucoamylases of the invention to catalyze the hydrolysis of a starch-comprising composition, e.g., a corn starch, the approximately 20,000 MW fragments are produced. These approximately 20,000 MW fragments can be rapidly and fully converted to glucose. Thus, in one aspect, saccharified syrups resulting from *Bacillus* amylase liquefaction contain less dextrose than saccharified syrups from liquefaction using glucoamylases of the invention.

Processes to produce homogenous maltodextrins.

[0460] The disclosure provides processes to produce homogenous maltodextrins using enzymes of the invention. The homogenous maltodextrins produced by the methods of the disclosure can be used in a wide variety of food, drug and coating applications. In one aspect, glucoamylases of the invention can be used to generate an extremely uniform maltodextrin composition (conventional manufacturing processes using either acid or enzymatic hydrolysis of starch result in a broad, typically bimodal MW distribution of oligosaccharides). The homogenous maltodextrins produced by the methods of the disclosure have a homogenous MW distribution and can be used in a variety of maltodextrin-comprising products, resulting in lower viscosity, clear (no haze) solutions, better coating properties, better film-forming properties, and the like.

[0461] In one aspect, glucoamylases of the invention are used to liquefy corn starch to produce a uniform maltodextrin-comprising composition. In one aspect, the liquefication is conducted at a pH of between about pH 4.5 to about pH 6.5, e.g., pH 5.0 or 5.5, at temperatures up to about 105°C. The uniform maltodextrin composition can be produced at DE's ranging from about 5 to as high as about 20. The syrups produced by these glucoamylases of the invention can be filtered, treated with charcoal and/or spray-dried to yield the maltodextrin-comprising product.

[0462] In one aspect, one or more other enzymes are used in conjunction with a composition comprising enzymes of the invention for use in starch to fructose processing, liquefaction of granular starch, and processes to produce homogenous maltodextrins or high MW dextrose syrups, e.g., including amylases beta-galactosidases, catalases, laccases, cellulases, endoglycosidases, endo-beta-1,4-laccases, amyloglucosidases, glucosidases, glucose isomerases, glycosyltransferases, lipases, phospholipases, lipooxygenases, beta-laccases, endo-beta-1,3(4)-laccases, cutinases, peroxidases, amylases, glucoamylases, pectinases, reductases, oxidases, decarboxylases, phenoloxidases, ligninases, pullulanases, arabinanases, hemicellulases, mannanases, xylolaccases, xylanases, pectin acetyl esterases, rhamnogalacturonan acetyl esterases, proteases, peptidases, proteinases, polygalacturonases, rhamnogalacturonases, galactanases, pectin lyases, transglutaminases, pectin methylesterases, cellobiohydrolases and/or transglutaminases.

Enzymatic dry milling processes

[0463] The invention provides enzymatic dry milling processes using a glucoamylase of the invention; exemplary processes are illustrated in Figure 12. In dry milling, whole grain is ground and combined with water. The germ is optionally removed by flotation separation or equivalent techniques. The resulting mixture, which contains polysaccharide, e.g., starch, fiber, protein and other components of the grain, is liquefied using amylase. In one aspect, enzymatic liquefaction is done at lower temperatures than the polysaccharide, e.g., starch, liquification processes discussed above. In one aspect, after gelatinization the polysaccharide, e.g., starch, solution is held at an elevated temperature in the presence of amylase until a DE of 10-20 is achieved. In one aspect, this is a period of about 1-3 hours. Dextrose equivalent (DE) is the industry standard for measuring the concentration of total reducing sugars, calculated as D-glucose on a dry weight basis. Unhydrolyzed granular starch has a DE of virtually zero, whereas the DE of D-glucose is defined as 100.

[0464] Enzymes of the invention can be used in biomass wet or dry milling processes. For example, in one aspect of an exemplary dry milling process whole grain is ground and combined with water and treated with an enzyme of the invention. The germ can be removed by flotation separation or equivalent techniques. In one aspect, the resulting mixture, which contains polysaccharide, e.g., starch, fiber, protein and other components of the grain, is liquefied using a glucoamylase of the invention. In one aspect, this enzymatic liquefaction is at a relatively lower temperature when using the dry milling process; however, low temperature liquefaction is believed to be less efficient than high temperature liquefaction in converting polysaccharide, e.g., starch, to soluble dextrins. Thus, in one aspect, the invention provides a further step wherein after gelatinization the polysaccharide, e.g., starch, solution is held at an elevated temperature in the presence of a glucoamylase of the invention, which in one aspect is until a DE of about 10 to 20 is achieved, usually a period of about 1 to 3 hours (dextrose equivalent (DE) is the industry standard for measuring the concentration of total reducing sugars, calculated as D-glucose on a dry weight basis; unhydrolyzed granular starch has a DE of virtually zero, whereas the DE of D-glucose is defined as 100).

[0465] In alternative aspects, use of glucoamylases of the invention in dry mill ethanol processes can provide operational advantages, for example: rapid reduction in viscosity of slurried corn flour, making an increase in dissolved solids and throughput possible without additional capital investment; superior thermal stability to best competitor, which eliminates split dosing; some glucoamylases of the invention are thermostable enzymes - and this eliminates the need to dose before jet cooking and after; lower viscosities are obtained at higher process temperatures, and provides improved microbial control in slurry tank (process is run at higher temperature, so unwanted microbes are killed); lower liquefaction pH, which eliminates need for pH adjustment, decreases scale formation (calcium oxalate precipitate forms on hardware, etc.; if liquefaction done at low pH, there is a higher potential for scale formation) and reduces byproduct formation.

[0466] In summary, in alternative aspects glucoamylases of the invention can be thermostable enzymes that can meet key industry needs, for example, under certain conditions, rapidly reduces viscosity of high dry solids corn flour slurry, can be thermostable (optimum temperature 95°C), can be calcium independent, can be active under low pH optimum, and can tolerate up to 30% recycled backset. In one aspect, the recommended dose is in the range of between about 0.4 to 0.6 kg/ MT starch.

Enzymatic wet milling processes

[0467] The invention provides wet milling processes, e.g., corn wet milling, using an enzyme, e.g., a glucoamylase, of the invention. Corn wet milling is a process which produces corn oil, gluten meal, gluten feed and polysaccharide, e.g., starch. Thus, the invention provides methods of making corn oil, gluten meal, gluten feed and polysaccharide, e.g., starch, using an enzyme of the invention. In one aspect, a alkaline-glucoamylase of the invention is used in the liquefaction of polysaccharide, e.g., starch. In one aspect, a glucoamylase of the invention is used in saccharification to produce glucose. An exemplary corn wet milling process of the invention (using at least one enzyme of the invention) is illustrated in Figure 6. Figure 6 illustrates an exemplary corn oil process of the invention comprising steeping, de-germing, de-fibering and gluten separation, followed by liquefaction using an enzyme of the disclosure (e.g., an alpha amylase), and saccharification using an enzyme of the invention (e.g., glucoamylase).

[0468] In one aspect, corn (a kernel that consists of a outer seed coat (fiber), polysaccharide, e.g., starch, a combination of starch and glucose and the inner germ), is subjected to a four step process, which results in the production of starch. In one aspect, the corn is steeped, de-germed, de-fibered, and the gluten is separated. In a steeping process the solubles are taken out. The product remaining after removal of the solubles is de-germed, resulting in production of corn oil and production of an oil cake, which is added to the solubles from the steeping step. The remaining product is de-fibered and the fiber solids are added to the oil cake/solubles mixture. This mixture of fiber solids, oil cake and solubles forms a gluten feed. After de-fibering, the remaining product is subjected to gluten separation. This separation results in a gluten meal and starch. The starch is then subjected to liquefaction and saccharification using polypeptides of the invention to produce glucose.

[0469] Figure 6 illustrates an exemplary corn wet milling process of the invention (using at least one enzyme of the

invention). Figure 7, Figure 8 and Figure 9 illustrate alternative exemplary starch processing methods (e.g., industrial processes), including starch liquefaction processes, of the invention (using at least one enzyme of the invention).

[0470] Enzymes of the invention can be used in biomass wet dry milling processes; e.g., a corn wet milling, to produces a vegetable (e.g., a corn) oil, a gluten meal, a gluten feed and/or a starch. In one aspect, an alkaline-amylase of the disclosure is used in the liquefaction of starch and a glucoamylase of the invention is used in saccharification, producing glucose. In one aspect, the biomass (e.g., a corn kernel, which consists of an outer seed coat (fiber), starch, a combination of starch and glucose and the inner germ) is subjected to a four step process to produce starch. The biomass (e.g., a corn) is steeped, de-germed, de-fibered, and finally the gluten is separated. In the steeping process, the solubles are taken out. The product remaining after removal of the solubles is de-germed, resulting in production of a vegetable oil (e.g., a corn oil) and production of an oil cake, which is added to the solubles from the steeping step. The remaining product is de-fibered and the fiber solids are added to the oil cake/solubles mixture. This mixture of fiber solids, oil cake and solubles forms a gluten feed. After de-fibering, the remaining product is subjected to gluten separation. This separation results in a gluten meal and starch. In one aspect, the starch then subjected to liquefaction and saccharification (e.g., using enzymes of the invention) to produce glucose.

[0471] The invention also provides a high yield process for producing high quality corn fiber gum by treatment of corn fiber with an enzyme of the invention followed by hydrogen peroxide treatment to obtain an extract of milled corn fiber. See, e.g., U.S. Patent No. 6,147,206.

[0472] In one aspect, one or more other enzymes are used in conjunction with a composition comprising enzymes of the invention for use with these dry or wet milling processes, e.g., including amylases, beta-galactosidases, catalases, laccases, cellulases, endoglycosidases, endo-beta-1,4-laccases, amyloglucosidases, glucosidases, glucose isomerases, glycosyltransferases, lipases, phospholipases, lipooxygenases, beta-laccases, endo-beta-1,3(4)-laccases, cutinases, peroxidases, amylases, other glucoamylases, pectinases, reductases, oxidases, decarboxylases, phenoloxidases, ligninases, pullulanases, arabinanases, hemicellulases, mannanases, xylo laccases, xylanases, pectin acetyl esterases, rhamnogalacturonan acetyl esterases, proteases, peptidases, proteinases, polygalacturonases, rhamnogalacturonases, galactanases, pectin lyases, transglutaminases, pectin methylesterases, cellobiohydrolases and/or transglutaminases.

Anti-staling processes

[0473] The disclosure provides anti-staling processes (e.g., of baked products such as bread) using a glucoamylase of the invention. The disclosure provides methods to slow the increase of the firmness of the crumb (of the baked product) and a decrease of the elasticity of the crumb using a glucoamylase of the invention. Staling of baked products (such as bread) is more serious as time passes between the moment of preparation of the bread product and the moment of consumption. The term staling is used to describe changes undesirable to the consumer in the properties of the bread product after leaving the oven, such as an increase of the firmness of the crumb, a decrease of the elasticity of the crumb, and changes in the crust, which becomes tough and leathery. The firmness of the bread crumb increases further during storage up to a level, which is considered as negative. Glucoamylases of the invention are used to retard staling of the bread as described e.g., in U.S. Patent Nos. 6,197,352, 2,615,810; 3,026,205; Silberstein (1964) Baker's Digest 38:66-72.

[0474] In one aspect, an enzyme of the invention is used to retard the staling of baked products while not hydrolyzing starch into the branched dextrins. Branched dextrins are formed by cleaving off the branched chains of the dextrins generated by α-amylase hydrolysis which cannot be degraded further by the α-amylase. This can produce a gummy crumb in the resulting bread. Accordingly, the disclosure provides a process for retarding the staling of baked products (e.g., leavened baked products) comprising adding an enzyme of the invention comprising exoamylase activity to a flour or a dough used for producing a baked product. Glucoamylases of the invention can have glucoamylase activity.

[0475] The disclosure also provides a process for preparing a dough or a baked product prepared from the dough which comprises adding a glucoamylase of the invention to the dough in an amount which is effective to retard the staling of the bread. The disclosure also provides a dough comprising an amylase and a premix comprising flour together with said amylase. Finally, the disclosure provides an enzymatic baking additive, which contains said amylase.

Animal feeds and additives

[0476] The invention provides feeds, foods, food additives, feed additives, nutritional supplements and/or dietary supplements comprising a polypeptide of this invention for humans and animals; and the invention provides methods for treating humans and animals using feeds, foods, food additives, feed additives, nutritional supplements and/or dietary supplements comprising a polypeptide of this invention; and/or using a glucoamylase enzyme of the invention. The invention provides human and/or animal feeds, foods, food additives, feed additives, nutritional supplements and/or dietary supplements comprising glucoamylases of the invention. In one aspect, treating human and/or animal feeds, foods, additives, food additives, feed additives, nutritional supplements and/or dietary supplements using glucoamylases

enzymes of the invention can help in the availability of polysaccharide, e.g., starch, in the human and/or animal feed, food, additive, food additive, feed additive, nutritional supplement and/or dietary supplement. This can result in release of readily digestible and easily absorbed sugars.

[0477] Use of a glucoamylase of the invention can increase the digestive capacity of animals and birds. Use of a glucoamylase of the invention can ensure availability of an adequate nutrient supply for better growth and performance. In one aspect, the enzymes of the invention can be added as feed additives, or in feeds, foods, additives, food additives, feed additives, nutritional supplements and/or dietary supplements, for animals. In another aspect, the feeds, foods, additives, food additives, feed additives, nutritional supplements and/or dietary supplements, e.g., animal feed, can be treated with a glucoamylase of the invention prior to animal consumption. In another aspect, a glucoamylase of the invention may be supplied by expressing the enzymes directly in transgenic feed crops (as, e.g., transgenic plants, seeds and the like), such as corn. As discussed above, the disclosure provides transgenic plants, plant parts and plant cells comprising a nucleic acid sequence encoding a polypeptide of the invention. In one aspect, the nucleic acid is expressed such that a glucoamylase of the invention is produced in recoverable quantities. The glucoamylase of the invention can be recovered from any plant or plant part. Alternatively, the plant or plant part containing the recombinant polypeptide can be used as such for improving the quality of a food or feed, feed additive, nutritional supplement and/or dietary supplement, and the like, e.g., improving nutritional value, palatability, and rheological properties, or to destroy an antinutritive factor.

Paper or pulp treatment

[0478] The enzymes of the invention can be in paper or pulp treatment or paper deinking. For example, in one aspect, the invention provides a paper treatment process using glucoamylases of the invention. In one aspect, the enzymes of the invention can be used to modify polysaccharide, e.g., starch, in the paper thereby converting it into a liquefied form. In another aspect, paper components of recycled photocopied paper during chemical and enzymatic deinking processes. In one aspect, glucoamylases of the invention can be used in combination with cellulases. The paper can be treated by the following three processes: 1) disintegration in the presence of an enzyme of the invention, 2) disintegration with a deinking chemical and an enzyme of the invention, and/or 3) disintegration after soaking with an enzyme of the invention. The recycled paper treated with amylase can have a higher brightness due to removal of toner particles as compared to the paper treated with just cellulase. While the invention is not limited by any particular mechanism, the effect of a glucoamylase of the invention may be due to its behavior as surface-active agents in pulp suspension.

[0479] The invention provides methods of treating paper and paper pulp using one or more polypeptides of the invention. The polypeptides of the invention can be used in any paper- or pulp-treating method, which are well known in the art, see, e.g., U.S. Patent No. 6,241,849; 6,066,233; 5,582,681. For example, in one aspect, the invention provides a method for deinking and decolorizing a printed paper containing a dye, comprising pulping a printed paper to obtain a pulp slurry, and dislodging an ink from the pulp slurry in the presence of an enzyme of the invention (other enzymes can also be added). In another aspect, the invention provides a method for enhancing the freeness of pulp, e.g., pulp made from secondary fiber, by adding an enzymatic mixture comprising an enzyme of the invention (can also include other enzymes, e.g., pectinase enzymes) to the pulp and treating under conditions to cause a reaction to produce an enzymatically treated pulp. The freeness of the enzymatically treated pulp is increased from the initial freeness of the secondary fiber pulp without a loss in brightness.

[0480] In one aspect, one or more other enzymes are used in conjunction with a composition comprising enzymes of the invention for use with these pulp and/or paper treatment methods, e.g., including amylases, beta-galactosidases, catalases, laccases, cellulases, endoglycosidases, endo-beta-1,4-laccases, amyloglucosidases, glucosidases, glucose isomerases, glycosyltransferases, lipases, phospholipases, lipooxygenases, beta-laccases, endo-beta-1,3(4)-laccases, cutinases, peroxidases, amylases, glucoamylases, pectinases, reductases, oxidases, decarboxylases, phenoloxidases, ligninases, pullulanases, arabinanases, hemicellulases, mannanases, xylolaccases, xylanases, pectin acetyl esterases, rhamnogalacturonan acetyl esterases, proteases, peptidases, proteinases, polygalacturonases, rhamnogalacturonases, galactanases, pectin lyases, transglutaminases, pectin methylesterases, cellobiohydrolases and/or transglutaminases.

Repulping: treatment of lignocellulosic materials

[0481] The invention also provides a method for the treatment of lignocellulosic fibers, wherein the fibers are treated with a polypeptide of the invention, in an amount which is efficient for improving the fiber properties. The glucoamylases of the invention may also be used in the production of lignocellulosic materials such as pulp, paper and cardboard, from polysaccharide, e.g., starch, reinforced waste paper and cardboard, especially where repulping occurs at pH above 7and where amylases can facilitate the disintegration of the waste material through degradation of the reinforcing polysaccharide, e.g., starch. The glucoamylases of the invention can be useful in a process for producing a papermaking pulp from starch-coated printed paper. The process may be performed as described in, e.g., WO 95/14807.

[0482] An exemplary process comprises disintegrating the paper to produce a pulp, treating with a polysaccharide-degrading, e.g., starch-degrading, enzyme before, during or after the disintegrating, and separating ink particles from the pulp after disintegrating and enzyme treatment. See also U.S. Patent No. 6,309,871 and other US patents cited herein. Thus, the invention includes a method for enzymatic deinking of recycled paper pulp, wherein the polypeptide is applied in an amount which is efficient for effective deinking of the fiber surface.

Waste treatment

[0483] The enzymes of the invention can be used in a variety of other industrial applications, e.g., in waste treatment. For example, in one aspect, the invention provides a solid waste digestion process using enzymes of the invention. The methods can comprise reducing the mass and volume of substantially untreated solid waste. Solid waste can be treated with an enzymatic digestive process in the presence of an enzymatic solution (including an enzyme of the invention) at a controlled temperature. This results in a reaction without appreciable bacterial fermentation from added microorganisms. The solid waste is converted into a liquefied waste and any residual solid waste. The resulting liquefied waste can be separated from said any residual solidified waste. See e.g., U.S. Patent No. 5,709,796.

[0484] In one aspect, one or more other enzymes are used in conjunction with a composition comprising enzymes of the invention for use with these waste treatment methods, e.g., including amylases, beta-galactosidases, catalases, laccases, cellulases, endoglycosidases, endo-beta-1,4-laccases, amyloglucosidases, glucosidases, glucose isomerases, glycosyltransferases, lipases, phospholipases, lipooxygenases, beta-laccases, endo-beta-1,3(4)-laccases, cutinases, peroxidases, amylases, glucoamylases, pectinases, reductases, oxidases, decarboxylases, phenoloxidases, ligninases, pullulanases, arabinanases, hemicellulases, mannanases, xylolaccases, xylanases, pectin acetyl esterases, rhamnogalacturonan acetyl esterases, proteases, peptidases, proteinases, polygalacturonases, rhamnogalacturonases, galactanases, pectin lyases, transglutaminases, pectin methylesterases, cellobiohydrolases and/or transglutaminases.

Oral care products

[0485] The invention provides oral care product comprising a glucoamylase of the invention. Exemplary oral care products include toothpastes, dental creams, gels or tooth powders, odontics, mouth washes, pre- or post brushing rinse formulations, chewing gums, lozenges, or candy. See, e.g., U.S. Patent No. 6,264,925.

[0486] In one aspect, one or more other enzymes are used in conjunction with an oral care composition comprising enzymes of the invention, and for use with these methods, e.g., including amylases, beta-galactosidases, catalases, laccases, cellulases, endoglycosidases, endo-beta-1,4-laccases, amyloglucosidases, glucosidases, glucose isomerases, glycosyltransferases, lipases, phospholipases, lipooxygenases, beta-laccases, endo-beta-1,3(4)-laccases, cutinases, peroxidases, amylases, glucoamylases, pectinases, reductases, oxidases, decarboxylases, phenoloxidases, ligninases, pullulanases, arabinanases, hemicellulases, mannanases, xylolaccases, xylanases, pectin acetyl esterases, rhamnogalacturonan acetyl esterases, proteases, peptidases, proteinases, polygalacturonases, rhamnogalacturonases, galactanases, pectin lyases, transglutaminases, pectin methylesterases, cellobiohydrolases and/or transglutaminases.

Brewing and fermenting

[0487] The invention provides methods of brewing (e.g., fermenting) beer comprising a glucoamylase of the invention. In one exemplary process, starch-containing raw materials are disintegrated and processed to form a malt. A glucoamylase of the invention can be used at any point in a fermentation process. For example, glucoamylases of the invention can be used in the processing of barley malt. The major raw material of beer brewing is barley malt. This can be a three stage process. First, the barley grain can be steeped to increase water content, e.g., to around about 40%. Second, the grain can be germinated by incubation at 15-25°C for 3 to 6 days when enzyme synthesis is stimulated under the control of gibberellins. During this time amylase levels rise significantly. In one aspect, glucoamylases of the invention are added at this (or any other) stage of the process. The action of the amylase results in an increase in fermentable reducing sugars. This can be expressed as the diastatic power, DP, which can rise from around 80 to 190 in 5 days at 12°C.

[0488] Glucoamylases of the invention can be used in any beer producing process, as described, e.g., in U.S. Patent No. 5,762,991; 5,536,650; 5,405,624; 5,021,246; 4,788,066.

[0489] In one aspect, one or more other enzymes are used in conjunction with a feed, feed or drink comprising enzymes of the invention, e.g., including amylases, beta-galactosidases, catalases, laccases, cellulases, endoglycosidases, endo-beta-1,4-laccases, amyloglucosidases, glucosidases, glucose isomerases, glycosyltransferases, lipases, phospholipases, lipooxygenases, beta-laccases, endo-beta-1,3(4)-laccases, cutinases, peroxidases, amylases, glucoamylases, pectinases, reductases, oxidases, decarboxylases, phenoloxidases, ligninases, pullulanases, arabinanases, hemicellulases, mannanases, xylolaccases, xylanases, pectin acetyl esterases, rhamnogalacturonan acetyl esterases, proteases, peptidases, proteinases, polygalacturonases, rhamnogalacturonases, galactanases, pectin lyases, transglutaminases,

pectin methylesterases, cellobiohydrolases and/or transglutaminases.

Use in drilling well and mining operations

[0490] The invention also includes methods using enzymes of the invention in well and drilling operations, e.g., gas, oil or other drilling or mining operations. For example, in one aspect, enzymes of the invention are used to increase the flow of production fluids from a subterranean formation, e.g., a well or a mine. In one aspect, the enzymes of the invention are used to remove viscous polysaccharide-containing and/or starch-containing fluids that can be damaging, e.g., fluids formed during production operations. These polysaccharide-containing and/or starch-containing fluids can be found within a subterranean formation which surrounds a completed well bore. In one aspect, an a glucoamylase of the invention is used in an oil well drilling fluid to aid in the carrying away of drilling mud.

[0491] The invention provides methods for changing the viscosity of a composition comprising: providing a composition and the polypeptide of the invention, and a composition; and treating the composition with the polypeptide of the invention; and in one aspect, of the method, the composition comprises a soil or a drilling mud.

[0492] In one aspect, the use of these methods of the invention allows production fluids (comprising enzymes of the invention) to flow from the well bore or a mine. The methods can comprise reducing the flow of production fluids from the formation below expected flow rates and formulating an enzyme treatment by blending together an aqueous fluid and a polypeptide of the invention. The methods can comprise pumping the enzyme treatment to a desired location within the well bore or other drilled shaft and allowing the enzyme treatment to degrade the viscous, starch-containing, damaging fluid. The methods can comprise removing the fluid from the subterranean formation to the well or shaft surface. In one aspect, the enzyme treatment is effective to attack the alpha glucosidic linkages in the starch-containing fluid. In one aspect, glucoamylases of the invention are used in mine drilling, well drilling (e.g., gas or oil well drilling), and the like to carry away drilling mud, e.g., while drilling the hole (well bore or shaft).

[0493] The enzymes of the invention can be used in any well, shaft or mine drilling operation, many of which are well known in the art. For example, the invention provides methods of introducing an enzyme of the invention, which in one aspect can also comprise an oil or gas field production chemical, into a rock formation comprising oil and/or gas, which comprises passing a microemulsion comprising the enzyme (and, in one aspect, the chemical) down a production well and then into the formation. In one aspect, a production well is subjected to a "shut-in" treatment whereby an aqueous composition comprising an enzyme of the invention is injected into the production well under pressure and "squeezed" into the formation and held there. See, e.g., U.S. Patent No. 6,581,687.

[0494] In one aspect, the glucoamylases of the invention used in gas, oil or other drilling or mining operations are active at high or low pH and/or high or low temperatures, e.g., glucoamylases of the invention used in these processes are active under conditions comprising about pH 6.5, pH 6, pH 5.5, pH 5, pH 4.5 or pH 4, or lower, or, under conditions comprising about pH 7, pH 7.5 pH 8.0, pH 8.5, pH 9, pH 9.5, pH 10, pH 10.5 or pH 11 or higher. In one aspect, the glucoamylases of the invention used in these processes are active under conditions comprising a temperature range of anywhere between about 0°C to about 37°C, or, between about 37°C to about 95°C or more, or, between about 80°C to about 120°C, e.g., 85°C, 90°C, 95°C, 98°C, 100°C, 105°C, 110°C, 115°C, 120°C or more.

[0495] In one aspect, one or more other enzymes are used in conjunction with a composition comprising enzymes of the invention for use with these methods, e.g., including amylases, beta-galactosidases, catalases, laccases, cellulases, endoglycosidases, endo-beta-1,4-laccases, amyloglucosidases, glucosidases, glucose isomerases, glycosyltransferases, lipases, phospholipases, lipooxygenases, beta-laccases, endo-beta-1,3(4)-laccases, cutinases, peroxidases, amylases, glucoamylases, pectinases, reductases, oxidases, decarboxylases, phenoloxidases, ligninases, pullulanases, arabinanases, hemicellulases, mannanases, xylolaccases, xylanases, pectin acetyl esterases, rhamnogalacturonan acetyl esterases, proteases, peptidases, proteinases, polygalacturonases, rhamnogalacturonases, galactanases, pectin lyases, transglutaminases, pectin methylesterases, cellobiohydrolases and/or transglutaminases.

[0496] In one aspect, enzymes or enzyme cocktails of the invention that are used in these gas, oil or other drilling or mining operations, or including any oil and gas well washing and/or fracturing processes, are active at high or low pH and/or high or low temperatures, e.g., polymer-degrading or polysaccharide-degrading ("polymer breaker") enzymes of this invention, which include using "cocktails" of these and other enzymes such as amylase, glucoamylase, xanthanase, glycosidase and/or cellulase enzymes, or a lignin degrading enzyme, alpha amylase, beta amylase, glucoamylase, dextrinase, cellulase, cellobiohydrolase, avicelase, carboxymethylcellulase, beta-glucanase, glucosidase, xylanase, mannanase, arabinofuranosidase, laccase, lignin peroxidase, pectinase, pectate lyase, xanthanase, xanthan lyase, xanthan depolymerase, pullulanase, lichenase, pachymanase, lipase, protease, proteinase, phytase, peptidase and catalase, which include using "cocktails" of these and other enzymes, are used in these processes are active under conditions comprising about pH 6.5, pH 6, pH 5.5, pH 5, pH 4.5, pH 4.0, pH 3.5, pH 3.0 or less (more acidic), or, under conditions comprising about pH 7, pH 7.5 pH 8.0, pH 8.5, pH 9, pH 9.5, pH 10, pH 10.5, pH 11.0, pH 11.5, pH 12, pH 12.5 or more (more basic). In one aspect, enzymes or enzyme cocktails of the invention used in these processes are active under conditions comprising a temperature range of anywhere between about -100°C to about -80°C, about -80°C

to about -40°C, about -40°C to about-20°C, about -20°C to about 0°C, about 0°C to about 37°C, about 0°C to about 5°C, about 5°C to about 15°C, about 15°C to about 25°C, about 25°C to about 37°C, about 37°C to about 45°C, about 45°C to about 55°C, about 55°C to about 70°C, about 70°C to about 75°C, about 75°C to about 85°C, about 85°C to about 90°C, about 90°C to about 95°C, about 95°C to about 100°C, about 100°C to about 105°C, about 105°C to about 110°C, about 110°C to about 120°C, or 95°C, 96°C, 97°C, 98°C, 99°C, 100°C, 101°C, 102°C, 103°C, 104°C, 105°C, 106°C, 107°C, 108°C, 109°C, 110°C, 111°C, 112°C, 113°C, 114°C, 115°C, 120°C or more.

[0497] In one embodiment, the "pH trigger mechanism" comprises use of a thermophilic enzymes, for example a "pyrolase" such as the polypeptide SEQ ID NO:4 and/or SEQ ID NO:6 (encoded, e.g., by SEQ ID NO:3 and SEQ ID NO:5, respectively). In one aspect, the disclosure provides a system comprising one or more enzymes entrained in a mud or its ingredients in a dry form, e.g., a guar gum powder, sand or a buffer salt. In one aspect, the enzyme remains dormant or "less active" because of low temperature of the mud, or remains dormant or "less active" because the amount of enzyme loading is adjusted such that no or less "premature" (unwanted) degradation (of substrate) occurs until the mud is heated to higher temperatures conducive to the activation of the enzyme(s). This embodiment can be referenced as "an entrained enzyme system with a temperature trigger mechanism".

[0498] In one aspect, the composition and methods of the invention are used to degrade polymers in a "mud", which in alternative embodiments comprises a water-containing medium in which enzyme, polysaccharide and other components are mixed. In one aspect, the composition and methods of the invention are used as a solution or a dry powder, which can be mixed with an ingredient or component of the "mud". In one aspect, the enzyme is incorporated/entrained into the ingredients of the mud prior to the actual mud preparation. For example, an exemplary formulation or mixture used to practice this invention comprises a starch, xanthan or cellulose powder mixed with one or more enzyme(s), or a mixture of buffer salts and enzyme(s), wherein each of such enzyme-containing ingredients can then be used to prepare the mud.

Use of free and immobilized enzymes in hydraulic fracturing and drilling operations:

[0499] The disclosure provides compositions and methods comprising the inclusion of polymer-breaking (polymer-degrading), e.g., polysaccharide-degrading, enzymes in a free form or in an immobilized form, e.g., in an immobilized form as on a coating, e.g., of a particle, e.g., of a sand grain or a ceramic material such as a sintered bauxite.

[0500] In one aspect, the compositions and methods comprising the inclusion of polymer-breaking (polymer-degrading), e.g., polysaccharide-degrading, enzymes in or on a resin or similar material that coats particles, e.g., sand grains or a ceramic material such as a sintered bauxite; these particles (e.g., sand grains) can be used as the proppant or with a proppant (e.g., a resin-coated sand or high-strength ceramic materials) in a hydraulic fracturing fluid. In one aspect, a proppant is a sized particles mixed with a fracturing fluid to hold fractures open after a hydraulic fracturing treatment. In addition to naturally occurring sand grains, man-made or specially engineered proppants, such as resin-coated sand or high-strength ceramic materials like sintered bauxite, can also be used. Proppant materials can be sorted for size and sphericity to provide an efficient conduit for production of fluid from the reservoir to the wellbore. After the settling of the sand in the well fissures and fractures, the resin-bound enzymes can diffuse out and work on the concentrated and unbroken polymer that is often deposited on the formation surface at the completion of fracturing operations. Thus, this aspect of the disclosure can effectively remove a polysaccharide, a xanthan or a guar, e.g., a guar filter cake, from fractured oil and gas wells, and/or can enhance the permeability of the fractured zone.

[0501] In one embodiment, during the hydraulic fracturing operations, large volumes of water, sand, auxiliary chemicals (including enzymes and the mixtures of enzymes of this invention) and a polysaccharide-based polymer (e.g., a guar and/or its derivatives) are mixed and injected under pressure into the oil and/or gas wells to 'fracture' the surrounding formation and enhance the flow of gas or oil into the wellbore. Enzymes and enzyme mixtures as described herein can be used to hydrolyze these polysaccharide polymers and reduce the viscosity of the fluid (used in the hydraulic fracturing operations) for better penetration into the formation and more effective flow back at the end of the operation.

[0502] In one embodiment, the compositions and methods of this disclosure are used in enzymatic hydrolysis of base polymers (e.g., polysaccharide-based polymers, such as guar, xanthan and/or their derivatives); practicing this disclosure can solve the problem where enzymatic hydrolysis of these base polymers may be incomplete to leave some "unbroken" polymer in the fluid used in the hydraulic fracturing operations. As the fluid water content is lost to the formation the fluid becomes more concentrated and the unbroken polymers form a thick filter cake; this filter cake plugs the formation pores and reduces the flow of oil or gas into the wellbore - in one embodiment, the compositions and methods of this invention are used to break up these filter cake plugs.

[0503] Fracturing fluids contain large amounts of sand, commonly referred to as the proppant. As the fluid is pumped into the well, the proppant settles into the fissures and fractures and prevents them from closing. This helps enhance the porosity and permeability of the formation for better gas/oil flow. The sand grains are often coated with different industrial resins to increase their mechanical strength and prevent them from crushing under formation pressure. Thus, in one embodiment, the disclosure provides compositions and methods using free or immobilized polymer-degrading

("polymer-breaking") enzymes around, in or on the coating material of the sand. In one aspect, this is done by entrapment of the enzyme in the resin or by immobilization on the coating surface. Thus, in this aspect, enzyme(s) used can remain in contact with the filter cake thereby providing continual hydrolysis of the concentrated polymer, removing the cake from the fractures, and enhancing the permeability of the fractured formation.

[0504] In on aspect, the disclosure provides methods using these described enzyme in drilling operations, e.g., a typical drilling operation, where a well is created by drilling a hole 5 to 30 inches (13-76 cm) diameter into the earth with an oil rig, which rotates a drill bit. After the hole is drilled, a steel pipe (casing) slightly smaller than the hole is placed in the hole, and secured with cement. The casing provides structural integrity to the newly drilled wellbore in addition to isolating potentially dangerous high pressure zones from each other and from the surface.

[0505] With these zones safely isolated and the formation protected by the casing, the well can be drilled deeper (into potentially more-unstable and violent formations) with a smaller bit, and also cased with a smaller size casing. A wells can have 2 to 5 sets of subsequently smaller hole sizes drilled inside one another, each cemented with casing.

[0506] To drill the well, the drill bit, aided by rotary torque and the compressive weight of drill collars above it, breaks up the earth. Drilling fluid, or "mud", comprising the inclusion of polymer-breaking (polymer-degrading), e.g., polysaccharide-degrading, enzymes and enzyme mixtures of this invention, in a free form or in an immobilized form, is pumped down the inside of the drill pipe. The fluid exits at the drill bit and aids to break up the rock, keeping pressure on top of the bit, as well as cleaning, cooling and lubricating the bit.

[0507] The generated rock "cuttings" are swept up by the drilling fluid as it circulates back to surface outside the drill pipe. Fluid comprising polymer-breaking (polymer-degrading), e.g., polysaccharide-degrading, enzymes and enzyme mixtures of this invention, in a free form or in an immobilized form, can be added at this stage, too.

[0508] The fluids then go over "shakers" which shakes out the cuttings over screens allowing the good fluid to return back into the pits. Fluid comprising polymer-breaking (polymer-degrading), e.g., polysaccharide-degrading, enzymes and enzyme mixtures of this invention, in a free form or in an immobilized form, can be added at this stage, too.

[0509] These processes of the disclosure can be facilitated by addition of polymer-breaking (polymer-degrading), e.g., polysaccharide-degrading, enzymes and enzyme mixtures of this invention, in a free form or in an immobilized form. The drilling rig can contain all necessary equipment to circulate the drilling fluid, hoist and turn the pipe, control downhole pressures, remove cuttings from the drilling fluid, and generate onsite power for these operations.

[0510] The enzymes, enzyme mixtures, and methods of the invention can be practiced with any drilling mud or drilling fluid (some prefer to reserve the term "drilling fluid" for more sophisticated and well-defined "muds"), or any fluid used in operations to drill boreholes into the earth. The enzymes, enzyme mixtures, and methods of the invention can be practiced while drilling oil and/or natural gas wells and on exploration drilling rigs, including use with simpler holes.

[0511] The enzymes, enzyme mixtures, and methods of the invention can be used in, mixed with and/or practiced together with any well or drilling operation, e.g., where any mud is used, including use of any of the three main classification schemes of mud, where "mud" is used broadly and is separated into 3 categories based on the main component that makes up the mud: (1) "Water Based Mud" (WBM), which can be subdivided into dispersed and non-dispersed muds; (2) "Non Aqueous" or more commonly "Oil Based Mud" (OBM), including synthetic oils (SBM); and/or (3) Gaseous or Pneumatic mud.

[0512] The enzymes, enzyme mixtures, and methods of the invention can be used in, mixed with and/or practiced together with any well or drilling operation, e.g., can also be used in or with:

- *production wells* when they are drilled primarily for producing oil or gas, once the producing structure and characteristics are established,
- *appraisal wells when* they are used to assess characteristics (such as flowrate) of a proven hydrocarbon accumulation,
- *exploration wells* when they are drilled purely for exploratory (information gathering) purposes in a new area,
- *wildcat wells* when a well is drilled, based on a large element of hope, in a frontier area where very little is known about the subsurface.

[0513] The enzymes, enzyme mixtures, and methods of the invention can be used in, mixed with and/or practiced together with any well or drilling operation, e.g., can also be used in conjunction with methods, equipment and/or drilling operations as described, e.g., in U.S. Patent Application Publication No. 20070089910, Hewson, et al., describing, e.g., methods of forming a supported subterranean well bore, and uses, e.g., a positive displacement mud motor.

[0514] The enzymes, enzyme mixtures, and methods of the invention can be used in, mixed with and/or practiced together with methods, equipment and/or drilling operations as described, e.g., in U.S. Patent Application Publication No. 20070084638, Bohnsack; C., et al., describing, e.g., a system for facilitating flow of settled solids with drilling fluid from a container, the system including pressure nozzle apparatus with at least one nozzle from which is flowable fluid under pressure, powered rotation apparatus for selectively rotating the pressure nozzle apparatus so that the at least one nozzle is movable within the container as fluid is pumped through the at least one nozzle into the container; and,

in one aspect, translation apparatus for moving the pressure nozzle apparatus with respect to the container as fluid under pressure is pumped to the at least one rotating nozzle. Mud tanks and mud pits are also described, and the enzymes, enzyme mixtures, and methods of the invention can be used in or with any of these fluids, and/or in any mud tanks and mud pits used in these types of operations.

[0515] The enzymes, enzyme mixtures, and methods of the invention can be used in, mixed with and/or practiced together with methods, equipment and/or drilling operations as described, e.g., in U.S. Patent Application Publication No. 20070081157, Csutak; S., et al., describing, e.g., apparatus for estimating a property of a fluid downhole comprising an ultraviolet (UV) light source for inducing light into the fluid at a wavelength that produces Raman scattered light at wavelengths that are shorter than wavelengths of substantial fluorescence reflected from the fluid in response to the induced light; a detector that detects a spectrum of the Raman scattered light and provides signals in response to the detected spectrum; and a processor that processes the signals to provide an estimate of the a property of the fluid. The enzymes, enzyme mixtures, and methods of the invention can be used in or with any of these fluids, and/or in operations to estimate filtrate contamination in a formation fluid. For example, these methods include detecting Raman scatters at a plurality of wavelengths of at least one component present in an oilbased mud that is not naturally present in the formation, and enzymes, enzyme mixtures, and methods of the invention can be used to aid in the accuracy of this detection.

[0516] The enzymes, enzyme mixtures, and methods of the invention can be used in, mixed with and/or practiced together with methods, equipment and/or drilling operations as described, e.g., in U.S. Patent Application Publication No. 20070075706, Chen, S. , et al., describing, e.g., methods of evaluating an earth formation comprising making measurements with a downhole tool in a borehole in the earth formation; measuring a Quality factor of an antenna of the downhole tool at depths where the measurements are made; and using the measured Q and a resistivity of a mud in the borehole and a formation resistivity, and/or a borehole size indicator (BSI), for estimating the other of the formation resistivity and BSI, including measuring the resistivity of the mud in the borehole. The enzymes, enzyme mixtures, and methods of the invention can be used in or with any of these fluids, and/or in operations to evaluate an earth formation.

[0517] The enzymes, enzyme mixtures, and methods of the invention can be used in, mixed with and/or practiced together with methods, equipment and/or drilling operations as described, e.g., in U.S. Patent Application Publication No. 20070068675, Barry, M., et al, describing, e.g., methods for drilling and completing a gravel packed well, comprising drilling a wellbore with a drilling fluid, conditioning the drilling fluid, running the gravel packing assembly tools to depth in the wellbore with the conditioned drilling-fluid, and gravel packing a wellbore interval with a completion-fluid. The completion fluid may be the same as the drilling-fluid. This method may be combined with alternate-path sand screen technology to ensure proper distribution of the gravel pack. The proper fluids for drilling, gravel packing and sand screens installation are essential for well completion success. Careful planning, well preparation and completion execution are required to increase completion productivity and longevity. Usually, a minimum of three fluids have been used to drill and complete gravel packed wells. The first fluid is a solids-laden drilling-fluid used to drill the completion interval. The second fluid is a solids-free completion-fluid used to displace the solids-laden drilling-fluid and to run sand-exclusion equipment and gravel packing tools in a generally solids-free environment. The third fluid is a carrier fluid for the gravel during gravel packing of the completion interval. The enzymes, enzyme mixtures, and methods of the invention can be used in or with any of these fluids (including solids-laden drilling-fluids, solids-free completion-fluids and/or carrier fluids), and/or in operations for drilling and completing a gravel packed well.

[0518] The enzymes, enzyme mixtures, and methods of the invention can be used in, mixed with and/or practiced together with methods, equipment and/or drilling operations as described, e.g., in U.S. Patent Application Publication No. 20070066491, Bicerano; J., et al., use of particles in the construction, drilling, completion and/or fracture stimulation of oil and natural gas wells; for example, as a proppant partial monolayer, a proppant pack, an integral component of a gravel pack completion, a ball bearing, a solid lubricant, a drilling mud constituent, and/or a cement additive, including use of thermoset polymer particles for use in applications requiring lightweight particles possessing high stiffness, strength, temperature resistance, and/or resistance to aggressive environments. The enzymes, enzyme mixtures, and methods of the invention can be used in or with any of these gravel packs, ball bearings, solid lubricants, drilling mud constituents, cement additives and/or the described thermoset polymer particles. The enzymes, enzyme mixtures, and methods of the invention can be used in or with nanofillers and/or nanocomposites, including heterogeneous nanocomposite morphologies.

[0519] The enzymes, enzyme mixtures, and methods of the invention can be used in, mixed with and/or practiced together with methods, equipment and/or drilling operations as described, e.g., in U.S. Patent Application Publication No. 20070039735, Robertson; B., et al., describing, e.g., methods of sealing a permeable zone within a subterranean formation, comprising: preparing a plugging composition comprising oil, clay, magnesium chloride, and magnesium oxide powder; and contacting the plugging composition with water in the subterranean formation such that the plugging composition forms a sealing mass, thereby substantially sealing a permeable zone within the subterranean formation.

[0520] The enzymes, enzyme mixtures, and methods of the invention can be used in, mixed with and/or practiced together with variable density drilling muds comprising compressible particulate materials, e.g., as described in U.S.

Patent Application Publication No. 20070027036, Polizzotti; R., et al. The enzymes, enzyme mixtures, and methods of the invention can be used in or with, e.g., drilling muds comprising a compressible particulate material in the drilling mud, wherein density of the drilling mud changes due to a volume change of the compressible particulate material in response to pressure or temperature changes and wherein the compressible particulate material is configured to maintain the density of the drilling mud between a pore pressure gradient and a fracture gradient based on the volume change of the compressible particulate material in response to pressure changes at certain depths.

[0521] The enzymes, enzyme mixtures, and methods of the invention can be used to modify the viscosity of the drilling mud alone or in conjunction with the described (see Polizzotti; R., et al.) compressible materials, e.g., to place the fluid viscosity within pumpability requirements, and/or to adjust the pore pressure gradient and the fracture gradient. The enzymes, enzyme mixtures, and methods of the invention can be used to effect a volume change in the drilling mud, e.g., where the drilling mud rheology is configured to achieve a desired composite drilling mud rheology.

[0522] In one aspect, the enzymes, enzyme mixtures, and methods of the invention are used to alter the properties of the drilling mud to provide a desired composite a mud gel point, e.g., a mud gel point that can suspend rock cuttings in an annulus of a wellbore during drilling operations; and/or to alter the viscosity of the drilling mud in conjunction with, or alone (without), compressible hollow objects (see Polizzotti; R., et al.) to alter pumpability requirements.

[0523] In one aspect, the enzymes, enzyme mixtures, and methods of the invention are used to alter the properties well fluids comprising drilling muds, well cleanup fluids, workover fluids, spacer fluids, gravel pack fluids, acidizing fluids and/or fracturing fluids. In one aspect, the enzymes, enzyme mixtures, and methods of the invention are used to facilitate drilling, completing and/or stimulating a subterranean formation using a variable density fluid, and to modify the variable density fluid.

[0524] In one aspect, the enzymes, enzyme mixtures, and methods of the invention are used in methods of drilling, completing and/or stimulating subterranean formations using a variable density fluid, e.g., by modifying and/or "adjusting" the density of the fluid; for example, a method (see Polizzotti; R., et al.) comprising the steps of: introducing a fluid having a density that varies as a function of pressure into the subterranean formation, where the fluid comprises a base fluid and a portion of elastic particles; and drilling, completing and/or stimulating a subterranean formation using the variable density fluid (which can comprise the enzymes, enzyme mixtures of the invention, or have been modified by the methods of the invention).

[0525] In one aspect, the enzymes, enzyme mixtures, and methods of the invention are used with the methods and compositions as described in U.S. Pat. No. 4,099,583, describing, e.g., a dual gradient drilling system, where a lighter fluid is injected into the mud return annulus (typically in the riser) or other pathway to reduce the mud density from the injection point upwards, and the enzymes, enzyme mixtures, and methods of the invention can modify and/or "adjust" the density of this fluid.

[0526] In one aspect, the enzymes, enzyme mixtures, and methods of the invention are used with the methods and compositions as described in U.S. Pat. No. 6,530,437 and U.S. Pat. No. 6,588,501, describing a multi-gradient drilling method and an apparatus for reduction of hydrostatic pressure in sub sea risers; and U.S. Pat. No. 6,422,326, U.S. Pat. No. 6,156,708, U.S. Pat. Nos. 5,910,467 and 5,881,826, describing the addition of various fluid aphrons to drilling mud formulations.

[0527] In one aspect, the enzymes, enzyme mixtures, and methods of the invention are used with the methods and compositions as described in U.S. Pat. No. 6,497,289, describing use of solid expandable liners, e.g., as tubular systems that are run into a well and expanded.

[0528] In alternative embodiments, the enzymes, enzyme mixtures, and methods of the invention are used to tailor drilling mud density with depth so that the effective mud weight remains between the pore pressure and the fracture gradient at all depths. The required variation in mud density can be achieved by changing the properties of fluids with the enzymes, enzyme mixtures, and methods of the invention to modify/ change volume and density, to effect a change in response to pressure. The enzymes, enzyme mixtures, and methods of the invention can be used with any particulate components, e.g., various shapes, such as spheres, cubes, pyramids, oblate or prolate spheroids, cylinders, pillows and/or other shapes or structures. The enzymes, enzyme mixtures, and methods of the invention can be used with any particulate components, e.g., compressible hollow objects which are filled with pressurized gas, or compressible solid materials or objects as described in Polizzotti; R., et al., supra.

[0529] In alternative aspects, the enzymes, enzyme mixtures, and methods of the invention can be used in or with any well or drilling operation, e.g., including directional drilling, sometimes known as slant drilling, to drill non-vertical wells; including used in any of directional drillings three main groups; Oilfield Directional Drilling, Utility Installation Directional Drilling (commonly known as H.D.D./Horizontal Directional Drilling/Directional boring); and/or in-seam directional drilling (Coal-Bed methane).

[0530] In one aspect, the enzymes, enzyme mixtures, and methods of the invention can be used in conjunction with well logging, a technique used in the oil and gas industry for recording rock and fluid properties to find hydrocarbon zones in the geological formations within the Earth's crust. Logging can be performed to measure the effect of practicing the methods of this invention, e.g., pumping fluids comprising the enzymes or enzyme mixtures of this invention into a

well. A logging procedure may consist of lowering a 'logging tool' on the end of a wireline into an oil well (or hole) to measure the rock and fluid properties of the formation. An interpretation of these measurements is then made to locate and quantify potential depth zones containing oil and gas (hydrocarbons). Logging tools developed over the years measure the electrical, acoustic, radioactive, electromagnetic, and other properties of the rocks and their contained fluids. Logging is usually performed as the logging tools are pulled out of the hole. This data is recorded to a printed record called a 'Well Log' and is normally transmitted digitally to office locations. Well logging is performed at various intervals during the drilling of the well and when the total depth is drilled, which could range in depths from 300 m to 8000 m (1000 ft to 25,000 ft) or more.

[0531] In addition to the methods, enzymes or enzymes mixtures described herein, the methods, the enzyme muds or other drilling fluids used to practice this invention can comprise (use of) a water-based drilling mud that can comprise a bentonite clay (gel), and in some aspects, also comprising additives such as barium sulfate (barite), calcium carbonate (chalk) or hematite. Various thickeners also can be used to influence the , viscosity of the fluid, e.g., lignosulfonates, xanthan gum, guar gum, glycol, carboxymethylcellulose, polyanionic cellulose (PAC), or starch. The enzymes or enzymes mixtures described herein, used to practice this invention can be used to modify the properties of (e.g., the viscosity of) the fluids, e.g., to modify the properties of lignosulfonates, xanthan gum, guar gum, glycol, carboxymethylcellulose, polyanionic cellulose (PAC), or starch.

[0532] The methods, enzymes or enzymes mixtures described herein, used to practice this invention can be used to modify the properties of deflocculants, which are used to reduce viscosity of clay-based muds; anionic polyelectrolytes, e.g., acrylates, polyphosphates, lignosulfonates (Lig) or tannic acid derivates such as Quebracho (red mud was the name for a Quebracho-based mixture, named after the color of the red tannic acid salts; it was commonly used in 1940s to 1950s, then became obsolete when lignosulfates became available).

[0533] The methods, enzymes or enzymes mixtures described herein, used to practice this invention can be used in (e.g., added to) water injectors for injecting water into a formation, either to maintain reservoir pressure or simply to dispose of water produced with a hydrocarbon (e.g., because even after treatment, it would be too oily and too saline to be considered clean for dumping, e.g., dumping overboard or into a fresh water source in the case of onshore wells). Thus, the methods and compositions (e.g., mixtures of enzymes, immobilized enzymes) of this invention are used with water injection as an element of reservoir management and produced water disposal.

[0534] The methods, enzymes or enzymes mixtures described herein, used to practice this invention can be used in (e.g., added to) aquifer producers, e.g., as in intentionally producing reservoir water for re-injection (e.g., in a well bore) to manage pressure; this is in effect moving reservoir water from where it is not as useful, to where it is more useful. These wells will generally only be used if produced water from the oil or gas producer is insufficient for reservoir management purposes. Thus, in one aspect, the methods and compositions (e.g., mixtures of enzymes, immobilized enzymes) of this invention are used with aquifer produced water and/or sea water.

Delayed release compositions

[0535] The invention provides delayed release or "controlled release" compositions comprising a desired composition coated by a latex polymer, e.g., a latex paint, or equivalent. The delayed release/ controlled release compositions of the invention can comprise any desired composition, including enzymes or any active ingredient, including small molecules, drugs, polysaccharides, lipids, nucleic acids, vitamins, antibiotics, insecticides, and the like. In one aspect, the coating will not readily dissolve at a relatively low temperature but will decompose to release the desired composition (e.g., enzyme) at a relatively higher temperature.

[0536] The disclosure provides methods for the delayed release/ controlled release of compositions wherein the composition is coated by a latex polymer, e.g., a latex paint, or equivalent.

[0537] The delayed release/ controlled release compositions of the invention can be used for a variety of medical and industrial applications, for example, in one aspect, delayed release/ controlled release enzyme compositions of the invention comprise enzymes involved in guar fracturing fluids in enhanced oil recovery operations. The oilfield guar degrading application of the invention is facilitated by a coating that will not readily dissolve at low temperature but will decompose to release the enzyme at higher temperatures.

[0538] In another aspect, the delayed release/ controlled release enzyme compositions of the invention comprise animal feeds or nutritional supplements comprising, e.g., enzymes, vitamins, antibiotics and/or other food, drug or nutritional supplements. These active compounds in the animal feeds or nutritional supplements are protected from pelleting conditions or gastric digestion by the coating on a delayed release/ controlled release composition of the invention.

[0539] In one aspect, the release is a temperature activated release, e.g., the desired composition (e.g., enzyme) is released at an elevated temperature, e.g., between about 37°C to about 95°C or more, e.g., 85°C, 90°C, 95°C, 98°C, 100°C or more. The rate of release can be controlled by the thickness or amount of "barrier" or latex polymer, applied to the desired composition, e.g., a pellet or matrix comprising the desired composition. Thus, the invention provides

pellets or matrices having a range of thicknesses of latex polymer or equivalent and methods of using them.

[0540] The invention provides delayed release/ controlled release enzyme compositions, e.g., in one aspect, comprising an enzyme of the invention. In one aspect, the invention provides an enzyme (e.g., an enzyme of the invention), or a pelleted composition comprising an enzyme (e.g., an enzyme of the invention), coated with a latex polymer, e.g., a latex paint, or equivalent. In one aspect, the disclosure provides methods of making delayed release enzyme compositions comprising coating an enzyme (e.g., an enzyme of the invention), or a pelleted composition comprising an enzyme (e.g., an enzyme of the invention), with a latex polymer, e.g., a latex paint, or equivalent. In one aspect, the disclosure provides methods of making delayed release/ controlled release compositions comprising coating a desired compound with a latex polymer, e.g., a latex paint, or equivalent.

[0541] Latex polymers that are used in the delayed release/ controlled release compositions (e.g., delayed release/ controlled release enzyme compositions) of the invention include, but are not limited to, various types such as the following: acrylics; alkyds; celluloses; coumarone-indenes; epoxys; esters; hydrocarbons; maleics; melamines; natural resins; oleo resins; phenolics; polyamides; polyesters; rosins; silicones; styrenes; terpenes; ureas; urethanes; vinyls; and the like. Latex polymers that are used in the delayed release compositions and methods of the invention also include, but are not limited to, one or more homo- or copolymers containing one or more of the following monomers: (meth)acrylates; vinyl acetate; styrene; ethylene; vinyl chloride; butadiene; vinylidene chloride; vinyl versatate; vinyl propionate; t-butyl acrylate; acrylonitrile; neoprene; maleates; fumarates; and the like, including plasticized or other derivatives thereof.

[0542] The amount of latex polymer used in the latex composition of the invention is not critical, but may be any amount following well established procedures using latex polymers. In alternative aspects, the amount of dry latex polymer is at least about 1, or, from about 2 to about 50, or, from about 3 to about 40 weight percent of the total latex composition. The latex composition of the invention may optionally contain other components such as those generally used in latex compositions. These additional components include, but are not limited to, one or more of the following: solvents such as aliphatic or aromatic hydrocarbons, alcohols, esters, ketones, glycols, glycol ethers, nitroparaffins or the like; pigments; fillers, dryers; flatting agents; plasticizers; stabilizers; dispersants; surfactants; viscosifiers including polymeric associative thickeners, polysaccharide-based thickeners and so on; suspension agents; flow control agents; defoamers; anti-skinning agents; preservatives; extenders; filming aids; crosslinkers; surface improvers; corrosion inhibitors; and other ingredients useful in latex compositions. In one aspect, latex compositions of the invention having improved rheology and stability are provided by combining the latex polymer and a polysaccharide with water following established procedures. See, e.g., U.S. Patent Nos. 6,372,901; 5,610,225.

[0543] In one aspect, in making a pelleted or matrix-comprising composition of the invention comprising an active composition, e.g., an enzyme (e.g., an enzyme of the invention, coated with a latex polymer, e.g., a latex paint, or equivalent, the active composition (e.g., enzyme) is embedded in the body of the pellet (in one aspect, a majority, or all, of the active composition (e.g., enzyme) is embedded in the pellet. Thus, harsh chemicals, e.g., the latex coating, which may be an inactivator of the desired, active ingredient, can be used to coat the surface of the pellet or matrix. The composition of the coating can be broken down by agents such as heat, acid, base, pressure, enzymes, other chemicals and the like, to have a controlled release of the desired enzymatic activity triggered by the exposure to the coating-degrading agent.

[0544] In one aspect, an active composition, e.g., an enzyme (e.g., an enzyme of the invention, or another enzyme, e.g., a mannanase), is dispersed in a corn term meal and/or a corn starch matrix (e.g., as a pellet). This mixture (e.g., pellet) disintegrates within ten minutes in room temperature (e.g., about 22°C) water to release all (100%) of the active composition, e.g., releases all of the enzymatic activity. At higher temperatures, the rate of release increases. This is not an acceptable rate of disintegration for many uses.

[0545] However, as a delayed release/ controlled release composition of the invention, i.e., when this mixture is coated with a latex polymer, e.g., a latex paint, or equivalent, the disintegration of the mixture (e.g., pellet, matrix) is delayed. The rate and extent of release can be controlled by the thickness of the coating (barrier) applied to the pellet or matrix. For example, a coated particle will release only 30% of the activity after six hours in 22°C water. At 60°C, 50% of the enzyme is released in 90 minutes. At 80°C, 80% of the enzyme is released during one hour.

[0546] In one aspect, one or more other enzymes are added to a delayed release/ controlled release composition of the invention, e.g., including amylases, beta-galactosidases, catalases, laccases, cellulases, endoglycosidases, endo-beta-1,4-laccases, amyloglucosidases, glucosidases, glucose isomerases, glycosyltransferases, lipases, phospholipases, lipooxygenases, beta-laccases, endo-beta-1,3(4)-laccases, cutinases, peroxidases, amylases, glucoamylases, pectinases, reductases, oxidases, decarboxylases, phenoloxidases, ligninases, pullulanases, arabinanases, hemicellulases, mannanases, xylolaccases, xylanases, pectin acetyl esterases, rhamnogalacturonan acetyl esterases, proteases, peptidases, proteinases, polygalacturonases, rhamnogalacturonases, galactanases, pectin lyases, transglutaminases, pectin methylesterases, cellobiohydrolases and/or transglutaminases.

Biomass conversion and production of clean bio fuels

[0547] The invention provides enzymes and methods for the conversion of biomass to fuels (e.g., bioethanol, biopropanol, biobutanol, or biodiesel) and chemicals. Thus, the compositions and methods of the invention provide effective and sustainable alternatives to use of petroleum-based products. The invention provides organisms expressing enzymes of the invention for participation in chemical cycles involving natural biomass conversion. In one aspect, enzymes and methods for the conversion are used in enzyme ensembles for the efficient depolymerization of biomass polymers to metabolizable carbon moieties. As discussed above, the invention provides methods for discovering and implementing the most effective of enzymes to enable these important new "biomass conversion" and alternative energy industrial processes.

[0548] In one aspect, the polypeptides of the invention are used in processes for converting lignocellulosic and/or starch biomass to ethanol. The invention also provides processes for making ethanol ("bioethanol"), propanol ("biopropanol"), butanol ("biobutanol"), or diesel fuel ("biodiesel"), from compositions comprising starch and/or lignocellulosic biomass. The lignocellulose and/or starch biomass material can be obtained from agricultural crops, as a byproduct of food or feed production, or as lignocellulosic waste products, such as plant residues and waste paper. Examples of suitable plant residues for treatment with polypeptides of the invention include stems, leaves, hulls, husks, cobs and the like, as well as wood, wood chips, wood pulp, and sawdust. Examples of paper waste suitable for treatment with polypeptides of the invention include discard photocopy paper, computer printer paper, notebook paper, notepad paper, typewriter paper, and the like, as well as newspapers, magazines, cardboard, and paper-based packaging materials.

[0549] In one aspect, the enzymes and methods of the invention can be used in conjunction with more "traditional" means of making ethanol from biomass, e.g., as methods comprising hydrolyzing lignocellulosic materials by subjecting dried lignocellulosic material in a reactor to a catalyst comprised of a dilute solution of a strong acid and a metal salt; this can lower the activation energy, or the temperature, of cellulose hydrolysis to obtain higher sugar yields; see, e.g., U.S. Patent Nos. 6,660,506; 6,423,145.

[0550] Another exemplary method that incorporates use of enzymes of the invention comprises catalyzing the hydrolysis of polysaccharides comprising glucose monomers, such as starch (a polymer of glucose monomers joined by 1,4-alpha or 1,6- alpha linkages), into sugars; this can be used in conjunction with enzyme for hydrolyzing lignocellulosic material containing hemicellulose, cellulose and lignin. In one aspect, the biomass is subjected to a first stage hydrolysis step in an aqueous medium at a temperature and a pressure chosen to effect primarily depolymerization of hemicellulose without major depolymerization of cellulose to glucose. This step results in a slurry in which the liquid aqueous phase contains dissolved monosaccharides resulting from depolymerization of hemicellulose and a solid phase containing cellulose and lignin. A second stage hydrolysis step can comprise conditions such that at least a major portion of the cellulose is depolymerized, such step resulting in a liquid aqueous phase containing dissolved/ soluble depolymerization products of cellulose, which can be hydrolyzed with enzymes of this invention. See, e.g., U.S. Patent No. 5,536,325. Enzymes of the invention can be added at any stage of this exemplary process.

[0551] Another exemplary method incorporating enzymes of the invention comprises processing a lignocellulose-containing biomass material by one or more stages of dilute acid hydrolysis with about 0.4% to 2% strong acid; and treating an unreacted solid lignocellulosic component of the acid hydrolyzed biomass material by alkaline delignification to produce precursors for biodegradable thermoplastics and derivatives. See, e.g., U.S. Patent No. 6,409,841. Enzymes of the invention can be added at any stage of this exemplary process.

[0552] Another exemplary method that incorporated use of enzymes of the invention comprises prehydrolyzing lignocellulosic material in a prehydrolysis reactor; adding an acidic liquid to the solid lignocellulosic material to make a mixture; heating the mixture to reaction temperature; maintaining reaction temperature for time sufficient to fractionate the lignocellulosic material into a solubilized portion containing at least about 20% of the lignin from the lignocellulosic material and a solid fraction containing cellulose; removing a solubilized portion from the solid fraction while at or near reaction temperature wherein the cellulose in the solid fraction is rendered more amenable to enzymatic digestion; and recovering a solubilized portion. See, e.g., U.S. Patent No. 5,705,369. Enzymes of the invention can be added at any stage of this exemplary process.

[0553] The invention provides methods for making motor fuel compositions (e.g., for spark ignition motors) based on liquid hydrocarbons blended with a fuel grade alcohol made by using an enzyme or a method of the invention. In one aspect, the fuels made by use of an enzyme of the invention comprise, e.g., coal gas liquid- or natural gas liquid-ethanol blends. In one aspect, a co-solvent is biomass-derived 2-methyltetrahydrofuran (MTHF). See, e.g., U.S. Patent No. 6,712,866.

[0554] Methods of the invention for the enzymatic degradation of lignocellulose, e.g., for production of ethanol from lignocellulosic material, can also comprise use of ultrasonic treatment of the biomass material; see, e.g., U.S. Patent No. 6,333,181.

[0555] Another exemplary process for making a biofuel comprising, e.g., a bioethanol, biopropanol, biobutanol, or a biodiesel, using enzymes of the invention comprises pretreating a starting material comprising a lignocellulosic feedstock

comprising at least hemicellulose and cellulose. In one aspect, the starting material comprises potatoes, soybean (rapeseed), barley, rye, corn, oats, wheat, beets or sugar cane or a component or waste or food or feed production byproduct. The starting material ("feedstock") is reacted at conditions which disrupt the plant's fiber structure to effect at least a partial hydrolysis of the hemicellulose and cellulose. Disruptive conditions can comprise, e.g., subjecting the starting material to an average temperature of 180°C to 270°C at pH 0.5 to 2.5 for a period of about 5 seconds to 60 minutes; or, temperature of 220°C to 270°C, at pH 0.5 to 2.5 for a period of 5 seconds to 120 seconds, or equivalent. This generates a feedstock with increased accessibility to being digested by an enzyme, e.g., a glucoamylase, of this invention. U.S. Patent No. 6,090,595.

[0556] Exemplary conditions for enzyme hydrolysis of lignocellulosic material include reactions at temperatures between about 30°C and 48°C, and/or a pH between about 4.0 and 6.0. Other exemplary conditions include a temperature between about 30°C and 60°C and a pH between about 4.0 and 8.0.

[0557] In one aspect of these biofuel (such as a bioethanol, biopropanol, biobutanol, or a biodiesel) generating processes of the invention using at least one enzyme of the invention, one or more other enzymes are added, e.g., amylases, beta-galactosidases, catalases, laccases, cellulases, endoglycosidases, endo-beta-1,4-laccases, amyloglucosidases, glucosidases, glucose isomerases, glycosyltransferases, lipases, phospholipases, lipooxygenases, beta-laccases, endo-beta-1,3(4)-laccases, cutinases, peroxidases, amylases, glucoamylases, pectinases, reductases, oxidases, decarboxylases, phenoloxidases, ligninases, pullulanases, arabinanases, hemicellulases, mannanases, xylolaccases, xylanases, pectin acetyl esterases, rhamnogalacturonan acetyl esterases, proteases, peptidases, proteinases, polygalacturonases, rhamnogalacturonases, galactanases, pectin lyases, transglutaminases, pectin methylesterases, cellobiohydrolases and/or transglutaminases.

[0558] Thus, the invention provides methods for processing a biomass material comprising lignocellulose comprising contacting a composition comprising a polypeptide of the invention, wherein optionally the biomass material comprises or is derived from an agricultural crop, or is a byproduct of a food or a feed production, or is a lignocellulosic waste product, or is a plant residue or a waste paper or waste paper product, and the polypeptide has activity comprising glucoamylase activity, and optionally the plant residue comprise stems, leaves, hulls, husks, cobs, wood, wood chips, wood pulp and sawdust, and optionally the paper waste comprises discarded or used photocopy paper, computer printer paper, notebook paper, notepad paper, typewriter paper, newspapers, magazines, cardboard and paper-based packaging materials, and optionally the processing of the biomass material generates a bioethanol. The invention provides biomass material comprising a polypeptide of the invention.

[0559] The invention provides methods for making biofuel (such as a bioethanol, biopropanol, biobutanol, or a biodiesel) comprising contacting a composition comprising a fermentable sugar with a polypeptide of the invention, wherein optionally the composition comprising a fermentable sugar comprises a plant, plant product or plant derivative, and optionally the plant or plant product comprises cane sugar plants or plant products, beets or sugarbeets, wheat, corn, soybeans, potato, rice or barley, and the polypeptide has activity comprising glucoamylase activity.

[0560] The invention provides methods for making a fuel (such as a bioethanol, biopropanol, biobutanol, or a biodiesel) comprising contacting a composition comprising a fermentable sugar with a polypeptide of the invention, wherein optionally the composition comprising a fermentable sugar comprises a plant, plant product or plant derivative, and optionally the plant or plant product comprises cane sugar plants or plant products, beets or sugarbeets, wheat, corn, soybeans, potato, rice or barley, and the polypeptide has activity comprising glucoamylase activity, and optionally the fuel comprises a bioethanol or a gasoline-ethanol mix. The invention provides fuels comprising a polypeptide of the invention, wherein the polypeptide has activity comprising glucoamylase activity, wherein optionally the fuel is derived from a plant material, which optionally comprises potatoes, soybean (rapeseed), barley, rye, corn, oats, wheat, beets or sugar cane, and optionally the fuel comprises a bioethanol, biopropanol, biobutanol, biodiesel and/or a gasoline-ethanol mix.

[0561] In another aspect, plant material comprising the enzymes described herein can be used in an industrial process to produce fuel or energy. Enzymes expressed in plants can be added to, mixed into or sprayed onto feedstock material. Alternatively, the enzymes could be directly expressed in the feedstock material. In one embodiment, plant material expressing enzymes could be ground, milled, heated or the like, in order to disrupt the physical integrity of the plant cells or organs that contain the enzyme, thereby releasing the enzyme to come in contact with the substrate. Exemplary sources of plant material include, but are not limited to, maize, alfalfa, sunflower, *Brassica,* soybean, cotton, safflower, peanut, sorghum, wheat, oat, rye, millet, barley, rice, conifers, grasses, e.g., switch grass and *Miscanthus*, legume crops, e.g., pea, bean and soybean, starchy tuber/roots, e.g., potato, sweet potato, cassava, taro, canna and sugar beet and the like.

[0562] The invention provides polypeptide, including glucoamylases of the invention, and methods for the conversion of a biomass or any lignocellulosic material (e.g., any composition comprising cellulose, hemicellulose and lignin), to a fuel (e.g., bioethanol, biopropanol, biobutanol, biopropanol, biomethanol, biodiesel), in addition to feeds, foods and chemicals. For example, in one aspect, an enzyme of the disclosure has ß-glucosidase activity to liberate D-glucose from cellobiose dimers. In one aspect, the enzymes have exo- or endo-beta-glucanase activity.

[0563] Thus, the compositions and methods of the invention provide effective and sustainable alternatives or adjuncts

to use of petroleum-based products, e.g., as a mixture of a biofuel such as biomethanol, bioethanol, biopropanol, biobutanol, and the like, to diesel fuel, gasoline, kerosene and the like. The invention provides organisms expressing enzymes of the invention for participation in chemical cycles involving natural biomass conversion. In one aspect, enzymes and methods for the conversion are used in enzyme ensembles for the efficient depolymerization of polysaccharides, cellulosic and/or hemicellulosic polymers to metabolizeable (e.g., fermentable) carbon moieties. The invention provides methods for discovering and implementing the most effective of enzymes to enable these important new "biomass conversion" and alternative energy industrial processes.

[0564] The compositions and methods of the invention can be used to provide effective and sustainable alternatives or adjuncts to use of petroleum-based products, e.g., as a mixture of bioethanol, biopropanol, biobutanol, biopropanol, biomethanol and/or biodiesel and gasoline. The invention provides organisms expressing enzymes of the invention for participation in chemical cycles involving natural biomass conversion. The invention provides methods for discovering and implementing the most effective of enzymes to enable these important new "biomass conversion" and alternative energy industrial processes.

[0565] The invention provides methods, enzymes and mixtures of enzymes or "cocktails" of the invention, for processing a material, e.g. a biomass material, comprising a cellooligsaccharide, an arabinoxylan oligomer, a lignin, a lignocellulose, a xylan, a glucan, a cellulose and/or a fermentable sugar comprising contacting the composition with a polypeptide of the invention, or a polypeptide encoded by a nucleic acid of the invention, wherein optionally the material is derived from an agricultural crop (e.g., wheat, barley, potatoes, switchgrass, poplar wood), is a byproduct of a food or a feed production, is a lignocellulosic waste product, or is a plant residue or a waste paper or waste paper product, and optionally the plant residue comprise stems, leaves, hulls, husks, corn or corn cobs, corn stover, corn fiber, hay, straw (e.g. rice straw or wheat straw), sugarcane bagasse, sugar beet pulp, citrus pulp, and citrus peels, wood, wood thinnings, wood chips, wood pulp, pulp waste, wood waste, wood shavings and sawdust, construction and/or demolition wastes and debris (e.g. wood, wood shavings and sawdust), and optionally the paper waste comprises discarded or used photocopy paper, computer printer paper, notebook paper, notepad paper, typewriter paper, newspapers, magazines, cardboard and paper-based packaging materials, and recycled paper materials. In addition, urban wastes, e.g. the paper fraction of municipal solid waste, municipal wood waste, and municipal green waste, along with other materials containing sugar, starch, and/or cellulose can be used. Optionally the processing of the material, e.g. the biomass material, generates a bioalcohol, e.g., a bioethanol, biomethanol, biobutanol or biopropanol.

[0566] Alternatively, the polypeptide of the invention may be expressed in the biomass plant material or feedstock itself.

[0567] The methods of the invention also include taking the converted lignocellulosic material (processed by enzymes of the invention) and making it into a fuel (e.g. a bioalcohol, e.g., a bioethanol, biomethanol, biobutanol or biopropanol, or biodiesel) by fermentation and/or by chemical synthesis. In one aspect, the produced sugars are fermented and/or the non-fermentable products are gasified.

[0568] The methods of the invention also include converting algae, virgin vegetable oils, waste vegetable oils, animal fats and greases (e.g. tallow, lard, and yellow grease), or sewage, using enzymes of the invention, and making it into a fuel (e.g. a bioalcohol, e.g., a bioethanol, biomethanol, biobutanol or biopropanol, or biodiesel) by fermentation and/or by chemical synthesis or conversion.

[0569] The enzymes of the invention (including, for example, organisms, such as microorganisms, e.g., fungi, yeast or bacteria, making and in some aspects secreting recombinant enzymes of the invention) can be used in or included/integrated at any stage of any biomass conversion process, e.g., at anyone step, several steps, or included in all of the steps, or all of the following methods of biomass conversion processes, or all of these biofuel alternatives:

Direct combustion: the burning of material by direct heat and is the simplest biomass technology; can be very economical if a biomass source is nearby.

Pyrolysis: is the thermal degradation of biomass by heat in the absence of oxygen. In one aspect, biomass is heated to a temperature between about 800 and 1400 degrees Fahrenheit, but no oxygen is introduced to support combustion resulting in the creation of gas, fuel oil and charcoal.

Gasification: biomass can be used to produce methane through heating or anaerobic digestion. Syngas, a mixture of carbon monoxide and hydrogen, can be derived from biomass.

Landfill Gas: is generated by the decay (anaerobic digestion) of buried garbage in landfills. When the organic waste decomposes, it generates gas consisting of approximately 50% methane, the major component of natural gas.

Anaerobic digestion: converts organic matter to a mixture of methane, the major component of natural gas, and carbon dioxide. In one aspect, biomass such as waterwaste (sewage), manure, or food processing waste, is mixed with water and fed into a digester tank without air.

Fermentation

[0570] Alcohol Fermentation: fuel alcohol is produced by converting cellulosic mass and/or starch to sugar, fermenting

the sugar to alcohol, then separating the alcohol water mixture by distillation. Feedstocks such as dedicated crops (e.g., wheat, barley, potatoes, switchgrass, poplar wood), agricultural residues and wastes (e.g. rice straw, corn stover, wheat straw, sugarcane bagasse, rice hulls, corn fiber, sugar beet pulp, citrus pulp, and citrus peels), forestry wastes (e.g. hardwood and softwood thinnings, hardwood and softwood residues from timber operations, wood shavings, and sawdust), urban wastes (e.g. paper fraction of municipal solid waste, municipal wood waste, municipal green waste), wood wastes (e.g. saw mill waste, pulp mill waste, construction waste, demolition waste, wood shavings, and sawdust), and waste paper or other materials containing sugar, starch, and/or cellulose can be converted to sugars and then to alcohol by fermentation with yeast. Alternatively, materials containing sugars can be converted directly to alcohol by fermentation.

[0571] Transesterification: An exemplary reaction for converting oil to biodiesel is called transesterification. The transesterification process reacts an alcohol (like methanol) with the triglyceride oils contained in vegetable oils, animal fats, or recycled greases, forming fatty acid alkyl esters (biodiesel) and glycerin. The reaction requires heat and a strong base catalyst, such as sodium hydroxide or potassium hydroxide.

[0572] Biodiesel: Biodiesel is a mixture of fatty acid alkyl esters made from vegetable oils, animal fats or recycled greases. Biodiesel can be used as a fuel for vehicles in its pure form, but it is usually used as a petroleum diesel additive to reduce levels of particulates, carbon monoxide, hydrocarbons and air toxics from diesel-powered vehicles.

[0573] Hydrolysis: includes hydrolysis of a compound, e.g., a biomass, such as a lignocellulosic material, catalyzed using an enzyme of the instant invention.

[0574] Congeneration: is the simultaneous production of more than one form of energy using a single fuel and facility. In one aspect, biomass cogeneration has more potential growth than biomass generation alone because cogeneration produces both heat and electricity.

[0575] In one aspect, the polypeptides of the invention have enzymatic activity (a glucoamylase activity) for generating a fuel (e.g. a bioalcohol, e.g., a bioethanol, biomethanol, biobutanol or biopropanol, or biodiesel) from an organic material, e.g., a biomass, such as compositions derived from plants and animals, including any agricultural crop or other renewable feedstock, an agricultural residue or an animal waste, the organic components of municipal and industrial wastes, or construction or demolition wastes or debris, or microorganisms such as algae or yeast.

[0576] In one aspect, polypeptides of the invention are used in processes for converting lignocellulosic biomass to a fuel (e.g. a bioalcohol, e.g., a bioethanol, biomethanol, biobutanol or biopropanol, or biodiesel), or otherwise are used in processes for hydrolyzing or digesting biomaterials such that they can be used as a fuel (e.g. a bioalcohol, e.g., a bioethanol, biomethanol, biobutanol or biopropanol, or biodiesel), or for making it easier for the biomass to be processed into a fuel.

[0577] In an alternative aspect, polypeptides of the invention, including the mixture of enzymes or "cocktails" of the invention, are used in processes for a transesterification process reacting an alcohol (like ethanol, propanol, butanol, propanol, methanol) with a triglyceride oil contained in a vegetable oil, animal fat or recycled greases, forming fatty acid alkyl esters (biodiesel) and glycerin. In one aspect, biodiesel is made from soybean oil or recycled cooking oils. Animal's fats, other vegetable oils, and other recycled oils can also be used to produce biodiesel, depending on their costs and availability. In another aspect, blends of all kinds of fats and oils are used to produce a biodiesel fuel of the invention.

[0578] Enzymes of the invention, including the mixture of enzymes or "cocktails" of the invention, can also be used in glycerin refining. The glycerin by-product contains unreacted catalyst and soaps that are neutralized with an acid. Water and alcohol are removed to produce 50% to 80% crude glycerin. The remaining contaminants include unreacted fats and oils, which can be processes using the polypeptides of the invention. In a large biodiesel plants of the invention, the glycerin can be further purified, e.g., to 99% or higher purity, for the pharmaceutical and cosmetic industries.

[0579] Fuels (including bioalcohols such as bioethanols, biomethanols, biobutanols or biopropanols, or biodiesels) made using the polypeptides of the invention, including the mixture of enzymes or "cocktails" of the invention, can be used with fuel oxygenates to improve combustion characteristics. Adding oxygen results in more complete combustion, which reduces carbon monoxide emissions. This is another environmental benefit of replacing petroleum fuels with biofuels (e.g., a fuel of the invention). A biofuel made using the compositions and/or methods of this invention can be blended with gasoline to form an E10 blend (about 5% to 10% ethanol and about 90% to 95% gasoline), but it can be used in higher concentrations such as E85 or in its pure form. A biofuel made using the compositions and/or methods of this invention can be blended with petroleum diesel to form a B20 blend (20% biodiesel and 80% petroleum diesel), although other blend levels can be used up to B100 (pure biodiesel).

[0580] The invention also provides processes for making biofuels (including bioalcohols such as bioethanols, biomethanols, biobutanols or biopropanols, or biodiesels) from compositions comprising lignocellulosic biomass. The lignocellulose biomass material can be obtained from agricultural crops, as a byproduct of food or feed production, or as lignocellulosic waste products, such as plant residues, waste paper or construction and/or demolition wastes or debris. Examples of suitable plant sources or plant residues for treatment with polypeptides of the invention include kelp, algae, grains, seeds, stems, leaves, hulls, husks, corn cobs, corn stover, straw, grasses (e.g., Indian grass, such as *Sorghastrum nutans*; or, switch grass, e.g., *Panicum* species, such as *Panicum virgatum*), and the like, as well as wood, wood chips, wood pulp, and sawdust. Examples of paper waste suitable for treatment with polypeptides of the invention include

discard photocopy paper, computer printer paper, notebook paper, notepad paper, typewriter paper, and the like, as well as newspapers, magazines, cardboard, and paper-based packaging materials. Examples of construction and demolition wastes and debris include wood, wood scraps, wood shavings and sawdust.

[0581] In one embodiment, the enzymes, including the mixture of enzymes or "cocktails" of the invention, and methods of the invention can be used in conjunction with more "traditional" means of making ethanol, methanol, propanol, butanol, propanol and/or diesel from biomass, e.g., as methods comprising hydrolyzing lignocellulosic materials by subjecting dried lignocellulosic material in a reactor to a catalyst comprised of a dilute solution of a strong acid and a metal salt; this can lower the activation energy, or the temperature, of cellulose hydrolysis to obtain higher sugar yields; see, e.g., U.S. Patent Nos. 6,660,506 and 6,423,145.

[0582] Another exemplary method that incorporated use of enzymes of the invention, including the mixture of enzymes or "cocktails" of the invention, comprises hydrolyzing lignocellulosic material containing hemicellulose, cellulose and lignin, or any other polysaccharide that can be hydrolyzed by an enzyme of this invention, by subjecting the material to a first stage hydrolysis step in an aqueous medium at a temperature and a pressure chosen to effect primarily depolymerization of hemicellulose without major depolymerization of cellulose to glucose. This step results in a slurry in which the liquid aqueous phase contains dissolved monosaccharides resulting from depolymerization of hemicellulose and a solid phase containing cellulose and lignin. A second stage hydrolysis step can comprise conditions such that at least a major portion of the cellulose is depolymerized, such step resulting in a liquid aqueous phase containing dissolved/soluble depolymerization products of cellulose. See, e.g., U.S. Patent No. 5,536,325. Enzymes of the invention (including the invention's mixtures, or "cocktails" of enzymes) can be added at any stage of this exemplary process.

[0583] Another exemplary method that incorporated use of enzymes of the invention, including the mixture of enzymes or "cocktails" of the invention, comprises processing a lignocellulose-containing biomass material by one or more stages of dilute acid hydrolysis with about 0.4% to 2% strong acid; and treating an unreacted solid lignocellulosic component of the acid hydrolyzed biomass material by alkaline delignification to produce precursors for biodegradable thermoplastics and derivatives. See, e.g., U.S. Patent No. 6,409,841. Enzymes of the invention can be added at any stage of this exemplary process.

[0584] Another exemplary method that incorporated use of enzymes of the invention, including the mixture of enzymes or "cocktails" of the invention, comprises prehydrolyzing lignocellulosic material in a prehydrolysis reactor; adding an acidic liquid to the solid lignocellulosic material to make a mixture; heating the mixture to reaction temperature; maintaining reaction temperature for time sufficient to fractionate the lignocellulosic material into a solubilized portion containing at least about 20% of the lignin from the lignocellulosic material and a solid fraction containing cellulose; removing a solubilized portion from the solid fraction while at or near reaction temperature wherein the cellulose in the solid fraction is rendered more amenable to enzymatic digestion; and recovering a solubilized portion. See, e.g., U.S. Patent No. 5,705,369. Enzymes of the invention can be added at any stage of this exemplary process.

[0585] The invention provides methods for making motor fuel compositions (e.g., for spark ignition motors) based on liquid hydrocarbons blended with a fuel grade alcohol made by using an enzyme or a method of the invention. In one aspect, the fuels made by use of an enzyme of the invention comprise, e.g., coal gas liquid- or natural gas liquid-ethanol blends. In one aspect, a co-solvent is biomass-derived 2-methyltetrahydrofuran (MTHF). See, e.g., U.S. Patent No. 6,712,866.

[0586] In one aspect, methods of the invention for the enzymatic degradation of lignocellulose, e.g., for production of biofuels (including bioalcohols such as bioethanols, biomethanols, biobutanols or biopropanols, or biodiesels) from lignocellulosic material, can also comprise use of ultrasonic treatment of the biomass material; see, e.g., U.S. Patent No. 6,333,181.

[0587] In another aspect, methods of the invention for producing biofuels (including bioalcohols such as bioethanols, biomethanols, biobutanols or biopropanols, or biodiesels) from a cellulosic substrate comprise providing a reaction mixture in the form of a slurry comprising cellulosic substrate, an enzyme of this invention and a fermentation agent (e.g., within a reaction vessel, such as a semi-continuously solids-fed bioreactor), and the reaction mixture is reacted under conditions sufficient to initiate and maintain a fermentation reaction (as described, e.g., in U.S. Pat. App. No. 20060014260). In one aspect, experiment or theoretical calculations can determine an optimum feeding frequency. In one aspect, additional quantities of the cellulosic substrate and the enzyme are provided into the reaction vessel at an interval(s) according to the optimized feeding frequency.

[0588] One exemplary process for making biofuels (including bioalcohols such as bioethanols, biomethanols, biobutanols or biopropanols, or biodiesels) of the invention is described in U.S. Pat. App. Pub. Nos. 20050069998; 20020164730; and in one aspect comprises stages of grinding the lignocellulosic biomass (e.g., to a size of 15-30 mm), subjecting the product obtained to steam explosion pre-treatment (e.g., at a temperature of 190-230°C) for between 1 and 10 minutes in a reactor; collecting the pre-treated material in a cyclone or related product of manufacture; and separating the liquid and solid fractions by filtration in a filter press, introducing the solid fraction in a fermentation deposit and adding one or more enzymes of the invention, e.g., aglucoamylase enzyme (e.g., dissolved in citrate buffer pH 4.8).

[0589] Another exemplary process for making bio fuels (including bioalcohols such as bioethanols, biomethanols,

biobutanols or biopropanols, or biodiesels) of the invention comprising bioethanols, biomethanols, biobutanols or biopropanols using enzymes of the invention comprises pretreating a starting material comprising a lignocellulosic feedstock comprising at least hemicellulose and cellulose. In one aspect, the starting material comprises potatoes, soybean (rapeseed), barley, rye, corn, oats, wheat, beets or sugar cane or a component or waste or food or feed production byproduct. The starting material ("feedstock") is reacted at conditions which disrupt the plant's fiber structure to effect at least a partial hydrolysis of the hemicellulose and cellulose. Disruptive conditions can comprise, e.g., subjecting the starting material to an average temperature of 180°C to 270°C at pH 0.5 to 2.5 for a period of about 5 seconds to 60 minutes; or, temperature of 220°C to 270°C, at pH 0.5 to 2.5 for a period of 5 seconds to 120 seconds, or equivalent. This generates a feedstock with increased accessibility to being digested by an enzyme, e.g., a cellulase enzyme of the invention. U.S. Patent No. 6,090,595.

[0590] Exemplary conditions for using enzymes of the invention in the hydrolysis of lignocellulosic material include reactions at temperatures between about 30°C and 48°C, and/or a pH between about 4.0 and 6.0. Other exemplary conditions include a temperature between about 30°C and 60°C and a pH between about 4.0 and 8.0.

[0591] Glucoamylases of the invention can be used in the conversion of biomass to fuels, and in the production of ethanol, e.g., as described in PCT Application Nos. WO0043496 and WO8100857. Glucoamylases of the invention can be used to produce fermentable sugars and glucan-containing biomass that can be converted into fuel ethanol.

Pharmaceutical compositions, disinfectants and dietary supplements

[0592] The invention also provides pharmaceutical compositions, disinfectants and dietary supplements (e.g., dietary aids) comprising enzymes of the invention (e.g., enzymes having glucoamylase activity). In one aspect, the pharmaceutical compositions and dietary supplements (e.g., dietary aids) are formulated for oral ingestion, e.g., to improve the digestibility of foods and feeds having a high starch, cellulose or lignocellulosic component.

[0593] Periodontal treatment compounds can comprise an enzyme of the invention, e.g., as described in U.S. patent no. 6,776,979. Compositions and methods for the treatment or prophylaxis of acidic gut syndrome can comprise an enzyme of the invention, e.g., as described in U.S. patent no. 6,468,964.

[0594] In another aspect, wound dressings, implants and the like comprise antimicrobial (e.g., antibiotic-acting) enzymes, including an enzyme of the invention. Enzymes of the invention can also be used in alginate dressings, antimicrobial barrier dressings, burn dressings, compression bandages, diagnostic tools, gel dressings, hydro-elective dressings, hydrocellular (foam) dressings, hydrocolloid dressings, I.V dressings, incise drapes, low adherent dressings, odor absorbing dressings, paste bandages, post operative dressings, scar management, skin care, transparent film dressings and/or wound closure. Enzymes of the invention can be used in wound cleansing, wound bed preparation, to treat pressure ulcers, leg ulcers, bums, diabetic foot ulcers, scars, IV fixation, surgical wounds and minor wounds. Enzymes of the invention can be used to in sterile enzymatic debriding compositions, e.g., ointments. In various aspects, the cellulase is formulated as a tablet, gel, pill, implant, liquid, spray, powder, food, feed pellet or as an encapsulated formulation.

Biodefense applications

[0595] In other aspects, enzymes of the invention, e.g., glucoamylases, can be used in biodefense, e.g., destruction of spores or bacteria. Use of enzymes of the invention in biodefense applications offers a significant benefit, in that they can be very rapidly developed against any currently unknown or biological warfare agents of the future. In addition, enzymes of the invention can be used for decontamination of affected environments. In aspect, the invention provides a biodefense or bio-detoxifying agent comprising a polypeptide of the invention having glucoamylase activity.

[0596] The invention will be further described with reference to the following examples; however, it is to be understood that the invention is not limited to such examples.

EXAMPLES

Example 1: Identification and Characterization of Thermostable α-Amylases

[0597] Screening programs can be carried out under neutral and low pH conditions. DNA sequence and bioinformatic analyses can classify amylases.

Biochemical studies

[0598] Biochemical analysis of amylase genomic clones can be used to determine if any have a pH optima of less than pH 6. Lysates of these genomic clones can be tested for thermal tolerance by incubation at 70°C, 80°C, 90°C or

100°C for 10 minutes and measurement of residual activity at pH 4.5. Those clones retaining >50% activity after heat treatment at 80°C are chosen for further analysis. These clones can be incubated at 90°C for 10 minutes at pH 6.0 and 4.5 and tested for residual activity at pH 4.5. Thermal activity of the clones with residual activity after heat treatment at 90°C at pH 4.5 can be measured at room temperature, 70°C and 90°C at pH 4.5.

[0599] In one aspect, starch-degrading enzymes are screened for activity on raw starch and 'resistant' starch for any form of hydrolase activity, including amylase, pullulanase, cyclodextrin glycosyltransferase, glucoamylases other any other glucosidase activity. In one aspect, the identified active enzymes are characterized, e.g., by specific activity and specificity for branched sugars and/or longer oligosaccharides.

[0600] In one aspect, fungal isolates are investigated for novel enzymes using, e.g., probes and/or discovery processes of the disclosure, for example, screening gDNA and/or cDNA libraries made from animals, microorganisms or insects, e.g., the gut contents of insects that attack and consume stored grains, including screening environmental libraries for under-represented enzymes. Combinations of enzymes can be evaluated.

[0601] In one aspect, stability tests are performed: e.g., purified enzymes in their own storage buffer, as compared to activity of fresh enzyme, if tested; e.g., aliquots of 20 ul stored at lower temperatures, e.g., at between about 4°C to -20°C to -80 °C; and in one aspect, activity is retested in granular and soluble starch monthly

[0602] Amylases can be evaluated under a variety of conditions. In the following protocols N°2 yellow dent corn can be used as a starch source.

Exemplary Liquefaction Assay

[0603] A starch slurry comprising 35% dry solids ("DS") is subjected to primary liquefaction for five minutes under various temperatures in the range of 95°C to 119°C (e.g., at about 110°C), with an enzyme concentration of between 0.2 to 0.8 gram/kilogram (g/kg) starch DS, with added calcium in the range of between zero and 30 parts per million (ppm), at pH 4.0 to pH 5.6. Secondary liquefaction comprised conditions of 120 minutes at 95°C.

Exemplary Saccharification Assay

[0604] Saccharification is initially tested using 35% dry solids ("DS") (starch slurry) and glucoamylase AMG 300L (Novozymes A/S, Denmark) at 0.225 AGU/gram DS (AGU= amyloglucosidase, or glucoamylase, units), pH 4.3, at 60°C for 44 hours.

[0605] In one aspect, exemplary glucoamylases of the invention are used in a dosage range of between 0.5 to 0.7 kg / MT DS starch.

[0606] The invention provides methods for making nutritive sweeteners using enzymes of the invention, e.g., processes comprising the above described liquefaction and saccharification protocols using any glucoamylase of the invention. In one aspect, the dosage range for an enzyme of the invention in these processes is between about 0.5 to 0.7 gram per kg starch DS, a jet temperature (e.g., using a jet cooker) of about 110°C, pH 4.5, no added calcium.

Dry Mill Ethanol Production

[0607] The invention provides methods for Dry Mill Ethanol Production using enzymes of the invention. In evaluating enzymes of the invention for use in Dry Mill Ethanol Production, particularly, liquefaction of dry mill corn flour, a bench scale reactor can be used with corn flour sourced from commercial dry mill. TERMAMYL™ SC (Novozymes A/S, Denmark) amylase can be used as a competitive benchmark. In alternative aspects, optimum conditions are 85°C, pH 5.7. Five independent variables can be studied: temperature (in a range of between 80°C to 100°C), enzyme dose of between 0.2 to 1.0 g/kg starch, pH 4.4 to 6.0, calcium in a range between 0 ppm to 200 ppm, and a recycled backset between about 0% to 40%.

[0608] At 95°C, in some embodiments, glucoamylases of the invention can reduce viscosity of dry mill corn flour more rapidly than TERMAMYL™ SC (Novozymes A/S, Denmark) amylase at its optimum conditions, including at 85°C. The rate of viscosity reduction by amylases can be influenced most by enzyme dose and temperature. Alternative optimal ranges can be in the range of 0.4 to 0.6 g/kg starch, with an optimum temperature at 95°C.

[0609] In some embodiments, glucoamylases of the invention can be effective at a lower pH and a higher temperature than TERMAMYL™ SC (Novozymes A/S, Denmark) amylase at a pH in the range between pH 4.4 and pH 5.6. Calcium addition can have a minimal effect on rate of viscosity reduction at 95°C.

[0610] The activities of the purified enzymes were compared in different storage buffers, as listed below, after 1 week of incubation at 37°C. The buffer with the lowest loss of activity compared to the activity of the same enzyme kept at +4°C was selected as the storage buffer of choice. Exemplary assay conditions to test for amylase activity, e.g., to determine if a polypeptide of the invention retains activity under particular conditions, include (MPB: Methylparaben):

- 20% Glucose, 0.1% MPB in PBS, pH 7.2@37°C
- 20% Glucose, 0.1% MPB in Acetate Buffer, pH 5@37°C
- 20% Sucrose, 0.1% MPB in PBS, pH 7.2@37°C
- 20% Sucrose, 0.1% MPB in Acetate Buffer, pH 5@37°C
- 0.1% MPB in PBS, pH 7.2@37°C
- 0.1% MPB in Acetate Buffer, pH 5@37°C
- PBS, pH 7.2@37°C
- Acetate Buffer, pH 5@37°C
- 20% Glucose, 0.1% MPB in PBS, pH 7.2@4°C
- 20% Glucose, 0.1% MPB in Acetate Buffer, pH 5@4°C
- 20% Sucrose, 0.1% MPB in PBS, pH 7.2@4°C
- 20% Sucrose, 0.1% MPB in Acetate Buffer, pH 5@4°C
- 20% Maltose in PBS (50mM sodium phosphate pH7.5; 100mM NaCl)
- 0.1% MPB in PBS, pH 7.2@4°C
- 0.1% MPB in Acetate Buffer, pH 5@4°C
- PBS, pH 7.2@4°C
- Acetate Buffer, pH 5@4°C

[0611] Exemplary enzymes that have been tested under these exemplary assay conditions include: SEQ ID NO:26; SEQ ID NO:18; SEQ ID NO:4; SEQ ID NO:28; SEQ ID NO:48; SEQ ID NO:76; SEQ ID NO:52; SEQ ID NO:70; SEQ ID NO:66. For SEQ ID NO:26 and SEQ ID NO:4, 20% sucrose in 1X PBS, pH 7 with 0.1% methyl paraben (SMP*) was chosen, and for SEQ ID NO:18, 20% sucrose in 50 mM sodium acetate with 150 mM sodium chloride and 0.1% methyl paraben (SMA**) was chosen. Figure 13 illustrates an initial rate comparison (using Vmax) of the exemplary enzyme SEQ ID NO:70 in different storage buffers (as indicated in the figure); 0.15 ug total protein per reaction; Figure 14 illustrates an initial rate comparison (using Vmax) of the exemplary enzyme SEQ ID NO:76 in different storage buffers (as indicated in the figure); 0.5 ug total protein per reaction; Figure 15 illustrates an initial rate comparison (using Vmax) of the exemplary enzyme SEQ ID NO:4 in different storage buffers (as indicated in the figure); Figure 16 illustrates an initial rate comparison (using Vmax) of the exemplary enzyme SEQ ID NO:66 in different storage buffers (as indicated in the figure); 0.03 ug total protein per reaction; Figure 17 illustrates an initial rate comparison (using Vmax) of the exemplary enzyme SEQ ID NO:52 in different storage buffers (as indicated in the figure); 0.025 ug total protein per reaction; Figure 18 illustrates an initial rate comparison (using nmole of glucose per min per ug of enzyme) of the exemplary enzyme SEQ ID NO:28 in different storage buffers (as indicated in the figure); Figure 19 illustrates an initial rate comparison (using nmole of glucose per min per ug of enzyme) of the exemplary enzyme SEQ ID NO:26 in different storage buffers (as indicated in the figure); Figure 20 illustrates an initial rate comparison (using nmole of glucose per min per ug of enzyme) of the exemplary enzyme SEQ ID NO:18 in different storage buffers (as indicated in the figure).

[0612] Bradford and BCA assays as well as SDS PAGE and Absorbance at A280 were used to determine the concentration of the purified proteins. The standardized amylase solution (Sigma A6211) was used as a reference in these assays, and BSA. Concentration by BCA and A280 was similar, but not by Bradford using BSA as a standard.

[0613] In these assays, protein (enzyme) was purified by precipitation; hydrophobic interaction; size exclusion; ion exchange; affinity; and in one exemplary protocol, protein (enzyme) was purified by precipitation, hydrophobic interaction, ion exchange and as a last step, affinity (chromatography).

[0614] The exemplary SEQ ID NO:18, SEQ ID NO:4 and SEQ ID NO:26 were purified by either ammonium sulfate (SEQ ID NO:26) or ethanol precipitation (SEQ ID NO:18 and SEQ ID NO:4). Lyophilized supernatants from cultures expressing the enzymes of interest were resuspended in water at a concentration of about 1g / 5ml. SEQ ID NO:18 and SEQ ID NO:4 were precipitated by addition of cold ethanol (2 volumes of protein solution). SEQ ID NO:26 was precipitated in 80% ammonium sulfate solution. After precipitation the protein suspensions were dialyzed once against water and subsequently against storage buffer.

[0615] Stability of purified exemplary enzymes of this disclosure was tested in either PBS at pH 7 or PBS pH 7.5 (in this case, the parameters were 50 mM sodium phosphate; 100 mM NaCl) or in 50 mM sodium acetate pH 5.2 with 150 mM NaCl added. Glucose (20%) or sucrose (20%) was added to the buffers as well as an antimicrobial, 0.1% methyl paraben (Sigma). The enzymes were incubated in their respective buffers at 37°C for 1 week and the enzyme activity was tested and compared to the activity of the same enzyme stored at +4 °C.

[0616] The concentration of each purified enzyme was estimated using 4 different methods: Bradford assay with amylase Sigma A-6211 as standard, BCA assay with Sigma A-6211 as standard, absorbance at 280nm in 8M urea, and densitometric measurement of stained protein after SDS PAGE, using Sigma A-6211 amylase as the reference. The numbers obtained with these different methods for each of the purified proteins are presented in Table 3.1. For final quantification, the concentration obtained by measuring absorbance at 280 nm in 8M urea was used.

[0617] A summary of the large scale purification for these assays is:

Summary of Recover Amount based on Absorbance Value			
	mg/ml	Volume (ml)	Total mg
SEQ ID NO:18	53.94	111	5987.34
SEQ ID NO:26	39.23	86.8	3405.164
SEQ ID NO:4	70.01	55.4	3878.554

Summary of Purity				
	Before Purification		After Purification	
	A260/A280	Expression Ratio	A260/A280	Expression Ratios
SEQ ID NO:18	0.52	91%	0.59	92%
SEQ ID NO:26	0.56	85%	0.65	86%
SEQ ID NO:4	0.67	91%	0.73	85%

Summary of Activity				
	Raw Starch		Soluble	
	Purified	Unpurified	Purified	Unpurified
SEQ ID NO:18	10.7228 ±0.7925	6.4828 ± 0.5681	40.6725 ± 1.7016	24.7354 ± 3.821
SEQ ID NO:26	5.7837 ± 0.2271	4.0691 ± 0.2864	17.4833 ± 0.7831	14.1849 ± 0.3761
SEQ ID NO:4	6.47 ± 0.34	4.074 ± 0.35	127.37 ± 1.78	116.52 ± 1.27

Estimation of Enzyme Concentration by Different Methods

	Bradford	BCA (A6211)	Absorbance (Urea)
SEQ ID NO:18	71.10	57.64	53.94
SEQ ID NO:26	43.98	57.64	39.29
SEQ ID NO:4	69.20	57.64	70.01

Example 2: Thermostable Amylases Active at Alkaline pH

[0618] The following example describes an exemplary method for determining if a polypeptide is a thermostable amylase.

[0619] Commercial automatic dish wash (ADW) formulations can be used to determine if a polypeptide is a thermostable amylase. Studies can include the identification of high pH amylases; and enzymes having the ability to degrade starch. DNA sequence and bioinformatics analyses can classify many of these genes as amylases, or having other enzyme specificities, e.g., neopullulanases, amylopullulanases and amylomaltases.

Biochemical studies

[0620] One exemplary method for determining if a polypeptide is a thermostable amylase, is to test for activity where the enzyme can hydrolyze starch at alkaline pH, e.g., up to pH 10, and about 50°C.

[0621] Soluble protein is purified to homogeneity and specific activity (units/mg, where 1 unit = μmol reducing sugars/min) is measured at pH 8 and pH 10 (40°C and 50°C) using 2% starch in buffer. Specific activity can be determined by removing samples at various time points during a 30 minute reaction and analyzing for reducing sugars. The initial rate can be determined by fitting the progress curves to a linear equation.

Stability studies

[0622] Stability in the presence of the ADW formulation can be measured by biochemical analysis. The benchmark for these studies can be a commercial enzyme in the formulation matrix. The measured activity after the incubation can be expressed as a percentage of the original activity.

Wash tests

[0623] Wash tests using starch coated slides can be carried out to gauge the performance of a purified enzyme of the disclosure as compared to a commercial amylase. Spaghetti starch coated slides can be prepared according to protocol: two pre-weighed starch coated slides are placed back to back in a 50 mL conical tube and 25 mL of ADW solution, +/enzyme are added per tube. The tubes are incubated for 20 minutes at 50°C with gentle rotation on a vertical carousel. Following the incubation period, the slides are immediately rinsed in water and oven dried overnight. All trials are run in duplicate and the commercial enzyme was run as a positive control. The results can be expressed as net % starch removed, e.g. % of starch removed in ADW with enzyme, *minus* the % of starch removed in ADW alone.

Example 3: Gene Optimization

[0624] The following example describes an exemplary method for assessing enzyme performance in the presence of ADW performance.

[0625] The properties of enzymes may be improved by various evolution strategies, including Gene Site Saturation Mutagenesis™ (GSSM™) and GeneReassembly™ technologies (Diversa Corporation, San Diego, CA). Such techniques will be applied to the amylase nucleic acids of the disclosure in order to generate pools of variants that can be screened for improved performance. In one aspect, parental molecules for evolution include any nucleic acid of the disclosure, e.g., sequences encoding SEQ ID NO:2; SEQ ID NO:4; SEQ ID NO:6, etc.

[0626] A high throughput screen (HTS) can be used to assess enzyme performance in the presence of ADW performance. HTS can be automated and has showed consistent results for the parental amylases.

Example 4: Characterization of α-Amylases having Activity at Alkaline pH

[0627] The following example describes exemplary methods for determining if a polypeptide has alpha-amylase activity at alkaline pH.

[0628] Glucoamylases of the invention having activity at alkaline pH can be characterized using kinetics on 2% starch at pH 8 and 10 (40°C and 50°C). 1 unit of activity can be defined as release of 1 μmol reducing sugars per minute.

EXAMPLE 5: Amylase Activity Assay: BCA Reducing Ends Assay

[0629] The following example describes an exemplary method for a BCA reducing ends assay. Amylase activity of clones of interest can be determined using the following methodology.

1. Prepare 2 substrate solutions, as follows:

   a) 2% soluble starch (potato or granular corn starch) pH 8 solution by dissolving 2 gm potato starch in 100 ml 100 mM sodium phosphate pH 8).
   b) 2% soluble starch (potato) pH 10 solution by dissolving 2 gm potato starch in 100 ml 100 mM sodium carbonate. Heat both solutions in a boiling water bath, while mixing, for 30-40 minutes until starch dissolves.

2. Prepare Solution A from 64 mg/ml sodium carbonate monohydrate, 24 mg/ml sodium bicarbonate and 1.95 mg/ml BCA(4,4'-dicarboxy-2,2'-biquinoline disodium salt (Sigma Chemical cat # D-8284). Added above to dH2O.
3. Prepare solution B by combining 1.24 mg/ml cupric sulfate pentahydrate and 1.26 mg/ml L-serine. Add mixture to dH2O.
4. Prepare a working reagent of a 1:1 ration of solutions A and B.
5. Prepare a Maltose standard solution of 10 mM Maltose in dH2O, where the 10 mM maltose is combined in 2% soluble starch at desired pH to a final concentration of 0, 100, 200, 300, 400, 600 μM. The standard curve will be generated for each set of time-points. Since the curve is determined by adding 10 ul of the standards to the working reagent it works out to 0, 1, 2, 3, 4, 6 nmole maltose.
6. Aliquot 1 ml of substrate solution into microcentrifuge tubes, equilibrate to desired temperature (5 min) in heat block or heated water bath. Add 50 ul of enzyme solution to the inside of the tube lid.

7. While solution is equilibrating mix 5 ml of both solution A & B. Aliquot 100 ul to 96 well PCR plate. Set plate on ice.
8. After 5 minute temperature equilibration, close lid on tubes, invert and vortex 3 times. Immediately aliquot 10 ul into plate as t=0 (zero time point). Leave enzyme mixture in heat block and aliquot 10 ul at each desired time point (e.g. 0, 5, 10,15, 20, 30 minutes).
9. Ensure that 12 wells are left empty (only working reagent aliquotted) for the addition of 10 ul of standards, for the standard curve.
10. When all time points are collected and standards are added, cover plate and heated to 80°C for 35 min. Cool plate on ice for 10 min. Add 100 ul H2O to all wells. Mix and aliquot 100 ul into flat bottomed 96-well plate and read absorbance at 560 nm.
11. Zero each sample's time points against its own t=0 (subtract the average t=0 A560 value from other average A560 values). Convert the $A560_{(experimental)}$ to umole (Divide $A560_{(experimental)}$ by the slope of the standard curve (A560/umole). Generate a slope of the time points and the umole (in umole/min), multiply by 100 (as the umole value only accounts for the 10 ul used in the assay, not the amount made in the 1ml rxn). To get the specific activity divide the slope (in umole/min) by the mg of protein. All points should be done at a minimum in duplicate with three being best.

Divide protein concentration (mg/ml) by any dilution to get mg used in assay.
Divide the above slope by mg used in assay to get specific activity
Specific Activity =24.93 umole/min/mg
See for example, Dominic W.S. Wong, Sarah B. Batt, and George H. Robertson (2000) J. Agric. Food Chem. 48:4540-4543; Jeffrey D. Fox and John F. Robyt, (1991) Anal. Biochem. 195, 93-96.

Example 6: Screening for α-Amylase activity

[0630] Amylase activity of clones can be assessed by a number of methods known in the art. The following is an exemplary methodology. The number of plaques screened, per plate, can be approximately 10,000 pfu's. For each DNA library: at least 50,000 plaques per isolated library and 200,000 plaques per non-isolated library should be screened depending upon the pfu titer for the λ Zap Express amplified lysate.

Titer determination of Lambda Library

[0631]

1) μL of Lambda Zap Express amplified library stock added to 600μL *E. coli* MRF' cells (OD_{600}=1.0). To dilute MRF' stock, 10mM $MgS0_4$ is used.
2) Incubate at 37 °C for 15 minutes.
3) Transfer suspension to 5-6mL of NZY top agar at 50 °C and gently mix.
4) Immediately pour agar solution onto large (150mm) NZY media plate.
5) Allow top agar to solidify completely (approximately 30 minutes), then invert plate.
6) Incubate the plate at 39 °C for 8-12 hours.
7) Number of plaques is approximated. Phage titer determined to give 10,000 pfu/plate. Dilute an aliquot of Library phage with SM buffer if needed.

Substrate screening

[0632]

1) Lambda Zap Express (50,000 pfu) from amplified library added to 600μL of *E. coli* MRF' cells (OD600=1.0). For non-environment libraries, prepare 4 tubes (50,000 pfu per tube).
2) Incubate at 37 °C for 15 minutes.
3) While phage/cell suspensions are incubating, 1.0mL of red starch substrate (1.2% w/v) is added to 6.0mL NZY top agar at 50 °C and mixed thoroughly. Keep solution at 50°C until needed.
4) Transfer 1/5 (10,000 pfu) of the cell suspension to substrate/top agar solution and gently mixed.
5) Solution is immediately poured onto large (150mm) NZY media plate.
6) Allow top agar to solidify completely (approximately 30 minutes), then invert plate.
7) Repeat procedures 4-6 4 times for the rest of the cell suspension (1/5 of the suspension each time).
8) Incubate plates at 39 °C for 8-12 hours.
9) Plate observed for clearing zones (halos) around plaques.

10) Plaques with halos are cored out of agar and transferred to a sterile micro tube. A large bore 200μL pipette tip works well to remove (core) the agar plug containing the desired plaque.
11) Phages are re-suspended in 500μL SM buffer. 20μL Chloroform is added to inhibit any further cell growth.
12) Pure phage suspension is incubated at room temperature for 4 hours or overnight before next step.

Isolation of pure clones

[0633]

1) 10μL of re-suspended phage suspension is added to 500μL of *E. coli* MRF' cells (OD600=1.0).
2) Incubate at 37 °C for 15 minutes.
3) While phage/cell suspension is incubating, 1mL of red starch substrate (1.2% w/v) is added to 6.0mL NZY top agar at 50 °C and mixed thoroughly. Keep solution at 50 °C until needed.
4) Cell suspension is transferred to substrate/top agar solution and gently mixed.
5) Solution is immediately poured onto large (150mm) NZY media plate.
6) Allow top agar to solidify completely (approximately 30 minutes), then invert plate.
7) Plate incubated at 39 °C for 8-12 hours.
8) Plate observed for a clearing zone (halo) around a single plaque (pure clone). If a single plaque cannot be isolated, adjust titer and re-plate phage suspension.
9) Single plaque with halo is cored out of agar and transferred to a sterile micro tube. A large bore 200μL pipette tip works well to remove (core) the agar plug containing the desired plaque. To amplify the titer, core 5 single active plaques into a micro tube.
10) Phages are re-suspended in 500μL SM buffer. 20μL Chloroform is added to inhibit any further cell growth.
11) Pure phage suspension is incubated at room temperature for 4 hours or overnight before next step. The pure phage suspension is stored at -80 °C by adding DMSO into the phage suspension (7% v/v).

Excision of pure clone

[0634]

1) 100μL of pure phage suspension is added to 200μL *E. coli* MRF' cells (OD600=1.0). To this, 1.0μL of EXASSIST helper phage (>1 x 106 pfu/mL; Stratagene) is added. Use 2059 Falcon tube for excision.
2) Suspension is incubated at 37 °C for 15 minutes.
3) 3.0 mL of 2 x YT media is added to cell suspension.
4) Incubate at 30 °C for at least 6 hours or overnight while shaking.
5) Tube transferred to 70°C for 20 minutes. The phagemid suspension can be stored at 4 °C for 1 to 2 months.
6) 100 μL of phagemid suspension transferred to a micro tube containing 200μL of *E. coli* Exp 505 cells (OD600=1.0).
7) Suspension incubated at 37 °C for 15 minutes.
8) 300μL of SOB is added to the suspension.
9) Suspension is incubated at 37 °C for 30 to 45 minutes.
10) 100μL of suspension is transferred to a small (90mm) LB media plate containing Kanamycin (LB media with Kanamycin 50μg/mL) for Zap Express DNA libraries or Ampicillin (LB media with Kanamycin 100μg/mL) for Zap II DNA libraries.
11) The rest of suspension is transferred to another small LB media plate.
12) Use sterile glass beads to evenly distribute suspension on the plate.
13) Plates are incubated at 30 °C for 12 to 24 hours.
14) Plate observed for colonies.
15) Inoculate single colony into LB liquid media containing suitable antibiotic and incubate at 30 °C for 12 to 24 hours.
16) Glycerol stock can be prepared by adding 80% glycerol into liquid culture (15% v/v) and stored at -80 °C.

Activity verification

[0635]

1) 50μL of liquid culture is transferred to a micro tube. Add 500μL of 8% pH7 Amylopectin Azure into the same tube. Prepare 2 tubes for each clone.
2) Activity is tested at 50 °C for 3 hours and overnight. Use pH 7 buffer as control.
3) Cool the test specimen at ice-water bath for 5 minutes.

4) Add 750μL of Ethanol and mixed thoroughly.
5) Centrifuge at 13000 rpm (16000 g's) for 5 minutes.
6) Measure OD of the supernatant at 595nm.

RFLP analysis

[0636]

1) 1.0mL of liquid culture is transferred to a sterile micro tube.
2) Centrifuge at 13200 rpm (16000 g's) for 1 minute.
3) Discard the supernatant. Add another 1.0 mL of liquid culture into the same sterile micro tube.
4) Centrifuge at 13200 rpm (16000 g's) for 1 minute.
5) Discard the supernatant.
6) Follow QIAPREP™ spin mini kit protocol for plasmid isolation.
7) Check DNA concentration using BioPhotometer.
8) Use Sac I and Kpn I for first double digestion. Incubate at 37 °C for 1 hour.
9) Use Pst I and Xho I for second double digestion. Incubate at 37 °C for 1 hour.
10) Add Loading dye into the digested sample.
11) Run the digested sample on a 1.0% agarose gel for 1-1.5 hours at 120 volts.
12) View gel with gel imager. All clones with a different digest pattern can be sequence analyzed.

Example 7: Assay for Amylases

Preparation of Host Cultures

[0637]

1. Start an overnight culture of XL1-BLUE™ MRF' host cells. Use a single colony from a streak plate to inoculate 10 mL LB supplemented with 20 ug/mL tetracycline. Grow overnight culture shaking at 37°C for at least 16 hours.
2. Using aseptic technique, inoculate a fresh 100 mL of LB_{Tet} day culture with XL1-BLUE™ MRF' host from the overnight LB_{Tet} culture.
3. Grow in a 37°C shaker until the OD reaches 0.75 - 1.0.
4. Pellet host cells at 1000 x g for 10 minutes and gently resuspend in 10 mM $MgSO_4$ at OD5.
5. Dilute a small amount of host cells to OD1 for use in titering and pintooling.
6. Host preparations can be used for up to 1 week when stored on ice or at 4°C.

- To shorten growth time for the day culture, use ½X the usual Tet concentration in LB (½X = 10 ug/mL), or omit the antibiotic altogether.
- Do not use NZY when selecting with Tetracycline. The high Mg^{++} concentration in NZY medium renders Tet inactive.

Titering Lambda Libraries

[0638]

7. Place three sterile microfuge tubes in a rack.
8. Aliquot 995 uL prepared host cells in one tube and 45 uL prepared OD1 host cells into each of the two remaining tubes.
9. Add 5 uL of lambda library to the tube containing 995 uL host cells and mix by vortexing. This results in a dilution factor of 200.
10. Prepare 1/2,000 and 1/20,000 dilutions by consecutively adding 5 uL of previous dilution to the remaining two tubes containing 45 uL prepared host cells. Mix by vortexing after each dilution was made.
11. Allow phage to adsorb to host by incubating at 37°C for 15 minutes.
12. Meanwhile, pipet 100 uL of prepared OD1 host cells to each of three Falcon 2059 tubes.
13. Add 5 uL of each dilution to a separate 2059 tube containing host cells.
14. Plate each by adding 3 mL top agar to each tube and quickly pour over 90 mm NZY plates. Ensure a smooth, even distribution before the top agar hardens.
15. Invert plates and incubate at 37°C overnight.

16. Count plaques and calculate titer of the library stock (in plaque forming units (pfu) per uL).

Lambda Microtiter Screening For Amylases

Preparation

[0639]

1. Prepare a sufficient amount of XL1-Blue MRF' host culture, as described above, for the amount of screening planned. A culture of 100 mL is usually sufficient for screening 2-3 libraries.
2. Autoclave several bottles compatible with the QFill2 dispenser. These are the wide-mouth Coming bottles, 250 mL containing a sealing ring around the lip.
3. Make sure there are sufficient amounts of plates, top agar, BODIPY starch, red starch solution, etc. available for the screen.
4. Schedule the Day 2 robot run with a representative from Automation.

Day 1

[0640]

1. Label the 1536-well plates (black) with library screen and plate number. Tough-Tags™ tube stickers, cut in half width-wise, are ideal for labeling 1536 well plates.
2. Calculate volumes of library, host cells and NZY medium necessary for the screen. This is easily done with an Excel spreadsheet.
3. Combine the calculated volumes of lambda library and OD5 host cells in a sterile 250 mL wide-mouth Corning bottle (containing a sealing ring).
4. Allow adsorption to occur at 37°C for 15 minutes.
5. Add the calculated volume of NZY medium and mix well. This is referred to as the cell-phage-medium suspension.
6. Perform a concomitant titer by combining 50 uL of the cell-phage-medium suspension with 250 uL of OD1 host cells in a Falcon 2059 tube, then plating with 9 mL of top agar onto a 150 mm NZY plate. Incubate concomitant titer plate at 37°C overnight.
7. Load the dispenser with the remainder of the suspension and array each labeled 1536-well plate at 4 uL per well. If the dispenser leaves air bubbles in some wells, they can be removed by centrifuging the plates at 200 x g for 1 minute.
8. Add 0.5 uL of positive control phage to well position AD46 of at least two of the assay plates. Use a strong amylase-positive lambda clone for this purpose.
9. Incubate assay plates at 37°C overnight in a humidified (≥95%) incubator.

Day 2

[0641]

1. Count the pfu on the concomitant titer plate and determine the average seed density per well (in pfu per well).
2. Pintool at least 2 plates of each library screen (preferably the 2 containing positive controls) as follows:

    a) Prepare 2 host lawn plates to act as a surface on which to pintool: combine 250 uL of OD1 host cells with 2 mL 2% red starch and plate with 9 mL top agar onto 150 mm NZY plates. Hold each plate as level as possible as the top agar solidifies in order to produce an even hue of red across the plate.
    b) Using a twice flame-sterilized 1536 position pintool, replicate at least 2 of the screening plates onto the host lawn plates.
    c) Place the pintooled recipient plates in a laminar flow hood with the lids off for about 15-30 minutes (to vent off excess moisture).
    d) Replace the lids and incubate inverted at 37°C overnight.

3. Prepare the 2X BODIPY starch substrate buffer as follows:

    a) Calculate the total volume of 2X substrate buffer solution needed for all screening plates at 4 uL per well (including any extra deadspace volume required by the dispenser) and measure this amount of 100 mM CAPS pH 10.4 into a vessel appropriate for the dispenser used.

b) Retrieve enough 0.5 mg tubes of BODIPY starch to produce the required volume of 2X substrate buffer [calculated in step a) above] at a final concentration of 20-30 ug/mL.
c) Dissolve each 0.5 mg tube in 50 uL DMSO at room temperature, protected from light, with frequent vortexing. This takes more than 15 minutes; some production lots of BODIPY starch dissolve better than others.
d) Add 50 uL 100mM CAPS buffer pH 10.4 to each tube and mix by vortexing.
e) Pool the contents of all tubes and remove any undissolved aggregates by centrifuging for 1 minute at maximum speed in a microfuge.
f) Add the supernatant to the rest of the 100 mM CAPS buffer measured in step a) above.
g) Protect the 2X substrate buffer from light by wrapping in foil.

4. Take plates and substrate buffer to the automation room and program the robot with the following parameters:

a) dispense 4 uL substrate buffer per well
b) 1st read at 1 hour post-substrate, 2nd read at 9 hours, and third read at 17 hours; with 37°C incubation between reads
c) excitation filter: 485 nm; emission filter: 535 nm
d) set the Spectrafluor gain at 70, or the optimal gain for the batch of 2X substrate buffer prepared.
e) ensure that the incubator used will protect assay plates from light.

Day 3

[0642]

1. Check pintooled plates for clearings in the bacterial lawn at all positions corresponding to wells on the associated assay plate. Also check for clearings in the red starch in any of the pin positions. If plates containing positive controls were used for pintooling, you should be able to see a large clearing zone in the red background. Be wary of contaminants that also form clearing zones in red starch (see comment "Contaminants That Form Clearing Zones in Red Starch" at end of Example 7).
2. Identify putative hits from the data file produced by the robot computer. The KANAL program produced by Engineering simplifies data analysis. As a rule of thumb, a putative hit is characterized as a well having signal intensity rising at least 1.5 fold over background.
3. For each putative, remove 2 uL from the well and add to a tube containing 500 uL SM buffer and 50 uL CHCl3. Vortex to mix and store at 4°C. This solution will be referred to hereafter as the 4e-3 stock. The original screening plates should be stored at 4°C, protected from light, at least until breakouts are complete.

[0643] This is an exemplary method of breaking out putative hits. It is a liquid phase assay that relies on confirmation of activity on BODIPY starch. Alternatively, putative hits can be plated directly onto solid phase plates containing red starch such that 2,000-3,000 pfu per hit are examined for clearing zones. However, inability to observe clearing zones on red starch is not necessarily an indication that a putative hit was a false positive. It would then need to be assayed using the format in which it was originally identified (i.e., liquid phase using BODIPY starch as substrate). In addition, very weak positives are more easily identified using the method detailed below.

Day 1

[0644]

1. In a sterile 50 mL conical tube, combine 0.5 mL OD5 host cells with 45.5 mL NZY. This will be referred to as the host-medium suspension.
2. For each putative hit to be analyzed, aliquot 1 mL of host-medium suspension into each of 3 three sterile microfuge tubes.
3. Set the 12-channel pipetman in multidispense mode with an aliquot size of 20 uL and an aliquot number of 2x. Mount the pipetman with a clean set of sterile tips.
4. Pour about 1 mL of host-medium suspension into a new sterile solution basin and load the multichannel pipetman.
5. Dispense 20 uL per well into the last row (row P) of a black 384-well plate (12 channels x 2 = 24 wells). This row will be used later for the controls.
6. Expel the remaining liquid in the tips by touching the tips against the surface of the basin and pressing the RESET button on the pipetman. Lay the pipetman down in a way to prevent contamination of the tips. There is no need to change the tips at this point.

7. Pour the remainder of the fluid in the basin into a waste container (like a beaker) taking care to avoid splash-back contamination.
8. For the first putative to be analyzed, take 111 uL of the 4e-3 stock (see Day 2 in *Lambda Microtiter Screening for Amylases*) and add it to the first in a set of three tubes containing 1 mL host-medium suspension (step 2). Vortex to mix. This is *Dilution A.*
9. Take 111 uL of Dilution A and add to the next tube in the set. Vortex to mix. This is *Dilution B.*
10. Take 111 uL of Dilution B and add to the last tube in the set. Vortex to mix. This is *Dilution C*. You should now have three dilutions of phage, where concentrations of each differ by a factor of 10.
11. Pour the contents of Dilution C (the most dilute of the 3 samples) into the solution basin and load the multichannel pipetman.
12. Dispense 20 uL per well into the first row of the 384-well plate (12 channels x 2 = 24 wells).
13. Expel the remaining liquid in the tips by touching the tips against the surface of the basin and pressing the RESET button on the pipetman. Lay the pipetman down in a way to prevent contamination of the tips. There is no need to change the tips at this point.
14. Empty the basin as described above.
15. Pour the contents of Dilution B into the same basin and load the multichannel pipetman.
16. Dispense 20 uL per well into the second row of the 384-well plate.
17. Perform steps 13-16 similarly to dispense Dilution A into the third row of the plate.
18. After all three dilutions have been arrayed into the first 3 rows of the plate, discard all tips and the solution basin into the biohazardous waste container.
19. Mount the pipetman with a clean set of sterile tips and open a new sterile solution basin.
20. Repeat steps 8-19 for each remaining putative hit, using remaining rows on the plate up to row O. Five putative hits can be analyzed on one 384-well plate, with the last row (row P) saved for the controls.
21. Add 0.5 uL of each control to a separate well. Use at least 2-3 separate controls, preferably covering a range of activity.
22. Incubate assay plates at 37°C overnight in a humidified (≥95%) incubator.

Day 2

[0645]

1. Pintool all breakout plates onto a host lawn with red starch using the same method described for Day 2 in *Lambda Microtiter Screening for Amylases,* except that a 384 position pintool is used.
2. Prepare the 2X BODIPY starch substrate buffer as follows:

    a) Calculate the total volume of 2X substrate buffer solution needed for all breakout plates at 20 uL per well (including any extra deadspace volume required by the dispenser) and measure this amount of 100 mM CAPS pH 10.4 into a vessel appropriate for the dispenser used.
    b) Retrieve enough 0.5 mg tubes of BODIPY starch to produce the required volume of 2X substrate buffer [calculated in step a) above] at a final concentration of 20-30 ug/mL.
    c) Dissolve each 0.5 mg tube in 50 uL DMSO at room temperature, protected from light, with frequent vortexing. This takes more than 15 minutes; some production lots ofBODIPY starch dissolve better than others.
    d) Add 50 uL 100mM CAPS buffer pH 10.4 to each tube and mix by vortexing.
    e) Pool the contents of all tubes and remove any undissolved aggregates by centrifuging for 1 minute at maximum speed in a microfuge.
    f) Add the supernatant to the rest of the 100 mM CAPS buffer measured in step a) above.
    g) Protect the 2X substrate buffer from light by wrapping in foil.

3. Dispense 20 uL per well into all breakout plates.
4. Wrap all plates in aluminum foil and incubate at room temperature for 2-6 hours.
5. Read each plate in the Spectrafluor with the following settings:

    a) fluorescence read (excitation filter: 485 nm; emission filter: 535 nm)
    b) plate definition: 384 well black
    c) read from the top
    d) optimal gain
    e) number of flashes: 3

6. On the resulting Excel spreadsheet, chart each putative's 3 rows in a separate graph and check for activity. Ensure that the positives controls produced signals over background.
7. For each putative that appears to have a real signal among the wells, harvest a sample from a positive well as follows:

a) Select a positive well from a row representing the highest initial dilution.
b) Transfer 2 uL from that well into a tube containing 500 uL SM and 50 uL $CHCl_3$. This is referred to as the breakout stock.
c) Store at 4°C.

8. Using methods previously described, plate about 10 uL of each breakout stock onto 150 mm NZY plates using red starch. The objective is to obtain several (at least 20) well-separated plaques from which to core isolates.

Day 3

[0646]

1. Check pintooled plates for an acceptable incidence of clearings in the bacterial lawn corresponding to wells on the associated assay plate. Also check for clearings in the red starch in the positive controls and in any tested putatives. Be wary of contaminants that also form clearing zones in red starch (see below).
2. From the solid phase plates containing dilutions of breakout stocks, core several isolated plaques, each into 500 uL SM with 50 uL $CHCl_3$. This is referred to as the isolate stock.
3. The isolate stocks can then be individually tested on BODIPY starch using methods described above. This step can be skipped if the plaque that was cored in step 2 produced a clearing zone in the red starch background. The isolate stocks were then be individually tested on BODIPY starch using methods described above. However, this step may be skipped if the plaque that was cored in step 2 produced a clearing zone in the red starch background.

Excisions

Day 1

[0647]

1. In a Falcon 2059 tube, mix 200 uL OD1 XL1-Blue MRF' host, 100 uL lambda isolate stock and 1 uL EXASSIST™ phage stock.
2. Incubate in 37°C shaker for 15 minutes.
3. Add 3 mL NZY medium.
4. Incubate in 30°C shaker overnight.

Day 2

[0648]

1. Heat to excision tube to 70°C for 20 minutes.
2. Centrifuge 1000 x g for 10 minutes.
3. In a Falcon 2059 tube, combine 50 uL supernatant with 200 uL EXP505™ OD1 host.
4. Incubate in 37°C shaker for 15 minutes.
5. Add 300 uL SOB medium.
6. Incubate in 37C shaker for 30-45 minutes.
7. Plate 50 uL on large LB_{Kan50} plate using sterile glass beads. If the plates are "dry", extra SOB medium can be added to help disburse the cells.
8. Incubate plate at 30°C for at least 24 hours.
9. Culture an isolate for sequencing and/or RFLP.

[0649] Growth at 30°C reduces plasmid copy number and is used to mitigate the apparent toxicity of some amylase clones.

Contaminants That Form Clearing Zones in Red Starch

[0650] When using red starch on solid medium to assay phage for amylase activity, it is common to see contaminating colony forming units (cfu) that form clearing zones in the red starch. For pintooled plates, it is important to distinguish amylase-positive phage clones from these contaminants whenever they align with a particular well position. The source of the contaminating microbes is presumably the 2% red starch stock solution, which cannot be sterilized by autoclaving or by filtering after preparation. It is thought that they are opportunistic organisms that survive by metabolizing the red starch. In order to reduce these contaminants, sterile techniques can be used when making 2% red starch solutions and store the stocks either at 4°C or on ice.

Example 8: Characterization of alpha amylase pH optimum and specific activity determination

[0651] The following example describes an exemplary method for alpha amylase activity pH optimum and specific activity determination.

[0652] Enzymes of this invention can be used, for example, for both starch liquefaction for corn wet milling and desizing for textiles; e.g., in some embodiments enzymes of the invention have a pH optimum of 4.5 to 5.0; at this lower pH, it is possible to use little or no calcium which lowers overall operating costs and less byproduct formation. In addition, at this low pH, there is decreased chemical usage and ion exchange load. The industry standard *B. licheniformis* amylase is suboptimal in both thermostability and pH optimum. In some embodiments enzymes of the invention have a higher application specific activity compared to *B. licheniformis* amylase and therefore much less enzyme is required to hydrolyze a ton of starch (e.g., in some embodiments, as much as 20-fold less enzyme can be used).

[0653] The pH optimum for the hydrolysis of starch can be determined by reacting 50 uL of enzyme, 0.35 U/ml, with a 100ml of 1% soluble starch solution (0.0175U/g of starch) for 30 minutes at 95 degrees C. The reducing ends generated in the liquefied starch solution can be measured by the neocupronine assay, described herein. The percent hydrolysis of cornstarch can be determined by measuring the number of sugar reducing ends produced with the neocupronine assay. Seventy grams of buffer solution (pH4-7) was weighed and 100 ppm of calcium can be added. Thirty grams of cornstarch can be mixed into the buffer solution to form a starch slurry. The enzyme can be added and the vessels sealed and incubated at 95 degrees C for 30 minutes with an initial heating rate of six degrees C per minute. A1 ml sample can be extracted from the reaction beakers and analyzed by the neocupronine assay. In some embodiments enzymes of the invention have an optimum between pH 4.5 and 5, while the commercial *B. licheniformis* amylase performs optimally at about pH 6.0.

Example 9: Amylase Activity Assays

Assay Using RBB-starch

[0654] 75 ul ofRBB-starch substrate (1% RBB-insoluble corn starch in 50mM NaAc buffer, pH=4.5) is added into each well of a new 96-well plate (V-bottom). Five micro-liters of enzyme lysate is transferred into each well with substrate using BIOMEK™ or ZYMARK™. The plates are sealed with aluminum sealing tape and shaken briefly on the shaker. The plates are incubated at 90°C for 30 minutes, followed by cooling at room temperature for about 5 to 10 minutes. One hundred micro-liters of 100% ethanol is added to each well, the plates sealed and shaken briefly on the shaker. The plates are then centrifuged 4000 rpm for 20 minutes using bench-top centrifuge. 100 ul of the supernatant is transferred into a new 96-well plate (flat bottom) by BIOMEK™ and read OD_{595}. Controls should be used.

Assay using FITC-starch

[0655] Add 50 ul of substrate (0.01% FITC-starch in 100mM NaAc buffer, pH=4.5) into each well of a new 384-well plate. Transfer 5 ul of enzyme lysate into each well with substrate and incubate the plate at room temperature overnight. The polarization change of the substrate, excitation 485nm, emission 535nm, is read for each well. Controls should be used. 96 well plates can be used for all assays.

Confirmation of new active clones

[0656] Each positive clone from screening is grown and induced using a standard protocol. Each clone is examined for growth (i.e., cell density over time), activity at per cell level (RBB-starch assay and liquefaction assay), expression (protein gel) and solubility of protein (by microscope analysis). The confirmed new elevated clones are transferred for fermentation.

Example 10: Exemplary protocol for liquefying starch and measuring results

[0657] The following example described and exemplary protocol for liquefying starch using glucoamylases of the invention. One exemplary assay uses liquefied starch at pH 4.5 or 6.5 using the reaction conditions show below:

Reaction Conditions: 100 mM PO_4 pH 6.5, 1% (w/w) liquefied starch DE 12 at 55°C. Both TLC and HPLC assays are done to verify activity. pH profiles for the amylases to be tested are run using phosphate buffer pHed from 3.0 - 6.5, at 55°C. From the amount of observable hydrolysis, it can be visually demonstrated that some clones are more (or less) active at certain pH values than at other values at the above indicated reaction conditions.

[0658] An exemplary protocol for the saccharification of liquefied starch at pH 6.5:

Adjust the pH of the liquefied starch to the pH at which the saccharification(s) is performed. Liquefy starch in 100 mM sodium acetate buffer, pH 4.5 with 100 mM sodium phosphate salts added so that before saccharification, the pH is adjusted to pH 6.5.
Weigh 5 gram samples of liquefied starch into tared bottles.
Use 0.04% (w/w) OPTIDEX L-400™ or approximately 400 mL of 1-10 diluted stock OPTIDEX L-400™ per 100 grams of liquefied starch.
Calculate the milligrams of OPTIDEX L-400™ contained in the 400 mL of 1-10 diluted stock OPTIDEX L-400™. Next, calculate the volume of lysates needed to give the same concentration of enzyme as the OPTIDEX L-400™. Add enzymes to liquefied starch samples and incubate at desired temperature (50C°). After 18 hours determine DE and prepare a sample for HPLC analysis.

An exemplary DE Determination:

Exemplary Neocuproine Assay:

[0659] A 100ml sample is added to 2.0ml of neocuproine solution A (40g/L sodium carbonate, 16g/L glycine, 0.45g/L copper sulfate). To this is added 2.0 ml of neocuproine solution B (1.2g/L neocuproine hydrochloride-Sigma N-1626). The tubes are mixed and heated in a boiling water bath for 12 minutes; cooled, diluted to 10ml volume with DI water and the OD read at 450nm on the spectrophotometer. The glucose equivalent in the sample is extrapolated from the response of a 0.2mg/ml glucose standard run simultaneously.

Exemplary HPLC Analysis:

[0660] Saccharification carbohydrate profiles are measured by HPLC (Bio-Rad Aminex HPX-87A column in silver form, 80°C) using refractive index detection. Mobile phase is filtered Millipore water used at a flow rate of 0.7 ml/min. Saccharification samples are diluted 1-10 with acidified DI water (5 drops of 6 M HCl into 200 mL DI water) then filtered through a 0.45 mm syringe filter. Injection volume is 20 uL.

Exemplary TLC:

[0661] Reaction products were w/d at hourly timepoints and spotted and dried on a TLC plate. The Plate was then developed in 10:90 water:isopropanol and visualized with either a vanillin stain or CAM stain and then heated to show reducible sugars. The liquefied starch was partially hydrolyzed to glucose in cases where activity was observed.

Example 11: Starch Liquefaction using glucoamylases of the invention

[0662] This example describes an exemplary method of the invention for liquefying starch using glucoamylases of the invention.

[0663] Amylase concentrate can be prepared from fermentation broths by heat treatment, cell washing, alkaline extraction using microfiltration and ultrafiltration (48% overall yield). The UF concentrate can be neutralized with acetic acid and formulated with 30% glycerol at pH 4.5. The activity level of the slurry formulation can be representative of a commercial product ($120U^1$/g - 0.5kg/ ton starch).

Standard Amylase Activity Assay

[0664] A 1 mL cuvette containing 950 μL of 50 mM MOPS pH 7.0 containing 5 mM PNP-α- D-hexa-(1→4)-glucopyra-

noside is placed in the Peltier temperature controller of the Beckman DU-7400 spectrophotometer preheated to 80°C. The spectrophotometer is blanked at 405nm and 50 μL of the enzyme solution is added to the cuvette, mixed well and the increase in the OD_{405nm} is monitored over a one-minute interval. The $\Delta OD_{405nm/min}$ rate is converted to a standard unit of μmole/minute from the OD_{405nm} response of 50 μL of 1 μmole/mL PNP in 950 mL 50 mM MOPS at pH 7.0 -80°C. One standard unit of thermostable alpha amylase (DTAA) is equal to the amount of enzyme that will catalyze the release of 1 μmole/mL/minute of pNP under the defined conditions of the assay.

Standard Glucoamylase Activity Assay

[0665] A 1 mL cuvette containing 950 μL of 50 mM MOPS pH 7.0 containing 5 mM pNP-α- D-glucopyranoside is placed in a Peltier temperature controller of a DU-7400™ spectrophotometer (Beckman) preheated to 60°C. The spectrophotometer is blanked at 405nm and 50 μL of the enzyme solution is added to the cuvette, mixed well and the increase in the OD_{405nm} is monitored over a one-minute interval. The ΔOD_{405nm}/min rate is converted to a standard unit of μmole/minute from the OD_{405nm} response of 50 μL of 1 μmole/mL pNP in 950 mL 50 mM MOPS at pH 7.0-60°C. One standard unit of glucoamylase (DGA) is equal to the amount of enzyme that will catalyze the release of 1 μmole/mL/minute of pNP under the defined conditions of the assay.

Dextrose Equivalent Determination

[0666] The neocuproine assay method can be used to measure the DE. Selected samples can be measured by a procedure described herein, and/or by a GPC analyst using the GPC Fehlings procedure.

Neocuproine Assay

[0667] A 100 μl sample is added to 2.0 ml of neocuproine solution A (40 g/L sodium carbonate, 16g/L glycine, 0.45g/L copper sulfate). To this is added 2.0 ml of neocuproine solution B (1.2 g/L neocuproine hydrochloride-Sigma N-1626). The tubes are mixed and heated in a boiling water bath for 12 minutes; cooled, diluted to 10ml volume with DI water and the OD read at 450 nm on the spectrophotometer. The glucose equivalent in the sample is extrapolated from the response of a 0.2mg/ml glucose standard run simultaneously.

[0668] The starch sample is diluted ~1 to 16 with DI water with the exact dilution recorded. Ten milliliters of the diluted sample is added to 20 mls of DI water. Ten milliliters of Fehlings solution A and B are added to the diluted starch. The sample is boiled for 3 minutes and cooled on ice. Ten milliliters of 30% KI and 10ml of 6N H_2SO_4 is added. The solution is titrated against 0.1N sodium thiosulfate. The titrant volume is recorded and used to calculate the DE.

Residual Starch Determination

[0669] Post-saccharification samples can be checked for residual starch using the Staley iodine procedure. Twenty grams of sample is weighed into a large weigh dish. 45 μL of Iodine solution is added to the weigh dish and the starch solution is mixed well. Dark blue indicates the presence of starch, a light blue-green indicates slight starch, light green indicates a trace of starch and yellow-red, absence of starch. Iodine solution is prepared by dissolving 21.25 grams of iodine and 40.0 grams of potassium iodide in one liter of water.

Oligosaccharide Profile

[0670] Liquefaction and saccharification carbohydrate profiles can be measured by HPLC (e.g., an AMINEX HPX-87C™ column (Bio-Rad) in calcium form - 80°C) using refractive index detection.

Gel Permeation Chromatography

[0671] The molecular weight distribution can be determined by chromatography, e.g., on a PL AQUAGEL-OH column™ with mass detection by refractive index (Waters Model 2410). A Model T60™ (Viscotek) detector can be used for continuous viscosity and light scattering measurements.

Capillary Electrophoresis

[0672] A Beckman Coulter P/ACE MDQ™ Glycoprotein System for separation of APTS derivatized oligosaccharides on a fused silica capillary can be used; in one aspect, detection by laser-induced fluorescence is used.

Primary Liquefaction

[0673] Line starch directly from the GPC process is pumped into a 60 liter feed tank where pH, DS (dry solids) and calcium level can be adjusted before liquefaction. The amylase is added to the slurry. The 32% DS slurry is pumped at 0.7 liter/minute by a positive displacement pump to the jet - a pressurized mixing chamber where the starch slurry is instantaneously heated to greater than 100°C by steam injection. The gelatinized partially liquefied starch is pumped through a network of piping (still under pressure) to give the desired dwell time (5 minutes) at temperature. The pressure is released into a flash tank and samples can be taken. Samples were taken in duplicate.

Secondary Liquefaction

[0674] The liquefied starch is collected in one liter glass bottles and held in a water bath at 95C for 90 minutes.

Saccharification

[0675] Liquefied starch is cooled to 60°C, the pH adjusted to 4.5 and the samples treated with glucoamylase. Saccharification progress can be monitored over time, e.g., by HPLC.

[0676] The liquefied syrups produced with each amylase are adjusted to approximately pH 2.5 with 6N HCl immediately after the 90 minute secondary liquefaction to inactivate any residual amylase. The syrups are then adjusted to pH 4.5, placed in a 60°C water bath and saccharified with three levels of glucoamylase. The extent of saccharification is monitored by HPLC at 18-88 hour time points.

[0677] The liquefied syrups are saccharified with the standard dosage - 0.04% of a double-strength glucoamylase - and two lower dosages (50% and 25%) to monitor any differences in the saccharification progress.

[0678] Saccharification progress data can be analyzed by % dextrose development vs time, e.g., with 0.04% glucoamylases; or, % dextrose development vs time, e.g., with 0.02% glucoamylases.

Post-Saccharification sugar profile

Molecular weight distribution

[0679] The molecular weight distribution of syrups liquefied to DE's of 12 and 18 by glucoamylases of the invention, and in some aspects, using controls, e.g., commercial enzymes, e.g., *Bacillus licheniformis* or commercial *Bacillus stearothermophilus,* can be measured by gel permeation chromatography using detection by refractive index, light scattering and viscosity. Both the *B. licheniformis* and *B. stearothermophilus* amylases generate a bimodal distribution - the primary peak centered at 2000, a secondary peak at 32,000 with a shoulder extending past the 160,000 range. The lower molecular weight peak represents approximately 60% of the total mass of the sample. In some embodiments, glucoamylases of the invention can exhibit a single peak at 2000 with very little above 30,000.

HPLC

[0680] The DE 12 and 18 syrups produced by glucoamylases of the invention (and the control commercial enzymes, e.g., amylases from *Bacillus licheniformis* and/or commercial *Bacillus stearothermophilus* amylases) can be analyzed by HPLC. Both techniques produce fingerprints characteristic of each class of amylase; the oligosaccharide patterns are different for *B. licheniformis* amylase vs *B. stearothermophilus* amylase, and glucoamylases of the invention are also expected to produce fingerprints characteristic of their enzyme classes. The liquefied syrups of the invention (e.g., syrups made by methods of the invention and/or made by enzymes of the invention) exhibit evidence of greater branching in the oligosaccharides. HPLC only resolve the oligosaccharides in the <DP 15 range - larger fragments are not visible in these techniques. *Bacillus* amylases are known to liquefy starch in a manner such that the amylopectin fraction is hydrolyzed less extensively than the amylose fraction. These >DP30 amylopectin fragments are contained in the high molecular weight fraction centered at 32,000 and consequently, little evidence of branching is seen in the HPLC analyses of the *Bacillus* liquefied syrups. In one aspect, <DP15 oligosaccharides in liquefied syrups made using glucoamylases of the invention contain fragments from both amylose and amylopectin.

Example 12: Starch Liquefaction at acidic conditions using glucoamylases of the invention

[0681] The invention provides methods for liquefying starch using glucoamylases of the invention. In one embodiment, the conversion of starch to glucose can be catalyzed by the sequence action of two enzymes: an endoamylase of the disclosure, e.g., an amylase, such as an alpha-amylases, and an exoamylase, e.g., a glucoamylase of the invention, to

liquefy the starch (e.g., the hydrolysis of high molecular weight glucose polymers to oligosaccharides consisting of 2 to 20 glycose units, typically a dextrose equivalent of 10 to 12, by an amylase of the disclosure), followed by saccharification with an exoamylase, e.g., a glucoamylase (which can be a glucoamylase of the invention).

[0682] In one aspect, processing is in a corn wet milling plant producing a starch slurry having a pH or about 4.0 to 4.5. In one aspect, the pH is raised, e.g., to 5.8 to 6.0 before liquefaction to accommodate an alpha amylase with a low pH activity and stability. In one aspect, glucoamylases of the invention can liquefy starch at pH 4.5 to dextrose equivalents ranging from 12 to 18; in one aspect, using glucoamylases of the invention at levels of about 3 to 6 grams per ton of starch. In this aspect, use of glucoamylases of the invention enables starch liquefaction to be conducted at pH 4.5.

[0683] In one aspect, starch liquefaction is conducted at pH 4.5 for 5 minutes at 105°C to 90 minutes at 95°C using glucoamylases of the invention. The quantity of enzyme can be adjusted in order to adjust a target DE of 12 to 15 after liquefaction. In one aspect, the liquefied starch is then saccharified with a glucoamylase, e.g., an *Aspergillis* glucoamylase, for about 48 hours at about pH 4.5 and 60°C. If the saccharified syrup does not contain at least 95% glucose, the target liquefaction DE is raised and the saccharification repeated until the liquefaction eventually does produce a saccharified syrup containing more than 95% glucose. The amylase protein required to produce a suitable liquefied feedstock for saccharification can be determined, e.g., by PAGE or HPLC.

Example 13: Starch Liquefaction using glucoamylases of the invention

[0684] This example describes an exemplary method for liquefying starch using glucoamylases of the invention; and described use of commercial *Bacillus licheniformis* and *Bacillus stearothermophilus* amylases as controls. These assays can compare the saccharification progress and final dextrose levels from syrups generated by enzymes of the invention and commercial amylases.

[0685] Dextrose equivalent (DE) is the industry standard for measuring the concentration of total reducing sugars, calculated as D-glucose on a dry weight basis. Unhydrolyzed granular starch has a DE of virtually zero, whereas the DE of D-glucose is defined as 100. One exemplary process of the invention uses an enzyme dosage of about 60 to 70 Units/kilo starch at pH 4.5 to reach a 19 DE.

[0686] Oligosaccharide patterns generated by glucoamylases of the invention and commercial amylases can be analyzed by molecular weight (MW) distribution using, e.g., gel permeation chromatography with detection by light scattering and viscosity. In one aspect, glucoamylases of the invention can generate an 18DE and a uniform oligosaccharide MW distribution, with nothing greater than 20,000. This is consistent with the lower viscosity for syrups of the invention (e.g., syrups made by methods of the invention, or, made using enzymes of the invention). The DP (degrees of polymerization) profiles as measured by HPLC also can be used to analyze differences in action pattern.

[0687] Amylase concentrate are prepared from fermentation broths by heat treatment, cell washing, alkaline extraction using microfiltration and ultrafiltration (UF). The UF concentrate is neutralized with acetic acid and formulated with 30% glycerol at pH 4.5. The activity level of the slurry formulation can be analyzed, e.g., 120U1/g - 0.5kg/ ton starch is representative of a commercial product.

Example 14: Alkaline Amylases for Laundry and Autodishwash Applications

[0688] In one aspect, the invention provides detergents comprising glucoamylases of the invention, including glucoamylases active under alkaline conditions, and methods of making and using them.

[0689] The invention provides alkali-stable glucoamylase enzymes, which can be compared to commercial benchmark enzyme(s) with respect to features important in laundry and automatic dishwashing (ADW) applications:

- An ADW wash test on starch-coated slides.
- Amylase and/or glucoamylase enzyme activity testing in the presence of a laundry/ADW formulation using a soluble substrate.
- In the presence of chelators, amylase and/or glucoamylase enzyme activity testing.
- Amylase and/or glucoamylase enzyme activity testing and alkaline pH optima ranges determined (e.g., from pH 10 to 11).
- o Amylase and/or glucoamylase enzyme activity testing for thermophilic properties, e.g., performance at about 65° to 70°C.

[0690] Amylase and/or glucoamylase activity can be measured either by a reducing sugar assay or by monitoring the fluorescence at 520 nm (485 nm excitation) when BODIPY-starch was used. Initial rates can be calculated and converted to a percentage of the maximum rate.

Application testing

[0691] Experiments can be designed to assess the activity and stability of alkaline glucoamylases of the invention in laundry/ADW formulations and with the components individually. Amylase and/or glucoamylase activity can be assessed under conditions comprising the chelator EDTA and/or hydrogen peroxide; commercial benchmark enzymes can be controls.

[0692] For example, purified proteins are incubated at 50°C in the presence or absence of 5mM EDTA for a desired time(s), after which residual amylase activity is measured using soluble substrate. Activity in the presence of EDTA is expressed as the % of activity in the absence of chelator. Alternatively, enzyme activity in the presence of peroxide hydroxide can be assessed. Purified proteins are incubated at 50°C in the presence or absence of 1M H_2O_2 for a desired time, after which amylase activity is measured using soluble starch. Activity in the presence of peroxide hydroxide is presented as the % of activity in the absence of H_2O_2. Enzyme activity can be tested in an ADW solution (distilled water, hardening solution, bleach, chelators, surfactants) with soluble substrate (BODIPY-starch). Purified proteins can be reacted with soluble starch at 40°C in the presence of laundry/ADW formulation. Initial rates are calculated over 5 minutes and expressed as fluorescent units (FU)/s per ng of protein.

[0693] Wash tests with starch-coated slides can be performed as follows: purified proteins are incubated with slides at 50°C for 30 min in the presence of ADW solution (distilled water, water hardening solution, bleach, chelators, surfactants). Starch removal is measured comparing weight loss after the enzyme treatment to the initial weight of the slide.

Characterization of exemplary amylases

[0694] The gene encoding the amylase can be modified to comprise a Starch/ Carbohydrate Binding Domain. The proteins can be expressed with and without a C-terminal histidine tag, and in non-glycosylating and a glycosylating host. Enzymes can be expressed in Host/His tag combinations, and pH and temperature optimas determined. Enzymes expressed in a glycosylating host with a His tag can be used for test experiments. The presence of the His tag should not affect specific activity, however, glycosylation may result in a slightly lower specific activity than that without glycosylation.

Example 15: Identification and characterization of a thermostable amylase

[0695] The following example describes exemplary protocols for the identification and characterization of thermostable amylases.

[0696] In one study, 350 fungal isolates were screened on solid medium containing granular starch as the sole source of carbon. Strains that completely hydrolyzed starch or displayed significant growth were submitted for cDNA isolation and proteomics analysis of their secreted proteins. A combination of Sequence Based Discovery and Proteomic Analysis was employed for recovering DNA sequences encoding amylases, glucoamylases and glucosidases that were shown by proteomics to be secreted during growth on granular starch.

[0697] The recovered full-length cDNA sequences were subcloned for expression in *Pichia pastoris* and the expressed proteins were further characterized with the BCA assay (which was used to determine the increase in concentration of reducing ends during hydrolysis of starch by the amylases) and the Glucose Oxidase (GO) assay (which was used for detecting glucose released from starch by the glucoamylases), exemplary protocols for both of these assays are described herein.

Table 1

SEQ ID NOs:	Enzyme activity class	CBM20	Source (as determined by 18S RNA)
SEQ ID NO:50 (encoded by, e.g., SEQ ID NO:49)	amylase	no	*Aspergillus terreus*
SEQ ID NO:52 (encoded by, e.q., SEQ ID NO:51)	amylase	CBM20	*Aspergillus terreus*
SEQ ID NO:54 (encoded by, e.g., SEQ ID NO:53)	amylase	no	*Aspergillus terreus*
SEQ ID NO:4 (encoded by, e.q., SEQ ID NO:3)	amylase	CBM20	*Cochliobolus heterostrophus*

(continued)

SEQ ID NOs:	Enzyme activity class	CBM20	Source (as determined by 18S RNA)
SEQ ID NO:2 (encoded by, e.q., SEQ ID NO:1)	amylase	CBM20	*Cochliobolus heterostrophus*
SEQ ID NO:32 (encoded by, e.g., SEQ ID NO:31)	amylase	no	*Penicillium chrysogenum 100%*
SEQ ID NO:46 (encoded by, e.g., SEQ ID NO:45)	amylase	no	*Fusarium equiseti 100%*
SEQ, ID NO:22 (encoded by, e.g., SEQ ID NO:21)	amylase	no	*Penicillium expansum* 99%
SEQ ID NO:24 (encoded by, e.g., SEQ ID NO:23)	amylase	no	*Penicillium chrysogenum* 100%
SEQ ID NO:8 (encoded by, e.g., SEQ ID NO:7)	glucoamylase	no	*Cochliobolus heterostrophus*
SEQ ID NO:16 (encoded by, e.g., SEQ ID NO:15)	glucoamylase	CBM20	*Cochliobolus heterostrophus*
SEQ ID NO:14 (encoded by, e.g., SEQ ID NO:13)	glucoamylase	CBM20	*Fusarium verticillioides* GZ3639
SEQ ID NO:18 (encoded by, e.g., SEQ ID NO:17)	glucoamylase	CBM20	*Fusarium verticillioides* GZ3639
SEQ ID NO:10 (encoded by, e.g., SEQ ID NO:9)	glucoamylase	no	*Penicillium expansum 99%*
SEQ ID NO:12 (encoded by, e.g., SEQ ID NO:11)	glucoamylase	CBM20	*Fusarium equiseti 100%*
SEQ ID NO:26 (encoded by, e.g., SEQ ID NO:25)	glucoamylase	CBM20	*Penicillium verruculosum 100%*
SEQ ID NO:20 (encoded by, e.g., SEQ ID NO:19)	glucoamylase	no	*Penicillium chrysogenum* 100%
SEQ ID NO:28 (encoded by, e.g., SEQ ID NO:27)	glucoamylase	CBM20	*Fusarium merismoides 99%*
SEQ ID NO:30 (encoded by, e.q., SEQ ID NO:29)	glucoamylase	CBM20	*Phoma herbarum 99*
SEQ ID NO:34 (encoded by, e.g., SEQ ID NO:33)	glucoamylase	no	*Penicillium herquei 99%*
SEQ ID NO:36 (encoded by, e.g., SEQ ID NO:35)	glucoamylase	CBM20	*Fusarium oxysporum 100%*
SEQ ID NO:38 (encoded by, e.q., SEQ ID NO:37)	glucoamylase	CBM20	*Cordyceps ophioglossoides 99%*
SEQ ID NO:40 (encoded by, e.q., SEQ ID NO:39)	glucoamylase	CBM20	*Penicillium chrysogenum 100%*
SEQ ID NO:42 (encoded by, e.g., SEQ ID NO:41)	glucoamylase	no	*Cucurbitaria berberidis 98%*
SEQ ID NO:48 (encoded by, e.g., SEQ ID NO:47)	glucoamylase	CBM20	*Aspergillus versicolor 99*

(continued)

SEQ ID NOs:	Enzyme activity class	CBM20	Source (as determined by 18S RNA)
SEQ ID NO:44 (encoded by, e.g., SEQ ID NO:43)	α-glucosidase		*Cochliobolus heterostrophus*
SEQ ID NO:6 (encoded by, e.g., SEQ ID NO:5)	α-glucosidase		*Cochliobolus heterostrophus*

[0698] Fungal isolates were screened on modified solid media according to Marlida (2000) World J. Microbiol. Biotechnol. 16:573-578. The medium contained Czapek Dox salts and 1% MIMAIZE^{260}™ (HiMaize^{260}, National Starch & Chemical, Bridgewater, NJ) resistant starch as the sole source of carbon. The MIMAIZE^{260}™starch was added to the cool agar without sterilization in order to preserve the granular structure of the starch. Additionally the same fungal strains were screened on a medium with 0.5% red starch (Megazyme, Ireland) in order to identify strains that secreted amylolytic enzymes.

[0699] After 5 days of growth, isolates that grew well on resistant starch and secreted starch - degrading enzymes, visualized by a clearing zone on the red starch medium, were chosen as "primary hits". In the second step, primary hits were grown in liquid medium with Czapek Dox salts and 1% resistant starch (MIMAIZE^{260}™) as the sole source of carbon for approximately 2 weeks. Strains that completely cleared the starch solution or displayed significant growth in this medium were submitted for cDNA isolation and proteomics analysis of culture supernatants. The combination of Sequence Based Discovery and proteomics was employed for recovering sequences encoding amylases and glucoamylases.

[0700] Sequence Based Discovery was performed using two approaches. In one, universal degenerate primers for PCR recovery of amylase and glucoamylase genes were designed based on alignment of known fungal protein sequences. In the other approach, universal degenerate primers were combined in PCR with primers based on the peptide sequences obtained from the proteomics analysis of culture supernatants from fungal isolates grown on granular starch. In both cases the primers were used for PCR with template DNA comprising cDNA made from the same starch-digesting fungal strains. By this means, partial gene sequences were recovered. Peptide data were also used to facilitate recovery of full-length versions of the partial sequences using 5' and 3' RACE PCR.

[0701] For known fungal species with pre-sequenced genomes, proteomics analysis was used to determine the sequences of peptides derived from putative amylases using the following approach: 1) Following SDS-PAGE analysis of samples derived from fungal extracts grown on resistant starch as the sole source of carbon, individual bands were subjected to protease digestion; 2) Mass spectroscopy analysis of recovered peptides was carried out on an LCQ instrument; 3) Peptide sequences were determined by SEQUEST™ (Sage-N Research, Inc. and Thermo Scientific) database searches.

[0702] For unknown fungal species, the peptide sequences where determined by a method similar to the one described above with the exception that the accurate masses of peptides and their constituent amino acids were determined using a QTOF instrument. The sequences of the recovered peptides were used as a template to produce degenerate oligonucleotide primers for PCR amplification of the genes from cDNA made from the relevant fungal species grown on MIMAIZE^{260}™granular starch.

[0703] The recovered full-length cDNA sequences encoding amylases and glucoamylases were subcloned for expression in *Pichia pastoris* and the expressed proteins were further characterized using the BCA assay for measuring the increase in concentration of reducing ends during starch hydrolysis by amylases, and the GO assay for the detection of glucose released during starch hydrolysis by glucoamylases.

[0704] For determining the activity of both amylases and glucoamylases, starch hydrolysis reactions were performed as follows: Assays were performed in triplicate in an Eppendorf tabletop incubator with constant shaking (800 rpm), at 37°C and pH 5.0 in 50 mM sodium acetate buffer containing 1% raw granular starch. Reactions were started by adding the enzyme to the reaction mix. At different time points aliquots of the reactions were withdrawn and quenched either by addition of 1 M Tris pH 7.5 (glucoamylases) or BCA reagent (amylases).

[0705] BCA assay for measuring the increase in concentration of reducing ends. The activity of alpha-amylases was measured by the appearance of reducing groups formed during the hydrolysis of starch. The BCA -Bicincochinic Acid (Copper-BCA) assay of Reducing Sugars was performed according to Wong (2000) Microassay for rapid screening of alpha-amylase activity, J. Agric. Food Chem. 48:4540-4543; and, Fox (1991) Miniaturization of three carbohydrate analyses using a micro sample plate reader, Anal. Biochem. 195:93-96, was used.

[0706] A 10 μl aliquot of amylase starch hydrolysis reaction was quenched into 100 μl of BCA reagent (consisting of

64 mg/mL sodium carbonate monohydrate, 24 mg/mL sodium bicarbonate, 1.95 mg/mL BCA, 1.24 mg/mL cupric sulfate pentahydrate, 1.26 mg/mL L-serine). Color development occurred during incubation of the quenched reaction at 80°C for 35 minutes, and was followed by absorbance determination at 560nm. Initial rates were calculated over a 50 min reaction time. A standard curve using maltose was constructed to correlate A_{560nm} with the concentration of generated reducing sugars (nmoles). Specific activity was expressed as nmoles/min/μg enzyme.

[0707] Glucose oxidase / peroxidase (GO) assay for the quantification of glucose released during starch hydrolysis: The activity of glucoamylases was measured by the modified GO (coupled glucose oxidase) assay according to Bergmeyer, In Determination with Glucose Oxidase and Peroxidase; Bergmeyer, H. U., Ed.; Methods of. Enzymatic Analysis. 2nd Ed.; 1974; pp 1205-1212).

[0708] GO reactions were started by adding 10 μl of the quenched starch hydrolysis reaction to 90 μl of PBS containing glucose oxidase (0.1 U/ml), peroxidase (0.25 U/ml) and 0.05 mM Amplex Red, in black Nunc 96 well plates. Plates were kept at room temperature in the dark for 30 min prior to reading on a fluorescence plate reader with Ex/Em 545/590 nm. A standard curve constructed with glucose was used to assess the amount of glucose produced in the hydrolysis reactions. Initial rates of starch hydrolysis (nmols of glucose released from 1% granular starch / min/ μg glucoamylase) were determined by plotting the amount of glucose released over time, and calculating the slope of the best linear fit through the data points.

[0709] The influence of pH in the range of between about pH 3.5 to 6.0 on the hydrolysis of starch by seven (7) exemplary glucoamylases and two exemplary amylases of this disclosure is illustrated in Figure 10 and Figure 11. Figure 10 illustrates data showing the influence of pH on granular starch hydrolysis by seven (7) exemplary glucoamylases of this disclosure at 37°C; initial rates were calculated over 15 min and converted to the percentage of the maximum observed rate. Figure 11 illustrates data showing the influence of pH on granular starch hydrolysis by SEQ ID NO:4 (encoded by, e.g., SEQ ID NO:3) and SEQ ID NO:52 (encoded by, e.g., SEQ ID NO:51) amylases at 37°C; initial rates were calculated over 50 min and converted to the percentage of the maximum observed rate.

[0710] Other assays can also be used to characterize an enzyme of the invention, and some exemplary protocols are described, below:

Exemplary Nucleic Acid Extraction Protocol: Microorganisms, e.g., filamentous fungus, are grown in liquid culture. Biomass is collected and high molecular weight genomic DNA is isolated using DNEASY™ (DNeasy) Plant Maxi Kit (Qiagen, Valencia, CA) using standard protocols. Total RNA can be isolated using RNEASY™ (RNeasy) Plant Mini Kit (Qiagen) using standard protocols.

[0711] Exemplary Library Construction Protocol: genomic DNA can be partially digested with restriction enzymes and fragments between 1-10 kb can be purified for construction of a genome library. The fragments can be ligated into the vector Lambda Zap Express™ (Stratagene, San Diego, CA) and packaged into infectable phage as per manufacturer's instructions.

[0712] Exemplary Library Screening Protocol: Lambda libraries can be used to infect XL1 Blue MRF™ cells (Stratagene) in top agar. Approximately 50,000 pfu of phage can be added to 600 ul of cells OD600=1. The mixture is incubated at 37°C for 15 minutes in a water bath and then added to 6 ml melted 0.7% top agar and plated onto NZY agar plates. The plate is then incubated overnight at 39°C. A nylon circle (F. Hoffmann-La Roche Ltd., Basel Switzerland) can be laid on top of the resulting plaque lawn and lifted back up with some of the phage adhering to the nylon. The nylon can be submerged in 1.5M NaCl, 0.5M NaOH for 2 minutes, 1.5M NaCl, 0.5M Tris pH 7.6 for 5 minutes and 2X SSC, 0.2M Tris pH7.6 for 30 seconds. The nylon filter is then UV crosslinked, e.g., in a Stratagene crosslinker.

[0713] PCR fragments from amylase, e.g., glucoamylases, genes can be used probes, e.g., using an Expand High Fidelity PCR Kit™ (Roche) using 30 cycles of 95°C for 20 seconds, 55°C for 30 seconds, and 72°C for 1 minute in a thermal cycler. The isolated PCR fragment can be prepared as a radioactive probe using the Prime It Kit™ (Stratagene) following manufacturer's instructions.

[0714] The library filter lifts are washed in a prehybridization solution (e.g., DIG EASY HYB™, Roche) for two hours at 42°C in a hybridization oven (Robbins). The probe can be added to 15ml fresh DIG EASY HYB™ and used to replace the prehybridization solution. The filter is washed with probe overnight, e.g., at 45°C. The probe can be removed and the filter washed once with 2X SSC, 0.1% SDS for 15 minutes, and twice with 0.1X SSC, 0.1% SDS for 15 minutes each. The nylon filter can be exposed to x-ray film overnight at -80C. Following developing, hybridization spots on the x-ray film can be used to identify clones from the original plate. An agar plug can be taken from the plate where the spots lined up and suspended in SM buffer to release the phage into solution. Several isolated plaques corresponding to genomic fragments containing all or part of an amylase gene can be thus isolated.

[0715] 100 ul of isolated phage stock can be added to 200ul XL-1 BLUE MRF™ cells (Stratagene) and 1 ul EXASSIST™ helper phage (Stratagene). The mixture can be incubated at 37C for 15 minutes, and 3 ml of 2X YT media can be added. This can be incubated at 37°C with shaking for 2.5 hours. The mix can be heated for 20 minutes at 70°C and cooled on ice. 100 ul of the mix can be removed and added to 200 ul SOLR cells (Stratagene) and incubated at 37°C for 15 minutes.

50 ul can be plated on LB kanamycin (50 ug/ml) plates and incubated overnight at 37°C. Resulting colonies may contain cloned genomic fragments in the plasmid pBK-CMV.

[0716] Exemplary Sequencing Protocol: DNA sequencing on candidate clones can be performed with the BIGDYE TERMINATOR™ cycle sequencing VERSION 2.0 Kit™ (Applied Biosystems, Foster City, CA) and a 3700 DNA Analyzer™ (Applied Biosystems) using manufacturer's protocols. Potential introns can be identified by comparing this sequence with consensus sequences for introns in known amylases.

[0717] Exemplary cDNA Synthesis Protocol: PCT primers are used in a cDNA synthesis reaction using a THERMOSCRIPT™ rtPCR Kit™ (Invitrogen) using manufacturer's protocols.

[0718] Exemplary Expression Cloning Protocol: PCT primers are used to generate a PCR fragment using the cDNA clone as a template using 30 cycles of 95°C for 20 seconds, 55°C for 30 seconds, 72°C for 2 minutes, using EXPAND HIGH FIDELITY PCR Kit™ (Roche) and manufacturer's protocols. The PCR fragments are digested with the restriction enzymes and ligated into the corresponding restriction sites of a plasmid, e.g., pPIC Z™ alpha (Invitrogen). The construct can be transformed into a yeast, e.g., *Pichia pastoris* Strain X-33™ (Invitrogen) where the construct integrates stably into the *Pichia* chromosome. Selection can be based on resistance to zeocin. The construct can be designed such that the *Pichia* clone can be induced with methanol to secrete the mature amylase into the media. A 1-liter culture of the expression clone can be inoculated with an overnight yeast starter culture in BMGY and grown overnight at 30°C in a shake flask. The yeast cells are collected by centrifugation the following day and resuspended in 1 liter of BMMY. The cells are cultured at 30°C in a shake flask for 3 days with methanol added to 0.5% final every 24 hours. The media containing the expressed glucoamylase enzyme are then collected and tested in a glucoamylase activity assay and SDS PAGE electrophoresed using standard protocols to determine the protein size.

[0719] Primers also can be designed for overexpression in *Escherichia coli.* PCR primers are used to generate a PCR product as before, from the cDNA template. The PCR fragment can be digested with the restriction enzymes and ligated into corresponding restriction sites of the plasmid, e.g., a pSE420 (Invitrogen). The construct can be transformed into *Escherichia coli,* e.g., Strain XL-1 Blue MR (Stratagene). Selection for the plasmid can be based on ampicillin resistance. The amylase gene can be under the control of a lac-z promoter and can be induced with IPTG (isopropyl-thiogalactopyranoside). The construct can be designed such that the mature glucoamylase gene will be expressed within the *Escherichia* cell and will contain an extra methionine residue at the N-terminus.

[0720] Exemplary "Standard" assay: Enzyme aliquots can be added to a solution of 5 mM buffer, 3 mM malto-oligosaccharides (Sigma, M-3639) in a waterbath. 100 ul aliquots can be removed at time points to 200 ul glucose oxidase reagent (Sigma, GAGO-20) and incubated 37°C, 30 min. The reaction can be stopped with addition of 12 N sulfuric acid and the absorbance at 540 nm determined. The full-length version of the enzyme can be tested for pH, temperature and substrate utilization.

[0721] Exemplary "Activity" Assay: Enzyme activity can be measured by the release of free glucose from an oligodextrin substrate. The liberated glucose can be oxidized in a coupled reaction resulting in a colored product. An enzyme aliquot can be added to solution of 5mM buffer, 3mM malto-oligosaccharides (Sigma, M-3639) in a water bath. 100 ul aliquots can be removed at time points to 200ul glucose oxidase reagent (Sigma, GAGO-20) and incubated 37°C, 30 min. The reaction is stopped with addition of 12 N sulfuric acid and the absorbance at 540 nm determined. Time points are then plotted to determine the relative rate for the reaction.pH Profile: Acetate buffer (pH 4.0, 4.5, 5.0, and 5.4) as well as phosphate buffer (pH 6.2, 7.0, 8.1) can be used in an activity assay to determine the relative rate for the glucoamylase at each pH.

[0722] Temperature Profile: The relative rate of the enzyme at various temperatures (e.g., 50°C, 60°C, 70°C, 80°C, and 85°C) can be determined in acetate buffer pH 5.3.

[0723] Temperature Stability Data: Enzyme can be added to 5 mM acetate buffer at a desired indicated temperature. Enzyme aliquots can be removed to ice at 4 minute intervals. The aliquots are then tested for activity on substrate for 20 minutes at 70°C.

[0724] Substrate Utilization: The dextrins maltose, maltotriose, panose, maltotetraose, and maltoheptaose can be substituted for the malto-oligosaccharides in the activity assay to test for substrate utilization of an amylase, e.g., a glucoamylase. Rate of glucose release for various substrates can be tested in 5 mM acetate buffer, 70°C.

Example 16: Amylase Activity Assay: BCA Reducing Ends Assay

[0725] The following example describes an exemplary method for a BCA reducing ends assay. Amylase (including, e.g., glucoamylase) activity can be determined using the following methodology.

1. Prepare 2 substrate solutions, as follows:

   a) 2% soluble starch (potato or granular corn starch) pH 8 solution by dissolving 2 gm potato starch in 100 ml 100 mM sodium phosphate pH 8).

b) 2% soluble starch (potato) pH 10 solution by dissolving 2 gm potato starch in 100 ml 100 mM sodium carbonate.

Heat both solutions in a boiling water bath, while mixing, for 30-40 minutes until starch dissolves.

2. Prepare Solution A from 64 mg/ml sodium carbonate monohydrate, 24 mg/ml sodium bicarbonate and 1.95 mg/ml BCA (4,4'-dicarboxy-2,2'- biquinoline disodium salt (Sigma Chemical cat # D-8284). Added above to dH_2O.

3. Prepare solution B by combining 1.24 mg/ml cupric sulfate pentahydrate and 1.26 mg/ml L-serine. Add mixture to dH_2O.

4. Prepare a working reagent of a 1:1 ration of solutions A and B.

5. Prepare a Maltose standard solution of 10 mM Maltose in dH_2O, where the 10 mM maltose is combined in 2% soluble starch at desired pH to a final concentration of 0, 100, 200, 300, 400, 600 μM. The standard curve will be generated for each set of timepoints. Since the curve is determined by adding 10 ul of the standards to the working reagent it works out to 0, 1, 2, 3, 4, 6 nmole maltose.

6. Aliquot 1 ml of substrate solution into microcentrifuge tubes, equilibrate to desired temperature (5 min) in heat block or heated water bath. Add 50 ul of enzyme solution to the inside of the tube lid.

7. While solution is equilibrating mix 5 ml of both solution A & B. Aliquot 100 ul to 96 well PCR plate. Set plate on ice.

8. After 5 minute temperature equilibration, close lid on tubes, invert and vortex 3 times. Immediately aliquot 10 ul into plate as t=0 (zero time point). Leave enzyme mixture in heat block and aliquot 10 ul at each desired time point (e.g. 0, 5, 10,15, 20, 30 minutes).

9. Ensure that 12 wells are left empty (only working reagent aliquotted) for the addition of 10 ul of standards, for the standard curve.

10. When all time points are collected and standards are added, cover plate and heated to 80° C for 35 min. Cool plate on ice for 10 min. Add 100 ul H2O to all wells. Mix and aliquot 100 ul into flat bottomed 96-well plate and read absorbance at 560 nm.

11. Zero each sample's time points against its own t=0 (subtract the average t=0 A560 value from other average A560 values). Convert the $A560_{(experimental)}$ to umole (Divide $A560_{(experimental)}$ by the slope of the standard curve (A560/umole). Generate a slope of the time points and the umole (in umole/min), multiply by 100 (as the umole value only accounts for the 10 ul used in the assay, not the amount made in the 1ml rxn). To get the specific activity divide the slope (in umole/min) by the mg of protein. All points should be done at a minimum in duplicate with three being best. Divide protein concentration (mg/ml) by any dilution to get mg used in assay. Divide the above slope by mg used in assay to get specific activity. See for example, Wong (2000) J. Agric. Food Chem. 48:4540-4543; Fox (1991) Anal. Biochem. 195, 93-96.

Example 17: Screening for Amylase activity

[0726] Amylase (e.g., glucoamylase) activity of clones can be assessed by a number of methods known in the art. The following is an example of methodology that can be used.

[0727] The number of plaques screened, per plate, can be approximately 10,000 pfu's. For each DNA library: about 50,000 plaques per isolated library and 200,000 plaques per non-isolated library can be screened depending upon the pfu titer for the λ Zap Express amplified lysate.

Titer determination of Lambda Library

[0728]

1) μL of Lambda Zap Express amplified library stock added to 600μL *E. coli* MRF' cells (OD_{600}=1.0). To dilute MRF' stock, 10mM $MgSO_4$ is used.

2) Incubate at 37°C for 15 minutes.

3) Transfer suspension to 5-6mL of NZY top agar at 50 °C and gently mix. Immediately pour agar solution onto large (150mm) NZY media plate.

4) Allow top agar to solidify completely (approximately 30 minutes), then invert plate.

5) Incubate the plate at 39 °C for 8-12 hours.

6) Number of plaques is approximated. Phage titer determined to give 10,000 pfu/plate.

7) Dilute an aliquot of Library phage with SM buffer if needed.

Substrate screening

[0729]

- Lambda Zap Express (50,000 pfu) from amplified library added to 600μL of *E. coli* MRF' cells (OD600=1.0). For non-environment libraries, prepare 4 tubes (50,000 pfu per tube).
- Incubate at 37 °C for 15 minutes.
- While phage/cell suspension are incubating, 1.0mL of red starch substrate (1.2% w/v) is added to 6.0mL NZY top agar at 50 °C and mixed thoroughly. Keep solution at 50°C until needed.
- Transfer 1/5 (10,000 pfu) of the cell suspension to substrate/top agar solution and gently mixed.
- Solution is immediately poured onto large (150mm) NZY media plate.
- Allow top agar to solidify completely (approximately 30 minutes), then invert plate.
- Repeat procedures 4-6 four times for the rest of the cell suspension (1/5 of the suspension each time).
- Incubate plates at 39°C for 8-12 hours.
- Plate observed for clearing zones (halos) around plaques.
- Plaques with halos are cored out of agar and transferred to a sterile micro tube.

[0730] A large bore 200μL pipette tip works well to remove (core) the agar plug containing the desired plaque.

- Phages are re-suspended in 500μL SM buffer. 20μL chloroform is added to inhibit any further cell growth.
- Pure phage suspension is incubated at room temperature for 4 hours or overnight before next step.

Isolation of pure clones

[0731]

- 10μL of re-suspended phage suspension is added to 500μL of *E. coli* MRF' cells (OD600=1.0).
- Incubate at 37°C for 15 minutes.
- While phage/cell suspension is incubating, 1mL of red starch substrate (1.2% w/v) is added to 6.0mL NZY top agar at 50 °C and mixed thoroughly. Keep solution at 50 °C until needed.
- Cell suspension is transferred to substrate/top agar solution and gently mixed.
- Solution is immediately poured onto large (150mm) NZY media plate.
- Allow top agar to solidify completely (approximately 30 minutes), then invert plate.
- Plate incubated at 39°C for 8-12 hours.
- Plate observed for a clearing zone (halo) around a single plaque (pure clone). If a single plaque cannot be isolated, adjust titer and re-plate phage suspension.
- Single plaque with halo is cored out of agar and transferred to a sterile micro tube. A large bore 200μL pipette tip works well to remove (core) the agar plug containing the desired plaque. To amplify the titer, core 5 single active plaques into a micro tube.
- Phages are re-suspended in 500μL SM buffer. 20μL Chloroform is added to inhibit any further cell growth.
- Pure phage suspension is incubated at room temperature for 4 hours or overnight before next step. The pure phage suspension is stored at -80 °C by adding DMSO into the phage suspension (7% v/v).

Excision of pure clone

[0732]

- 100μL of pure phage suspension is added to 200μL *E. coli* MRF' cells (OD600=1.0). To this, 1.0μL of EXASSIST™ helper phage (>1 x 106 pfu/mL; Stratagene) is added. Use 2059 Falcon tube for excision.
- Suspension is incubated at 37°C for 15 minutes.
- 3.0 mL of 2 x YT media is added to cell suspension.
- Incubate at 30 °C for at least 6 hours or overnight while shaking.
- Tube transferred to 70°C for 20 minutes. The phagemid suspension can be stored at 4°C for 1 to 2 months.
- 100 μL of phagemid suspension transferred to a micro tube containing 200μL of *E. coli* Exp 505 cells (OD600=1.0).
- Suspension incubated at 37 °C for 15 minutes.
- 300μL of SOB is added to the suspension.
- Suspension is incubated at 37°C for 30 to 45 minutes.
- 100μL of suspension is transferred to a small (90mm) LB media plate containing Kanamycin (LB media with Kanamycin 50μg/mL) for Zap Express DNA libraries or Ampicillin (LB media with Kanamycin 100μg/mL) for Zap II DNA libraries.
- The rest of suspension is transferred to another small LB media plate.
- Use sterile glass beads to evenly distribute suspension on the plate.

- Plates are incubated at 30°C for 12 to 24 hours.
- Plate observed for colonies.
- Inoculate single colony into LB liquid media containing suitable antibiotic and incubate at 30 °C for 12 to 24 hours.
- Glycerol stock can be prepared by adding 80% glycerol into liquid culture (15% v/v) and stored at -80 °C.

Activity verification

[0733]

- 50μL of liquid culture is transferred to a micro tube. Add 500μL of 8% pH 7 Amylopectin Azure into the same tube. Prepare 2 tubes for each clone.
- Activity is tested at 50°C for 3 hours and overnight. Use pH 7 buffer as control.
- Cool the test specimen at ice-water bath for 5 minutes.
- Add 750 μL of Ethanol and mixed thoroughly.
- Centrifuge at 13000 rpm (16000 g's) for 5 minutes.
- Measure OD of the supernatant at 595nm.

RFLP analysis

[0734]

- 1.0mL of liquid culture is transferred to a sterile micro tube.
- Centrifuge at 13200 rpm (16000 g's) for 1 minute.
- Discard the supernatant. Add another 1.0 mL of liquid culture into the same sterile micro tube.
- Centrifuge at 13200 rpm (16000 g's) for 1 minute.
- Discard the supernatant.
- Follow QIAPREP™ spin mini kit protocol for plasmid isolation.
- Check DNA concentration using BioPhotometer.
- Use Sac I and Kpn I for first double digestion. Incubate at 37 °C for 1 hour.
- Use Pst I and Xho I for second double digestion. Incubate at 37 °C for 1 hour.
- Add Loading dye into the digested sample.
- Run the digested sample on a 1.0% agarose gel for 1-1.5 hours at 120 volts.
- View gel with gel imager. All clones with a different digest pattern will be sent for sequence analysis.

Example 18: Exemplary Protocols for Purifying Enzymes

[0735] The following example describes exemplary protocols for purifying enzymes of this invention.

[0736] SEQ ID NO:52: 6g of lyophilized supernatant of *P. pastoris* culture (see discussion in Example 25, below) expressing SEQ ID NO:52 was suspended in 24 mL of H_2O and precipitated with cold ethanol. Precipitated pellet was re-suspended in approximately 40 mL of H_2O and dialyzed O/N against water. After dialysis a concentrated acetate buffer pH 6.0 was added to the sample to get a final concentration of 50 mM. The protein bound to the column (Q SEPHAROSE™; Amersham Pharmacia resin poured in a XK 50™ column) in 50mM Na acetate pH6.0 and was eluted during a gradient between 0 and 400 mM NaCl in Na acetate pH6.0. Contaminating proteins were removed from the column with 1M NaCl in Na acetate pH6.0. Elution of amylase from the Q SEPHAROSE™ was tracked with SDS PAGE and activity assays using BODIPY-starch as a substrate. Multiple purifications were run to obtain enough protein to meet the requirement of approximately 1g. The purified fractions from these runs were pooled together and concentrated by stir cell concentrator.

[0737] SEQ ID NO:48: 24 g of lyophilized supernatant of *P. pastoris* culture expressing SEQ ID NO:48 was suspended in 20 mL of 50 mM carbonate buffer pH 10.0; 100 mM NaCl and precipitated with cold ethanol. Precipitated pellet was re-suspended in approximately 20mL of 50 mM acetate buffer pH 5.2; 500 mM NaCl, and dialyzed O/N against 50 mM malic buffer pH 3.5; 500 mM NaCl. The protein bound to the 100 mL agarose - amylose (NEB) column in 50 mM malic buffer pH3.5 ; 500 mM NaCl and was eluted with 0.5% corn dextrin in 50 mM carbonate buffer pH 10.0; 50 mM NaCl. Contaminating proteins were removed from the column with 50 mM sodium phosphate buffer pH 7.5; 100 mM NaCl. Elution of glucoamylase from the agarose-amylose was tracked with SDS PAGE.

[0738] Other enzymes can be purified using these protocols or variations thereof, or analogous protocols.

[0739] Figure 26 illustrates tables summarizing the efficiency of these purification protocols for the exemplary SEQ ID NO:4, SEQ ID NO:18, SEQ ID NO:26, and corresponding activity data on, inter alia, raw starch and soluble starch comparing purified and unpurified enzyme.

[0740] Purified enzyme preparations of the exemplary SEQ ID NO:52 and SEQ ID NO:26 were analyzed for activity (the ability to hydrolyze) in raw starch fermentations; this data on the purity (determined by densitometric analysis), concentration, quantity and storage buffer is summarized:

	SEQ ID NO:52	SEQ ID NO:48
Class	Alpha-amylase	Glucoamylase
Storage Buffer	GMP*	GMA**
Concentration	25 mg /ml	25 mg/ml
Volume Sent	40 ml	20
Total Amount	1. 0 g	0.5 g
Purity	>90%	> 95%
*GMP- 20% glucose; 0.1% methyl paraben, PBS pH 7.0 **GMA - 20% glucose; 0.1% methyl paraben; 50 mM sodium acetate pH 5.2; 100 mM NaCl.		

Example 19: Exemplary protocol for liquefying starch and measuring results

[0741] The following example describes exemplary protocols for liquefying starch using, e.g., enzymes of this invention. Reaction Conditions: 100 mM PO_4 pH 6.5, 1 % (w/w) liquefied starch DE 12 at 55°C. Both TLC and HPLC assays can be done to verify activity.

[0742] An exemplary protocol for the saccharification of liquefied starch at pH 6.5:

· Adjust the pH of the liquefied starch to the pH at which the saccharification(s) will be performed. Liquefy starch in 100 mM sodium acetate buffer, pH 4.5 with 100 mM sodium phosphate salts added so that before saccharification, the pH could be adjusted to pH 6.5.
Weigh 5 gram samples of liquefied starch into tared bottles.

Use 0.04% (w/w) OPTIDEX L-400™ or approximately 400 mL of 1-10 diluted stock OPTIDEX L-400™ per 100 grams of liquefied starch.
Calculate the milligrams of OPTIDEX L-400™contained in the 400 mL of 1-10 diluted stock OPTIDEX L-400™.
Next, calculate the volume of lysates needed to give the same concentration of enzyme as the OPTIDEX L-400™.
· Add enzymes to liquefied starch samples and incubate at desired temperature (50C°). After 18 hours determine DE and prepare a sample for HPLC analysis.

An exemplary DE Determination:

Exemplary Neocuproine Assay:

[0743] A 100ml sample can be added to 2.0ml of neocuproine solution A (40g/L sodium carbonate, 16g/L glycine, 0.45g/L copper sulfate). To this can be added 2.0 ml of neocuproine solution B (1.2g/L neocuproine hydrochloride-Sigma N-1626). The tubes can be mixed and heated in a boiling water bath for 12 minutes; cooled, diluted to 10ml volume with DI water and the OD read at 450nm on the spectrophotometer. The glucose equivalent in the sample can be extrapolated from the response of a 0.2mg/ml glucose standard run simultaneously.

Exemplary HPLC Analysis:

[0744] Saccharification carbohydrate profiles are measured by HPLC (Bio-Rad Aminex HPX-87A column in silver form, 80°C) using refractive index detection. Mobile phase is filtered Millipore water used at a flow rate of 0.7 ml/min. Saccharification samples are diluted 1-10 with acidified DI water (5 drops of 6 M HCl into 200 mL DI water) then filtered through a 0.45 mm syringe filter. Injection volume is 20 uL.

Exemplary TLC:

[0745] Reaction products can be w/d at hourly timepoints and spotted and dried on a TLC plate. The plate can be then developed in 10:90 water: isopropanol and visualized with either a vanillin stain or CAM stain and then heated to show

reducible sugars. The liquefied starch can be partially hydrolyzed to glucose in cases where activity was observed.

Example 20: Starch Liquefaction using glucoamylases

[0746] This example describes an exemplary method of the invention for liquefying starch using glucoamylases of the invention. Glucoamylase concentrate can be prepared from fermentation broths by heat treatment, cell washing, alkaline extraction using microfiltration and ultrafiltration (48% overall yield). The UF concentrate can be neutralized with acetic acid and formulated with 30% glycerol at pH 4.5. The activity level of a commercial product can be about 120 U^1/g - 0.5 kg/ ton starch.

Exemplary glucoamylase activity assay

[0747] A 1 mL cuvette containing 950 μL of 50 mM MOPS pH 7.0 containing 5 mM PNP-α- D-hexa-(1→4)-glucopyranoside is placed in the Peltier temperature controller of the Beckman DU-7400 spectrophotometer preheated to 80°C. The spectrophotometer is blanked at 405nm and 50 μL of the enzyme solution is added to the cuvette, mixed well and the increase in the OD_{405nm} is monitored over a one-minute interval. The $\Delta OD_{405nm/min}$ rate is converted to a standard unit of μmole/minute from the OD_{405nm} response of 50 μL of 1 μmole/mL PNP in 950 mL 50 mM MOPS at pH 7.0 -80°C. One standard unit of thermostable alpha glucoamylase (DTAA) is equal to the amount of enzyme that will catalyze the release of 1 μmole/mL/minute of pNP under the defined conditions of the assay.

Standard Glucoamylase Activity Assay

[0748] A 1 mL cuvette containing 950 μL of 50 mM MOPS pH 7.0 containing 5 mM pNP-α- D-glucopyranoside is placed in the Peltier temperature controller of the Beckman DU-7400 spectrophotometer preheated to 60°C. The spectrophotometer is blanked at 405nm and 50 μL of the enzyme solution is added to the cuvette, mixed well and the increase in the OD_{405nm} is monitored over a one-minute interval. The ΔOD_{405nm}/min rate is converted to a standard unit of μmole/minute from the OD_{405nm} response of 50 μL of 1 μmole/mL pNP in 950 mL 50 mM MOPS at pH 7.0-60°C. One standard Diversa unit of glucoamylase (DGA) is equal to the amount of enzyme that will catalyze the release of 1 μmole/mL/minute of pNP under the defined conditions of the assay.

Dextrose Equivalent Determination

[0749] The neocuproine method is used to measure the DE. Selected samples were measured by both the procedure described above, and by a GPC analyst using the GPC Fehlings procedure.

Neocuproine Assay

[0750] A 100 μl sample is added to 2.0 ml of neocuproine solution A (40 g/L sodium carbonate, 16g/L glycine, 0.45g/L copper sulfate). To this is added 2.0 ml of neocuproine solution B (1.2 g/L neocuproine hydrochloride-Sigma N-1626). The tubes were mixed and heated in a boiling water bath for 12 minutes; cooled, diluted to 10ml volume with DI water and the OD read at 450 nm on the spectrophotometer. The glucose equivalent in the sample is extrapolated from the response of a 0.2mg/ml glucose standard run simultaneously.

[0751] The starch sample is diluted ~1 to 16 with DI water with the exact dilution recorded. Ten milliliters of the diluted sample is added to 20 mls of DI water. Ten milliliters of Fehlings solution A and B were added to the diluted starch. The sample is boiled for 3 minutes and cooled on ice. Ten milliliters of 30% KI and 10ml of 6N H_2SO_4 is added. The solution is titrated against 0.1N sodium thiosulfate. The titrant volume is recorded and used to calculate the DE.

Residual Starch Determination

[0752] Post-saccharification samples were checked for residual starch using the Staley iodine procedure.

[0753] Twenty grams of sample is weighed into a large weigh dish. 45 μL of Iodine solution is added to the weigh dish and the starch solution is mixed well. Dark blue indicates the presence of starch, a light blue-green indicates slight starch, light green indicates a trace of starch and yellow-red, absence of starch. Iodine solution is prepared by dissolving 21.25 grams of iodine and 40.0 grams of potassium iodide in one liter of water.

Oligosaccharide Profile

[0754] Liquefaction and saccharification carbohydrate profiles were measured by HPLC (Bio-Rad AMINEX HPX-87C™

column in calcium form -80°C) using refractive index detection.

Gel Permeation Chromatography

[0755] The molecular weight distribution is determined by chromatography on a PL AQUAGEL-OH™ column with mass detection by refractive index (Waters Model 2410). A Viscotek Model T60™ detector is used for continuous viscosity and light scattering measurements.

Capillary Electrophoresis

[0756] Beckman Coulter P/ACE MDQ™ Glycoprotein System - separation of APTS derivatized oligosaccharides on a fused silica capillary - detection by laser-induced fluorescence.

Primary Liquefaction

[0757] Line starch directly from the GPC process is pumped into a 60 liter feed tank where pH, DS (dry solids) and calcium level can be adjusted before liquefaction. The glucoamylase is added to the slurry. The 32% DS slurry is pumped at 0.7 liter/minute by a positive displacement pump to the jet - a pressurized mixing chamber where the starch slurry is instantaneously heated to greater than 100°C by steam injection. The gelatinized partially liquefied starch is pumped through a network of piping (still under pressure) to give the desired dwell time (5 minutes) at temperature. The pressure is released into a flash tank and samples can be taken. Samples were taken in duplicate.

Secondary Liquefaction

[0758] The liquefied starch is collected in one liter glass bottles and held in a water bath at 95°C for 90 minutes.

Saccharification

[0759] Liquefied starch is cooled to 60°C, the pH adjusted to 4.5 and the samples treated with glucoamylase. Saccharification progress is monitored over time by HPLC.

Saccharification

[0760] The liquefied syrups produced with each glucoamylase were adjusted to approximately pH 2.5 with 6N HCl immediately after the 90 minute secondary liquefaction to inactivate any residual glucoamylase. The syrups were then adjusted to pH 4.5, placed in a 60°C water bath and saccharified with three levels of glucoamylase. The extent of saccharification is monitored by HPLC at 18 to 88 hour time points.

[0761] The liquefied syrups were saccharified with the standard dosage - 0.04% of a double-strength glucoamylase - and two lower dosages (50% and 25%) to monitor any differences in the saccharification progress.

[0762] Saccharification Progress - % dextrose development vs time - 0.04% glucoamylase.

Example 21: Exemplary Starch Liquefaction Process

[0763] This example describes an exemplary starch liquefaction process of the invention comprising use of enzymes of the invention. The conversion of starch to glucose can be catalyzed by the sequence action of two enzymes: amylases (e.g., alpha-amylases), to liquefy the starch (e.g., the hydrolysis of high molecular weight glucose polymers to oligosaccharides consisting of 2 to 20 glycose units, typically a dextrose equivalent of 10 to 12, by a glucoamylase of the invention), followed by saccharification with a glucoamylase (which can be a glucoamylase of the invention). In one aspect, processing is in a corn wet milling plant producing a starch slurry having a pH or about 4.0 to 4.5. In one aspect, the pH is raised, e.g., to 5.8 to 6.0 before liquefaction to accommodate a glucoamylase with a low pH activity and stability. In one aspect, glucoamylases of the invention can liquefy starch at pH 4.5 to dextrose equivalents ranging from 12 to 18; in one aspect, using glucoamylases of the invention at levels of about 3 to 6 grams per ton of starch. In this aspect, use of glucoamylases of the invention enables starch liquefaction to be conducted at pH 4.5.

[0764] In one aspect, starch liquefaction is conducted at pH 4.5 for 5 minutes at 105°C to 90 minutes at 95°C using glucoamylases of the invention. The quantity of enzyme is adjusted in order to adjust a target DE of 12 to 15 after liquefaction. In one aspect, the liquefied starch is then saccharified with a glucoamylase, e.g., an *Aspergillis* glucoamylase, for about 48 hours at about pH 4.5 and 60°C. If the saccharified syrup did not contain at least 95% glucose, the target liquefaction DE is raised and the saccharification repeated until the liquefaction eventually did produce a saccharified

syrup containing more than 95% glucose. The glucoamylase protein required to produce a suitable liquefied feedstock for saccharification is determined by PAGE.

Example 22: Identification of peptides resulting from protease proteolysis in Simulated Gastric Fluid (SGF) test

[0765] This example describes the identification of small peptides resulting from pepsin proteolysis of SEQ ID NO:52 (encoded, e.g., by SEQ ID NO:51). This example also describes the evaluation of activity of exemplary enzymes in *in vitro* "Simulated Gastric Fluid" (SGF) tests. The SGF tests showed that all the enzymes were quickly digested by the gastric protease pepsin. In one case a small pepsin-resistant fragment was observed after 60 minutes of treatment.

[0766] Figure 21 illustrates an SDS PAGE showing the results of proteolysis (*in vitro* digestibility, the SGF test) of SEQ ID NO:52 by pepsin at pH 1.3; pepsin cuts at the C-terminus of Lys (K) and Phe (F) residues (the upper arrow indicates a 36.5 K band, the middle arrow a 5 K band (see discussion, below), and the lower arrow a 3 K band). SEEBLUE PLUS2™ prestained Standard from Invitrogen, and MARKER12™ from Invitrogen was used. All the samples were run on 16% Tricine gels. A common band present in all tests is pepsin.

[0767] Figure 22 illustrates the characterization of the peptides generated in this digest of SEQ ID NO:52, as identified using LC MS/MS (Liquid Chromatography/ Mass Spectrometry/Mass Spectrometry) analysis; an N-linked glycosylation site is identified.

[0768] Figure 23A illustrates the small peptide isolation scheme used (for the peptides generated by the proteolysis of SEQ ID NO:52 by pepsin). Figure 23B is discussed, below, and illustrates an SDS PAGE of the results of the small peptide isolation scheme, where lane 1 is pepsin only, lane 2 is undigested amylase SEQ ID NO:52, lane 3 is the amylase SEQ ID NO:52 digested by pepsin, lane 4 is the 30 kd cut-off top as illustrated in Figure 23A, lane 5 is the 30 kd flow-through as illustrated in Figure 23A, lane 6 is the sample captured by C18 RP (C18 reverse phase column chromatography) as illustrated in Figure 23A.

[0769] Figure 23C illustrates the LC/MS profile of the C18 RP eluted fraction, and is discussed, below.

[0770] Figure 23D illustrates the sequence of the peptides identified by the LC MS/MS analysis; Figure 23E and Figure 23F illustrate the "Asn-Xaa-Ser/Thr" sequins (motifs) in the sequence output (highlighted in blue); asparagines predicted to be N-glycosylated are highlighted in red.

[0771] In Vitro Digestibility Assays: No full-length versions of any of the enzymes tested were detected by SDS-PAGE after SGF treatment, indicating that pepsin had digested each of the full-length proteins; after 60 minutes of SGF treatment, small proteolytic fragments, about 6 kDa in size, were observed in digests of α-amylase SEQ ID NO:52 (see discussion in Example 22).

[0772] Activity Test after Pepsin Treatment: To determine if there is any residual activity remaining after pepsin treatment, the SGF test was modified and activity tests were performed with dextrin as substrate for glucoamylases and BODIPY-starch as substrate for α-amylases. No residual activity was observed for any of the enzymes tested, after SGF treatment for 60 minutes. The loss of activity was caused either by pepsin digestion of the test proteins (e.g. SEQ ID NO:4, SEQ ID NO:76 and SEQ ID NO:2), by the inactivation of the enzyme under the low pH reaction conditions, or both.

[0773] To identify the pepsin resistant peptide band, migrating at 5 kDa range on a SDS-PAGE, small peptides where enriched by filtering the sample over a 30 kDa filter membrane. The flow through was further purified using a C18 solid phase extraction column. The recovered peptides where then analyzed by Edmond degradation N-terminal sequencing, LC-MS, and LC tandem mass spectroscopy to determine the peptide sequence. The samples analyzed by LC tandem mass spectroscopy were first treated with trypsin protease.

[0774] Peptide purification: To determine the sequence of the 5 kDa peptide band, the peptide was enriched by first passing the pepsin treated SEQ ID NO:52 reaction over a 30 KDa filter membrane. The flow-through was then purified using a C18 solid phase extraction column. The recovered peptides formed a band at the 5kDa range on an SDS-PAGE. Figure 23B illustrates an SDS-PAGE analysis of the pepsin resistant alpha-amylase SEQ ID NO:52 peptide. This gel was Coomassie stained. Lane 1 is the pepsin protease. Lane 2 is the amylase SEQ ID NO:52. Lane 3 is the amylase SEQ ID NO:52 treated with pepsin protease. Lane 4 is same as sample 3 which did not flow through a 30 kDa filter membrane. Lane 5 is the flow through of sample in lane 3 after passed through a 30 kDa filter membrane. Lane 6 is sample 5 after recovery from a C18 solid phase extraction column.

[0775] To determine the peptide masses in the recovered sample the peptides where analyzed by LC-MS analysis over a C18 reverse phase column. The m/z profile of this sample showed that there are multiple masses of various m/z values that are present in this sample, as illustrated in Figure 23C, an LC-MS analysis of the amylase SEQ ID NO:52 small peptides. The solid phase was a C18 RP material. A gradient of 5%-80% ACN (15-90 minutes) was used as the mobile phase in the experiment.

[0776] Edmond degradation N-terminal sequencing: To determine the sequence of the SEQ ID NO:52 small peptides, the purified peptides where sent for N-terminal sequencing using Edmond degradation method. The results for this analysis were inconclusive, since, a number of different amino acids were released at each cleavage step. This result was consistent with the LC/MS analysis that showed that there are more than one prominent species of peptides present

in this sample.

[0777] LC tandem mass spectroscopy analysis: To determine the sequence of peptides present in the amylase SEQ ID NO:52 purified peptide fraction, these where treated with trypsin protease and analyzed by LC MS/MS. This analysis resulted in the identification of the following seven peptides; these peptide sequences were identified by SEQUEST™ searches from the LC MS/MS analysis of the amylase SEQ ID NO:52 small tryptic peptides.:

Peptide	Peptide sequence	Residue location within SEQ ID NO:52
1	AGQEQHYSGGSDPANR	349-364
2	VFSGDPAYTCPYQN	251-264
3	SGDPAYTCPYQN	253-264
4	SLLLLLSVFGQATHA	6-20
5	YENTGDGTSYHG	90-101
6	VYCGGSWQGIINHLD	56-70
7	GYSAGATLVETYTCT	448-462

[0778] It also determined that there were a number of peptide peaks in the LC-MS spectrum that their MS/MS profile did not match neither SEQ ID NO:52 nor other peptides in the database. One possible reason for this could be the complex N-linked glycosylation modification of the parent protein that takes place in the host organism, *Pichia pastoris.* To test this hypothesis, the extracted peptides were first treated with PNGase F to remove their N-linked glycosyl groups. The resulting peptides where then trypsin treated and where subjected to LC MS/MS analysis to determine their peptide composition. This analysis determined that there where at least 6 different peptide species present in the sample (see table summary above). The resolution of these peptides, post deglycosylation treatment, suggests that these peptides where part of other peptides that where glycosylated in the sample.

[0779] In conclusion, N-terminal sequencing and LC-MS analysis showed that the 5 KDa peptide band resulting from the pepsin protease digestion of amylase SEQ ID NO:52 is composed of many different peptide species. This is most likely due to incomplete digestion of the amylase SEQ ID NO:52 by the pepsin protease partly due to glycosylation state of these peptides. Thus, there is no one prominent peptide species in the MS spectra - multiple peptides are present; multiple different peptides species were sequenced in the sample; N-terminal chemical sequencing also provided evidence for the presence of multiple peptides; there is one peptide species that only appears after PNGase F deglycosylation of the sample, suggesting a glycosylation-related event is responsible for the appearance of this peptide.

[0780] This exemplary series of protocols can be used on any polypeptide of the invention, e.g., an exemplary sequence of the invention, to determine sequence, motifs, including glycosylation motifs, active sites and the like.

Example 23: Low temperature-active amylases

[0781] This example describes making and characterizing enzymes SEQ ID NO:56, SEQ ID NO:52; SEQ ID NO:62; SEQ ID NO:70, that are active at low temperatures, including having the ability to hydrolyze starch at low temperatures. This example also describes the development of an enzyme cocktail that can hydrolyze > 95% of the starch in milled corn into fermentable sugars in no more than 60 hours at about 30 to 40°C and about pH 3.5 to pH 5.5 in the presence of yeast. In one aspect, the total quantity of enzyme protein required is not greater than 50 grams/ton of corn, e.g., in alternative embodiments 0.05% w/w, or 0.005% w/w, or anywhere in the range of between 0.05% w/w to 0.005% w/w.

[0782] The initial reaction rates for starch hydrolysis were determined; and the influence of pH at varying ranges, e.g., in the range of about pH 3.5 - 7.0, and temperatures at varying ranges, e.g., in the range of about 30 - 40°C, on activity of enzymes were studied; the bond-type specificity of glucoamylases was also determined.

Methods:

1. Determination of Protein Concentration

[0783] Lyophilized supernatants of *P. pastoris* cultures expressing glucoamylases and α-amylases were suspended in water at a concentration of ~10 mg of powder / ml. After protein content determination by the Bradford protocol, 5 μg of protein sample and standardized BSA solution were run on a 4 -20% Tris-Glycine gradient gel. The gel was scanned on a BioRad GS800™ gel scanner following Coomassie blue staining. The Bio-Rad QUANTITY ONE™ software was used for the quantification of the BSA and glucoamylase (or α-amylases) bands, and the actual enzyme concentration

was then calculated. Protein concentration was adjusted accordingly and confirmed by additional SDS PAGE.

2. Determination of Initial Reaction Rates.

[0784] Unless mentioned otherwise, assays were performed in triplicate at 37°C and pH 5.0 in buffer (50 mM $NaCH_3CO_2$, 10 mM $CaCl_2$; 10 mM NaN_3 and 0.01% Triton X-100) containing 1% raw starch, or 0.5% dextrin or 1% "soluble corn starch". Assays were performed at 0.5ml scale for glucoamylase and 0.25 ml scale for alpha-amylase (α-amylase) in an Eppendorf tabletop incubator with constant shaking (800 rpm).

[0785] For glucoamylases, reactions were started by adding the enzyme (final concentration 0.25 μg /ml) to the reaction mix. At 0, 2.5, 5, 7.5, 10, 15, 20 and 30 min, 50 μl aliquots of the reactions were withdrawn and quenched by addition to 100 μl of 1M Tris buffer, pH 7.5. For alpha-amylases (α-amylases), reactions were started by adding the enzyme (final concentration 0.4 μg total protein/ml for SEQ ID NO:56, 13434 and SEQ ID NO:52; 2 μg/ml for SEQ ID NO:62; 4 μg/ml for SEQ ID NO:70) to the reaction mix, and 10 μl aliquots of reactions were withdrawn and quenched in BCA reagent at 2, 5, 10, 15, 20, 25, 30, and 40 min. For determination of temperature profiles, assays were performed at 30, 34, 37 and 40°C.

[0786] The effect of pH on glucoamylase and amylase activities was evaluated at pH 3.5, 4, 5, 6 and 7, using the broad pH range Britton - Robinson buffer (50 mM CH_3COOH; H_3PO_4; H_3BO_3). Parallel reactions at pH 4, 5 and 6 were also performed in the presence of 50mM acetate buffer to ensure that the buffer used did not influence the results. For the determination of pH profile of two calcium-dependent alpha-amylases (α-amylases) SEQ ID NO:56 and SEQ ID NO:62, malic acid / acetate / MES buffers were used instead of Britton-Robinson.

[0787] Preparation of "soluble corn starch" for reaction with alpha-amylases. Dextrin (Sigma D2006) could not be used as a substrate in the BCA alpha-amylase reactions due to the high reducing ends background. Therefore a heated corn starch (Syngenta material) was employed as a substrate. 2% corn starch was dissolved in deionized water and heated with mixing in a boiling water bath for 30-40 minutes, until the starch had dissolved and the solution appeared milky, but translucent. The solution of heated starch was used for 2 days, after which time some signs of retrogradation were observed (appearance of starch clumps), and the solution was discarded.

3. Glucose oxidase / peroxidase (GO) assay for the quantification of glucose released during starch hydrolysis.

[0788] A coupled glucose oxidase/ peroxidase (GO) assay was used to determine the amount of glucose released by glucoamylase during starch hydrolysis. GO reactions were started by adding 10 μl of the quenched starch hydrolysis reaction to 90 μl of PBS containing glucose oxidase (0.1 U/ml), peroxidase (0.25 U/ml) and 0.05 mM Amplex Red, in black Nunc 96-well plates. Plates were kept at room temperature in the dark for 30 min prior to reading on a fluorescent plate reader with Ex/Em 545/590 nm. A standard curve with glucose concentrations of 0 to 100 μM was used to assess the amount of glucose produced in the hydrolysis reactions. Initial rates of starch hydrolysis (nmols of glucose released from 1% granular starch / min/ μg glucoamylase) were determined by plotting the amount of glucose released over time, and calculating the slope of the best linear fit through the data points.

4. BCA assay for determining the increase in concentration of reducing ends during starch hydrolysis.

[0789] A 10 μl aliquot of amylase starch hydrolysis reaction was quenched into 100 μl of BCA reagent (consisting of 64 mg/mL sodium carbonate monohydrate, 24 mg/mL sodium bicarbonate, 1.95 mg/mL BCA, 1.24 mg/mL cupric sulfate pentahydrate, 1.26 mg/mL L-serine). Color development occurred during incubation of the quenched reaction at 80°C for 35 minutes and was followed by absorbance determination at 560nm. Initial rates were calculated over a 40 min reaction time. A standard curve using maltose (0 - 54 μM) was constructed to correlate A_{560nm} with the concentration of generated reducing sugars (nmoles). Specific activity was expressed as nmoles/min/μg enzyme.

5. Bond-type specificity of glucoamylases with maltose and isomaltose as substrates.

[0790] Reactions were started by adding the enzyme (final concentration 5 μg /ml for maltose, and 30μg /ml for isomaltose) to the reaction mix. At 2, 5, 10, 15, 20, 25, 30 and 40 min, 5 μl aliquots of the reactions were withdrawn and quenched by addition to 10 μl of 1M Tris buffer, pH 7.5. Nine substrate concentrations were used in the studies, ranging from 0 to 12 mM for maltose and 2.5 to 120 mM for isomaltose. The reactions were performed in triplicate at 37°C and pH 5.0 in buffer (50 mM $NaCH_3CO_2$, 10 mM $CaCl_2$), at 50 ul scale in an Eppendorf tabletop incubator with constant shaking (800 rpm). Glucose production was measured at the end of the reaction using the glucose oxidase / peroxidase (GO) assay.

Results

1. Characterization of glucoamylases:

[0791] 1.1 Initial reaction rates: Initial rates for granular and soluble starch hydrolysis are presented in Table 2, illustrated in Figure 24. As can be seen from Table 2, amylases and/or glucoamylases displayed up to 3x better activity (SEQ ID NO:48) against granular starch, with similar or slightly better activity on soluble starch when compared to the benchmark *A. niger* enzyme. SEQ ID NO:20 did not appear to display any activity against granular starch under the conditions tested (probably due to the lack of a Starch Binding Domain). Table 2 (Figure 24) summarizes data comparing initial rates of granular corn starch and soluble starch (dextrin) hydrolysis by exemplary enzymes (including those with glucoamylases activity) and a benchmark enzyme *A. niger* glucoamylase at 37°C, pH 5.0; initial rates are expressed as nmoles of glucose /min/μg of glucoamylase protein released from 1% granular starch or from 0.5% dextrin. Each number is the average value from 6-10 data points.

[0792] 1.2 Temperature profile: The effect of temperature on the activity of exemplary enzymes, including glucoamylases, on granular starch as a substrate was determined; a "benchmark" enzyme, the commercially available *A. niger* glucoamylase, was used. Glucose release was measured 30, 32, 34, 36, 38 and 40°C at pH 5.0. Activities of glucoamylases increased with temperature; they were most active at 40°C but retained approximately 50% of peak activity at 30°C.

[0793] Figure 25 illustrates the effect of temperature on the activity of the exemplary glucoamylase SEQ ID NO:20 and the *A. niger* "benchmark" with soluble starch (dextrin) as a substrate. Glucose release was measured at the indicated temperature at pH 5.0.

[0794] 1.3 pH profile: The influence of pH on starch hydrolysis was tested with both granular and soluble starch, respectively. All glucoamylases hydrolyzed both substrates best at lower pH, with the exemplary SEQ ID NO:26 being the most acidic in character. For both substrates, glucose release was measured at the indicated pH at 37°C. Initial rates were calculated over 20 min and converted to a percentage of the maximum rate.

[0795] 1.4 Bond-type cleavage specificity: The kinetic parameters for the hydrolysis of maltose (α-1,4-linkage) and isomaltose (α-1,6-linkage) were determined for 7 selected glucoamylases and the "benchmark" *A. niger* glucoamylase. The experiments were conducted with lyophilized *P. pastoris* lysates and the proteins were not purified; therefore only data independent of protein concentration are reported herein. Table 3, below, summarizes values of K_M for maltose and isomaltose and the ratio of k_{cat}/K_M for maltose compared to k_{cat}/K_M for isomaltose. These parameters determined for *A. niger* glucoamylase are in good agreement with published data; e.g., K_M for maltose is reported to be 1.2 - 2.1mM); K_M for isomaltose is reported to be 19.8 - 42.0 and k_{cat}/K_M for maltose over k_{cat}/K_M for isomaltose is reported to be between 300 - 600, according to Frandesen (1995) Biochemistry. 34:10162-10169; Sierks and Svensson (1996) Biochemistry 35:1865-1871; Fagerstrom and Kalkkinen (1995) Biotechnol. Appl. Biochem. 21:223-231.

[0796] Table 3, summarizes the kinetic parameters for hydrolysis of maltose and isomaltose by seven exemplary glucoamylases and a "benchmark" *A. niger* glucoamylases; each number is the average value from 5 different experiments:

Table 3

	Maltose	Isomaltose	
	K_M(mM)	K_M(mM)	k_{cat}/K_M (maltose) /k_{cat}/K_M (isomaltose)
SEQ ID NO:28	0.61 ± 0.06	11.94 ± 4.99	750
SEQ ID NO:74	1.87 ± 0.17	11.55 ± 2.92	481
SEQ ID NO:20	2.62 ± 0.19	53.97 ± 23.17	897
SEQ ID NO:14	2.67 ± 0.15	41.5 ± 5.05	116
SEQ ID NO:26	0.98 ± 0.33	12.18 ± 0.64	456
SEQ ID NO:48	2.26 ± 0.12	21.69 ± 3	565
SEQ ID NO:18	1.01 ± 0.09	11.74 ± 7.74	415
A. niger glucoamylase	0.93 ± 0.1	18.72 ± 3.95	249

[0797] As can be seen from Table 3, the exemplary glucoamylase SEQ ID NO:20 was most strongly selective for maltose and had nearly 900-fold higher specificity towards α-1,4- linkages relative to α-1,6- bonds. The least selective glucoamylase was SEQ ID NO:14 with ~100-fold higher specificity towards α-1,4- bonds relative to α-1,6- bonds.

Example 24: Characterization of alpha-Amylases and/or glucoamylases

[0798] This example describes the characterization of alpha-Amylases and/or glucoamylases.

[0799] Initial reaction rates: The initial rates of hydrolysis of granular and soluble starch by eight (8) exemplary α-amylases were compared with a "benchmark" α -amylase from *A. oryzae,* and the results are summarized in Table 4, below. Due to the relatively high background seen in BCA assays with *A. oryzae* α -amylase from Sigma (A6211), a second preparation of the same amylase from Megazyme (E-ANAAM™) was also evaluated. Results obtained with both preparations were very similar. As can be seen from Table 34, all amylases tested displayed significantly higher activity against soluble starch when compared with granular starch. However, this difference was less marked for the exemplary Amylases and/or glucoamylases than for the "benchmark" *A. oryzae* enzyme.

[0800] Table 4 shows data comparing initial rates of hydrolysis of granular corn starch and soluble corn starch by eight (8) exemplary α-Amylases and/or glucoamylases and the "benchmark" α-amylase from *A. oryzae* at 37°C and pH 5 (note: *Initial rates are expressed as nmole of reducing ends released from 1% starch /min/μg of α-amylase protein for SEQ ID NO:56, SEQ ID NO:62, SEQ ID NO:2, and SEQ ID NO:52; **Initial rates for SEQ ID NO:70, SEQ ID NO:66 and SEQ ID NO:76 are expressed as nmoles of reducing ends released from 1% starch/min/μg of total protein; each number is an average value from 5 data points:

Table 4

Enzyme	Initial rate* ± SD Granular starch	Initial rate* ± SD Soluble starch
SEQ ID NO:56	15.7 ± 1.67	1607.9 ± 518.22
SEQ ID NO:70**	2.1 ± 0.24	28.4 ± 3.81
SEQ ID NO:62	3.5 ± 0.37	139.6 ± 55.96
SEQ ID NO:66**	0.8 ± 0.09	81.3 ± 31.11
SEQ ID NO:2	7 ± 0.75	381.2 ± 74.15
SEQ ID NO:52	10.3 ± 1.75	248.2 ± 28.46
SEQ ID NO:76**	2.6 ± 0.41	160.3 ± 42.62
A. oryzae amylase (MegazymeE-ANAAM)	0.4 ± 0.07	498.7 ± 64.78

[0801] Temperature profile: The effect of temperature on starch hydrolysis by the characterized α-amylases was determined. Activity was measured at pH 5.0 during 40 min incubation at the indicated temperature, and initial rates were calculated and plotted against time. Activities of amylases were affected by temperature to a different degree. Five of the exemplary Amylases and/or glucoamylases SEQ ID NO:56, SEQ ID NO:70, SEQ ID NO:76 and SEQ ID NO:66, were most active at 40°C and retained approximately 30% of activity at 30°C. Activities of SEQ ID NO:2, SEQ ID NO:52 and SEQ ID NO:62 were only marginally affected by changes in temperature over the range investigated.

[0802] pH profile: The influence of pH on exemplary Amylases and/or glucoamylases on starch hydrolysis was tested with both granular and soluble starch substrates, and the results were determined. The increase in reducing ends was measured at the indicated pH at 37°C. Initial rates were calculated over 40 min and converted to a percentage of the maximum rate. The exemplary SEQ ID NO:52 (originally of fungal origin) had the lowest pH optimum (approximately pH 4). Another exemplary enzyme SEQ ID NO:2 (also originally of fungal origin) also displayed preference for acidic pH, with an apparent optimum at approximately pH 4.5 - 5. The exemplary amylase SEQ ID NO:66 (originally of Archaeal origin) had an apparent optimum of approximately pH 5.0, retaining approximately 70% of peak activity at pH 4.0. The remaining exemplary enzymes had apparent optima between pH 5.0 and 6.0, and were almost inactive at pH 4.0 and 3.5.

Example 25: Expression of Amylases and/or glucoamylases of the disclosure in host cells

[0803] This example describes the evaluation of expression of exemplary enzymes of the disclosure in different hosts cells; in particular, the expression of three (3) exemplary amylases in different expression hosts: *Pichia pastoris, Hansenula polymorpha*, and *Cochliobolus heterostrophus* were studied.

[0804] *H. polymorpha*: Two expression vectors (Artes Biotechnology GmbH, Erkrath, Germany) were used: pFPMT-Mfa™ with a formate dehydrogenase promoter and pTPS1-Mfa™ with a trehalose-6-phosphate synthase promoter. In both vectors the secretion signal used was the pre-prosequence of the mating-factor MFa1 from *Saccharomyces cerevisiae.*

[0805] Plasmids with subcloned amylase genes were introduced into *H. polymorpha* competent cells by electroporation.

96 colonies from each transformation were selected and grown for approximately 30 (3 passages) to 80 (8 passages) generations by various sub-culturing steps, under selective conditions in liquid medium (YNB-Glucose). These steps allow for increasing the copy number and facilitate the integration of the plasmid into the chromosome. After these 3 or 8 passaging steps, transformants were cultivated under non-selective conditions (YPD medium) to test for plasmid stabilization (loss of any non-integrated plasmid). Screening for positive clones was performed on Red Starch - agar plates. Clones with larger than the control (same gene expressed in *P. pastoris*) clearing zone were selected as primary hits. Amylase expression by the selected clones was confirmed by assaying culture supernatants on BODIPY-starch substrate, and SDS PAGE was used for visualization of the protein produced. 1 L cultures were prepared for further testing.

[0806] *Cochliobolus heterostrophus.* cDNA and gDNA of amylase from *C. heterostrophus* were subcloned into pCh-ubi (ubiquitin promoter) and pCh-GPD (GPD promoter) and targeted to the PKS18 locus of the C5 strain of *C. heterostrophus* by PEG-mediated protoplast transformation, using linearized construct DNA. One of the constructs, a genomic version under an ubi (ubiquitin) promoter, was also introduced into the JMD3 strain from which the amylase - encoding gene had been deleted. Similarly, as in the case of *P. pastoris*, screening of the transformants was performed on red starch plates and confirmed by BODIPY-starch assays and SDS PAGE with culture supernatants. No difference was observed in expression between the cDNA and the genomic versions. Similarly no significant difference was observed with the two different promoters.

[0807] Results: Strains of: *H. polymorpha* and *C. heterostrophus* expressing, for comparison, 3 exemplary amylase-encoding and glucoamylase-encoding genes were constructed, in addition to 3 original *P. pastoris* strains. All strains used for comparisons and preliminary fermentation yields obtained with these strains are presented in Table 5, summary estimated expression yields (g/L) of exemplary Amylases and/or glucoamylases of the disclosure produced in *Pichia*:

Table 5

Origin of the amylase gene	*Pichia pastoris*
fungal	SEQ ID NO:2 0.01*
bacterial	SEQ ID NO:70 0.07*
archaeal	SEQ ID NO:66 0.13*
*fermentation yield	

Following the expression evaluation, protein purity, specific activity and performance in granular starch hydrolysis assays were compared. These comparisons led to the following conclusions:

- No significant biochemical differences were observed between enzymes expressed in the different hosts.
- Expression in *Hansenula* did not appear to result in an improvement over the expression observed in *P. pastoris*. Furthermore, the expressed proteins appeared to be highly glycosylated.

Example 26: Raw starch fermentation using amylases of this disclosure

[0808] This example describes exemplary methods for raw starch fermentation using amylases of this disclosure.

[0809] Fifteen (15) exemplary enzymes consisting of eight (8) alpha-amylases and seven (7) glucoamylases were initially identified. The "lead" exemplary enzymes were determined based on activity at temperatures from 30° to 40°C, pH range from 3.5 - 7.0, and hydrolysis of 1% raw starch. Unpurified microbial enzyme was first analyzed for amount of proteins and expression ratios; and then evaluated in raw starch fermentations. The total enzyme loading and ratios of alpha-amylases to glucoamylases were then determined.

[0810] Note: for the raw starch fermentation work, all enzymes were obtained by expression in *Pichia pastoris,* except for SEQ ID NO:78 (encoded, e.g., by SEQ ID NO:77), which was expressed in *Pseudomonas fluorescens* (e.g., as described in JBC, 2002, 277(29):26501-26507).

Table 1.1. Enzyme Expression and Relative Purity of "lead" exemplary alpha-amylases and glucoamylases

Enzyme***	protein/powder (g/g)*	Expression ratio**
SEQ ID NO:56	0.12±0.42	0.6

(continued)

Enzyme***	protein/powder (g/g)*	Expression ratio**
SEQ ID NO:52	0.09±0.003	0.6
SEQ ID NO:66	0.05±0.005	0.1 (0.5****)
SEQ ID NO:66	0.05	0.1 (0.5****)
SEQ ID NO:66	0.059±0.002	0.5****
SEQ ID NO:66	0.085	0.5****
SEQ ID NO:2	0.03±0.005	0.3
SEQ ID NO:2	0.03±0.002	0.3
SEQ ID NO:70	0.09±0.003	0.05(0.25****)
SEQ ID NO:70	0.126±0.002	0.25****
SEQ ID NO:70	0.131	0.25****
SEQ ID NO:62	0.2±0.03	0.75
SEQ ID NO:78	10 (mgs/ml)	0.25
SEQ ID NO:76	0.1±0.01	0.2
SEQ ID NO:26	0.2±0.026	0.8
SEQ ID NO:28	0.15±0.076	0.45
SEQ ID NO:28	0.36±0.042	0.45
SEQ ID NO:18	0.24±0.096	0.8
SEQ ID NO:74	0.21±0.016	0.8
SEQ ID NO:48	0.28±0.098	0.5
SEQ ID NO:20	0.28±0.066	0.7
SEQ ID NO:14	0.21±0.155	0.2
Control (empty vector)	0.0063	

*protein content was determined using Bradford's protocol.
**expressed as percentage of total protein in clarified lysate; determined by SDS PAGE using 5 μg protein loadings, and the Bio-Rad Quantity One software.
*** where an enzyme appears several times in this list, different lots were used.
**** value after de-glycosylation.

Experimental Methods

Raw Starch Fermentation

[0811] All fermentations were done using Yellow Dent II corn, milled on the Ultra Centrifugal Mill by Glen Mills at 12,000 rpm through a 0.5 mm screen. The corn flour was analyzed on a Mettler HB-43™ moisture balance to determine moisture content and then 10 g dry weight of corn flour was measured into a 50 ml flask with stir bar. Water was then added followed by the addition of tetracycline (0.5 mg), H2SO4 (175 μl of 0.9 M solution), unpurified/purified enzyme stocks, and yeast to bring the total slurry to 33% total solids.. The flasks were placed on a stir plate in 30°C for 72 hours. Samples were taken at 50 or 72 hours of fermentation for analyses. Sigma *Aspergillus niger* glucoamylase (A-7095) was used as the benchmark added at 2.065 Units (approximately 0.707 mg total protein, or 0.07% total protein loading) /g dw of flour and the test enzyme total loading (%) is based on the total weight of expressed enzyme (g) / dry weight of corn flour in fermentation (g).

[0812] To analyze ethanol content and sugar profiles, 1.5 ml of fermentation slurry was taken from each flask at 50 or 72 hours, placed in a 1.8 ml microfuge tube, and spun down at 13000 rpm for 5 minutes. The supernatant was poured into a 0.45 μm spin-x column, spun down at 7000 rpm for 5 minutes, and 200 μl of the spin through was aliquoted into

an HPLC vial and analyzed by HPLC. A Waters HPLC equipped with a refractive index detector was used. The column used was the Bio-Rad AMINEX HPX-87H™ (Cat. #: 125-0140). The mobile phase used was 0.005 M H_2SO4 at 0.6 mL / minute.

Preparation of Standards

[0813] The standards used for the analysis described herein contain glucose, maltose, maltotriose, maltodextrin, fructose, glycerol, lactic acid, acetic acid, succinic acid, ethanol and methanol. One composite standard is prepared and subsequently diluted to produce the varying concentrations needed to establish standard curves for each compound. All reagents used for standard preparation should be stored in a dessicator cabinet, refrigerated or stored per manufacturers' recommendations. The most accurate means of measuring the components should be employed. Into a clean dry 100 mL volumetric flask add all dry reagents.

- Add -35 mL water and swirl to dissolve solids.
- Place a 50 mL Falcon tube on the balance add 25 mL water and then tare.
- To the water add glycerol, ethanol, methanol, lactic and acetic acids.
- Gently vortex to mix and add contents to volumetric flask with sugar solution. Rinse 50 mL Falcon tube 3 times with ~5 mL water each time and add rinse to volumetric flask.
- Bring entire volume of flask to 100 mL. This solution represents the mixed 20% ethanol standard. The solution is sterilized using a Millipore STERIFLIP™ 0.22 or 0.45 μm filter.
- Dilutions of this standard are used to create the mixed 5, 10, and 15% ethanol standards.
- A mixed 5% standard consists of 1 part mixed 20% standard and 3 parts water.
- A mixed 10% standard is a simple 1:1 dilution of the mixed 20% standard.
- A mixture of 3 parts mixed 20% standard and 1 part water will create the mixed 15% standard.
- A certified 10.3% v/v ethanol standard (Sigma Cat.# E2385) should be run each time a new standard is prepared or monthly to verify the accuracy of the Mixed standard set.

Results

[0814] Several alpha-amylases and glucoamylases were tested in combinations in raw starch fermentations at different total enzyme loadings of 0.001, 0.01, and 0.1% (w/w). The alpha-amylases tested were SEQ ID NO:56, SEQ ID NO:70, SEQ ID NO:52, SEQ ID NO:2, SEQ ID NO:62, and SEQ ID NO:66, SEQ ID NO:76. The glucoamylases tested were SEQ ID NO:48, SEQ ID NO:26, SEQ ID NO:18, SEQ ID NO:74, SEQ ID NO:28, SEQ ID NO:14, and SEQ ID NO:20. All combinations were given an ID to be used in the experimental process. Total enzyme loading was calculated based on the expression ratio information provided herein. The alpha-amylase to glucoamylase ratio was determined based on previous data. Ethanol yield was measured after 50 or 72 hours of fermentation by HPLC analysis. Results indicated that all enzyme combinations produced various levels of ethanol and some combinations outperformed the Sigma GA benchmark (Table 1.2).

Table 1.2. Ethanol yield in raw starch fermentations of "lead" exemplary alpha-Amylases and Glucoamylases after 50 hours of fermentation[a].

				Ethanol Yield (% v/v)		
	Alpha-amylase (AA)	glucoamylase (GA)	AA: GA ratio	0.001% total enzyme loading	0.01% total enzyme loading	0.1% total enzyme loading
Combo 1	SEQ ID NO:56	SEQ ID NO:26	2:1	6.12	11.60	15.56
Combo 2	SEQ ID NO:56	SEQ ID NO:18	1:2	6.97	12.53	15.69
Combo 3	SEQ ID NO:56	SEQ ID NO:28	1:2	5.96	11.06	16.42
Combo 4	SEQ ID NO:56	SEQ ID NO:74	1:2	6.83	11.49	14.29
Combo 5	SEQ ID NO:56	SEQ ID NO:20	1:2	4.28	5.61	7.55
Combo 6	SEQ ID NO:56	SEQ ID NO:48	1:2	6.40	12.58	15.86
Combo 7	SEQ ID NO:66	SEQ ID NO:26	1:2	7.39	15.38	N/A
Combo 8	SEQ ID NO:66	SEQ ID NO:18	1:2	5.84	12.81	N/A

(continued)

	Alpha-amylase (AA)	glucoamylase (GA)	AA: GA ratio	Ethanol Yield (% v/v)		
				0.001% total enzyme loading	0.01% total enzyme loading	0.1% total enzyme loading
Combo 9	SEQ ID NO:66	SEQ ID NO:28	1:2	6.12	12.62	N/A
Combo 10	SEQ ID NO:66	SEQ ID NO:74	1:2	7.02	12.29	N/A
Combo 11	SEQ ID NO:66	SEQ ID NO:20	1:2	4.48	5.72	N/A
Combo 12	SEQ ID NO:66	SEQ ID NO:48	1:2	6.54	14.16	N/A
Combo 13	SEQ ID NO:70	SEQ ID NO:26	1:2	9.08	15.04	16.76
Combo 14	SEQ ID NO:70	SEQ ID NO:18	1:2	7.86	13.00	14.71
Combo 15	SEQ ID NO:70	SEQ ID NO:74	1:2	8.18	13.27	16.40
Combo 16	SEQ ID NO:70	SEQ ID NO:48	1:2	8.36	13.80	15.90
Combo 17	SEQ ID NO:52	SEQ ID NO:26	1:2	7.92	12.67	15.15
Combo 18	SEQ ID NO:52	SEQ ID NO:18	2:1	10.66	16.10	14.92
Combo 19	SEQ ID NO:52	SEQ ID NO:48	1:2	10.88	15.97	14.87
Combo 20	SEQ ID NO:62	SEQ ID NO:26	1:2	7.30	12.67	15.68
Combo 21	SEQ ID NO:62	SEQ ID NO:48	1:2	7.24	11.13	14.84
Combo 22	SEQ ID NO:2	SEQ ID NO:26	2:1	9.20	14.56	15.36
Combo 23	SEQ ID NO:2	SEQ ID NO:48	1:2	9.81	15.42	15.61
Combo 24	SEQ ID NO:56	SEQ ID NO:14	1:2	5.46	7.86	13.67
Combo 25	SEQ ID NO:70	SEQ ID NO:14	2:1	6.99	10.85	15.72
Combo 26	SEQ ID NO:78	SEQ ID NO:48	1:2	N/A	N/A	16.13[a]
Combo 27	SEQ ID NO:78	SEQ ID NO:26	1:2	N/A	N/A	16.88[a]
Combo 28	SEQ ID NO:76	SEQ ID NO:48	1:2	N/A	N/A	17.15[a]
Sigma GA control				N/A	N/A	16.82[b]

[a] Ethanol yield after 72 hours of fermentation.
[b] Total enzyme loading was estimated to be approximately 0.07%.

Example 27: Expression of enzymes in plants; raw starch fermentation

[0815] This example describes plant expression of nine exemplary enzymes of this disclosure (enzymes not having codon optimization), and raw starch fermentation using plant material.

[0816] Five (5) alpha-amylases, SEQ ID NO:52, SEQ ID NO:4, SEQ ID NO:70, SEQ ID NO:66, and SEQ ID NO:76 and 4 glucoamylases, SEQ ID NO:48, SEQ ID NO:18, SEQ ID NO:26, and SEQ ID NO:28 were chosen for plant expression based on high ethanol yield of the microbial enzymes in raw starch fermentation. This maize expression was carried out with the gene sequences without codon optimization. The objectives of this study were to evaluate the potential expression levels of these enzymes and to evaluate whether expression of these enzymes would have detrimental effect on maize agronomics through out its developmental stages.

Experimental Methods

Vector Construction

[0817] Table 2.1 summarized the abbreviations used for the DNA elements in the maps shown below.

Table 2.1. Abbreviations used in the construct maps

Name	Function	Description
cAMY(SEQ ID NO:66)-02	CDS	Alpha amylase, SEQ ID NO:66, minus the native leader, attached to a Gamma Zein signal sequence targeting to the apoplast
cAmy(SEQ ID NO:18)-02	CDS	Glucoamylase, SEQ ID NO:18, minus the native leader, attached to a Gamma Zein signal sequence targeting to the apoplast
cAmy(SEQ ID NO:52)-02	CDS	Alpha amylase, SEQ ID NO:52, minus the native leader, attached to a Gamma Zein signal sequence targeting to the apoplast
cAmy(SEQ ID NO:28)-02	CDS	Glucoamylase, SEQ ID NO:28, minus the native leader, attached to a Gamma Zein signal sequence targeting to the apoplast
cAmy(SEQ ID NO:26)-02	CDS	Glucoamylase, SEQ ID NO:26, minus the native leader, attached to a Gamma Zein signal sequence targeting to the apoplast
cAmy(SEQ ID NO:48)-02	CDS	Glucoamylase, SEQ ID NO:48, minus the native leader, attached to a Gamma Zein signal sequence targeting to the apoplast
cAmy(SEQ ID NO:76)-02	CDS	Alpha-amylase, SEQ ID NO:76, minus the native leader, attached to the gamma zein signal sequence, targeting to the apoplast
cAmy(SEQ ID NO:70)-02	CDS	Alpha-amylase, SEQ ID NO:70, minus the native leader, attached to a Gamma Zein signal sequence targeting to the apoplast

[0818] The exemplary vector designated "15745", , below, is a binary vector containing an alpha amylase, the exemplary SEQ ID NO:70 minus its native leader. The native leader was replaced with the Gamma Zein signal sequence for targeting to the apoplast. The Gamma Zein-SEQ ID NO:70 fusion is expressed using the Gamma Zein promoter. The binary vector also contains a Ubi-PMI-Nos cassette for mannose selection.

[0819] A fragment of cAmy(SEQ ID NO:70)-01, containing amino acids 86-525 (plus stop codon), was PCR amplified from plasmid 15649. The PCR amplification introduced an AfeI site at the 5' end and a BglII site at the 3' end of the fragment. The addition of the AfeI site added an alanine amino acid in front of the cAmy(SEQ ID NO:70) fragment (SY1709: 92-93). This PCR amplicon was cloned into pCR2.1-TOPO and sequenced to verify the presence of the new restriction enzyme sites (SY1709:111-112,122-124). Once verified, cAmy(SEQ ID NO:70) was digested out of the TOPO backbone via AfeI/BglII. Similarly, construct 15460ZeinAmyVN was digested with AfeI/BglII. This backbone and cAmy(SEQ ID NO:70) were ligated together. When ligated, cAmy(SEQ ID NO:70) stayed in frame with the signal peptide xGZein27ss-01, with only the addition of an alanine residue at the cloning junction. Transformants were screened for the presence of the amylase gene using primers ZeinAmy1199F and prGTL-03R. Positive clones produced an amplicon of ~1.6 kB (SY1709:130). Five clones were selected and were digested with SanDI and RsrII to remove prGZein-01:cAmy(SEQ ID NO:70):t35s-08. Construct 15468 was linearized with RsrII. The binary backbone and digested cassette from one of the positive clones (SanDI/RsrII) were ligated together at the RsrII restriction site. Following transformation into Top10 cells, transformants were screened for the presence of the cassette using primers ZeinAmy1199F and Mubi-5 (SY1709:146,150). Three positive transformants were selected and checked via a BamHI diagnostic digest. These clones were sequenced (SY1709:162; both cloning junctions as well as the entire cAmy(SEQ ID NO:70) coding sequence), it was confirmed that all three were correct in sequence.

[0820] The exemplary vector designated "15750", as illustrated in Figure 28, below, is a binary vector containing a glucoamylase, the exemplary SEQ ID NO:18, minus its native leader. The native leader was replaced with the Gamma Zein signal sequence for targeting to the apoplast. The Gamma Zein-SEQ ID NO:18 fusion is expressed using the Gamma Zein promoter. The binary vector also contains an Ubi-PMI-Nos cassette for mannose selection.

[0821] A fragment of cAmy(SEQ ID NO:18)-01, containing amino acids 88-712 (plus stop codon), was PCR amplified from plasmid 15652. The PCR amplification introduced an AfeI site at the 5' end and a BglII site at the 3' end of the fragment. The addition of the AfeI site resulted in amino acid 88 changing from a serine to an alanine (SY1709: 113-114,116). This PCR amplicon was cloned into pCR2.1-TOPO and sequenced to verify the presence of the new restriction enzyme sites (SY1709:116,131). Once verified, the cAmy(SEQ ID NO:18) fragment was digested out of the TOPO backbone via AfeI/BglII. Similarly, construct 15460ZeinAmyVN was digested with AfeIBglII. This backbone and the cAmy(SEQ ID NO:18) fragment were ligated together. When ligated, cAmy(SEQ ID NO:18) stayed in frame with the signal peptide xGZein27ss-01, with the addition of an alanine residue at the cloning junction. Six clones were selected and were digested with SanDI and RsrII to remove prGZein-01:cAmy(SEQ ID NO:18):t35s-08. Construct 15468 was

linearized with RsrII. The binary backbone and digested cassette from one of the positive clones (SanDI/RsrII) were ligated together at the RsrII restriction site. Following transformation into Top 10 cells, transformants were screened for the presence of the cassette using primers ZeinAmy1199F and Mubi-5 (SY1709:181). Additionally, all 6 transformants were selected and checked via a SacI diagnostic digest. Three of the positive clones were sequenced (SY1709:189-190) (both cloning junctions as well as the entire cAmy(SEQ ID NO:18) coding sequence), it was confirmed that all three were correct in sequence.

[0822] The exemplary vector designated "15751", as illustrated in Figure 29, below, is a binary vector containing a glucoamylase, the exemplary SEQ ID NO:48, minus its native leader. The native leader was replaced with the Gamma Zein signal sequence for targeting to the apoplast. The Gamma Zein-SEQ ID NO:48 fusion is expressed using the Gamma Zein promoter. The binary vector also contains a Ubi-PMI-Nos cassette for mannose selection.

[0823] Using 15651 as a template, mutagenesis primers were used in a multi-site mutagenesis reaction (QUICKCHANGE™, Stratagene) to remove an internal AfeI site, introduce an AfeI site at the 5' end (mutation was in signal sequence, no peptide change), and introduce an external 3' BglII site for cloning. Restriction enzyme analysis was performed and clones that were positive for having the internal AfeI site removed and the BglII site introduced were selected. A positive clone was sent for sequence analysis which was confirmed. This clone DNA was then used as template for PCR amplification to introduce an AfeI site at the 5' end of the glucoamylase domain. The mutation was in the native signal sequence and did not change the glucoamylase peptide sequence. The PCR product was digested sequentially with AfeI and then with BglII. The digested product was gel purified and ligated to the 15460AmyZeinVN backbone, creating cAmy(SEQ ID NO:48) with a gamma zein signal sequence targeting to the apoplast. This intermediate vector was sequence verified and then digested with SanDI and RsrII sequentially. This fragment was gel purified and then ligated into 15468 binary cut with RsrII and CIP treated. The positive clones were PCR size screened and then one of the positive clones was sent off for sequence analysis of the entire fragment ligated into the binary. Sequence was confirmed.

[0824] The exemplary vector designated "15761", as illustrated in Figure 30, below, is a binary vector containing an alpha amylase, the exemplary SEQ ID NO:66, minus its native leader. The native leader was replaced with the Gamma Zein signal sequence for targeting to the apoplast. The Gamma Zein-SEQ ID NO:66 fusion is expressed using the Gamma Zein promoter. The binary vector also contains a Ubi-PMI-Nos cassette for mannose selection.

[0825] A fragment of cAmy(SEQ ID NO:66)-01, containing amino acids 86-521 was mutagenized using site directed mutagenesis according to the Stratagene Quick Change protocol. The mutagenesis required 3 individual primers (all amplifying in the forward direction) and introduced three mutations: an AfeI site at the 5'end, a BglII site at the 3' end (external to the gene), and the removal of an internal AfeI site (SY1709:174). The mutagenized construct was transformed into Top 10 cells for screening to determine if any/all of the desired mutations were present. 15 transformants were selected for screening and were digested separately with AfeI and BglII (SY1709:185-186). It was determined from the restriction digests that there were 6 possible clones with all three of the desired mutations; three of these clones were sent for sequencing (SY 1709:190; SY1777:11). For all three clones, an AfeI site had been introduced at the 5' end and the internal site had been removed. In addition, the 3' BglII site had been introduced. Following confirmation, the alpha amylase was digested out of the mutagenized construct with AfeI/BglII and ligated into the 15460ZeinAmyVN backbone which had also been digested with AfeI and BglII. When ligated, cAmy(SEQ ID NO:66) stayed in frame with the signal peptide xGZein27ss with no amino acid changes at the cloning junction. Transformants were screened for the presence of the amylase gene using primers ZeinAmy1199F and prGTL-03R. Positive clones produced an amplicon of ~1.6kB (SY1777:24). Three clones were selected and digested with SanDI/RsrII to remove prGZein-01:cAmy(SEQ ID NO:66):t35s-08. Construct 15468 was linearized with RsrII. The binary backbone and digested cassette from one of the positive clones (SanDI/RsrII) were ligated together at the RsrII restriction site. Following transformation into Top 10 cells, transformants were screened for the presence of the cassette using primers ZeinAmy1199F and prGTL-03R (SY1777:47-48). Six positive transformants were selected and checked via a HindIII diagnostic digest. Three of these clones were sequenced (SY1777:56, 62) at both cloning junctions as well as the entire coding sequence; it was confirmed that all three were correct in sequence.

[0826] The exemplary vector designated "15756", as illustrated in Figure 31, below, is a binary vector containing a glucoamylase, the exemplary SEQ ID NO:26, minus its native leader. The native leader was replaced with the Gamma Zein signal sequence for targeting to the apoplast. The Gamma Zein-SEQ ID NO:26 fusion is expressed using the Gamma Zein promoter. The binary vector also contains a Ubi-PMI-Nos cassette for mannose selection.

[0827] Using exemplary vector 15653 as a template, mutagenesis primers were used in a multi-site mutagenesis reaction (QUICKCHANGE™, Stratagene) to remove an internal AfeI site, introduce an AfeI site at the 5' end (mutation was in signal sequence, no peptide change), and introduce an external 3' SacI site for cloning. The reaction was transformed into Top10 competent cells. Colonies were picked and screened via restriction enzyme analysis. Clones that were positive for having the internal AfeI site removed, the 5' Afe site inserted and the SacI site introduced were selected. A positive clone was sent off for sequence analysis which was confirmed. This clone DNA was then digested with SacI and AfeI. The digest was then gel purified and ligated to the 15460AmyZeinVN backbone creating cAmy(SEQ ID NO:26)

with a gamma zein signal sequence targeting to apoplast. This intermediate vector was sequence verified then digested with SanDI and RsrII. This fragment was gel purified and ligated into 15468 binary vector cut with RsrII and CIP treated. The positive clones were PCR size screened and then one of the positive clones was sent off for sequence analysis of the entire fragment ligated into the binary. Sequence was confirmed.

[0828] The exemplary vector designated "15742", as illustrated in Figure 32, below, is a binary vector harboring (comprising) the C-terminal 616 amino acids of a glucoamylase, the exemplary SEQ ID NO:28 (cAmy(SEQ ID NO:28)-01) fused in-frame with the Gamma Zein signal sequence (xGZein27ss-01), targeting the glucoamylase to the apoplast to create (cAmy(SEQ ID NO:28)-02). Expression is driven by the 5' region from Gamma Zein A (prGZein-01), which is a seed specific promoter.

[0829] Vector 15654 harboring cAmy(SEQ ID NO:28)-01 served as PCR DNA template modified by the addition of an NcoI site (5-prime end) and a BglII site (3-prime end). The PCR product was TOPO-cloned and Clone #1 was sequenced for validation (SY1533:181). The new component alias "cAmy(SEQ ID NO:28)" was digested with NcoI/BglII and gel purified. The fragment was ligated in-frame to the NcoI/BglII site of a cloning vector alias "15460ZeinAmyVN" thereby generating a cassette harboring prGZein-01:xGZein27ss-01:cAmy(SEQ ID NO:28):iPEPC9-01:t35s-08 (SY1533:183). The gene cassette was digested sequentially with SanDI/RsrII, gel purified and ligated into the RsrII site of binary vector 15468. Successful ligation was confirmed by PCR and DNA sequence (SY1533:185-187).

[0830] The exemplary vector designated "15749", as illustrated in Figure 33, below, is a binary vector containing an alpha amylase, the exemplary SEQ ID NO:52, minus its native leader. The native leader was replaced with the Gamma Zein signal sequence for targeting to the apoplast (cAmy(SEQ ID NO:52)-02). The Gamma Zein-SEQ ID NO:52 fusion is expressed using the Gamma Zein promoter. The binary vector also contains a Ubi-PMI-Nos cassette for mannose selection.

[0831] A fragment of cAmy(SEQ ID NO:52)-01, containing amino acids 88-674 (plus stop codon), was PCR amplified from plasmid 15648. The PCR amplification introduced an AfeI site at the 5' end and a SacI site at the 3' end of the fragment. The addition of the AfeI site resulted in amino acid 88 changing from a serine to an alanine (SY1709:95-96). The PCR amplicon was cloned into pCR2.1TOPO and sequenced to verify the presence of the new restriction enzyme sites (SY1709:103,111-112). Once verified, cAmy(SEQ ID NO:52) was digested out of the TOPO backbone via AfeI/SacI. Similarly, construct 15460ZeinAmyVN was digested with AfeI/SacI. This backbone and cAmy(SEQ ID NO:52) were ligated together. When ligated, cAmy(SEQ ID NO:52) stayed in frame with the signal peptide xGZein27ss-01 with only the first amino acid residue changed from a leucine to an alanine. Transformants were screened for the presence of the amylase gene using primers ZeinAmy1199F and prGTL-03R. Positive clones produced an amplicon of ~2.1kB (SY1709:130). Five clones were selected and were digested with SanDI and RsrII to remove prGZein-01:cAmy(SEQ ID NO:52):t35s08. Construct 15468 was linearized with RsrII. The binary backbone and digested cassette from one of the positive clones (SanDI/RsrII) were ligated together at the RsrII restriction site. Following transformation into Top10 cells, transformants were screened for the presence of the cassette using primers ZeinAmy1199F and Mubi-5 (SY1709: 146, 150). Three positive transformants were selected and checked via a BamHI diagnostic digest. These clones were sequenced (SY1709:162; both cloning junctions as well as entire cAmy(SEQ ID NO:52) coding sequence) and it was determined that there was a point mutation within the prGZein sequence. This mutation is a single base pair change-T to C- at nucleotide 340. The promoter within the original 15460ZeinAmy backbone was sequenced again to check for the mutation and it was not present in the backbone source. However, it was present when the positive clones were sequenced a second time (SY1709:182,189). This mutation may have occurred during bacterial replication.

[0832] The exemplary vector designated "15718", as illustrated in Figure 34, below, is a binary vector containing an alpha amylase, the exemplary SEQ ID NO:76, minus its native leader. The native leader was replaced with the Gamma Zein signal sequence for targeting to the apoplast. The Gamma Zein-SEQ ID NO:76 fusion (cAmy(SEQ ID NO:76)-02) is expressed using the Gamma Zein promoter. The binary vector also contains a Ubi-PMI-Nos cassette for mannose selection.

[0833] A fragment of cAmy(SEQ ID NO:76)-01, containing amino acids 87-508 (plus stop codon), was PCR amplified from plasmid 15650. The PCR amplification introduced an AfeI site at the 5' end and a BglII site at the 3' end of the fragment. The addition of the AfeI site changed a glycine amino acid to alanine. The PCR product was digested with AfeI and BglII and cloned into the 15460AmyZeinVN backbone cut with AfeI and BglII, creating cAmy(SEQ ID NO:76) fused to the gamma zein signal sequence. This intermediate vector was then digested with SanDI and RsrII sequentially. This fragment was gel purified and then ligated into 15468 binary vector cut with RsrII and CIP treated. The positive clones were PCR size screened and then one of the positive clones was sent for sequence analysis of the entire insert. Sequence was confirmed.

Enzymatic activity analysis

[0834] Twenty seeds from selected events were randomly selected and pooled. The seeds were then ground and assayed using the Megazyme CERALPHA HR™ assay for alpha-amylases or Megazyme's glucoamylase assay for

glucoamylases.

Activity Calculations

[0835] One CERALPHA™ Unit of α-amylase activity is defined as the amount of enzyme, in the presence of excess thermostable α-glucosidase, required to release one micromole of p-nitrophenol in one minute under the defined assay conditions. Our assay condition is at pH 5.5 (100 mM NaOAc, pH 5.5 buffer with 1mg/ml BSA), 60°C. Note that the activity calculated is a CERALPHA™ unit.

[0836] According to Megazyme, 1 mM PNP in 1% tri-sodium phosphate gives absorbance 18.1 at 400 nm. So 1M of PNP in tri-sodium phosphate gives absorbance 18,100 at 400 nm.

ΔA_{400} / 18,100 = [PNP] (mol/l or M) in the final plate.
[PNP] in plate (mol/l x 8 x 100 x 10^{-6}l x 1 x 10^{6} (umol/mol) = PNP (μmol/rxn) PNP (μmol/rxn) / (0.05 ml x 20 min) = units / ml of diluted α-Amylase
units / ml in diluted enzyme x dilution = Ceralpha Units /ml in α-Amylase sample or
ΔA_{400} x 0.0442 x dilution of enzyme = Ceralpha U/ml in α-amylase sample.

[0837] This SOP is adapted from a CERALPHA HR ™ assay method SOP and a Megazyme α-amylase assay procedure (CERALPHA™ method) using amylase HR™ Reagent, ICC Standard No. 303.

[0838] Figure 27 illustrates the theoretical basis of the CERALPHA™ α-amylase assay procedure; from Megazyme's alpha-amylase assay procedure (CERALPHA™ Method) using amylase HR™ reagent: the figure illustrates the overall reaction scheme: that after alpha-amylase cleaves a bond within the blocked p-nitrophenyl malto-saccharide substrate, the non-blocked reaction product containing the p-nitrophenyl constituent is instantly cleaved to glucose and free p-nitrophenyl by the excess quantities of thermostable alpha-glucosidase, which are integral parts of the substrate mixture, and free p-nitrophenyl is released. The reaction is terminated and the phenolate color is developed on addition of tri-sodium phosphate, pH at about 11.0.

Glucoamylase Extraction from Corn Flour and Activity Assay For Starch Hydrolysis: Glucoamylase Extraction and Assay from Single Corn Seed

[0839] To describe the procedure for glucoamylase extraction and assay from corn flour:

PREPARATION OF EXTRACTION BUFFER:

[0840] 25 mM Sodium borate, .01% Tween 20, pH 10 buffer

PROCEDURE:

Pooled seed grinding:

[0841] Pool 20 seeds. Add the seeds to a Kelco grinding vessel. Place a steel ball in each vessel on top of the seed. Place a rubber ring around the vessel and place the cap on top. Grind in the Kelco for 45 seconds. Using a scupula transfer the flour from the vessel to a weigh boat and then pour into a 15 mL conical tube. Wash the vessel, ring, and steel ball with soap and water.

Extraction

[0842]

1. Add 3 ml of extraction buffer to the samples.
2. Seal the tubes with caps using a hammer.
3. Shake vigorously until the flour is suspended in the buffer.
4. Rotate at room temperature on a Rugged rotor at 70% for 5 minutes.
5. Incubate at 45°C in a water bath for 30 minutes
6. Rotate the samples for another 5 minutes at room temperature after incubation.
7. Centrifuge the samples at 3000 rpm for 5 minutes.

GA Assay (reagent information is after the assay procedure)

[0843]

1. Dilute samples in the dilution buffer to get an assay in the range of 0.5 to 1.5 OD_{400}.
2. Turn on the PCR machine. Set the program to incubate at 40°C for 10 min and then ramp down to 4°C.
3. On ice, add 50 μl of each diluted Glucoamylase sample to a PCR plate
4. Add 150 μl of Stop solution to each well of a microtiter plate. There should be two wells of Stop solution for each sample assayed.
5. Mix 50 μl of substrate reagent with the sample. Pipette 2 times. Prepare substrate as indicated in the instructions from Megazyme (10 ml water / bottle).
6. Immediately, remove 20 μl of the mixture and mix with 150 μl of Stop solution. Pipette 3 times with swirling. This is the 0-time point.
7. Place plate in the incubator and press Start. Make sure the samples are in the machine as it ramps up. Incubate the PCR plate for 10 min at 40 °C.
8. Allow the machine to ramp down to 4°C while the samples are in the machine.
9. Wait 2 minutes.
10. Remove 20 μl of product and mix with 150 μl of Stop in a microtiter plate. This is the 10 min. time point.
11. Let the plate sit for 15 min.
12. Read OD_{400} in the plate reader. Set Pathcheck to ON (this will normalize the sample absorbance reading as pathlength 1cm).
13. Calculate ΔA_{400}, the change in A_{400} between the 0 and 10 min point.

PREPARATION OF STOCK SOLUTIONS:

[0844]

Substrate (Megazyme catalog #: R-AMGR3)	
p-nitrophenyl-α-maltoside	(4 mM)
Thermostable α-glucosidase	(5 U/ml)

[0845] Dissolve the entire contents of one vial in 10.0 ml of MilliQ water. Divide into aliquots and store frozen. Store on ice during use.

Stop solution: 2% trizma base solution

[0846]

2.0 g of trizma base (Sigma T-1503)

MilliQ water added to final volume of 100 ml

Dilution buffer: 200 mM Sodium acetate buffer (pH 4.5)

Activity Calculations

[0847] One Unit of α-glucoamylase activity is defined as the amount of enzyme, in the presence of excess thermostable α-glucosidase, required to release one micromole of p-nitrophenol in one minute under the defined assay conditions.

[0848] According to Megazyme, 1 mM PNP in 2% trizma base gives absorbance of 18.1 at 400 nm.

$$\text{GA activity in samples (U/ml)} = (A400/10) \times (170/10) \times (1/18.1) \times \text{Dilution}$$
$$= A400 \times 0.0939 \times \text{Dilution}$$

[0849] This SOP is adapted from Megazyme's assay of amyloglucosidase using p-nitrophenyl- α-maltoside plus thermostable α-glucosidase

[0850] Raw Starch Fermentation: carried out as described above.

Results

TAQMAN™ assay and enzymatic activity assay

[0851] Copy numbers of the transgenes were determined by primary and secondary TAQMAN™ assays. Primers specific for the selectable marker gene used in all the maize transformation vectors described above, pmi, were used in the primary TAQMAN™ assays. Primers specific for both pmi gene and the bacterial selectable marker gene, spec, were used in secondary TAQMAN™ assays.

[0852] Expression of alpha-amylases or glucoamylases in mature dried-down T1 transgenic seeds of selected constructs were also analyzed by enzymatic activity assays. Results of gene copy numbers in events generated from construct 15749 and expression of the exemplary alpha-amylase SEQ ID NO:52 in these events were summarized in Table 2.2.

Table 2.2. Transgene copy numbers and α-amylase activity in events generated from construct 15749

Plant Number	α-amylase Activity in Pooled Seed (U/g)
1	25.6
2	23.5
3	21.3
4	21.2
5	20.2
6	18.4
7	15.0
8	14.3
9	14.0
10	12.7
11	12.2
12	11.1
13	10.2
14	9.9
15	9.6
16	7.6
17	6.9
18	6.8
19	6.4
20	6.4
21	6.0
22	5.4
23	5.3
24	5.3
25	4.7
26	4.7
27	4.5
28	4.2
29	4.0

(continued)

Plant Number	α-amylase Activity in Pooled Seed (U/g)
30	3.9
31	3.7
32	3.6
34	3.1
35	3.0
36	2.8
37	2.5
38	2.4
39	2.0
40	1.5
41	0.4
42	0.4
43	0.3
44	0.2
45	0.1

[0853] Results of gene copy numbers in events generated from construct 15751 and expression of glucoamylase SEQ ID NO:48 in these events are summarized in Table 2.3:

Table 2.3. Transgene copy numbers and glucoamylase activity in events generated from construct 15751

Plant	Gluco-amylase Activity in Pooled Seed (U/g)
1	4.86
2	4.48
3	4.47
4	4.22
5	4.20
6	4.08
7	4.05
8	3.94
9	3.88
10	3.84
11	3.82
12	3.68
13	3.65
14	3.57
15	3.50
16	3.47
17	3.43
18	3.33

(continued)

Plant	Gluco-amylase Activity in Pooled Seed (U/g)
19	2.91
20	1.13
21	0.73

[0854] Table 2.4 summarizes results of gene copy numbers in events generated from construct 15756 and expression of glucoamylase SEQ ID NO:26 in these events. Seeds from multiple events of constructs 15761 and 15718 expressing alpha-amylases SEQ ID NO:66 and SEQ ID NO:76, respectively, were pooled and ground to make composite samples. The two composite samples were assayed for □-amylase activities. Similarly, seeds from multiple events of constructs 15742 and 15750 expressing glucoamylases SEQ ID NO:28 and SEQ ID NO:18, respectively, were also pooled and ground to make composite samples. The two composite samples were assayed for glucoamylase activities. The results were summarized in Table 2.5.

Table 2.4. Transgene copy numbers and glucoamylase activity in events generated from construct 15756

Plant number	Gluco-amylase Activity in Pooled Seed (U/g)
1	4.41
2	4.31
3	4.18
4	4.15
5	3.95
6	3.94
7	3.88
8	3.83
9	3.78
10	3.68
11	3.67
12	3.64
13	3.60
14	3.58
15	3.45
16	3.27
17	3.18
18	3.16
19	3.14
20	3.10
21	3.07
22	3.00
23	2.75

Table 2.5. α-amylase or glucoamylase activity in seeds generated from constructs 15718, 15761, 15742, and 15750

Construct	Variety	Enzyme Expressed	Enzyme Type	Pooled Seed Activity (U/g)
15718	JHAX707	SEQ ID NO:76	α -amylase	0.033
15761	JHAX708	SEQ ID NO:66	α -amylase	0.085
15742	JHAX709	SEQ ID NO:28	Glucoamylase	3.399
15750	JHAX710	SEQ ID NO:18	Glucoamylase	3.55

Raw starch fermentation using maize expressed enzymes

[0855] Seeds from multiple events of selected constructs expressing α -amylases or glucoamylases were pooled and ground to make composite samples for raw starch fermentations. In all experiments, corn flour containing α -amylases was used at the inclusion rate of 20% (w/w) and corn flour containing glucoamylases at the inclusion rate of 50% (w/w). Yellow dent II commodity corn flour made up the rest of the 30% (w/w) of the corn flour. Raw starch fermentations were carried out by following the standard SOP described above (Section I). The ability of these maize expressed α-amylase and glucoamylase combinations to produce ethanol in raw starch fermentation was summarized in Table 2.6.

Table 2.6. Ethanol yield after 72 hours of raw starch fermentation using "lead" exemplary enzymes expressed in maize without codon optimization*

Construct (α-amylase)	Construct (Glucoamylase)	Inclusion Rate (α-amylase) (% w/w)	Inclusio n Rate (Glucoa mylase) (% w/w)	Inclusion Rate (Commo dity) (% w/w)	Ethanol Yield (% v/v)
15749 (SEQ ID NO:52)	15756 (SEQ ID NO:26)	20	50	30	6.96
15749 (SEQ ID NO:52)	15742 (SEQ ID NO:28)	20	50	30	8.90
15761 (SEQ ID NO:66)	15751 (SEQ ID NO:48)	20	50	30	7.30
15718 (SEQ ID NO:76)	15750 (SEQ ID NO:18)	20	50	30	8.33
N/A	N/A	0	0	100	2.25

Example 28: Plant Expression of Exemplary Enzymes using Synthetic Maize Codon Optimized Genes and Raw Starch Fermentation Using Plant Material

[0856] Synthetic genes with codons optimized for maize expression were generated for 2 α-amylases, SEQ ID NO:52 (encoded, e.g., by SEQ ID NO:51) and SEQ ID NO:4 (encoded, e.g., by SEQ ID NO:3), and 2 glucoamylases, SEQ ID NO:48 (encoded, e.g., by SEQ ID NO:47) and SEQ ID NO:26 (encoded, e.g., by SEQ ID NO:25). The table below correlates the wild-type SEQ ID NO: with the SEQ ID NO: assigned to the codon-optimized sequence. The enzymes chosen for codon optimization were selected based on giving the highest ethanol yield in raw starch fermentation. The objectives of this study were to generate commercial events for product development and to generate enough material to evaluate the raw starch fermentation performance of the maize expressed enzymes.

Enzyme	Wild-type SEQ ID NO:	Codon-optimized SEQ ID NO:
Amylase	51	79
Amylase	3	80
Glucoamylase	47	81

(continued)

Enzyme	Wild-type SEQ ID NO:	Codon-optimized SEQ ID NO:
Glucoamylase	25	82

Experimental Methods

Synthetic Genes

[0857] The synthetic genes were made by GENEART™. The sequences were codon optimized using GENEART™'s proprietary GENEOPTIMIZER™ technology. Sequences of the synthetic genes are listed below. The codon usage was adapted to the codon bias of *Zea mays* genes.

Vector Construction

[0858] Table 3.1 summarized the abbreviations used for the DNA elements in the maps shown below.

Table 3.1. Abbreviations used in the commercial construct maps

Name	Function	Description
cAmy(SEQ ID NO:79)Apo-01	CDS	Apoplast targeted maize codon optimized synthetic alpha-amylase gene encoding enzyme SEQ ID NO:52
cAmy(SEQ ID NO:79)ER-01	CDS	ER targeted synthetic maize codon-optimized alpha-amylase gene with Gamma Zein signal sequence and KDEL sequence encoding enzyme SEQ ID NO:52
cAmy(SEQ ID NO:80)Apo-01	CDS	Apoplast targeted synthetic maize codon optimized alpha-amylase gene encoding enzyme SEQ ID NO:4
cAmy(SEQ ID NO:80)ER-01	CDS	ER targeted synthetic maize codon-optimized alpha-amylase gene with Gamma Zein signal sequence and KDEL sequence encoding enzyme SEQ ID NO:4
cGAmy(SEQ ID NO:82)Apo-01	CDS	Apoplast targeted synthetic maize codon optimized glucoamylase gene encoding enzyme SEQ ID NO:26
cGAmy(SEQ ID NO:82)ER-01	CDS	ER targeted synthetic maize codon optimized glucoamylase gene with gamma zein signal sequence and KDEL sequence encoding enzyme SEQ ID NO:26
cGAmy(SEQ ID NO:81)Apo-01	CDS	Apoplast targeted synthetic maize codon optimized gluco-amylase gene encoding enzyme SEQ ID NO:48
cGAmy(SEQ ID NO:81)ER-01	CDS	ER targeted (with addition of KDEL ER-retention signal) synthetic maize codon optimized glucoamylase
		gene encoding enzyme SEQ ID NO:48

[0859] The exemplary vector designated "15740", , below, is a binary vector for maize transformation harboring seed specific promoter prGTL-03 driving the apoplast targeted synthetic maize optimized version of the exemplary alpha-amylase SEQ ID NO:51, the optimized version is the exemplary SEQ ID NO:79 or, cAmy(SEQ ID NO:79)Apo-01. This binary also contains a Ubi-PMI-Nos cassette for selection.

[0860] Cloning Vector: Construct alias 15460ZeinAmyVN was digested with SacI and RsrII to remove iPEPC9-01 and t35s-08. Likewise, construct 15460 was digested with SacI and RsrII to remove tNOS-03-01. Terminator t35s-08/iPEPC9-01 was ligated into 15460 using these sites (SY1709:6). The ligation reaction was transformed into DH5-alpha cells; transformants were screened with colony PCR. Four positive clones were sequenced, and from these sequences both cloning junctions and restriction enzyme sites were determined to be correct (SY1709:15-16). To insert promoter prGTL-03, 15460 containing t35s08/iPEPC9-01 was digested with BamHI and HindIII and gel purified (SY1709:14). Construct 11267 containing prGTL-03 was also digested with BamHI and HindIII and the promoter was gel purified (SY1710:3-4). It was then ligated into the 15460 backbone using these sites and transformed into competent

DH5-alpha cells. Ten transformants were screened using HindIII and BamHI to determine if prGTL-03 was present in vector; all ten had correct banding pattern (SY1709:25). Two clones were sequenced to confirm cloning junctions and restriction enzyme sites, and both were correct without any sequence differences (SY1709:34-35). Glycerol stocks were prepared from clone #1 and stored at -80 degrees Celsius. Cloning vector was digested with BamHI/SacI and CIP treated. The synthetic maize codon optimized version of alpha amylase SEQ ID NO:51, known as SEQ ID NO:79 (also known as cAmy(SEQ ID NO:79)) was digested with BamHI and SacI and ligated into the BamHI/SacI site of prGTL-03 cloning vector to create alias GTL+SYN(SEQ ID NO:79) (Sy1773:48). Binary Vector: An AscI/BamHI fragment from vector 15468 was ligated into the AscI/BamHI site of binary vector 12678 to create alias "12678 RsrII" (SY1533:189). This clone was then digested with RsrII and CIP treated. Cloning vector "GTL+SYN(SEQ ID NO:79)" was digested with SanDI/RsrII and ligated into the RsrII site to create B-prGTL:(SEQ ID NO:79):t35S:PMI (SY1773:56). Positive clones were identified by PCR and confirmed by DNA sequencing.

[0861] The exemplary vector designated "15741", as illustrated in Figure 37, below, is a binary vector for maize transformation harboring seed specific promoter prGTL-03 driving the apoplast targeted synthetic maize optimized version of the exemplary glucoamylase SEQ ID NO:25, the optimized version is the exemplary SEQ ID NO:82 or cGAmy(SEQ ID NO:82)Apo-01. This binary also contains a Ubi-PMI-Nos cassette for selection.

[0862] Cloning Vector: Construct alias 15460ZeinAmyVN was digested with SacI and RsrII to remove iPEPC9-01 and t35s-08. Likewise, construct 15460 was digested with SacI and RsrII to remove tNOS-03-01. Terminator t35s-08/iPEPC9-01 was ligated into 15460 using these sites (SY1709:6). The ligation reaction was transformed into DH5-alpha cells; transformants were screened with colony PCR. Four positive clones were sequenced, and from these sequences both cloning junctions and restriction enzyme sites were determined to be correct (SY1709:15-16). To insert promoter prGTL-03, 15460 containing t35s08/iPEPC9-01 was digested with BamHI and HindIII and gel purified (SY1709:14). Construct 11267 containing prGTL-03 was also digested with BamHI and HindIII and the promoter was gel purified (SY1710:3-4). It was then ligated into the 15460 backbone using these sites and transformed into competent DH5-alpha cells. Ten transformants were screened using HindIII and BamHI to determine if prGTL-03 was present in vector; all ten had correct banding pattern (SY1709:25). Two clones were sequenced to confirm cloning junctions and restriction enzyme sites, and both were correct without any sequence differences (SY1709:34-35). Glycerol stocks were prepared from clone #1 and stored at -80 degrees Celsius. Cloning vector was digested with BamHI/SacI and CIP treated. The synthetic maize codon optimized version of glucoamylase SEQ ID NO:25, known as SEQ ID NO:82 (also known as cAmy(SEQ ID NO:82 or SYN(SEQ ID NO:82) was digested with BamHI and SacI and ligated into the BamHI/SacI site of prGTL-03 cloning vector to create alias GTL+SYN(SEQ ID NO:82) (SY1773:48). Binary Vector: An AscI/BamHI fragment from vector 15468 was ligated into the AscI/BamHI site of binary vector 12678 to create alias "12678 RsrII" (SY 1533:189). This clone was then digested with RsrII and CIP treated. Cloning vector "GTL+SYN(SEQ ID NO:82)" was digested with SanDI/RsrII and ligated into the RsrII site to create B-prGTL:(SEQ IDNO:82):t35S:PMI (SY1773:66). Positive clones were identified by PCR and confirmed by DNA sequencing.

[0863] The exemplary vector designated "15742", as illustrated in Figure 38, below, is a binary vector harboring (comprising) the seed specific promoter prGTL-03 driving the apoplast targeted synthetic maize optimized version of the exemplary glucoamylase SEQ ID NO:47, the optimized version is the exemplary SEQ ID NO:81. This binary also contains a Ubi:PMI:NOS cassette for selection.

[0864] Cloning Vector: Construct alias15460ZeinAmyVN was digested with SacI and RsrII to remove iPEPC9-01 and t35s-08. Likewise, construct 15460 was digested with SacI and RsrII to remove tNOS-03-01. Terminator t35s-08/iPEPC9-01 was ligated into 15460 using these sites (SY1709:6). The ligation reaction was transformed into DH5-alpha cells; transformants were screened with colony PCR. Four positive clones were sequenced, and from these sequences both cloning junctions and restriction enzyme sites were determined to be correct (SY1709:15-16). To insert promoter prGTL-03, 15460 containing t35s08/iPEPC9-01 was digested with BamHI and HindIII and gel purified (SY1709:14). Construct 11267 containing prGTL-03 was also digested with BamHI and HindIII and the promoter was gel purified (SY1710:3-4). It was then ligated into the 15460 backbone using these sites and transformed into competent DH5-alpha cells. Ten transformants were screened using HindIII and BamHI to determine if prGTL-03 was present in vector; all ten had correct banding pattern (SY 1709:25). Two clones were sequenced to confirm cloning junctions and restriction enzyme sites, and both were correct without any sequence differences (SY1709:34-35). Glycerol stocks were prepared from clone #1 and stored at -80 degrees Celsius. Cloning vector was digested with BamHI/SacI and CIP treated. The synthetic maize codon optimized version of glucoamylase SEQ ID NO:47, known as SEQ ID NO:81 (also known as cAmy(SEQ ID NO:81) was digested with BamHI and SacI and ligated into the BamHI/SacI site of prGTL-03 cloning vector to create alias GTL+SYN(SEQ ID NO:81)) (SY 1773:48). Binary Vector: An AsclBamHI fragment from vector 15468 was ligated into the AscI/BamHI site of binary vector 12678 to create alias "12678 RsrII" (SY1533:189). This clone was then digested with RsrII and CIP treated. Cloning vector "GTL+SYN(SEQ ID NO:81)" was digested with SanDI/RsrII and ligated into the RsrII site to create B-prGTL:(SEQ ID NO:81):t35S:PMI (SY1773:56). Positive clones were identified by PCR and confirmed by DNA sequencing (SY1773:58).

[0865] The exemplary vector designated "15743", as illustrated in Figure 39 below, is a binary vector harboring (com-

prising) the seed specific promoter prGTL-03 driving the apoplast targeted synthetic maize optimized version of the exemplary alpha-amylase SEQ ID NO:3, the optimized version is SEQ ID NO:80. This binary also contains a Ubi:PMI:NOS cassette for selection.

[0866] Cloning Vector: Construct alias 15460ZeinAmyVN was digested with SacI and RsrII to remove iPEPC9-01 and t35s-08. Likewise, construct 15460 was digested with SacI and RsrII to remove tNOS-03-01. Terminator t35s-08/iPEPC9-01 was ligated into 15460 using these sites (SY1709:6). The ligation reaction was transformed into DH5-alpha cells; transformants were screened with colony PCR. Four positive clones were sequenced, and from these sequences both cloning junctions and restriction enzyme sites were determined to be correct (SY1709:15-16). To insert promoter prGTL-03, 15460 containing t35s08/iPEPC9-01 was digested with BamHI and HindIII and gel purified (SY1709:14). Construct 11267 containing prGTL-03 was also digested with BamHI and HindIII and the promoter was gel purified (SY 1710:3-4). It was then ligated into the 15460 backbone using these sites and transformed into competent DH5-alpha cells. Ten transformants were screened using HindIII and BamHI to determine if prGTL-03 was present in vector; all ten had correct banding pattern (SY 1709:25). Two clones were sequenced to confirm cloning junctions and restriction enzyme sites, and both were correct without any sequence differences (SY1709:34-35). Glycerol stocks were prepared from clone #1 and stored at -80 degrees Celsius. Cloning vector was digested with BamHI/SacI and CIP treated. The synthetic maize codon optimized version of alpha-amylase SEQ ID NO:3, known as SEQ ID NO:80 (also known as cAmy(SEQ ID NO:80) was digested with BamHI and SacI and ligated into the BamHI/SacI site of prGTL-03 cloning vector to create alias GTL+SYN(SEQ ID NO:80) (SY1773:48). Binary Vector: An AscI/BamHI fragment from vector 15468 was ligated into the AscI/BamHI site of binary vector 12678 to create alias "12678 RsrII" (SY1533:189). This clone was then digested with RsrII and CIP treated. Cloning vector "GTL+SYN(SEQ ID NO:80)" was digested with SanDI/RsrII and ligated into the RsrII site to create B-prGTL:(SEQ ID NO:80):t35S:PMI (SY1773:56). Positive clones were identified by PCR and confirmed by DNA sequencing.

[0867] The exemplary vector designated "15862", as illustrated in Figure 40 below, is a binary vector for maize transformation harboring (comprising) the seed specific promoter prGTL-03 driving the synthetic maize optimized version of the exemplary glucoamylase SEQ ID NO:47, the optimized version is the exemplary SEQ ID NO:81 or cGAmy(SEQ ID NO:81)ER-01 that is targeted to the ER by the KDEL ER-retention signal. This binary also contains a Ubi-PMI-Nos cassette for selection.

[0868] The maize codon optimized version of SEQ ID NO:47, known as SYN(SEQ ID NO:81) (cAMY(SEQ ID NO:81)-03) was PCR replicated and TOPO cloned with primers such that an ER-retention signal KDEL was added to the 3-prime end (SY1773:53). The presence of the KDEL was confirmed by DNA sequencing (SY1773:54 & 60). DNA sequence data confirmed no discrepancies (SY1773:63). Clone #1 was digested with BamHI/BglII, purified by agarose gel and ligated into a cloning vector harboring the prGTL-03 promoter and t35S-08 to create alias "GTL+SYN(SEQ ID NO:81) kdel v2" (SY1773:66). A gene cassette prGTL-03:cAmy(SEQ ID NO:81)kdel:t35s-08 was ligated as a SanDI/RsrII fragment into the RsrII site of a modified version of binary vector 12678 to create B-prGTL-(SEQ ID NO:81)KDEL:PMI (SY1773:83). Integrity of the binary vector was confirmed by PCR and DNA sequencing (SY1773:88).

[0869] The exemplary vector designated "15880", as illustrated in Figure 41 below, is a binary vector containing (comprising) the maize codon-optimized version of the exemplary alpha amylase SEQ ID NO:51, the codon-optimized version is the exemplary SEQ ID NO:79, was expressed using the rice glutelin promoter (prGTL-03). The alpha amylase also contains a Gamma Zein signal sequence and KDEL sequence for ER retention. This vector contains an Ubi-PMI-Nos cassette for mannose selection.

[0870] The maize codon optimized alpha amylase SEQ ID NO:51, known as SEQ ID NO:79 or cAmy(SEQ ID NO:79) was PCR replicated with primers such that an ER-retention signal (KDEL) was added to the 3-prime end (SY 1777:180). The PCR product was then gel purified, TOPO cloned, and transformed into TOP10 competent cells. Presence of the KDEL without any discrepancies was confirmed via sequencing (SY 1773:73). TOPO clones were digested with BamHI and BglII to obtain the modified amylase gene which was then gel purified. The gel purified product was ligated into 15460 containing prGTL-03 (rice glutelin promoter) and t35s-08 to create alias "GTL + syn(SEQ ID NO:79) KDEL v2" (SY1773:80; SY1777:180). Following transformation into DH5á competent cells, clones were screened for insert orientation via PCR. In addition, the entire CDS as well as cloning junctions were sequenced prior to construction of binary vector (SY1777:189, 191). The gene cassette prGTL:cAmy(SEQ ID NO:79):t35s08 was digested with SanDI/RsrII and ligated into a modified version of construct 12678 at the RsrII site (SY1818:12). This construct was modified by ligating an AsclBamHI fragment from vector 15468 into the AsclBamHI site of binary vector 12678 to create alias "12678 RsrII" (SY1533:189). The ligation reaction was transformed into TOP 10 competent cells, and transformants were subsequently screened via colony PCR. Two of the screened transformants were positive for the gene cassette and were digested with BamHI to confirm its presence. Following the digest confirmation, the entire cassette from clone #5 was sequenced (SY1818:34). Data confirmed that the entire cassette, including all junctions, is present without any sequence discrepancies (SY1818:37-38).

[0871] The exemplary vector designated "15884", as illustrated in Figure 42 below, is a binary vector containing (comprising) the maize codon-optimized version of the exemplary SEQ ID NO:25, the maize codon-optimized version

is the exemplary SEQ ID NO:82, was expressed using the rice glutelin promoter (prGTL-03). The glucoamylase also contains a Gamma Zein signal sequence and ER retention signal (KDEL). This vector contains an Ubi-PMI-Nos cassette for mannose selection.

[0872] The maize codon optimized version glucoamylase SEQ ID NO:25, known as SEQ ID NO:82 or cAmy(SEQ ID NO:82) was PCR replicated with primers such that an ER-retention signal (KDEL) was added to the 3-prime end (SY1777:184). The PCR product was then gel purified, TOPO cloned, and transformed into TOP10 competent cells. Presence of the KDEL without any discrepancies was confirmed via sequencing (SY1777:190). TOPO clones were digested with BamHI and BglII to obtain the modified amylase gene which was then gel purified. The gel purified product was ligated into 15460 containing prGTL-03 (rice glutelin promoter) and t35s-08 to create alias "GTL + syn(SEQ ID NO:82) KDEL v2" (SY1818; 1-3). Following transformation into Top10 competent cells, clones were screened for insert orientation via PCR. In addition, the entire CDS as well as cloning junctions were sequenced prior to construction of binary vector (SY1818:29-30). It was determined from sequencing that there is a single base pair change within the coding sequence of cAmy(SEQ ID NO:82) at base pair 156. It is a T to C change that does not change the amino acid sequence of the protein (SY1818: 39-40). The gene cassette prGTL: syn(SEQ ID NO:82) KDEL: t35s08 was digested with SanDI/RsrII and ligated into a modified version of construct 12678 at the RsrII site (SY1818:31). This construct was modified by ligating an AsclBamHI fragment from vector 15468 into the AscI/BamHI site of binary vector 12678 to create alias "12678 RsrII" (SY1533:189). The ligation reaction was transformed into TOP10 competent cells, and transformants were subsequently screened using restriction enzyme digests. Two of the transformants were digested with various combinations of restriction enzymes including NotI, SacI, EcoRV, and PstI to confirm the presence of the gene cassette (SY1818:45). Following the digest confirmation, the entire cassette from clone #7 was sequenced (SY1818:46-47). Data confirmed that the entire cassette, including all junctions, is present and only contains the single base pair change described earlier (SY1818:50).

[0873] The exemplary vector designated "15890", as illustrated in Figure 43 below, is a binary vector for maize transformation containing the maize codon-optimized version of the exemplary alpha-amylase SEQ ID NO:3, the maize codon-optimized version is the exemplary SEQ ID NO:80 or cAmy(SEQ ID NO:80) was expressed using the rice glutelin promoter (prGTL-03). The glucoamylase also contains a Gamma Zein signal sequence and ER retention signal (KDEL). This vector contains an Ubi-PMI-Nos cassette for mannose selection.

[0874] The maize codon optimized version of the exemplary alpha-amylase SEQ ID NO:3, known as cAmy(SEQ ID NO:80) was PCR replicated with primers such that an ER-retention signal (KDEL) was added to the 3-prime end (SY1777:184). The PCR product was then gel purified, TOPO cloned, and transformed into TOP 10 competent cells. Presence of the KDEL without any discrepancies was confirmed via sequencing (SY 1777:190). TOPO clones were digested with BamHI and BglII to obtain the modified amylase gene which was then gel purified. The gel purified product was ligated into 15460 containing prGTL-03 (rice glutelin promoter) and t35s-08 to create alias "GTL + syn(SEQ ID NO:80) KDEL v2" (SY1818; 1-3). Following transformation into TOP10 competent cells, clones were screened for insert orientation via PCR. In addition, the entire CDS as well as cloning junctions were sequenced prior to construction of binary vector (SY1818:51). The gene cassette prGTL: syn(SEQ ID NO:80) KDEL: t35s08 was digested with SanDI/RsrII and ligated into a modified version of construct 12678 at the RsrII site (SY1818:52). This construct was modified by ligating an AscI/BamHI fragment from vector 15468 into the AscI/BamHI site of binary vector 12678 to create alias "12678 RsrII" (SY1533:189). The ligation reaction was transformed into TOP10 competent cells, and transformants were subsequently screened using PCR and restriction enzyme digests. Two of the transformants that had positive PCR results were digested with various combinations of restriction enzymes including NcoI, KpnI, EcoRV, XbaI, and BglII to confirm the presence of the gene cassette (SY1818:65-66). Following the digest confirmation, the entire cassette from clone #2 was sequenced (SY1818:67). Data confirmed that the entire cassette, including all junctions, is present without any sequence differences (SY1818:69-70).

[0875] The exemplary vector designated "15889", as illustrated in Figure 44 below, is a binary vector for maize transformation containing a molecular stack of the codon optimized version of the exemplary glucoamylase SEQ ID NO:25, the codon optimized version is the exemplary SEQ ID NO:82 or cGAmy(SEQ ID NO:82) was expressed by two different promoters. In the first cassette, cAmy(SEQ ID NO:82) is driven by the rice glutelin promoter and has a Gamma Zein signal sequence and KDEL signal for retention in the ER. In the second cassette, the gluco-amylase is driven by the alpha trypsin inhibitor promoter and is targeted to the apoplast with the Gamma Zein signal sequence. This construct also contains a Ubi-PMI-Nos cassette for mannose selection.

[0876] The rice promoter, prATI-01 was mutagenized through site-directed mutagenesis to remove an internal RsrII site, and was submitted as a new component in construct #15882. Following sequence confirmation of the removal of this site, the cloning vector "15460+prATI+t35s08" was digested with BamHI/SacI, CIP treated, and gel extracted (SY1777:162). The synthetic, codon optimized gluco-amylase Syn(SEQ ID NO:82) (BamHI/SacI) was ligated into the digested backbone and the resulting construct is known as "15460prATIm.syn(SEQ ID NO:82)" (SY1777:170). Transformants were screened for the presence of the gene, using both colony PCR and a diagnostic NcoI/BglII digest. Three positive clones were sequenced; cloning junctions were confirmed for the three clones (SY1777:177-179). Following

sequence confirmation, "15460prATIm.syn(SEQ ID NO:82)" was digested with SanDI/RsrII and the ATI: syn(SEQ ID NO:82):t35s08 cassette was gel purified (SY1773:52). Binary vector #15884 was linearized with RsrII, CIP treated, and gel extracted. Following purification, the ATI: syn(SEQ ID NO:82):t35s08 cassette was ligated into the binary backbone and transformed into TOP10 competent cells (SY1818:53). Ten transformants were selected for screening, via PCR, one of which was confirmed to have the cassette containing the ATI promoter (SY1818:56). The positive clone was then screened with diagnostic restriction enzyme digests using the following enzymes: EcoRI, BglII, NcoI, and HindIII (SY 1818:60, 66). Clone #9 was determined to be correct by analyzing the banding pattern from the various digests. In addition, the cloning junctions of clone #9 were sequenced and also confirmed to be correct (SY1818:65).

[0877] The exemplary vector designated "15934", as illustrated in Figure 45 below, is a binary vector for plant transformation containing a molecular stack of the codon optimized version of the exemplary glucoamylase SEQ ID NO:47, the codon optimized version is the exemplary SEQ ID NO:81 or cGAmy(SEQ ID NO:81) was expressed by two different promoters. In the first cassette, GAmy(SEQ ID NO:81) is driven by the rice glutelin promoter and has a KDEL signal for retention in the ER (component is cGAmy(SEQ ID NO:81)ER-01). In the second cassette, the GAmy(SEQ ID NO:81) is driven by the alpha trypsin inhibitor promoter and is targeted to the apoplast (component is cGAmy(SEQ ID NO:81)Apo-01). This construct also contains an Ubi-PMI-Nos cassette for mannose selection. NOTE: IT was determined by restriction digest and sequencing that one of the left border insertions was missing. The project elected to proceed with the vector.

[0878] The cloning vector "15460+prATI+t35s08" was digested with BamHI/SacI, CIP treated, and gel extracted (SY1777:162). The synthetic, codon optimized gluco-amylase cGAmy(SEQ ID NO:81)Apo-01 (BamHI/SacI) was ligated into the digested backbone and the resulting construct is known as "15460prATIm.syn(SEQ ID NO:81)" (SY1777:170). Transformants were screened for the presence of the gene, using both colony PCR and a diagnostic NcoI/BglII digest. Three positive clones were sequenced; cloning junctions were confirmed for the three clones (SY1777:177-179). Following sequence confirmation, "15460prATIm.syn(SEQ ID NO:81)" was digested with SanDI/RsrII and the ATI: cGAmy(SEQ ID NO:81)Apo-01 :t35s08 cassette was gel purified (SY1773:72). Binary vector "B-prGTL: (SEQ ID NO:81)KDEL: PMI" was constructed and labeled as construct #15862 (SY17773:92). It was linearized with RsrII, CIP treated, and gel extracted. Following purification, the ATI: cGAmy(SEQ ID NO:81)Apo-01:t35s08 cassette was ligated into the binary backbone and transformed into TOP10 competent cells (SY1818:22-23). Transformants were initially screened with colony PCR using primers which only annealed in the ATI promoter. Ten transformants were selected and confirmed to have the cassette containing the ATI promoter. Clone #8 was selected for restriction analyses to further confirm the presence of the cassette within the binary vector. Two enzymes which specifically cut in the ATI promoter as well as the backbone were selected to rule out the possibility of a double cassette. When clone #8 was digested with AvrII and NcoI, it was determined that there was only one copy of each cassette present (SY1818: 93-94).

Enzymatic activity analysis

[0879] Single seed analysis or pooled seed analysis was used to measure alpha-amylase or glucoamylase activity in transgenic seeds. For single seed analysis, 12 seeds from each event were randomly selected and ground individually. For pooled seed analysis, twenty seeds from selected events were randomly selected, pooled, and ground. The flours were then assayed using the Megazyme CERALPHA HR™ assay for alpha-amylases or Megazyme's glucoamylase assay for glucoamylases. The assay's standard operating procedures (SOPs) are described above, including the "enzymatic activity analysis", the alpha-amylase extraction from corn flour and activity assay, the glucoamylase extraction from corn flour and activity assay, and the raw starch fermentation protocol.

Results

Taqman assay and enzymatic activity assays

[0880] Copy numbers of the transgenes were determined by primary and secondary Taqman assays. Primers specific for the selectable marker gene used in all the maize transformation vectors described above, pmi, were used in the primary Taqman assays. Primers specific for pmi gene, the bacterial selectable marker gene, spec, and genes encoding the alpha-amylases or glucoamylases were used in secondary Taqman assays.

[0881] Expression of alpha-amylases or glucoamylases in mature dried-down T1 transgenic seeds of selected constructs was also analyzed by enzymatic activity assays. Results of gene copy numbers in selected events generated from construct 15840 were summarized in Table 3.2. Expression of the exemplary codon-optimized alpha-amylase SEQ ID NO:79 in these events were measured by analysis of enzymatic activity in 12 randomly selected seeds individually. The average activity of the 12 seeds for each event is also shown in Table 3.2, below.

[0882] Similarly, Table 3.3 shows gene copy numbers of selected events generated from construct 15841. Expression of the exemplary codon-optimized glucoamylase SEQ ID NO:82 in these events were also measured by analysis of enzymatic activity in 12 randomly selected seeds individually and average activity of the 12 seeds for each event is

summarized in Table 3.3, below.

[0883] Twenty (20) seeds from each selected events generated from constructs 15842 and 15843, on the other hand, were pooled for enzymatic assays to determine expression levels of the codon-optimized glucoamylase SEQ ID NO:81 and the exemplary codon-optimized alpha-amylase SEQ ID NO:80 in these events, respectively. Results were summarized in Tables 3.4 and 3.5, respectively, below. Transgene copy numbers are also shown.

Table 3.2. Transgene copy numbers and alpha-amylase activity in selected events generated from construct 15840

Plant number	Average Activity of 12 Seeds (U/g)
1	377.3
2	291.9
3	95.1
4	119.4
5	155.4
6	129.4
7	125.3
8	539.4
9	1362.4
10	195.1
11	88.4
12	458.2
13	722.9
14	151.7
15	164.8

Table 3.3. Transgene copy numbers and glucoamylase activity in selected events generated from construct 15841

Plant Number	Average Activity of 12 Seeds (U/g)
1	2.5
2	3.5
3	2.6
4	2.8
5	2.4
6	3.0
7	2.6
8	2.5
9	2.6
10	2.8
11	3.0
12	3.0
13	2.9
14	3.4
15	2.3

(continued)

Plant Number	Average Activity of 12 Seeds (U/g)
16	2.5
17	2.6
18	3.3
19	2.4
20	2.9
21	3.5
22	2.3
23	2.5
24	2.3

Table 3.4. Transgene copy numbers and glucoamylase activity in selected events generated from construct 15842

Plant Number	Pooled Seed Activity (U/g)
1	1.5
2	1.3
3	1.3
4	1.2
5	1.3
6	1.6
7	1.4
8	1.4
9	1.3
10	1.2
11	1.4
12	1.6
13	1.7
14	1.2
15	1.3
16	1.6
17	1.4
18	1.6
19	1.6
20	1.6

Table 3.5. Transgene copy numbers and alpha-amylase activity in selected events generated from construct 15843

Plant Number	Pooled Seed Activity (U/g)
1	116.14
2	107.07

(continued)

Plant Number	Pooled Seed Activity (U/g)
3	105.02
4	277.65
5	240.90
6	165.25
7	425.17
8	151.02
9	139.74
10	245.27
11	197.68
12	179.77
13	368.59
14	350.91
15	225.03
16	233.18
17	366.24
18	220.36

Table 3.6. Ethanol yield after 72 hours of raw starch fermentation using "lead" exemplary enzymes expressed in maize using maize codon optimized synthetic genes

Construct (alpha-amylase)	Construct (Glucoamylase)	Inclusion Rate (alpha-amylase) (% w/w)	Inclusion Rate (Glucoamylase) (% w/w)	Inclusion Rate (Commidity) (% w/w)	Ethanol Yield (% v/v)
15840 (SEQ ID NO:79)	15841 (SEQ ID NO:82)	20	50	30	16.77
15840 (SEQ ID NO:79)	15842 (SEQ ID NO:81)	20	50	30	17.08
15843 (SEQ ID NO:80)	15841 (SEQ ID NO:82)	20	50	30	7.30
15843 (SEQ ID NO:80)	15842 (SEQ ID NO:81)	20	50	30	10.60
N/A	N/A	0	0	100	2.25

Raw starch fermentation using maize expressed enzymes

[0884] Seeds from events shown above expressing alpha-amylases or glucoamylases were pooled and ground to make composite samples for raw starch fermentations. In all experiments, corn flour containing alpha-amylases was used at the inclusion rate of 20% (w/w) and corn flour containing glucoamylases at the inclusion rate of 50% (w/w). Yellow dent II commodity corn flour made up the rest of the 30% (w/w) of the corn flour. Raw starch fermentations were carried out by following the standard SOP described above (Section I). The ability of these maize expressed alpha-amylase and glucoamylase combinations using maize codon optimized synthetic genes to produce ethanol in raw starch fermentation is summarized in Table 3.6, below.

Example 29: *Pichia pastoris* Expression Constructs

[0885] This example describes the expression of enzymes in yeast using, e.g., exemplary *Pichia* expression systems.

[0886] For construction of expression constructs in pPICZalpha and pAO815 vectors (both from Invitrogen, Carlsbad, CA), Xi-cloning technology was used. pPICZalpha was digested with EcoRI, then treated with Xi-cloning cocktails (according to manufacture protocol by Genlantis, a division of Gene Therapy Systems, Inc., San Diego, CA). The genes were amplified by PCR reactions with end matching to the vector sequences. The PCR products were mixed with the vectors and transformed into *E. coli* host TOP10 (Invitrogen) and selected under zeocin 25 (pPICZalpha) or carbenicillin 100 (pAO815). The final constructs were verified by sequencing. For transformation into *Pichia* hosts, the verified plasmid DNA was digested to make linear DNA and transform in to *Pichia* hosts. The transformants were selected under Zeocin (pPICZalpha) or histine deficient plates.

[0887] All enzymes were obtained by expression in *Pichia pastoris,* except for SEQ ID NO:78 (encoded by SEQ ID NO:77), which was expressed in *Pseudomonas fluorescens* (see JBC, 2002, 277(29):26501-26507).

I. Characterization of Glucoamylases and Amylases.

Methods:

6. Determination of Protein Concentration

[0888] Lyophilized supernatants of *P. pastoris* cultures expressing glucoamylases and α-amylases were suspended in water at a concentration of ~10 mg of powder / ml. After protein content determination by the Bradford protocol, 5μg of protein sample and standardized BSA solution were run on a 4 -20% Tris-Glycine gradient gel. The gel was scanned on the BioRad GS800 gel scanner following Coomassie blue staining. The BioRad Quantity One software was used for the quantification of the BSA and glucoamylase (or α-amylases) bands, and the actual enzyme concentration was then calculated. Protein concentration was adjusted accordingly and confirmed by additional SDS PAGE.

7. Determination of Initial Reaction Rates.

[0889] Unless mentioned otherwise, assays were performed in triplicate at 37°C and pH 5.0 in buffer (50 mM $NaCH_3CO_2$, 10 mM $CaCl_2$; 10 mM NaN_3 and 0.01% Triton X-100) containing 1% raw corn starch, or 0.5% dextrin or 1% "soluble corn starch" (see note below on the "soluble starch" preparation). Assays were performed at 0.5ml scale for glucoamylase and 0.25 ml scale for α-amylase in an Eppendorf tabletop incubator with constant shaking (800 rpm).

[0890] For glucoamylases, reactions were started by adding the enzyme (final concentration 0.25 μg /ml) to the reaction mix. At 0, 2.5, 5, 7.5, 10, 15, 20 and 30 min, 50 μl aliquots of the reactions were withdrawn and quenched by addition to 100μl of 1M Tris buffer, pH 7.5.

[0891] For α-amylases, reactions were started by adding the enzyme (final concentration 0.4μg total protein/ml for SEQ ID NO:56, SEQ ID NO:2 and SEQ ID NO:52; 2μg/ml for SEQ ID NO:62; 4μg/ml for SEQ ID NO:70 and SEQ ID NO:66) to the reaction mix, and 10 μl aliquots of reactions were withdrawn and quenched in BCA reagent at 2, 5, 10, 15, 20, 25, 30, and 40 min.

[0892] For determination of temperature profiles, assays were performed at 30, 34, 37 and 40°C.

[0893] The effect of pH on glucoamylase and amylase activities was evaluated at pH 3.5, 4, 5, 6 and 7, using the broad pH range Britton - Robinson buffer (50 mM CH_3COOH; H_3PO_4; H_3BO_3). Parallel reactions at pH 4, 5 and 6 were also performed in the presence of 50mM acetate buffer to ensure that the buffer used did not influence the results. For the determination of pH profile of two calcium-dependent α-amylases (SEQ ID NO:56 and SEQ ID NO:62), malic acid / acetate / MES buffers were used instead of Britton-Robinson.

[0894] Preparation of "soluble corn starch" for reaction with α-amylases. Dextrin (Sigma D2006) could not be used as a substrate in the BCA α-amylase reactions due to the high reducing ends background. Therefore a heated corn starch was employed as a substrate. Specifically, 2% corn starch was dissolved in deionized water and heated with mixing in a boiling water bath for 30-40 minutes, until the starch had dissolved and the solution appeared milky, but translucent. The solution of heated starch was used for 2 days, after which time some signs of retrogradation were observed (appearance of starch clumps), and the solution was discarded.

8. Glucose oxidase / peroxidase (GO) assay for the quantification of glucose released during starch hydrolysis.

[0895] A coupled glucose oxidase/ peroxidase (GO) assay was used to determine the amount of glucose released by glucoamylase during starch hydrolysis. GO reactions were started by adding 10 μl of the quenched starch hydrolysis reaction to 90 μl of PBS containing glucose oxidase (0.1 U/ml), peroxidase (0.25 U/ml) and 0.05 mM Amplex Red, in

black Nunc 96-well plates. Plates were kept at room temperature in the dark for 30 min prior to reading on a fluorescent plate reader with Ex/Em 545/590 nm. A standard curve with glucose concentrations of 0 to 100 μM was used to assess the amount of glucose produced in the hydrolysis reactions. Initial rates of starch hydrolysis (nmols of glucose released from 1% granular starch / min/ μg glucoamylase) were determined by plotting the amount of glucose released over time, and calculating the slope of the best linear fit through the data points.

9. BCA assay for determining the increase in concentration of reducing ends during starch hydrolysis.

[0896] A 10 μl aliquot of amylase starch hydrolysis reaction was quenched into 100 μl of BCA reagent (consisting of 64 mg/mL sodium carbonate monohydrate, 24 mg/mL sodium bicarbonate, 1.95 mg/mL BCA, 1.24 mg/mL cupric sulfate pentahydrate, 1.26 mg/mL L-serine). Color development occurred during incubation of the quenched reaction at 80°C for 35 minutes and was followed by absorbance determination at 560nm. Initial rates were calculated over a 40 min reaction time. A standard curve using maltose (0 - 54 μM) was constructed to correlate A_{560nm} with the concentration of generated reducing sugars (nmoles). Specific activity was expressed as nmoles/min/μg enzyme.

10. Bond-type specificity of glucoamylases with maltose and isomaltose as substrates.

[0897] Reactions were started by adding the enzyme (final concentration 5μg /ml for maltose, and 30μg /ml for isomaltose) to the reaction mix. At 2, 5, 10, 15, 20, 25, 30 and 40 min, 5 μl aliquots of the reactions were withdrawn and quenched by addition to 10μl of 1M Tris buffer, pH 7.5. Nine substrate concentrations were used in the studies, ranging from 0 to 12 mM for maltose and 2.5 to 120 mM for isomaltose. The reactions were performed in triplicate at 37°C and pH 5.0 in buffer (50mM $NaCH_3CO_2$, 10mM $CaCl_2$), at 50 ul scale in an Eppendorf tabletop incubator with constant shaking (800 rpm). Glucose production was measured at the end of the reaction using the glucose oxidase / peroxidase (GO) assay.

Results

2. Characterization of glucoamylases

1.1 Initial reaction rates:

[0898] Initial rates for granular and soluble starch hydrolysis are presented in Table 1. As can be seen from Table 1, exemplary glucoamylases of this disclosure displayed up to 3x better activity (SEQ ID NO:48) against granular starch, with similar or slightly better activity on soluble starch when compared to the benchmark *A. niger* enzyme. SEQ ID NO:48 was also expressed in *E. coli* (see above section on *Picha* expression constructs) - this *E. coli* expressed enzyme is labeled SEQ ID NO:48 (Ec) in the table below. SEQ ID NO:48(Ec) did not display any activity against granular starch under the conditions tested (probably due to the lack of a Starch Binding Domain).

[0899] Table 1: Comparison of initial rates of granular corn starch and soluble starch (dextrin) hydrolysis by "lead" exemplary amylases and/or glucoamylases and a benchmark enzyme *A. niger* glucoamylase (Sigma A7095) at 37°C, pH 5.0:

Table 1

Enzyme	Initial rate* ± SD Granular starch	Initial rate* ± SD Soluble starch
SEO ID NO:48	35.6 ± 3.8	60.7 ± 5.1
SEQ ID NO:26	28.8 ± 3.4	51.8 ± 7.4
SEQ ID NO:74	25.1 ± 2.5	84.3 ± 3.5
SEQ ID NO:18	24.3 ± 4.3	58.3 ± 3.0
SEQ ID NO:28	17.8 ± 4.3	33.8 ± 4.0
SEQ ID NO: 14	6.6 ± 1.2	53.9 ± 4.1
SEQ ID NO:48(Ec)	0	59.3 ± 8.5
A. niger	11.3 ± 2.7	43.3 ± 7.4

(continued)

Enzyme	Initial rate* ± SD Granular starch	Initial rate* ± SD Soluble starch
glucoamylase (Sigma A7095)		
*Initial rates are expressed as nmols of glucose /min/μg of glucoamylase protein released from 1% granular starch or from 0.5% dextrin. Each number is the average value from 6-10 data points.		

[0900] 1.2 Temperature profile: The effect of temperature (30°C - 40°C) on starch hydrolysis by the characterized glucoamylases is presented in Figures 1A and 1B. Activities of glucoamylases increased with temperature; they were most active at 40°C but retained ~50% of peak activity at 30°C.

[0901] Figure 28A: Effect of Temperature on the Activity of exemplary amylases and/or glucoamylases and the glucoamylase *A. niger* glucoamylase with Granular Starch as substrate (Benchmark *A. niger* glucoamylase (Sigma A7095). Glucose release was measured at the indicated temperature at pH 5.0. The exemplary SEQ ID NO:20 is not included in the plot because it did not display any activity against granular starch under these particular conditions as tested.

[0902] Figure 28B: Effect of Temperature on the Activity of the exemplary glucoamylase SEQ ID NO:20 and the glucoamylase *A. niger* glucoamylase (Sigma A7095) with Soluble Starch (Dextrin) as a substrate. Glucose release was measured at the indicated temperature at pH 5.0.

[0903] 1.3 pH profile: The influence of pH on starch hydrolysis was tested with both granular and soluble starch and the results are presented in Figure 2A and Figure 2B, respectively. All glucoamylases hydrolyzed both substrates best at lower pH with SEQ ID NO:26 being the most acidic in character.

[0904] Figure 29A: Effect of pH on the Activity of Glucoamylases with Granular Starch as substrate. Glucose release was measured at the indicated pH at 37 °C. Initial rates were calculated over 20 min and converted to a percentage of the maximum rate. The exemplary SEQ ID NO:20 is not included in the plot because it did not display any activity against granular starch, under the particular conditions tested here.

[0905] Figure 29B: Effect of pH on the Activity of Glucoamylases with Soluble Starch as substrate. Glucose released was measured at the indicated pH at 37 °C. Initial rates were calculated over 20 min and converted to a percentage of the maximum rate.

[0906] 1.4 Bond-type cleavage specificity: The kinetic parameters for the hydrolysis of maltose (alpha -1,4-linkage) (maltose is two alpha -D-glucoses) and isomaltose (alpha-1,6-linkage) were determined for 7 selected glucoamylases and the benchmark (*A. niger* glucoamylase (Sigma A7095). The experiments were conducted with lyophilized P. *pastoris* lysates and the proteins were not purified; therefore only data independent of protein concentration are reported in this document. Table 2, below, summarizes values of K_M for maltose and isomaltose and the ratio of k_{cat}/K_M for maltose compared to k_{cat}/K_M for isomaltose. These parameters determined for *A. niger* glucoamylase (Sigma A7095) are in good agreement with published data (K_M for maltose is reported to be 1.2 - 2.1mM); K_M for isomaltose is reported to be 19.8 - 42.0 and k_{cat}/K_M for maltose over k_{cat}/K_M for isomaltose is reported to be between 300 - 600 according to Frandesen *at al.* 1995; Sierks and Svensson;1996; Fagerstrom and Kalkkinen; 1995).

[0907] As can be seen from Table 2, the exemplary glucoamylase SEQ ID NO:20 was most strongly selective for maltose and had nearly 900-fold higher specificity towards alpha -1,4- linkages relative to alpha-1,6- bonds. The least selective glucoamylase was SEQ ID NO:14 with ~ 100-fold higher specificity towards alpha-1,4- bonds relative to alpha-1,6- bonds.

Table 2. Kinetic parameters for hydrolysis of Maltose and Isomaltose by 7 exemplary glucoamylases of this disclosure and a benchmark (*A. niger* glucoamylase (Sigma A7095)).

Enzyme	Ma ltose	Isomal tose	k_{cat}/K_M (maltose) /k_{cat}/K_M (isomaltose)
	K_M (mM)	K_M (mM)	
SEQ ID NO:29	0.61 ± 0.06	11.94 ± 4.99	750
SEQ ID NO:74	1.87 ± 0.17	11.55 ± 2.92	481
SEQ ID NO:20	2.62 ± 0.19	53.97 ± 23.17	897
SEQ ID NO:14	2.67 ± 0.15	41.5 ± 5.05	116
SEQ ID NO:26	0.98 ± 0.33	12.18 ± 0.64	456
SEQ ID NO:48	2.26 ± 0.12	21.69 ± 3	565

(continued)

Enzyme	Maltose K_M (mM)	Isomaltose K_M (mM)	k_{cat}/K_M (maltose) /k_{cat}/K_M (isomaltose)
SEQ ID NO:18	1.01 ± 0.09	11.74 ± 7.74	415
A. niger glucoamylase (Sigma A7095)	0.93 ± 0.1	18.72 ± 3.95	249

[0908] Each number is the average value from 5 different experiments.

References

[0909]

1. Frandsen TP, Christensen T, Stoffer B, Lehmbeck J, Dupont C, Honzatko RB, Svensson B (1995) Biochemistry. 34:10162-9.
2. Sierks MR and Svensson B. (1996) Biochemistry;35:1865-71.
3. Fagerstrom R and Kalkkinen N.(1995) Biotechnol Appl Biochem. 21:223-31.

Characterization of Amylases

[0910] Initial reaction rates: The initial rates of hydrolysis of granular and soluble starch are presented in Table 3. Eight exemplary alpha-amylases were compared with a benchmark alpha -amylase from *A. oryzae.* As can be seen from Table 3, below all amylases tested displayed significantly higher activity against soluble starch when compared with granular starch. However, this difference was less marked for Amylases and/or glucoamylases than for the benchmark enzyme.

[0911] Table 3: Comparison of initial rates of hydrolysis of granular corn starch and soluble corn starch by 8 α-amylases and a benchmark alpha-amylase from *A. oryzae* at 37°C and pH 5:

Table 3

Exemplary Enzyme	Initial rate* ± SD Granular starch	Initial rate* ± SD Soluble starch
SEQ ID NO:56	15.7 ± 1.67	1607.9 ± 518.22
SEQ ID NO:70**	20.5	109.1
SEQ ID NO:62	3.5 ± 0.37	139.6 ± 55.96
SEQ ID NO:66**	2.1	70.8
SEQ ID NO:2	7 ± 0.75	381.2 ± 74.15
SEQ ID NO:52	10.3 ± 1.75	248.2 ± 28.46
SEQ ID NO:78	0.4 ± 0.06	232 ± 52.63
SEQ ID NO:76**	25.2	809.1
A. oryzae amylase (MegazymeE-ANAAM)	0.4 ± 0.07	498.7 ± 64.78
*Initial rates are expressed as umole of reducing ends released from 1% starch /min/μg of alpha-amylase protein. **Data obtained using purified enzyme.		

-Each number is an average value from 5 data points.

[0912] Temperature profile: The effect of temperature on starch hydrolysis by the characterized α-amylases is presented in Figure 30. Activities of amylases were affected by temperature to a different degree. Five of the exemplary amylases of this disclosure (SEQ ID NO:56, SEQ ID NO:70, SEQ ID NO:78, SEQ ID NO:76 and SEQ ID NO:66) were most active at 40°C and retained ~30% of activity at 30°C. Activities of the exemplary SEQ ID NO:2, SEQ ID NO:52 and SEQ ID NO:62 were only marginally affected by changes in temperature over the range investigated.

[0913] Figure 30 illustrates: the Influence of Temperature on Starch Hydrolysis by 9 α-amylases. Activity was measured at pH 5.0 during 40 min incubation at the indicated temperature, and initial rates were calculated and plotted against time. In Figure 30:

* Rates for the exemplary SEQ ID NO:56, SEQ ID NO:2 and SEQ ID NO:52 are presented on the left axis; rates for the rest of enzymes are presented on the right axis.
*Initial rates are expressed as nmoles of reducing ends released from 1% granular corn starch /min/μg of enzyme in 250 μl reaction.
-Activities of the exemplary SEQ ID NO:70, SEQ ID NO:66 and SEQ ID NO:76 are expressed in nmoles/min/μg of total protein in *Pichia pastoris* supernatant.

[0914] pH profile: The influence of pH on starch hydrolysis was tested with both granular and soluble starch substrates and the results are presented in Figure 31A and Figure 31B, respectively. The exemplary SEQ ID NO:52 (of fixngal origin) had the lowest pH optimum (~pH 4). Another exemplary enzyme (SEQ ID NO:2) (of fungal origin) also displayed preference for acidic pH, with an apparent optimum at ~pH 4.5 - 5. The exemplary amylase SEQ ID NO:66 (of Archaeal origin) had an apparent optimum of ~pH 5.0, retaining ~ 70% of peak activity at pH 4.0. The remaining enzymes had apparent optima between pH 5.0 and 6.0, and were almost inactive at pH 4.0 and 3.5.

[0915] Figure 31A: Effect of pH on the activities of α-amylases with Granular Starch as substrate. The increase in reducing ends was measured at the indicated pH at 37 °C. Initial rates were calculated over 40 min and converted to a percentage of the maximum rate.

[0916] Figure 31B: Effect of pH on the activities of α-amylases with Soluble Starch as substrate. The increase in reducing ends was measured at the indicated pH at 37 °C. Initial rates were calculated over 40 min and converted to a percentage of the maximum rate.

A number of embodiments have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope. Accordingly, other embodiments are within the scope of the following claims.

SEQUENCE LISTING

[0917]

<110> VERENIUM CORPORATION
SYNGENTA PARTICIPATIONS AG

<120> AMYLASES AND GLUCOAMYLASES, NUCLEIC ACIDS ENCODING THEM AND METHODS FOR MAKING AND USING THEM

<130> V 574/MH

<140> 12 152 662.8
<141> 21.12.2007

<150> US 60/892,823
<151> 2007-03-02

<150> US 60/877,068
<151> 2006-12-21

<160> 82

<170> PatentIn 3.3

<210> 1
<211> 1710
<212> DNA
<213> Cochliobolus heterostrophus ATCC 48331

<400> 1

gccgacacca atgcttggaa gtcccgcagc atctactttg tcctgacgga tcgtattgcc 60

cgcaacagca gcgacacggg cggctcagcg tgcagcgacc tcggcaacta ctgcggtgga 120

actttccagg gcctcgagtc taagctcgac tacatcaagg gacttggatt cgatgccatt 180

tggatcaccc ccgtcgtctc aaacaaggct gctggatacc atggctactg ggccgaggac 240

ttgtatgccg tcaactcaaa ctacggcact gctgccgact tgaagagctt ggttgccgct 300

gcccatgcca agggcatcta catgatggtc gacgttgtcg caaaccacat gggtcctgga 360

gcaatcacaa acaaccgccc tgaacctctc aaccaggctt catcatacca ccctccttgc 420

aacatcgact acaacaacca aaccagtgtc gaggtatgtc aaatagccgg actccccgac 480

atctacacca ccaagagcga gatccgcacg ctcctcaaca cctgggtcaa ctggctcgta 540

aacgagtaca gcttcgacgg tgtccgcatc gacaccgtca agcacgtcga aaaggacttt 600

tggcctggct tctctgccgc taccggtgtc tacaacattg gcgaggtgtt tgacggagac 660

ccagcctacc ttgccccgta cgccaagctt atgcccggcc tcctcaacta cgcagtctac 720

tacccgatga acaactttta ccagcaaacg ggctcttccc aggcgcttgt agacatgatg 780

aacactgtca gcaacacctt ccctgaccca tctgccttgg gaaccttcct cgacaaccac 840

gacaacaagc gctggttgaa cgtcaagaac gaccagactc tgctcaagaa cgctcttgct 900

tatgtcatcc tcgcacgtgg tatccccatc ttgtactatg gtaccgagca gggatacgct 960

ggtggtgacg acccagctaa ccgagaggat ctgtggcgca gtggcttcaa caccaatgcc 1020

aacctctacc aagccatcaa gaaactgacc gccgcccgac aggctgccgg tggtctcgca 1080

ggaaacgacc acgtccacct gtacgtcgcc gacacggctt acgcctggag ccgtgccaac 1140

ggcaacctga ttgtcctcac caccaacgct ggcggcaact ccaacaccca gcactgcttc 1200

aacacgcaaa aggcaaacgg ccgctggacc aacgtctacg gcaacggcgc caccgtctct 1260

gccgatagca acggccaaat ctgcgtctcc gtcacaaacg gcgagcccgt tgtcctcctc 1320

gccggctccg ctacccccac cactggcact accctctcca cccgcaccgc cactgccacc 1380

gccacaccaa ccgcatgccc caccgccgtc tccgtctcct tcacccaccg cgtcaccact 1440

gttcccggtg acaccatcaa aatcactggc aacacggccc agctaggtaa ctggactccc 1500

gccaacggtc ttgccttgtc cgcagctagc tacacatcca gcaaccctat ctggaccatt 1560

accgtgcccc tggccgctgg atcctccatc tcgtacaagt ttgtcaagat tgacagtgga 1620

ggaactgtca cctgggagag tgaccccaac aggtcataca ctgcgccgag ctgccaggcg 1680

agtgccggtg tgaacagctc atggcaatag 1710

<210> 2
<211> 569
<212> PRT

<213> Cochliobolus heterostrophus ATCC 48331

<220>
<221> DOMAIN
<222> (13)...(353)
<223> Alpha amylase, catalytic domain

<220>
<221> DOMAIN
<222> (469)...(564)
<223> Starch binding domain

<220>
<221> SITE
<222> (22)...(25)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (148)...(151)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (558)...(561)
<223> N-glycosylation site. Prosite id = PS00001

<400> 2

```
Ala Asp Thr Asn Ala Trp Lys Ser Arg Ser Ile Tyr Phe Val Leu Thr
1               5                  10                  15

Asp Arg Ile Ala Arg Asn Ser Ser Asp Thr Gly Gly Ser Ala Cys Ser
            20                  25                  30

Asp Leu Gly Asn Tyr Cys Gly Gly Thr Phe Gln Gly Leu Glu Ser Lys
        35                  40                  45
```

Leu Asp Tyr Ile Lys Gly Leu Gly Phe Asp Ala Ile Trp Ile Thr Pro
50 55 60

Val Val Ser Asn Lys Ala Ala Gly Tyr His Gly Tyr Trp Ala Glu Asp
65 70 75 80

Leu Tyr Ala Val Asn Ser Asn Tyr Gly Thr Ala Ala Asp Leu Lys Ser
85 90 95

Leu Val Ala Ala Ala His Ala Lys Gly Ile Tyr Met Met Val Asp Val
100 105 110

Val Ala Asn His Met Gly Pro Gly Ala Ile Thr Asn Asn Arg Pro Glu
115 120 125

Pro Leu Asn Gln Ala Ser Ser Tyr His Pro Pro Cys Asn Ile Asp Tyr
130 135 140

Asn Asn Gln Thr Ser Val Glu Val Cys Gln Ile Ala Gly Leu Pro Asp
145 150 155 160

Ile Tyr Thr Thr Lys Ser Glu Ile Arg Thr Leu Leu Asn Thr Trp Val
165 170 175

Asn Trp Leu Val Asn Glu Tyr Ser Phe Asp Gly Val Arg Ile Asp Thr
180 185 190

Val Lys His Val Glu Lys Asp Phe Trp Pro Gly Phe Ser Ala Ala Thr
195 200 205

Gly Val Tyr Asn Ile Gly Glu Val Phe Asp Gly Asp Pro Ala Tyr Leu
210 215 220

Ala Pro Tyr Ala Lys Leu Met Pro Gly Leu Leu Asn Tyr Ala Val Tyr
225 230 235 240

Tyr Pro Met Asn Asn Phe Tyr Gln Gln Thr Gly Ser Ser Gln Ala Leu
245 250 255

Val Asp Met Met Asn Thr Val Ser Asn Thr Phe Pro Asp Pro Ser Ala
260 265 270

Leu Gly Thr Phe Leu Asp Asn His Asp Asn Lys Arg Trp Leu Asn Val
275 280 285

Lys Asn Asp Gln Thr Leu Leu Lys Asn Ala Leu Ala Tyr Val Ile Leu
290 295 300

```
Ala Arg Gly Ile Pro Ile Leu Tyr Tyr Gly Thr Glu Gln Gly Tyr Ala
305                 310                 315                 320

Gly Gly Asp Asp Pro Ala Asn Arg Glu Asp Leu Trp Arg Ser Gly Phe
                325                 330                 335

Asn Thr Asn Ala Asn Leu Tyr Gln Ala Ile Lys Lys Leu Thr Ala Ala
            340                 345                 350

Arg Gln Ala Ala Gly Gly Leu Ala Gly Asn Asp His Val His Leu Tyr
        355                 360                 365

Val Ala Asp Thr Ala Tyr Ala Trp Ser Arg Ala Asn Gly Asn Leu Ile
    370                 375                 380

Val Leu Thr Thr Asn Ala Gly Gly Asn Ser Asn Thr Gln His Cys Phe
385                 390                 395                 400

Asn Thr Gln Lys Ala Asn Gly Arg Trp Thr Asn Val Tyr Gly Asn Gly
                405                 410                 415

Ala Thr Val Ser Ala Asp Ser Asn Gly Gln Ile Cys Val Ser Val Thr
            420                 425                 430

Asn Gly Glu Pro Val Val Leu Leu Ala Gly Ser Ala Thr Pro Thr Thr
        435                 440                 445

Gly Thr Thr Leu Ser Thr Arg Thr Ala Thr Ala Thr Ala Thr Pro Thr
    450                 455                 460

Ala Cys Pro Thr Ala Val Ser Val Ser Phe Thr His Arg Val Thr Thr
465                 470                 475                 480

Val Pro Gly Asp Thr Ile Lys Ile Thr Gly Asn Thr Ala Gln Leu Gly
                485                 490                 495

Asn Trp Thr Pro Ala Asn Gly Leu Ala Leu Ser Ala Ala Ser Tyr Thr
            500                 505                 510

Ser Ser Asn Pro Ile Trp Thr Ile Thr Val Pro Leu Ala Ala Gly Ser
        515                 520                 525

Ser Ile Ser Tyr Lys Phe Val Lys Ile Asp Ser Gly Gly Thr Val Thr
    530                 535                 540

Trp Glu Ser Asp Pro Asn Arg Ser Tyr Thr Ala Pro Ser Cys Gln Ala
545                 550                 555                 560

Ser Ala Gly Val Asn Ser Ser Trp Gln
```

565

<210> 3
<211> 1770
<212> DNA
<213> Cochliobolus heterostrophus ATCC 48331

<400> 3

atgttgttgc tcaacatctt caccaccctc ttcttctaca tcacctgcat cgtctccgcc 60

gccgacacca atgcttggaa gtcccgcagc atctactttg tcctgacgga tcgtattgcc 120

cgcaacagca gcgacacggg cggctcagcg tgcagcgacc tcggcaacta ctgcggtgga 180

actttccagg gcctcgagtc taagctcgac tacatcaagg gacttggatt cgatgccatt 240

tggattaccc ccgtcgtctc aaacaaggct gctggatacc atggctactg ggccgaggac 300

ttgtatgccg tcaactcaaa ctacggcact gctgccgact tgaagagctt ggttgccgct 360

gcccatgcca agggcatcta catgatggtc gacgttgtcg caaaccacat gggtcctgga 420

gcaatcacaa acaaccgccc tgaacctctc aaccaggctt catcatacca ccctccttgc 480

aacatcgact acaacaacca aaccagtgtc gaggtatgtc aaatagccgg actccccgac 540

atctacacca ccaagagcga gatccgcacg ctcctcaaca cctgggtcaa ctggctcgta 600

aacgagtaca gcttcgacgg tgtccgcatc gacaccgtca agcacgtcga aaaggacttt 660

tggcctggct tctctgccgc taccggtgtc tacaacattg gcgaggtgtt tgacggagac 720

ccagcctacc ttgccccgta cgccaagctt atgcccggcc tcctcaacta cgcagtctac 780

tacccgatga acaactttta ccagcaaacg ggctcttccc aggcgcttgt agacatgatg 840

aacactgtca gcaacacctt ccctgaccca tctgccttgg gaaccttcct cgacaaccac 900

gacaacaagc gctggttgaa cgtcaagaac gaccagactc tgctcaagaa cgctcttgct 960

tatgtcatcc tcgcacgtgg tatccccatc ttgtactatg gtaccgagca gggatacgct 1020

ggtggtgacg acccagctaa ccgagaggat ctgtggcgca gtggcttcaa caccaatgcc 1080

aacctctacc aagccatcaa gaaactgacc gccgcccgac aggctgccgg tggtctcgca 1140

ggaaacgacc acgtccacct gtacgtcgcc gacacggctt acgcctggag ccgtgccaac 1200

ggcaacctga ttgtcctcac caccaacgct ggcggcaact ccaacaccca gcactgcttc 1260

aacacgcaaa aggcaaacgg ccgctggacc aacgtctacg gcaacggcgc caccgtctct 1320

gccgatagca acggccaaat ctgcgtctcc gtcacaaacg gcgagcccgt tgtcctcctc 1380

gccggctccg ctacccccac cactggcact accctctcca cccgcaccgc cactgccacc 1440

gccacaccaa ccgcatgccc caccgccgtc tccgtctcct tcacccaccg cgtcaccact 1500

gttcccggtg acaccatcaa aatcactggc aacacggccc agctaggtaa ctggactccc 1560

gccaacggcc ttgccttgtc cgcagctagc tacacatcca gcaaccctat ctggaccatt 1620

accgtgcccc tggccgctgg atcctccatc tcgtacaagt ttgtcaagat tgacagtgga 1680

ggaactgtca cctgggagag tgaccccaac aggtcataca ctgcgccgag ctgccaggcg 1740

agtgccagtg tgaacagctc atggcaatag 1770

<210> 4

<211> 589
<212> PRT
<213> Cochliobolus heterostrophus ATCC 48331

<220>
<221> SIGNAL
<222> (1)...(20)

<220>
<221> DOMAIN
<222> (33)...(373)
<223> Alpha amylase, catalytic domain

<220>
<221> DOMAIN
<222> (489)...(586)
<223> Starch binding domain

<220>
<221> SITE
<222> (42)...(45)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (168)...(171)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (578)...(581)
<223> N-glycosylation site. Prosite id = PS00001

<400> 4

```
Met Leu Leu Leu Asn Ile Phe Thr Thr Leu Phe Phe Tyr Ile Thr Cys
1               5                  10                  15

Ile Val Ser Ala Ala Asp Thr Asn Ala Trp Lys Ser Arg Ser Ile Tyr
            20                  25                  30

Phe Val Leu Thr Asp Arg Ile Ala Arg Asn Ser Ser Asp Thr Gly Gly
        35                  40                  45

Ser Ala Cys Ser Asp Leu Gly Asn Tyr Cys Gly Gly Thr Phe Gln Gly
    50                  55                  60

Leu Glu Ser Lys Leu Asp Tyr Ile Lys Gly Leu Gly Phe Asp Ala Ile
65                  70                  75                  80

Trp Ile Thr Pro Val Val Ser Asn Lys Ala Ala Gly Tyr His Gly Tyr
                85                  90                  95

Trp Ala Glu Asp Leu Tyr Ala Val Asn Ser Asn Tyr Gly Thr Ala Ala
            100                 105                 110
```

```
Asp Leu Lys Ser Leu Val Ala Ala Ala His Ala Lys Gly Ile Tyr Met
        115                 120                 125

Met Val Asp Val Val Ala Asn His Met Gly Pro Gly Ala Ile Thr Asn
    130                 135                 140

Asn Arg Pro Glu Pro Leu Asn Gln Ala Ser Ser Tyr His Pro Pro Cys
145                 150                 155                 160

Asn Ile Asp Tyr Asn Asn Gln Thr Ser Val Glu Val Cys Gln Ile Ala
                165                 170                 175

Gly Leu Pro Asp Ile Tyr Thr Thr Lys Ser Glu Ile Arg Thr Leu Leu
            180                 185                 190

Asn Thr Trp Val Asn Trp Leu Val Asn Glu Tyr Ser Phe Asp Gly Val
        195                 200                 205

Arg Ile Asp Thr Val Lys His Val Glu Lys Asp Phe Trp Pro Gly Phe
    210                 215                 220

Ser Ala Ala Thr Gly Val Tyr Asn Ile Gly Glu Val Phe Asp Gly Asp
225                 230                 235                 240

Pro Ala Tyr Leu Ala Pro Tyr Ala Lys Leu Met Pro Gly Leu Leu Asn
                245                 250                 255

Tyr Ala Val Tyr Tyr Pro Met Asn Asn Phe Tyr Gln Gln Thr Gly Ser
            260                 265                 270

Ser Gln Ala Leu Val Asp Met Met Asn Thr Val Ser Asn Thr Phe Pro
        275                 280                 285

Asp Pro Ser Ala Leu Gly Thr Phe Leu Asp Asn His Asp Asn Lys Arg
    290                 295                 300

Trp Leu Asn Val Lys Asn Asp Gln Thr Leu Leu Lys Asn Ala Leu Ala
305                 310                 315                 320

Tyr Val Ile Leu Ala Arg Gly Ile Pro Ile Leu Tyr Tyr Gly Thr Glu
                325                 330                 335

Gln Gly Tyr Ala Gly Gly Asp Asp Pro Ala Asn Arg Glu Asp Leu Trp
            340                 345                 350

Arg Ser Gly Phe Asn Thr Asn Ala Asn Leu Tyr Gln Ala Ile Lys Lys
        355                 360                 365
```

```
Leu Thr Ala Ala Arg Gln Ala Ala Gly Gly Leu Ala Gly Asn Asp His
370                 375                 380

Val His Leu Tyr Val Ala Asp Thr Ala Tyr Ala Trp Ser Arg Ala Asn
385                 390                 395                 400

Gly Asn Leu Ile Val Leu Thr Thr Asn Ala Gly Gly Asn Ser Asn Thr
                405                 410                 415

Gln His Cys Phe Asn Thr Gln Lys Ala Asn Gly Arg Trp Thr Asn Val
            420                 425                 430

Tyr Gly Asn Gly Ala Thr Val Ser Ala Asp Ser Asn Gly Gln Ile Cys
        435                 440                 445

Val Ser Val Thr Asn Gly Glu Pro Val Val Leu Leu Ala Gly Ser Ala
    450                 455                 460

Thr Pro Thr Thr Gly Thr Thr Leu Ser Thr Arg Thr Ala Thr Ala Thr
465                 470                 475                 480

Ala Thr Pro Thr Ala Cys Pro Thr Ala Val Ser Val Ser Phe Thr His
                485                 490                 495

Arg Val Thr Thr Val Pro Gly Asp Thr Ile Lys Ile Thr Gly Asn Thr
            500                 505                 510

Ala Gln Leu Gly Asn Trp Thr Pro Ala Asn Gly Leu Ala Leu Ser Ala
        515                 520                 525

Ala Ser Tyr Thr Ser Ser Asn Pro Ile Trp Thr Ile Thr Val Pro Leu
    530                 535                 540

Ala Ala Gly Ser Ser Ile Ser Tyr Lys Phe Val Lys Ile Asp Ser Gly
545                 550                 555                 560

Gly Thr Val Thr Trp Glu Ser Asp Pro Asn Arg Ser Tyr Thr Ala Pro
                565                 570                 575

Ser Cys Gln Ala Ser Ala Ser Val Asn Ser Ser Trp Gln
            580                 585
```

<210> 5
<211> 2541
<212> DNA
<213> Cochliobolus heterostrophus ATCC 48331

<400> 5

gtgaagatct acgacgcggc tgaacaggtc tttcagatcc aggaatccgt ctggcctcgc 60

cctgctgatg acgagggcac cgaccctgaa aagtcggctt tgaccttcac ttggaccgat 120

agcccgtttt cttttgccat taaacgtagg gccaccaatg agacgctctt cgatacttcg 180

gcagcttctc ttgttttcga gacgcagtac cttcgcttaa gaaccgctct accacctttg 240

ccaaacctgt acggtcttgg tgaatcaacg gatgctttcc atctcaacac caccaactat 300

acgcgaactc tttggaatcg agatgcctat ggcacgccac caggatccaa cctctacgga 360

gctcacccga tctactttga tcaccgcggt gagaatggta ctcacggtgt tttcttggct 420

agctctgagg gtatggacat caagattgat gatactgacg gccagttcct tgagtacaac 480

actctcggtg gtgttcttga cttctacttc cttgctggac ccggccctaa ggaagttgca 540

actcaatact cagccctttc tggcttgcct gctatgatgc cttactgggg tttcggctca 600

caccaatgca agtatggcta ccgtgatgtt tgggaggttg ccgaggttgt agcaaactac 660

tctgctgctg atatcccact tgagaccatg tggaccgata tcgactacat ggagcttcgc 720

cgcttgttca ccctggatcc cgagcgttac ccgcttgagc ttgttcgcca gcttgtagac 780

tatttgcatg ctcaccaaca gcactacatt ctgatggtca actccgctgt ctggagcggt 840

gactatgatg cctacaacaa cggtgcaaag cttgaagtct tccagaagaa gagcaacggc 900

tccttcgaac agggtgctgt ctggccaggc cctactgtct ttccagattg gttccatccc 960

aacacccaga aatactggga tgaggaattc gctcgcttct tcgaccccgc tactggtgtt 1020

gatatcgatg gactttggaa tgacatgaat gaacccgcca acttctgccc ataccctgc 1080

tcagaccctg aagcctattc tgaggagtcc aagaacccac ccgagccacc ggcggtgcgc 1140

acttctgcag gtcgccaaat ccctggcttc ccagcaggtt tccagccaca gtctaactct 1200

agcactgcaa ggcgttctgt tgtaaaggga ccatctagca tgcgtccttc caagcgccaa 1260

gcgcctaaca gcgctggaga tgctaagcac ctcggtcttc ccggtcgtga tctgatcaac 1320

cccaagtacc agatccacaa cgaagccggt tcaatcagca acaggaccct ggctacggat 1380

atcaagaatt acgatggctc ttatcactac gatacgcaca acttctgggg ctcgatgatg 1440

agcattacct ctcacaagtc tatgcaagct cgccgtcccg aaagacggcc attcattatc 1500

actaggtcat ctttccctgg cctcggttct tatctcggaa agtggcttgg tgacaacgtc 1560

tccgagtggg cacaataccg cttctcgatt gccggcatct tgaacttcaa caccatcttc 1620

cagatcccca tggtcggtcc agatatttgc ggtttcgccg gaaacacgac cgagactctc 1680

tgcgcccgct ggaccactct tggtgctttc tacccgttca tgaggaacca cgccggcgac 1740

acttccatca gccaagaata ctatcgctgg cctctcacca gggccgcagc caagaacgcc 1800

atcgcagtca ggtacaggct cttggactac ttctacacgg ccttccaccg ccaggccacc 1860

accggtctac ccagcttgaa ccccctcttc ttccactacc ccaccgacgc caaaaccttc 1920

ggcattgagc accagttctt ctacggagac agcatcctcg tctcgcccgt cctcgaagaa 1980

aactccacct cagtctccat ctacctcccc aaagatgtct tctacgacta ctggaccggc 2040

gagcgcatcc aaggaaacgg cgagaacatt aacctcactg acgtaggatt cgacaccatc 2100

cccctccacg tcaaaggtgg atccatcctc cctctccgcg ccgaatccgc aaacacaacc 2160

accgagctcc gcaaacaaaa ctttgtcctc tggatcgcac caaatgctac caaccaagcc 2220

tctggctcgc tctacctcga tgatggagat tccctcgagc agaagtctac ttcgctcatt 2280

aacttctcct tcaacaacgg cgccttcagc atgagcggcg atttcggatt cgagactgag 2340

cttgtcattc agaatatcac catcctgggt accteggcaga gcgtacaggg ccctgttgcg 2400

cttaccaagg gctgggaaca taactttggg gctggtgctg gtatgccgca gtttgatagt 2460

ggtgcagccg agtcgcgtcg agttgttgct gcagtttggg gtttggttgc gggggctgtc 2520

agtgtttggt tcagcttgta a 2541

<210> 6
<211> 846
<212> PRT
<213> Cochliobolus heterostrophus ATCC 48331

<220>
<221> DOMAIN
<222> (169)...(710)
<223> Glycosyl hydrolases family 31

<220>
<221> SITE
<222> (53)...(56)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (97)...(100)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (134)...(137)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (222)...(225)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (303)...(306)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (349)...(356)
<223> Glycosyl hydrolases family 31 active site. Prosite id = PS00129

<220>

<221> SITE
<222> (405)...(408)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (554)...(584)
<223> Glycosyl hydrolases family 31 signature 2. Prosite id = PS00707

<220>
<221> SITE
<222> (563)...(566)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (671)...(674)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (701)...(704)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (729)...(732)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (746)...(749)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (772)...(775)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (797)...(800)
<223> N-glycosylation site. Prosite id = PS00001

<400> 6

Met Lys Ile Tyr Asp Ala Ala Glu Gln Val Phe Gln Ile Gln Glu Ser
1 5 10 15

Val Trp Pro Arg Pro Ala Asp Asp Glu Gly Thr Asp Pro Glu Lys Ser
20 25 30

Ala Leu Thr Phe Thr Trp Thr Asp Ser Pro Phe Ser Phe Ala Ile Lys
35 40 45

Arg Arg Ala Thr Asn Glu Thr Leu Phe Asp Thr Ser Ala Ala Ser Leu
50 55 60

Val Phe Glu Thr Gln Tyr Leu Arg Leu Arg Thr Ala Leu Pro Pro Leu
65 70 75 80

Pro Asn Leu Tyr Gly Leu Gly Glu Ser Thr Asp Ala Phe His Leu Asn
85 90 95

Thr Thr Asn Tyr Thr Arg Thr Leu Trp Asn Arg Asp Ala Tyr Gly Thr

100 105 110

Pro Pro Gly Ser Asn Leu Tyr Gly Ala His Pro Ile Tyr Phe Asp His
115 120 125

Arg Gly Glu Asn Gly Thr His Gly Val Phe Leu Ala Ser Ser Glu Gly
130 135 140

Met Asp Ile Lys Ile Asp Asp Thr Asp Gly Gln Phe Leu Glu Tyr Asn
145 150 155 160

Thr Leu Gly Gly Val Leu Asp Phe Tyr Phe Leu Ala Gly Pro Gly Pro
165 170 175

Lys Glu Val Ala Thr Gln Tyr Ser Ala Leu Ser Gly Leu Pro Ala Met
180 185 190

Met Pro Tyr Trp Gly Phe Gly Ser His Gln Cys Lys Tyr Gly Tyr Arg
195 200 205

Asp Val Trp Glu Val Ala Glu Val Val Ala Asn Tyr Ser Ala Ala Asp
210 215 220

Ile Pro Leu Glu Thr Met Trp Thr Asp Ile Asp Tyr Met Glu Leu Arg
225 230 235 240

Arg Leu Phe Thr Leu Asp Pro Glu Arg Tyr Pro Leu Glu Leu Val Arg
245 250 255

Gln Leu Val Asp Tyr Leu His Ala His Gln Gln His Tyr Ile Leu Met
260 265 270

Val Asn Ser Ala Val Trp Ser Gly Asp Tyr Asp Ala Tyr Asn Asn Gly
275 280 285

Ala Lys Leu Glu Val Phe Gln Lys Lys Ser Asn Gly Ser Phe Glu Gln
290 295 300

Gly Ala Val Trp Pro Gly Pro Thr Val Phe Pro Asp Trp Phe His Pro
305 310 315 320

Asn Thr Gln Lys Tyr Trp Asp Glu Glu Phe Ala Arg Phe Phe Asp Pro
325 330 335

Ala Thr Gly Val Asp Ile Asp Gly Leu Trp Asn Asp Met Asn Glu Pro
340 345 350

Ala Asn Phe Cys Pro Tyr Pro Cys Ser Asp Pro Glu Ala Tyr Ser Glu
355 360 365

```
Glu Ser Lys Asn Pro Pro Glu Pro Pro Ala Val Arg Thr Ser Ala Gly
    370                 375                 380

Arg Gln Ile Pro Gly Phe Pro Ala Gly Phe Gln Pro Gln Ser Asn Ser
385                 390                 395                 400

Ser Thr Ala Arg Arg Ser Val Val Lys Gly Pro Ser Ser Met Arg Pro
                405                 410                 415

Ser Lys Arg Gln Ala Pro Asn Ser Ala Gly Asp Ala Lys His Leu Gly
            420                 425                 430

Leu Pro Gly Arg Asp Leu Ile Asn Pro Lys Tyr Gln Ile His Asn Glu
        435                 440                 445

Ala Gly Ser Ile Ser Asn Arg Thr Leu Ala Thr Asp Ile Lys Asn Tyr
    450                 455                 460

Asp Gly Ser Tyr His Tyr Asp Thr His Asn Phe Trp Gly Ser Met Met
465                 470                 475                 480

Ser Ile Thr Ser His Lys Ser Met Gln Ala Arg Arg Pro Glu Arg Arg
                485                 490                 495

Pro Phe Ile Ile Thr Arg Ser Ser Phe Pro Gly Leu Gly Ser Tyr Leu
            500                 505                 510

Gly Lys Trp Leu Gly Asp Asn Val Ser Glu Trp Ala Gln Tyr Arg Phe
        515                 520                 525

Ser Ile Ala Gly Ile Leu Asn Phe Asn Thr Ile Phe Gln Ile Pro Met
    530                 535                 540

Val Gly Pro Asp Ile Cys Gly Phe Ala Gly Asn Thr Thr Glu Thr Leu
545                 550                 555                 560

Cys Ala Arg Trp Thr Thr Leu Gly Ala Phe Tyr Pro Phe Met Arg Asn
                565                 570                 575

His Ala Gly Asp Thr Ser Ile Ser Gln Glu Tyr Tyr Arg Trp Pro Leu
            580                 585                 590

Thr Arg Ala Ala Ala Lys Asn Ala Ile Ala Val Arg Tyr Arg Leu Leu
        595                 600                 605

Asp Tyr Phe Tyr Thr Ala Phe His Arg Gln Ala Thr Thr Gly Leu Pro
    610                 615                 620
```

```
Ser Leu Asn Pro Leu Phe Phe His Tyr Pro Thr Asp Ala Lys Thr Phe
625                 630                 635                 640

Gly Ile Glu His Gln Phe Phe Tyr Gly Asp Ser Ile Leu Val Ser Pro
                645                 650                 655

Val Leu Glu Glu Asn Ser Thr Ser Val Ser Ile Tyr Leu Pro Lys Asp
            660                 665                 670

Val Phe Tyr Asp Tyr Trp Thr Gly Glu Arg Ile Gln Gly Asn Gly Glu
        675                 680                 685

Asn Ile Asn Leu Thr Asp Val Gly Phe Asp Thr Ile Pro Leu His Val
    690                 695                 700

Lys Gly Gly Ser Ile Leu Pro Leu Arg Ala Glu Ser Ala Asn Thr Thr
705                 710                 715                 720

Thr Glu Leu Arg Lys Gln Asn Phe Val Leu Trp Ile Ala Pro Asn Ala
                725                 730                 735

Thr Asn Gln Ala Ser Gly Ser Leu Tyr Leu Asp Asp Gly Asp Ser Leu
            740                 745                 750

Glu Gln Lys Ser Thr Ser Leu Ile Asn Phe Ser Phe Asn Asn Gly Ala
        755                 760                 765

Phe Ser Met Ser Gly Asp Phe Gly Phe Glu Thr Glu Leu Val Ile Gln
    770                 775                 780

Asn Ile Thr Ile Leu Gly Thr Ser Gln Ser Val Gln Gly Pro Val Ala
785                 790                 795                 800

Leu Thr Lys Gly Trp Glu His Asn Phe Gly Ala Gly Ala Gly Met Pro
                805                 810                 815

Gln Phe Asp Ser Gly Ala Ala Glu Ser Arg Arg Val Val Ala Ala Val
            820                 825                 830

Trp Gly Leu Val Ala Gly Ala Val Ser Val Trp Phe Ser Leu
        835                 840                 845
```

<210> 7
<211> 1572
<212> DNA
<213> Cochliobolus heterostrophus ATCC 48331

<400> 7

atgctccctc gcgtgttcct cctcacaggc ttcgtccacc atgcctacac attatcaata 60

cctcgtcttc cctccctcgc cacttttgct gcttccagcc gtgtgcagca acagcaaccg 120

cttcaagaca cacttgatgc ctggataaag catgaagagc gtattgcact cgataaactg 180

ctcgccaaca tcgcgcctgg aggtagcaat gtccaaggaa aaggtgtggc tgagggcacc 240

gtcatcgcca gccctagtca agacggtccg gactactggt tccaatgggt ccgtgacgct 300

gctatcacca tggataccct cgtcaacatc tatgccgatg acccctcgtc ctcgcgtgcg 360

tcttctctat ccaccatcct agacgcatac acctccctcc aaggtgacat tcagcgcact 420

tcaaacccgt ctggcacatt tgacgacctt tccggactag gtgagcccaa gttccaagtc 480

gatggcaagc catttaccgg ctcgtgggga cgacctcagc gcgatgggcc ggcccttcgc 540

gcactgacgc ttatgcatta tctccgcgag tacaatgcat cccatccctc actatggagc 600

tctcccaact cggaagactt ttttggctcg ttctacaccg ctgaaatgcc ccctcgtagc 660

atcatcaaag cagatctcga gtatgtcagc catttctgga accagtcagg gttcgatctc 720

tgggaagagg tcgagggcct gcacttcttc accctcatgg tcagcgcgag gagtctgagg 780

gaaggcagtc acctggcaag agtctttgga gacgtcggtg cggcggattg gtaccaaaag 840

caagctggct acattgagaa cctgttgggc aaattctgga atgcgcaaaa aggacatctt 900

gttgagacgc tttggagcaa gaggagtggc ctcgactgcg gactgcttct cggctctttg 960

cacgcccttc ctaagagcgg atctgaagat gatgatgttg tgtaccctcc gtggtctgac 1020

gagattcttg tttctctgct tgctttgact cgagaccagc gcgaccgttt ccccatcaac 1080

agcaatcctt ctggccagga tgacgacgat gacgacgtag acgagtccac attccaggga 1140

acaggccttg gtcgttatcc cgaggatgta tacgacggct atggaaactc gaaccgtggt 1200

ggaaacccat ggttcctttg cacctcttct gccgccgaga ttctttaccg caccgcctcg 1260

cacatctctg caactggcaa cttgactctt accgatgttg gccttccctt ttacgagtct 1320

ctcctcggta gctcctccct agatgtcgac gttggcacgt ttggccccac agacgccctt 1380

ttccactccg tcatcgagcg ccttcagagc accggcgacg agtttctgca agtcgtcaag 1440

acgcatgtgg atgccgaggg cagcatgagt gagcagtttg accgtgtaac tgggtacatg 1500

cgtggtgccc agcatttgac ttggagttat ggtgcctttt tgcaggctgc tagagcaaga 1560

aagatttctg tg 1572

<210> 8
<211> 524
<212> PRT
<213> Cochliobolus heterostrophus ATCC 48331

<220>
<221> DOMAIN

<222> (54)...(518)
<223> Glycosyl hydrolases family 15

<220>
<221> SITE
<222> (194)...(197)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (237)...(240)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (239)...(249)
<223> Glucoamylase active site region signature. Prosite id = PS00820

<220>
<221> SITE
<222> (433)...(436)
<223> N-glycosylation site. Prosite id = PS00001

<400> 8

```
Met Leu Pro Arg Val Phe Leu Leu Thr Gly Phe Val His His Ala Tyr
1               5                  10                  15

Thr Leu Ser Ile Pro Arg Leu Pro Ser Leu Ala Thr Phe Ala Ala Ser
            20                  25                  30

Ser Arg Val Gln Gln Gln Gln Pro Leu Gln Asp Thr Leu Asp Ala Trp
        35                  40                  45

Ile Lys His Glu Glu Arg Ile Ala Leu Asp Lys Leu Leu Ala Asn Ile
    50                  55                  60

Ala Pro Gly Gly Ser Asn Val Gln Gly Lys Gly Val Ala Glu Gly Thr
65                  70                  75                  80

Val Ile Ala Ser Pro Ser Gln Asp Gly Pro Asp Tyr Trp Phe Gln Trp
                85                  90                  95

Val Arg Asp Ala Ala Ile Thr Met Asp Thr Leu Val Asn Ile Tyr Ala
            100                 105                 110

Asp Asp Pro Ser Ser Ser Arg Ala Ser Ser Leu Ser Thr Ile Leu Asp
        115                 120                 125

Ala Tyr Thr Ser Leu Gln Gly Asp Ile Gln Arg Thr Ser Asn Pro Ser
    130                 135                 140

Gly Thr Phe Asp Asp Leu Ser Gly Leu Gly Glu Pro Lys Phe Gln Val
145                 150                 155                 160

Asp Gly Lys Pro Phe Thr Gly Ser Trp Gly Arg Pro Gln Arg Asp Gly
                165                 170                 175

Pro Ala Leu Arg Ala Leu Thr Leu Met His Tyr Leu Arg Glu Tyr Asn
            180                 185                 190
```

```
Ala Ser His Pro Ser Leu Trp Ser Ser Pro Asn Ser Glu Asp Phe Phe
        195                 200                 205

Gly Ser Phe Tyr Thr Ala Glu Met Pro Pro Arg Ser Ile Ile Lys Ala
    210                 215                 220

Asp Leu Glu Tyr Val Ser His Phe Trp Asn Gln Ser Gly Phe Asp Leu
225                 230                 235                 240

Trp Glu Glu Val Glu Gly Leu His Phe Phe Thr Leu Met Val Ser Ala
                245                 250                 255

Arg Ser Leu Arg Glu Gly Ser His Leu Ala Arg Val Phe Gly Asp Val
            260                 265                 270

Gly Ala Ala Asp Trp Tyr Gln Lys Gln Ala Gly Tyr Ile Glu Asn Leu
        275                 280                 285

Leu Gly Lys Phe Trp Asn Ala Gln Lys Gly His Leu Val Glu Thr Leu
    290                 295                 300

Trp Ser Lys Arg Ser Gly Leu Asp Cys Gly Leu Leu Leu Gly Ser Leu
305                 310                 315                 320

His Ala Leu Pro Lys Ser Gly Ser Glu Asp Asp Asp Val Val Tyr Pro
                325                 330                 335

Pro Trp Ser Asp Glu Ile Leu Val Ser Leu Leu Ala Leu Thr Arg Asp
            340                 345                 350

Gln Arg Asp Arg Phe Pro Ile Asn Ser Asn Pro Ser Gly Gln Asp Asp
        355                 360                 365

Asp Asp Asp Asp Val Asp Glu Ser Thr Phe Gln Gly Thr Gly Leu Gly
    370                 375                 380

Arg Tyr Pro Glu Asp Val Tyr Asp Gly Tyr Gly Asn Ser Asn Arg Gly
385                 390                 395                 400

Gly Asn Pro Trp Phe Leu Cys Thr Ser Ser Ala Ala Glu Ile Leu Tyr
                405                 410                 415

Arg Thr Ala Ser His Ile Ser Ala Thr Gly Asn Leu Thr Leu Thr Asp
            420                 425                 430

Val Gly Leu Pro Phe Tyr Glu Ser Leu Leu Gly Ser Ser Ser Leu Asp
        435                 440                 445
```

Val Asp Val Gly Thr Phe Gly Pro Thr Asp Ala Leu Phe His Ser Val
450 455 460

Ile Glu Arg Leu Gln Ser Thr Gly Asp Glu Phe Leu Gln Val Val Lys
465 470 475 480

Thr His Val Asp Ala Glu Gly Ser Met Ser Glu Gln Phe Asp Arg Val
485 490 495

Thr Gly Tyr Met Arg Gly Ala Gln His Leu Thr Trp Ser Tyr Gly Ala
500 505 510

Phe Leu Gln Ala Ala Arg Ala Arg Lys Ile Ser Val
515 520

<210> 9
<211> 1467
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 9

atgttgacat tgaatgtttt gacggcactg ttggcacctg gtgtgctatc atctgccttg 60

cctgctcgcg atctcaatgt gcgtgccgat tcaaagcctc ctgctcttca agccatcctg 120

aataacattg gcgccgatgg atctgctgtt tcgggagcat ctgccggcgt tgtggtagca 180

tccccatcta aatcggatcc cgattacttt tacacctgga cgcgtgatgc agccttgaca 240

tacaaggtct tgattgatga gctcattgcc ggagacacct cgctggaatc taccatccag 300

gactacatct ctgctcaagc aaagctgcag gccgtgtcta acccatctgg tgacttgtcc 360

gatggatcag gtcttgcaga gcccaagtac cacgtggact tgactgcctt cacggaggcc 420

tggggtcgtc ctcagcgcga tggacccgct ctgcgagcga cagctctgat tacctatggt 480

aactatttga tttcgaagga gaagacatct gtcgtcaagt cgaacatctg gcccattgtg 540

caaaatgatc tgaactatgt tgcgcaatac tggaaccaaa ccggattcga tctgtgggag 600

gaggtccagg gctcctcatt cttcaccatt gctgcacaac accgcgcatt ggtggaaggc 660

agcgcatttg ccaaggcgct gggagagtcc tgcgagggat gcgattccca ggcacctcag 720

gtcttatgct tccagcaatc tttctggaat ggcaaggctg ttgtttccaa ctttgccaac 780

aatggtcgaa ccggtcttga tgccaattct gtacttactt cgattgtaaa ctttgacccc 840

aaagctccat gcgatgacgt taccttccag ccttgctctg ctcgtgccct gtcgaaccac 900

aagctatacg ttgactcgtt ccgtaagatc tatcccgtga acagcggcaa ggaagctgga 960

actgccgttg ctgttggacg ttatgccgag gacacttaca tgggcggcaa cccatggtac 1020

ttgaccaccc tggccgcggc cgagcagttg tatgatgctc tttaccagtg gaagcaactg 1080

ggatcgctgg aaatcaccga ggccagtctt cctttcttca aggatcttgt ttcgtctgcc 1140

gccgctggga agtaccctag ctcctcggaa acttacacgt ccatcactgc cgcagtcaag 1200

aaatatgccg atggatttat ggctgttgtt aaggagcaca cgcctagcga tggatctcta 1260

tccgaacaat tcactcggga caacggcagc ccggcctctg ctaaggactt gacctggtcc 1320

tacgcggcgt tgctgtctgc tactcgacgt gaggctggaa cagtgccccc tagctggggc 1380

gcgtcgactg ccaataaagt gccctcaaag tgtgagggaa gctcggccaa gggaagctac 1440

acgactccat cggtcggcaa gtggtaa 1467

<210> 10
<211> 488
<212> PRT
<213> Unknown

<220>
<223> Obtained from an environmental sample

<220>
<221> SIGNAL

<222> (1)...(22)

<220>
<221> DOMAIN
<222> (33)...(449)
<223> Glycosyl hydrolases family 15

<220>
<221> SITE
<222> (194)...(197)
<223> N-glycosylation site. Prosite id = PS00001

<400> 10

```
Met Leu Thr Leu Asn Val Leu Thr Ala Leu Leu Ala Pro Gly Val Leu
1               5                  10                  15

Ser Ser Ala Leu Pro Ala Arg Asp Leu Asn Val Arg Ala Asp Ser Lys
            20                  25                  30

Pro Pro Ala Leu Gln Ala Ile Leu Asn Asn Ile Gly Ala Asp Gly Ser
        35                  40                  45

Ala Val Ser Gly Ala Ser Ala Gly Val Val Val Ala Ser Pro Ser Lys
    50                  55                  60

Ser Asp Pro Asp Tyr Phe Tyr Thr Trp Thr Arg Asp Ala Ala Leu Thr
65                  70                  75                  80

Tyr Lys Val Leu Ile Asp Glu Leu Ile Ala Gly Asp Thr Ser Leu Glu
                85                  90                  95

Ser Thr Ile Gln Asp Tyr Ile Ser Ala Gln Ala Lys Leu Gln Ala Val
            100                 105                 110
```

```
Ser Asn Pro Ser Gly Asp Leu Ser Asp Gly Ser Gly Leu Ala Glu Pro
        115                 120                 125

Lys Tyr His Val Asp Leu Thr Ala Phe Thr Glu Ala Trp Gly Arg Pro
    130                 135                 140

Gln Arg Asp Gly Pro Ala Leu Arg Ala Thr Ala Leu Ile Thr Tyr Gly
145                 150                 155                 160

Asn Tyr Leu Ile Ser Lys Glu Lys Thr Ser Val Val Lys Ser Asn Ile
                165                 170                 175

Trp Pro Ile Val Gln Asn Asp Leu Asn Tyr Val Ala Gln Tyr Trp Asn
            180                 185                 190

Gln Thr Gly Phe Asp Leu Trp Glu Glu Val Gln Gly Ser Ser Phe Phe
        195                 200                 205

Thr Ile Ala Ala Gln His Arg Ala Leu Val Glu Gly Ser Ala Phe Ala
    210                 215                 220

Lys Ala Leu Gly Glu Ser Cys Glu Gly Cys Asp Ser Gln Ala Pro Gln
225                 230                 235                 240

Val Leu Cys Phe Gln Gln Ser Phe Trp Asn Gly Lys Ala Val Val Ser
                245                 250                 255

Asn Phe Ala Asn Asn Gly Arg Thr Gly Leu Asp Ala Asn Ser Val Leu
            260                 265                 270

Thr Ser Ile Val Asn Phe Asp Pro Lys Ala Pro Cys Asp Asp Val Thr
        275                 280                 285

Phe Gln Pro Cys Ser Ala Arg Ala Leu Ser Asn His Lys Leu Tyr Val
    290                 295                 300

Asp Ser Phe Arg Lys Ile Tyr Pro Val Asn Ser Gly Lys Glu Ala Gly
305                 310                 315                 320

Thr Ala Val Ala Val Gly Arg Tyr Ala Glu Asp Thr Tyr Met Gly Gly
                325                 330                 335

Asn Pro Trp Tyr Leu Thr Thr Leu Ala Ala Ala Glu Gln Leu Tyr Asp
            340                 345                 350

Ala Leu Tyr Gln Trp Lys Gln Leu Gly Ser Leu Glu Ile Thr Glu Ala
        355                 360                 365

Ser Leu Pro Phe Phe Lys Asp Leu Val Ser Ser Ala Ala Ala Gly Lys
```

370 375 380

Tyr Pro Ser Ser Ser Glu Thr Tyr Thr Ser Ile Thr Ala Ala Val Lys
385 390 395 400

Lys Tyr Ala Asp Gly Phe Met Ala Val Val Lys Glu His Thr Pro Ser
405 410 415

Asp Gly Ser Leu Ser Glu Gln Phe Thr Arg Asp Asn Gly Ser Pro Ala
420 425 430

Ser Ala Lys Asp Leu Thr Trp Ser Tyr Ala Ala Leu Leu Ser Ala Thr
435 440 445

Arg Arg Glu Ala Gly Thr Val Pro Pro Ser Trp Gly Ala Ser Thr Ala
450 455 460

Asn Lys Val Pro Ser Lys Cys Glu Gly Ser Ser Ala Lys Gly Ser Tyr
465 470 475 480

Thr Thr Pro Ser Val Gly Lys Trp
485

<210> 11
<211> 1746
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 11

atgtttaatc aagttctcta tggcttggct gccactgcac tatggcaggg ccaagtcgtc 60

gcttcaccaa gcaaagacaa ctccctcgag aagttcatca ccaagcaagc cgatatttcc 120

atcaaaggtg tcctcgccaa tatcggctct gatggtaaac gtgcacaagg tgcagcgcct 180

ggtgccgtcg tcgcaagccc atcacgagaa gaccctgact attggtacac ttggacgcgt 240

gactcagcct tgacttataa ggtcctcgtc gagcgtctca tccacggcga aaagtctctc 300

cagcggaaga tagacgaata cgtctccgct caagccaaac ttcagaaaac cacaaaccca 360

tccggtagtc cagagtccgg tggtctcggc gagccaaagt tccacgtgaa cctcactgcc 420

ttcaccggat cttggggccg cccacagcgc gatggtcctc ctctgcgtgc tacagccttg 480

accctatacg cagagtggct catctcccac ggagacaagt ccaaggctgt gaacaaagtg 540

tggccagtga ttgagaagga tcttgcgtat actaccaagt tctggaatcg cactgggtat 600

gatctctggg aagaagtcaa tggatcttct ttctttacgc tatcggcttc gcatcgggct 660

ctcattgagg gagcggcttt ggcgaagaag ttgggcaagt cttgccctga ctgtgctgcc 720

aatgcccctc gtgtcctttg cttcatgcag agcttttgga ctggtggtta cattgactcg 780

aacatcaatg tgaaggatgg tcgcaagggc ttggatgcta actccattct gtcatcgatc 840

cacacatttg atcccaactc caaatgcacc gactcgacat tccagccttg ctcctcacga 900

gcgctcgcca accacaaggc ggtggtcgac tctttcaggt ccatctacat tgtcaacaag 960

aacagaggca aaggtaaagc cgcagctgtt ggcagataca gtgaggatgt gtactacaac 1020

ggcaaccctt ggtacttgac taccctcgcc gctgctgagc aattgtacgc cgcgctgtac 1080

caatggaaca aggttggggc tgtctcaatt gacgacgttt ctttaccttt cttccgggac 1140

cttgtgccta agggatccaa gggcacgtat aagaagaaca gcaagacgta caaggagatc 1200

gtcaaggctg tcaaggccta cgctgacggg tttgtcgctg ttgttcaaac ctacacaccc 1260

aaagatggtt cgctagccga gcagttcgac cgagccactg gtactcccaa atcggcggtc 1320

catctcactt ggtcttacgc ctccttcgtc agcgcgaccg aacgtcgctc cagcatcgtc 1380

tctccctctt ggggcgagag cagcgccaat aaggttcctg cagtatgcga agcagccccg 1440

gcttgtgaca cgacgataac cttcaacgtg aagaatgtgg aagtgtcttc tgaccagaag 1500

gtttacgtcg ttggctccgt gactgaactt tccaactggt cacctgatga gggcatacca 1560

ctcacggagg gtaccaaggg gttgtggagt gccaaggtta agattccttc tgatacaagc 1620

tttgagtata agtacatcaa gaagacaagt ggcggagatg ttacgtggtt gagcgacccg 1680

aacaatcggg ctgttacggg cagtaagtgt ggaagcgcga gtactcttga taatgagtgg 1740

aggtag 1746

<210> 12

<211> 581
<212> PRT
<213> Unknown

<220>
<223> Obtained from an environmental sample

<220>
<221> SIGNAL
<222> (1)...(21)

<220>
<221> DOMAIN
<222> (38)...(454)
<223> Glycosyl hydrolases family 15

<220>
<221> DOMAIN
<222> (483)...(576)
<223> Starch binding domain

<220>
<221> SITE
<222> (139)...(142)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (199)...(202)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (201)...(211)
<223> Glucoamylase active site region signature. Prosite id = PS00820

<220>
<221> SITE
<222> (210)...(213)
<223> N-glycosylation site. Prosite id = PS00001

<400> 12

```
Met Phe Asn Gln Val Leu Tyr Gly Leu Ala Ala Thr Ala Leu Trp Gln
1               5                  10                  15

Gly Gln Val Val Ala Ser Pro Ser Lys Asp Asn Ser Leu Glu Lys Phe
            20                  25                  30

Ile Thr Lys Gln Ala Asp Ile Ser Ile Lys Gly Val Leu Ala Asn Ile
        35                  40                  45

Gly Ser Asp Gly Lys Arg Ala Gln Gly Ala Ala Pro Gly Ala Val Val
    50                  55                  60

Ala Ser Pro Ser Arg Glu Asp Pro Asp Tyr Trp Tyr Thr Trp Thr Arg
65                  70                  75                  80

Asp Ser Ala Leu Thr Tyr Lys Val Leu Val Glu Arg Leu Ile His Gly
                85                  90                  95

Glu Lys Ser Leu Gln Arg Lys Ile Asp Glu Tyr Val Ser Ala Gln Ala
            100                 105                 110

Lys Leu Gln Lys Thr Thr Asn Pro Ser Gly Ser Pro Glu Ser Gly Gly
        115                 120                 125

Leu Gly Glu Pro Lys Phe His Val Asn Leu Thr Ala Phe Thr Gly Ser
    130                 135                 140

Trp Gly Arg Pro Gln Arg Asp Gly Pro Pro Leu Arg Ala Thr Ala Leu
145                 150                 155                 160

Thr Leu Tyr Ala Glu Trp Leu Ile Ser His Gly Asp Lys Ser Lys Ala
                165                 170                 175

Val Asn Lys Val Trp Pro Val Ile Glu Lys Asp Leu Ala Tyr Thr Thr
            180                 185                 190

Lys Phe Trp Asn Arg Thr Gly Tyr Asp Leu Trp Glu Glu Val Asn Gly
        195                 200                 205
```

```
Ser Ser Phe Phe Thr Leu Ser Ala Ser His Arg Ala Leu Ile Glu Gly
    210                 215                 220

Ala Ala Leu Ala Lys Lys Leu Gly Lys Ser Cys Pro Asp Cys Ala Ala
225                 230                 235                 240

Asn Ala Pro Arg Val Leu Cys Phe Met Gln Ser Phe Trp Thr Gly Gly
                245                 250                 255

Tyr Ile Asp Ser Asn Ile Asn Val Lys Asp Gly Arg Lys Gly Leu Asp
            260                 265                 270

Ala Asn Ser Ile Leu Ser Ser Ile His Thr Phe Asp Pro Asn Ser Lys
        275                 280                 285

Cys Thr Asp Ser Thr Phe Gln Pro Cys Ser Ser Arg Ala Leu Ala Asn
    290                 295                 300

His Lys Ala Val Val Asp Ser Phe Arg Ser Ile Tyr Ile Val Asn Lys
305                 310                 315                 320

Asn Arg Gly Lys Gly Lys Ala Ala Ala Val Gly Arg Tyr Ser Glu Asp
                325                 330                 335

Val Tyr Tyr Asn Gly Asn Pro Trp Tyr Leu Thr Thr Leu Ala Ala Ala
            340                 345                 350

Glu Gln Leu Tyr Ala Ala Leu Tyr Gln Trp Asn Lys Val Gly Ala Val
        355                 360                 365

Ser Ile Asp Asp Val Ser Leu Pro Phe Phe Arg Asp Leu Val Pro Lys
    370                 375                 380

Gly Ser Lys Gly Thr Tyr Lys Lys Asn Ser Lys Thr Tyr Lys Glu Ile
385                 390                 395                 400

Val Lys Ala Val Lys Ala Tyr Ala Asp Gly Phe Val Ala Val Val Gln
                405                 410                 415

Thr Tyr Thr Pro Lys Asp Gly Ser Leu Ala Glu Gln Phe Asp Arg Ala
            420                 425                 430

Thr Gly Thr Pro Lys Ser Ala Val His Leu Thr Trp Ser Tyr Ala Ser
        435                 440                 445

Phe Val Ser Ala Thr Glu Arg Arg Ser Ser Ile Val Ser Pro Ser Trp
    450                 455                 460

Gly Glu Ser Ser Ala Asn Lys Val Pro Ala Val Cys Glu Ala Ala Pro
```

```
465                 470                 475                 480

Ala Cys Asp Thr Thr Ile Thr Phe Asn Val Lys Asn Val Glu Val Ser
                485                 490                 495

Ser Asp Gln Lys Val Tyr Val Val Gly Ser Val Thr Glu Leu Ser Asn
            500                 505                 510

Trp Ser Pro Asp Glu Gly Ile Pro Leu Thr Glu Gly Thr Lys Gly Leu
        515                 520                 525

Trp Ser Ala Lys Val Lys Ile Pro Ser Asp Thr Ser Phe Glu Tyr Lys
    530                 535                 540

Tyr Ile Lys Lys Thr Ser Gly Gly Asp Val Thr Trp Leu Ser Asp Pro
545                 550                 555                 560

Asn Asn Arg Ala Val Thr Gly Ser Lys Cys Gly Ser Ala Ser Thr Leu
                565                 570                 575

Asp Asn Glu Trp Arg
            580
```

<210> 13
<211> 1749
<212> DNA
<213> Fusarium verticillioides GZ3639

<400> 13

atgtttactc agatcttgta tggcctcacg gccttatctg ccctccaagg tcaggtcact 60

gcatcaccag gcggttccag ccttgatcgc ttcatttcca aagaggccga catctccatc 120

aagggcgtgc ttgccaatat tggcgccgat ggcaagcgag cccaaggcgc tgcacctggc 180

gccgttgtag caagtccatc gagaacagat ccagactact ggtacacatg gacccgagac 240

tccgccttga catacaaagt ccttgttgag cgcttcattc acggagacaa gtctctccag 300

cgcaagatcg acgaatatgt ctctgcccaa gccaagctcc agggcgtcac caacccatct 360

ggcggccccg agtcaggcgg ccttggggaa cccaagtttc acgtcaacct cacagctttc 420

acaggatcct ggggtcgtcc tcaacgagat ggccctcctc tgagagctac tgcattgacg 480

ctgtacgcca actggcttgt ttctcacggc gaccgctcca aggccgtcaa caaggtttgg 540

cctgtcattg agaaggatct tgcatacacc gtcaagttct ggaacagaac cggttacgat 600

ctttgggagg aggttaacgg atcttcgttc ttcaccctct ctgcttcaca tcgtgctctg 660

gttgagggag ctgctcttgc taagaagctt ggcaagtctt gctccgactg cgcaaccaac 720

gccccccgtg ttctctgctt catgcaaagc ttctggaccg gcagctacat cgactcgaac 780

atcaatgtca acgatggccg caagggtctt gatgccaact ccattctgtc ttctattcac 840

acctttgatc cttcttcgaa gtgcacagac tctaccttcc agccttgttc ttcaagggct 900

cttgcgaacc acaaggaagt agtggactct ttccgctcca tctatggtgt caacaaaaac 960

agaggtaaag gtaaagctgc tgctgtcggt cgatacagtg aggatgtgta ctacgacggt 1020

aacccttggt acttggctac tcttgctgct gccgagcaac tgtacgctgc tgtctatcaa 1080

tggaacaaga tcggttccat cacagttgat agtgtgtcgc tccccttttt cagtgacctt 1140

gtaccaaagg tttccaaggg aacctatcgc aagaacagca agacatacaa ggctattatc 1200

aaggctgtca cttcatacgc tgatggcttt gtcgccgttg tgcagaccta tactcccaaa 1260

gatggctccc tcgcagagca gttcgataag tctactggaa ctcccaagtc agctgttcac 1320

ctaacctggt cctacgcctc ctttgtcggt gctgccgagc gtcgtactgg cgtcgttcct 1380

ccagcttggg gcgagagcaa cgccaacaag gtgcctgctg tttgcgaagc agctccagcc 1440

tgcgacacca ccatcacgtt caatgtgaag aacgttgatg tcacgtcgga ccagaaggtt 1500

tacattgttg gcgggatcac tcaactttcc aactgggccc ctgctgacgg cattgcgctt 1560

gaggaatcca cgagcaccaa gggcttgtgg actgtgaagg tcaagattcc atctgatacc 1620

agctttgagt ataagtatat aaagaagacg agtgatggaa ctgttacatg ggagagtgac 1680

cccaataaca gtgcggcgac gggcagcaag tgcggaagca gcagtaccat caacgatgag 1740

tggaggtag 1749

<210> 14
<211> 582

<212> PRT
<213> Fusarium verticillioides GZ3639

<220>
<221> SIGNAL
<222> (1)...(21)

<220>
<221> DOMAIN
<222> (37)...(453)
<223> Glycosyl hydrolases family 15

<220>
<221> DOMAIN
<222> (482)...(577)
<223> Starch binding domain

<220>
<221> SITE
<222> (138)...(141)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (200)...(210)
<223> Glucoamylase active site region signature. Prosite id = PS00820

<220>
<221> SITE
<222> (209)...(212)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (570)...(573)
<223> N-glycosylation site. Prosite id = PS00001

<400> 14

```
Met Phe Thr Gln Ile Leu Tyr Gly Leu Thr Ala Leu Ser Ala Leu Gln
1               5                  10                  15

Gly Gln Val Thr Ala Ser Pro Gly Gly Ser Ser Leu Asp Arg Phe Ile
            20                  25                  30

Ser Lys Glu Ala Asp Ile Ser Ile Lys Gly Val Leu Ala Asn Ile Gly
        35                  40                  45

Ala Asp Gly Lys Arg Ala Gln Gly Ala Ala Pro Gly Ala Val Val Ala
    50                  55                  60

Ser Pro Ser Arg Thr Asp Pro Asp Tyr Trp Tyr Thr Trp Thr Arg Asp
65                  70                  75                  80

Ser Ala Leu Thr Tyr Lys Val Leu Val Glu Arg Phe Ile His Gly Asp
                85                  90                  95

Lys Ser Leu Gln Arg Lys Ile Asp Glu Tyr Val Ser Ala Gln Ala Lys
            100                 105                 110

Leu Gln Gly Val Thr Asn Pro Ser Gly Gly Pro Glu Ser Gly Gly Leu
        115                 120                 125

Gly Glu Pro Lys Phe His Val Asn Leu Thr Ala Phe Thr Gly Ser Trp
    130                 135                 140

Gly Arg Pro Gln Arg Asp Gly Pro Pro Leu Arg Ala Thr Ala Leu Thr
145                 150                 155                 160

Leu Tyr Ala Asn Trp Leu Val Ser His Gly Asp Arg Ser Lys Ala Val
                165                 170                 175

Asn Lys Val Trp Pro Val Ile Glu Lys Asp Leu Ala Tyr Thr Val Lys
            180                 185                 190

Phe Trp Asn Arg Thr Gly Tyr Asp Leu Trp Glu Glu Val Asn Gly Ser
        195                 200                 205

Ser Phe Phe Thr Leu Ser Ala Ser His Arg Ala Leu Val Glu Gly Ala
    210                 215                 220

Ala Leu Ala Lys Lys Leu Gly Lys Ser Cys Ser Asp Cys Ala Thr Asn
225                 230                 235                 240
```

```
Ala Pro Arg Val Leu Cys Phe Met Gln Ser Phe Trp Thr Gly Ser Tyr
                245                 250                 255

Ile Asp Ser Asn Ile Asn Val Asn Asp Gly Arg Lys Gly Leu Asp Ala
            260                 265                 270

Asn Ser Ile Leu Ser Ser Ile His Thr Phe Asp Pro Ser Ser Lys Cys
        275                 280                 285

Thr Asp Ser Thr Phe Gln Pro Cys Ser Ser Arg Ala Leu Ala Asn His
    290                 295                 300

Lys Glu Val Val Asp Ser Phe Arg Ser Ile Tyr Gly Val Asn Lys Asn
305                 310                 315                 320

Arg Gly Lys Gly Lys Ala Ala Ala Val Gly Arg Tyr Ser Glu Asp Val
                325                 330                 335

Tyr Tyr Asp Gly Asn Pro Trp Tyr Leu Ala Thr Leu Ala Ala Ala Glu
            340                 345                 350

Gln Leu Tyr Ala Ala Val Tyr Gln Trp Asn Lys Ile Gly Ser Ile Thr
        355                 360                 365

Val Asp Ser Val Ser Leu Pro Phe Phe Ser Asp Leu Val Pro Lys Val
    370                 375                 380

Ser Lys Gly Thr Tyr Arg Lys Asn Ser Lys Thr Tyr Lys Ala Ile Ile
385                 390                 395                 400

Lys Ala Val Thr Ser Tyr Ala Asp Gly Phe Val Ala Val Val Gln Thr
                405                 410                 415

Tyr Thr Pro Lys Asp Gly Ser Leu Ala Glu Gln Phe Asp Lys Ser Thr
            420                 425                 430

Gly Thr Pro Lys Ser Ala Val His Leu Thr Trp Ser Tyr Ala Ser Phe
        435                 440                 445

Val Gly Ala Ala Glu Arg Arg Thr Gly Val Val Pro Pro Ala Trp Gly
    450                 455                 460

Glu Ser Asn Ala Asn Lys Val Pro Ala Val Cys Glu Ala Ala Pro Ala
465                 470                 475                 480

Cys Asp Thr Thr Ile Thr Phe Asn Val Lys Asn Val Asp Val Thr Ser
                485                 490                 495
```

Asp Gln Lys Val Tyr Ile Val Gly Gly Ile Thr Gln Leu Ser Asn Trp
500 505 510

Ala Pro Ala Asp Gly Ile Ala Leu Glu Glu Ser Thr Ser Thr Lys Gly
515 520 525

Leu Trp Thr Val Lys Val Lys Ile Pro Ser Asp Thr Ser Phe Glu Tyr
530 535 540

Lys Tyr Ile Lys Lys Thr Ser Asp Gly Thr Val Thr Trp Glu Ser Asp
545 550 555 560

Pro Asn Asn Ser Ala Ala Thr Gly Ser Lys Cys Gly Ser Ser Ser Thr
565 570 575

Ile Asn Asp Glu Trp Arg
580

<210> 15
<211> 1188
<212> DNA
<213> Cochliobolus heterostrophus ATCC 48332

<400> 15

atgttgtcca agatcttgct acccgttgtt gcacttgcag ccagtgccaa tgcgcatggc 60

tacctcactt cacccatgag ccgaacaggg ctgaatgcac agtctggggc tgatacgtgc 120

cccgaatgca caattctgga gccggtaaca gcatggcctg atcttgacgc cgcagctgtc 180

ggccgctcgg gtccttgcgg gtacaatgct cgtgtgtcag ttgactacaa ccagcccggg 240

cctcgctggg gttctgagcc cgtcattacc tacaaggctg gggatgtcgt cgatgtacag 300

tggtgtgtag atgccaacgg tgaccatggt ggcatgttca cctaccgcat ctgccaaaac 360

caagctcttg tcgacaagct cttgacccct ggatacctcc ctactgaagc agagaagcaa 420

gcagcagagg attgcttcca agctggagag ttgaagtgca cagatgtccc tggacagaca 480

tgcggattca actctgactg ccagcaaggc caagcctgct ggaggaatga ctggttcaca 540

tgcggtggct tcaacgacaa cctgaaatgc aggagcgtcg acaacgcccc cctaaattca 600

tgctacacca gcatcgcagg cggctacacc gtcagctcaa agatcaaaat cccaaactac 660

accagcaacc acaccctcct ctccttcaaa tggaactctt tccaaacccc gcaggtctac 720

ctcacctgcg ccgacatcaa aatcaccggc agcagctccg gcacctcccc acccccaact 780

tcgagcaaac cccctacgtc ctcctccaag cccacttcca cctccacctc cacctccgca 840

ccctccgcaa ccccccaccgc ctgcgccacc cccgtctcca ccgtcgccgt aaccttcaac 900

tccaaaacca ccacctcctt tggccaaacc gtcaaactcg caggttccat ctcccagctc 960

ggcagctgga acactgccaa tgcgcctgcc ctgtccgccg cccagtacac ctcgtccaac 1020

ccgctctgga caaccaccct caacctacct gcaggcacca gcttcgagta caagtacatc 1080

aaagttgaca gtagtggcgc cgtcacgtac gagagtgggg ccaatagaca gtacactgtg 1140

cctaacgggt gcgctaacac tgtcactgta gaagggactt ggaaataa 1188

<210> 16
<211> 395
<212> PRT
<213> Cochliobolus heterostrophus ATCC 48332

<220>
<221> SIGNAL
<222> (1)...(20)

<220>
<221> DOMAIN
<222> (294)...(390)
<223> Starch binding domain

<220>
<221> SITE
<222> (222)...(225)
<223> N-glycosylation site. Prosite id = PS00001

<220>

<221> SITE
<222> (226)...(229)
<223> N-glycosylation site. Prosite id = PS00001

<400> 16

```
Met Leu Ser Lys Ile Leu Leu Pro Val Val Ala Leu Ala Ala Ser Ala
1               5                  10                  15

Asn Ala His Gly Tyr Leu Thr Ser Pro Met Ser Arg Thr Gly Leu Asn
            20                  25                  30

Ala Gln Ser Gly Ala Asp Thr Cys Pro Glu Cys Thr Ile Leu Glu Pro
        35                  40                  45

Val Thr Ala Trp Pro Asp Leu Asp Ala Ala Ala Val Gly Arg Ser Gly
    50                  55                  60

Pro Cys Gly Tyr Asn Ala Arg Val Ser Val Asp Tyr Asn Gln Pro Gly
65                  70                  75                  80

Pro Arg Trp Gly Ser Glu Pro Val Ile Thr Tyr Lys Ala Gly Asp Val
                85                  90                  95

Val Asp Val Gln Trp Cys Val Asp Ala Asn Gly Asp His Gly Gly Met
            100                 105                 110

Phe Thr Tyr Arg Ile Cys Gln Asn Gln Ala Leu Val Asp Lys Leu Leu
        115                 120                 125

Thr Pro Gly Tyr Leu Pro Thr Glu Ala Glu Lys Gln Ala Ala Glu Asp
    130                 135                 140
```

```
Cys Phe Gln Ala Gly Glu Leu Lys Cys Thr Asp Val Pro Gly Gln Thr
145                 150                 155                 160

Cys Gly Phe Asn Ser Asp Cys Gln Gln Gly Gln Ala Cys Trp Arg Asn
            165                 170                 175

Asp Trp Phe Thr Cys Gly Gly Phe Asn Asp Asn Leu Lys Cys Arg Ser
        180                 185                 190

Val Asp Asn Ala Pro Leu Asn Ser Cys Tyr Thr Ser Ile Ala Gly Gly
    195                 200                 205

Tyr Thr Val Ser Ser Lys Ile Lys Ile Pro Asn Tyr Thr Ser Asn His
210                 215                 220

Thr Leu Leu Ser Phe Lys Trp Asn Ser Phe Gln Thr Pro Gln Val Tyr
225                 230                 235                 240

Leu Thr Cys Ala Asp Ile Lys Ile Thr Gly Ser Ser Ser Gly Thr Ser
            245                 250                 255

Pro Pro Pro Thr Ser Ser Lys Pro Pro Thr Ser Ser Ser Lys Pro Thr
        260                 265                 270

Ser Thr Ser Thr Ser Thr Ser Ala Pro Ser Ala Thr Pro Thr Ala Cys
    275                 280                 285

Ala Thr Pro Val Ser Thr Val Ala Val Thr Phe Asn Ser Lys Thr Thr
290                 295                 300

Thr Ser Phe Gly Gln Thr Val Lys Leu Ala Gly Ser Ile Ser Gln Leu
305                 310                 315                 320

Gly Ser Trp Asn Thr Ala Asn Ala Pro Ala Leu Ser Ala Ala Gln Tyr
            325                 330                 335

Thr Ser Ser Asn Pro Leu Trp Thr Thr Thr Leu Asn Leu Pro Ala Gly
        340                 345                 350

Thr Ser Phe Glu Tyr Lys Tyr Ile Lys Val Asp Ser Ser Gly Ala Val
    355                 360                 365

Thr Tyr Glu Ser Gly Ala Asn Arg Gln Tyr Thr Val Pro Asn Gly Cys
370                 375                 380

Ala Asn Thr Val Thr Val Glu Gly Thr Trp Lys
385                 390                 395
```

<210> 17
<211> 1938
<212> DNA
<213> Fusarium verticillioides GZ3639

<400> 17

atgtactttg tgtcttctgc ctttcttctc ggctcattcg ttcttcagaa cgtcttgggc 60

cgaccaacct tcgatgagag gagtctctta caagagagac agtcttcggt cgactccttt 120

atcaagtctg agagctcaat tgctattgag caactcctct gcaatatcgg ttccgatggc 180

tgcaactcca agaacgtagc cactggtatt gtcattgctt ctccagacac acaggatcct 240

gactactttt acacctggac tcgagatgct gctctagtct tcaagtacgt cgttgatagg 300

ttcatcaacc agtatgatgc tggcttgcag aggaagatcc aggagtacat cgcctcccaa 360

gccaagctcc agggtgtttc caacccttct gggtcgcttt cggatggctc aggtctggga 420

gaggccaagt tcaacgtcga catgagtgcc ttcactggtg gttggggtcg acctcagcga 480

gatggtccag ctctgcgtgc gactgctatg atcacctatg ccaactggct gattgccaac 540

ggctacacct ccacagccaa tgacattgtg tggcctgttg ttcgcaacga ccttaactat 600

gtggctcagt attggaatca aaccggattt gacttgtggg aggaggtcaa gggtagttcg 660

ttcttcacaa ctggttccca gtatcgagct ctcattgaag gcgccgctct ggccaagaag 720

ctcggcaagt cgggagacaa ctactccaac atcgctcctc aggctctctg cttcttgcag 780

acttactgga tctcttctgg caaatacgtc gactctaaca tcaatgtcaa tgacggccgc 840

actggcaagg acgccaacag tatcctgtcg tccatacaca atttcgaccc tgctctgaac 900

tgtgatcccg ccaccttcca gccatgcagt gacaaggccc tcgccaacca caaggcggtt 960

actgactctt tccgctcatg gaacatcaac aagggtatct ctcaaggctc agctgtcgcc 1020

gttggacgat acgtcgaaga tgtctactac aatggcaacc cctggtacct cgctacgctt 1080

gccgcggcag agcagctcta cgatgccatt tatgtctgga agcagcaggg atccatcact 1140

gtgtcggacg tctctctttc gttcttcaag gacctcgtct ctttggtctc taccggaaca 1200

tacgccagtg actctgccac cttcaagagc atcactgacg ccgtctccaa gtatgctgat 1260

gggtatgttg ccatcgttgc aaagtatgtc ggcacagatg gccacctcgc agagcagttt 1320

gacaagaacg acggccatcc tctttctgcc acagacttga cttggtcata tgccgcattc 1380

ctctcagctg ctgatcgtcg agctggtgtt attcctccct cttgggctgg aagcgtggct 1440

gctgtcccca accaatgcgg taccaatact gttgctggat cctactcatc ggctactgca 1500

acttcgttcc cagcatcgca aacacccaag ggtggtgtgc ccactccaac tggcgcccag 1560

acttccactt ccacttccac ttccacttcc agctcgtcca ctggtaccag ttgccctact 1620

gcaacctctg tggctgtcac tttccaagaa gttgtcacca ccaactttgg tgataccatc 1680

aagatcgttg gcaacatcgc tgctctcggt aactgggaca catcaaaggc tgttgccctg 1740

agcgcctccg actataccgc ctcgaaccct gtgtggaagg ccaccatttc cctaactgca 1800

ggacagtcca tccagtataa gtacatcaat gttaagaagg acggctctct tacctgggag 1860

aaggacccca accgcaccta cgctgttcct aagacatgtg ccacaacggc taccaagtct 1920

gacaagtggc agtcttga 1938

<210> 18
<211> 645
<212> PRT
<213> Fusarium verticillioides GZ3639

<220>
<221> DOMAIN
<222> (46)...(465)
<223> Glycosyl hydrolases family 15

<220>
<221> DOMAIN
<222> (543)...(639)
<223> Starch binding domain

<220>
<221> SITE
<222> (209)...(212)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (211)...(221)
<223> Glucoamylase active site region signature. Prosite id = PS00820

<220>
<221> SITE
<222> (250)...(253)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (633)...(636)
<223> N-glycosylation site. Prosite id = PS00001

<400> 18

```
Met Tyr Phe Val Ser Ser Ala Phe Leu Leu Gly Ser Phe Val Leu Gln
1               5                  10                  15

Asn Val Leu Gly Arg Pro Thr Phe Asp Glu Arg Ser Leu Leu Gln Glu
            20                  25                  30

Arg Gln Ser Ser Val Asp Ser Phe Ile Lys Ser Glu Ser Ser Ile Ala
        35                  40                  45

Ile Glu Gln Leu Leu Cys Asn Ile Gly Ser Asp Gly Cys Asn Ser Lys
    50                  55                  60

Asn Val Ala Thr Gly Ile Val Ile Ala Ser Pro Asp Thr Gln Asp Pro
65                  70                  75                  80

Asp Tyr Phe Tyr Thr Trp Thr Arg Asp Ala Ala Leu Val Phe Lys Tyr
```

85 90 95

Val Val Asp Arg Phe Ile Asn Gln Tyr Asp Ala Gly Leu Gln Arg Lys
100 105 110

Ile Gln Glu Tyr Ile Ala Ser Gln Ala Lys Leu Gln Gly Val Ser Asn
115 120 125

Pro Ser Gly Ser Leu Ser Asp Gly Ser Gly Leu Gly Glu Ala Lys Phe
130 135 140

Asn Val Asp Met Ser Ala Phe Thr Gly Gly Trp Gly Arg Pro Gln Arg
145 150 155 160

Asp Gly Pro Ala Leu Arg Ala Thr Ala Met Ile Thr Tyr Ala Asn Trp
165 170 175

Leu Ile Ala Asn Gly Tyr Thr Ser Thr Ala Asn Asp Ile Val Trp Pro
180 185 190

Val Val Arg Asn Asp Leu Asn Tyr Val Ala Gln Tyr Trp Asn Gln Thr
195 200 205

Gly Phe Asp Leu Trp Glu Glu Val Lys Gly Ser Ser Phe Phe Thr Thr
210 215 220

Gly Ser Gln Tyr Arg Ala Leu Ile Glu Gly Ala Ala Leu Ala Lys Lys
225 230 235 240

Leu Gly Lys Ser Gly Asp Asn Tyr Ser Asn Ile Ala Pro Gln Ala Leu
245 250 255

Cys Phe Leu Gln Thr Tyr Trp Ile Ser Ser Gly Lys Tyr Val Asp Ser
260 265 270

Asn Ile Asn Val Asn Asp Gly Arg Thr Gly Lys Asp Ala Asn Ser Ile
275 280 285

Leu Ser Ser Ile His Asn Phe Asp Pro Ala Leu Asn Cys Asp Pro Ala
290 295 300

Thr Phe Gln Pro Cys Ser Asp Lys Ala Leu Ala Asn His Lys Ala Val
305 310 315 320

Thr Asp Ser Phe Arg Ser Trp Asn Ile Asn Lys Gly Ile Ser Gln Gly
325 330 335

Ser Ala Val Ala Val Gly Arg Tyr Val Glu Asp Val Tyr Tyr Asn Gly
340 345 350

```
Asn Pro Trp Tyr Leu Ala Thr Leu Ala Ala Ala Glu Gln Leu Tyr Asp
        355                 360                 365

Ala Ile Tyr Val Trp Lys Gln Gln Gly Ser Ile Thr Val Ser Asp Val
    370                 375                 380

Ser Leu Ser Phe Phe Lys Asp Leu Val Ser Leu Val Ser Thr Gly Thr
385                 390                 395                 400

Tyr Ala Ser Asp Ser Ala Thr Phe Lys Ser Ile Thr Asp Ala Val Ser
                405                 410                 415

Lys Tyr Ala Asp Gly Tyr Val Ala Ile Val Ala Lys Tyr Val Gly Thr
            420                 425                 430

Asp Gly His Leu Ala Glu Gln Phe Asp Lys Asn Asp Gly His Pro Leu
        435                 440                 445

Ser Ala Thr Asp Leu Thr Trp Ser Tyr Ala Ala Phe Leu Ser Ala Ala
    450                 455                 460

Asp Arg Arg Ala Gly Val Ile Pro Pro Ser Trp Ala Gly Ser Val Ala
465                 470                 475                 480

Ala Val Pro Asn Gln Cys Gly Thr Asn Thr Val Ala Gly Ser Tyr Ser
                485                 490                 495

Ser Ala Thr Ala Thr Ser Phe Pro Ala Ser Gln Thr Pro Lys Gly Gly
            500                 505                 510

Val Pro Thr Pro Thr Gly Ala Gln Thr Ser Thr Ser Thr Ser Thr Ser
        515                 520                 525

Thr Ser Ser Ser Ser Thr Gly Thr Ser Cys Pro Thr Ala Thr Ser Val
    530                 535                 540

Ala Val Thr Phe Gln Glu Val Val Thr Thr Asn Phe Gly Asp Thr Ile
545                 550                 555                 560

Lys Ile Val Gly Asn Ile Ala Ala Leu Gly Asn Trp Asp Thr Ser Lys
                565                 570                 575

Ala Val Ala Leu Ser Ala Ser Asp Tyr Thr Ala Ser Asn Pro Val Trp
            580                 585                 590

Lys Ala Thr Ile Ser Leu Thr Ala Gly Gln Ser Ile Gln Tyr Lys Tyr
        595                 600                 605
```

Ile Asn Val Lys Lys Asp Gly Ser Leu Thr Trp Glu Lys Asp Pro Asn
610 615 620

Arg Thr Tyr Ala Val Pro Lys Thr Cys Ala Thr Thr Ala Thr Lys Ser
625 630 635 640

Asp Lys Trp Gln Ser
645

<210> 19
<211> 1467
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 19

atgttgacat tgaatgtttt gacagcactg ttggctccta ttgtgctgtc atctgccttg 60

cctgctcgcg atctcaatgc gcgtgccgat tcgaagcctg ctgctcttca agccatcctg 120

aacaacattg gcgccgatgg atctgctgtt tcgggagcat ctgcaggcgt tgtggtagca 180

tccccatcca aatcggatcc cgattacttt tacacctgga ctcgtgatgc agccttgaca 240

tacaaggtct tgattgatga gttcattgcc ggagacacct cgctggaacc taccatccag 300

gactacgtct ctgctcaagc aaagctgcag gccgtgtcta acccatctgg tgacttgtcc 360

gatggatcag gacttgcaga gcctaagtac cacgtggact tgactgcctt cacggaggcc 420

tggggtcgtc ctcagcgcga cggacccgct ctgcgagcca cagcactgat tacctacggt 480

aactatttga tttcgaagga gagaacatcc gtcgtcaagt cgaacatctg gcccattgtg 540

caaaatgatc tcaactatgt tgcgcaatac tggaaccaaa ccggattcga tctgtgggag 600

gaggtcgagg gctcctcgtt ttttaccatt gctgcacaac accgcgcatt ggtggagggc 660

agcgcatttg ccaaggcgct gggagagtcc tgcgagggat gcgattccca ggcacctcag 720

gtcctatgct tccagcagtc cttctgggat ggcaaggcta ttgtttctaa cttcgccaac 780

aatggccgaa ccggtctcga tgccaattcg gtgcttacct cgatcggaaa cttcgacccc 840

aaggctccct gcgatgacgt gaccttccag ccttgctctg ctcgtgccct gtcgaatcac 900

aagctatacg ttgactcgtt ccgtaagatc tatcctgtga acagtggtaa ggaagctgga 960

actgccgttg ctgttggacg ttattccgaa gacacttaca tgggcgggaa cccatggtat 1020

ttgaccaccc tggccgcggc cgagcagctg tacgatgctc tttaccagtg gaagcaactg 1080

ggatcgctag aaatcaccga gatcagtctt cctttcttca aggaccttgt ttcgtctgcc 1140

gccgccggaa agtatcccag ctcctcggaa acttacacgt ccatcactgc cgcagtcaaa 1200

aagtatgccg atggatttgt ggctgttgtt aaggagcaca cgcccagcga tgggtctctg 1260

gctgaacaat acactcggga caacggcagc ccggcctctg ctaaggactt gacctggtcc 1320

tacgcggcgc tcctgtctgc tacccgccgt gaggctggaa cagtgccccc tacctggggt 1380

gcgtcgactg ccaataaggt accctcaaag tgtgagggta gctcggccaa gggaagctac 1440

acgacaccat cagtcggcaa gtggtaa 1467

<210> 20
<211> 488
<212> PRT
<213> Unknown

<220>
<223> Obtained from an environmental sample

<220>
<221> SIGNAL
<222> (1)...(22)

<220>
<221> DOMAIN
<222> (33)...(449)
<223> Glycosyl hydrolases family 15

<220>
<221> SITE
<222> (194)...(197)
<223> N-glycosylation site. Prosite id = PS00001

<400> 20

```
Met Leu Thr Leu Asn Val Leu Thr Ala Leu Leu Ala Pro Ile Val Leu
1               5                  10                  15

Ser Ser Ala Leu Pro Ala Arg Asp Leu Asn Ala Arg Ala Asp Ser Lys
            20                  25                  30

Pro Ala Ala Leu Gln Ala Ile Leu Asn Asn Ile Gly Ala Asp Gly Ser
        35                  40                  45

Ala Val Ser Gly Ala Ser Ala Gly Val Val Val Ala Ser Pro Ser Lys
    50                  55                  60

Ser Asp Pro Asp Tyr Phe Tyr Thr Trp Thr Arg Asp Ala Ala Leu Thr
65                  70                  75                  80

Tyr Lys Val Leu Ile Asp Glu Phe Ile Ala Gly Asp Thr Ser Leu Glu
                85                  90                  95

Pro Thr Ile Gln Asp Tyr Val Ser Ala Gln Ala Lys Leu Gln Ala Val
            100                 105                 110

Ser Asn Pro Ser Gly Asp Leu Ser Asp Gly Ser Gly Leu Ala Glu Pro
        115                 120                 125

Lys Tyr His Val Asp Leu Thr Ala Phe Thr Glu Ala Trp Gly Arg Pro
    130                 135                 140
```

```
Gln Arg Asp Gly Pro Ala Leu Arg Ala Thr Ala Leu Ile Thr Tyr Gly
145                 150                 155                 160

Asn Tyr Leu Ile Ser Lys Glu Arg Thr Ser Val Val Lys Ser Asn Ile
                165                 170                 175

Trp Pro Ile Val Gln Asn Asp Leu Asn Tyr Val Ala Gln Tyr Trp Asn
            180                 185                 190

Gln Thr Gly Phe Asp Leu Trp Glu Glu Val Glu Gly Ser Ser Phe Phe
        195                 200                 205

Thr Ile Ala Ala Gln His Arg Ala Leu Val Glu Gly Ser Ala Phe Ala
    210                 215                 220

Lys Ala Leu Gly Glu Ser Cys Glu Gly Cys Asp Ser Gln Ala Pro Gln
225                 230                 235                 240

Val Leu Cys Phe Gln Gln Ser Phe Trp Asp Gly Lys Ala Ile Val Ser
                245                 250                 255

Asn Phe Ala Asn Asn Gly Arg Thr Gly Leu Asp Ala Asn Ser Val Leu
            260                 265                 270

Thr Ser Ile Gly Asn Phe Asp Pro Lys Ala Pro Cys Asp Asp Val Thr
        275                 280                 285

Phe Gln Pro Cys Ser Ala Arg Ala Leu Ser Asn His Lys Leu Tyr Val
    290                 295                 300

Asp Ser Phe Arg Lys Ile Tyr Pro Val Asn Ser Gly Lys Glu Ala Gly
305                 310                 315                 320

Thr Ala Val Ala Val Gly Arg Tyr Ser Glu Asp Thr Tyr Met Gly Gly
                325                 330                 335

Asn Pro Trp Tyr Leu Thr Thr Leu Ala Ala Ala Glu Gln Leu Tyr Asp
            340                 345                 350

Ala Leu Tyr Gln Trp Lys Gln Leu Gly Ser Leu Glu Ile Thr Glu Ile
        355                 360                 365

Ser Leu Pro Phe Phe Lys Asp Leu Val Ser Ser Ala Ala Ala Gly Lys
    370                 375                 380

Tyr Pro Ser Ser Ser Glu Thr Tyr Thr Ser Ile Thr Ala Ala Val Lys
385                 390                 395                 400
```

Lys Tyr Ala Asp Gly Phe Val Ala Val Val Lys Glu His Thr Pro Ser
405 410 415

Asp Gly Ser Leu Ala Glu Gln Tyr Thr Arg Asp Asn Gly Ser Pro Ala
420 425 430

Ser Ala Lys Asp Leu Thr Trp Ser Tyr Ala Ala Leu Leu Ser Ala Thr
435 440 445

Arg Arg Glu Ala Gly Thr Val Pro Pro Thr Trp Gly Ala Ser Thr Ala
450 455 460

Asn Lys Val Pro Ser Lys Cys Glu Gly Ser Ser Ala Lys Gly Ser Tyr
465 470 475 480

Thr Thr Pro Ser Val Gly Lys Trp
485

<210> 21
<211> 1488
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 21

atggttctag ctcggcttgc ctggcttgcc ggcctggtta gcactgccgt tgctgcgacc 60

ccagcggaat ggcgctcgca gtccatctac ttcatgctca cggatcgttt tgcccggacg 120

gatggttcaa ctactgctgc ctgcgatacc gctgacagaa aatactgtgg cggaacctgg 180

cagggaatca ttgacaagct ggactatatc caaggaatgg gctttacagc catttggatc 240

actcctgtga ccggtcaatt gagcggggaa accgcgtacg gagatgccta tcacggatac 300

tggcagcagg atatctattc tctcgattcc aactatggaa ccgcagacga tctcaaggcc 360

ctcgctgcgg ctttgcacga acgcaacatg tatctcatgg tcgatgtcgt agctaaccac 420

atgggctaca atggcccagg tactgacgtg gactacacca aattcaaccc cttcaacgat 480

gcaaagtatt tccactcgta ctgcccaatc accgattaca acgacgacac catgtcgcaa 540

aactgctggc ttggcgataa caaggtctcg ctaccggacc tgaatacaca gagcaaggag 600

gtccaggatc tatggtatga ctgggttggg tctttggtct ccaactactc tatcgacggt 660

ctccgtgtcg acacagtcaa acatgtccag aaagatttct ggcccggcta caacaaagcc 720

gcgggcgtct actgcgtagg cgagatcctt gatggtgacc cagattacac ctgtccatac 780

caggaggtaa tggacggagt gctcaactac ccgatttatt acccactcct caaggccttc 840

caatcgacct cgggaagcat gaccgatcta tacaacatga tcaacacggt gaaatcgacc 900

tgcaaggact cgacccttct tggaaatttc ttggagaacc acgataaccc ccgttttgcc 960

cacgtcaccg acgacattgc cctcgccaag aacgcagcta cgtttaccat tatggcagac 1020

ggcattccta ttgtctatgc aggacaggag cagcactaca gtggtggcga ggacccggct 1080

aatcgcgaag ctctgtggtt gtcaggatac aacacggaca gtgagctgta caagctcata 1140

gccaaggcca atggtgctag aagccaggcc attgctaagg gtaccaacta tacgatttac 1200

cagaaccaac caatctacaa agatgagagc accatcgcca tgcggaaggg cttcgacggt 1260

ggacagacaa tcactgtcct gacgaatctc ggcgcagggg gtaaagagga ctctgtttcg 1320

attcctgata ccggattcaa ggctggtgca aagttgactg aggtcgtctc ctgcgctagt 1380

gttactgtcg gtgacaatgg ggaggtgtct gtccctatgg cggctggagc gccgaggatt 1440

ttgctcccta ccttttgct tgagggctcg actctgtgtt catcatag 1488

<210> 22
<211> 495
<212> PRT
<213> Unknown

<220>
<223> Obtained from an environmental sample

<220>
<221> SIGNAL
<222> (1)...(18)

<220>
<221> DOMAIN
<222> (31)...(387)
<223> Alpha amylase, catalytic domain

<220>
<221> SITE
<222> (197)...(200)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (218)...(221)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (402)...(405)
<223> N-glycosylationsite. Prosite id = PS00001

<400> 22

Met Val Leu Ala Arg Leu Ala Trp Leu Ala Gly Leu Val Ser Thr Ala
1 5 10 15

Val Ala Ala Thr Pro Ala Glu Trp Arg Ser Gln Ser Ile Tyr Phe Met
20 25 30

Leu Thr Asp Arg Phe Ala Arg Thr Asp Gly Ser Thr Thr Ala Ala Cys
35 40 45

Asp Thr Ala Asp Arg Lys Tyr Cys Gly Gly Thr Trp Gln Gly Ile Ile
50 55 60

```
Asp Lys Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala Ile Trp Ile
65                  70                  75                  80

Thr Pro Val Thr Gly Gln Leu Ser Gly Glu Thr Ala Tyr Gly Asp Ala
                85                  90                  95

Tyr His Gly Tyr Trp Gln Gln Asp Ile Tyr Ser Leu Asp Ser Asn Tyr
            100                 105                 110

Gly Thr Ala Asp Asp Leu Lys Ala Leu Ala Ala Ala Leu His Glu Arg
        115                 120                 125

Asn Met Tyr Leu Met Val Asp Val Val Ala Asn His Met Gly Tyr Asn
    130                 135                 140

Gly Pro Gly Thr Asp Val Asp Tyr Thr Lys Phe Asn Pro Phe Asn Asp
145                 150                 155                 160

Ala Lys Tyr Phe His Ser Tyr Cys Pro Ile Thr Asp Tyr Asn Asp Asp
                165                 170                 175

Thr Met Ser Gln Asn Cys Trp Leu Gly Asp Asn Lys Val Ser Leu Pro
            180                 185                 190

Asp Leu Asn Thr Gln Ser Lys Glu Val Gln Asp Leu Trp Tyr Asp Trp
        195                 200                 205

Val Gly Ser Leu Val Ser Asn Tyr Ser Ile Asp Gly Leu Arg Val Asp
    210                 215                 220

Thr Val Lys His Val Gln Lys Asp Phe Trp Pro Gly Tyr Asn Lys Ala
225                 230                 235                 240

Ala Gly Val Tyr Cys Val Gly Glu Ile Leu Asp Gly Asp Pro Asp Tyr
                245                 250                 255

Thr Cys Pro Tyr Gln Glu Val Met Asp Gly Val Leu Asn Tyr Pro Ile
            260                 265                 270

Tyr Tyr Pro Leu Leu Lys Ala Phe Gln Ser Thr Ser Gly Ser Met Thr
        275                 280                 285

Asp Leu Tyr Asn Met Ile Asn Thr Val Lys Ser Thr Cys Lys Asp Ser
    290                 295                 300

Thr Leu Leu Gly Asn Phe Leu Glu Asn His Asp Asn Pro Arg Phe Ala
305                 310                 315                 320
```

```
His Val Thr Asp Asp Ile Ala Leu Ala Lys Asn Ala Ala Thr Phe Thr
                325                 330                 335

Ile Met Ala Asp Gly Ile Pro Ile Val Tyr Ala Gly Gln Glu Gln His
            340                 345                 350

Tyr Ser Gly Gly Glu Asp Pro Ala Asn Arg Glu Ala Leu Trp Leu Ser
        355                 360                 365

Gly Tyr Asn Thr Asp Ser Glu Leu Tyr Lys Leu Ile Ala Lys Ala Asn
    370                 375                 380

Gly Ala Arg Ser Gln Ala Ile Ala Lys Gly Thr Asn Tyr Thr Ile Tyr
385                 390                 395                 400

Gln Asn Gln Pro Ile Tyr Lys Asp Glu Ser Thr Ile Ala Met Arg Lys
                405                 410                 415

Gly Phe Asp Gly Gly Gln Thr Ile Thr Val Leu Thr Asn Leu Gly Ala
            420                 425                 430

Gly Gly Lys Glu Asp Ser Val Ser Ile Pro Asp Thr Gly Phe Lys Ala
        435                 440                 445

Gly Ala Lys Leu Thr Glu Val Val Ser Cys Ala Ser Val Thr Val Gly
    450                 455                 460

Asp Asn Gly Glu Val Ser Val Pro Met Ala Ala Gly Ala Pro Arg Ile
465                 470                 475                 480

Leu Leu Pro Thr Phe Leu Leu Glu Gly Ser Thr Leu Cys Ser Ser
                485                 490                 495
```

<210> 23
<211> 1389
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 23

atgaaactgt cccacaccct gacagctctt ctactccctc tgatatgcac tgtatcagca 60

gcggatgtga aagcatggaa gtcccgcaat atttactttg ccctgactga tcgcattgcc 120

cgtggaagcg atgatacagg tggagatgcc tgcggtaacc ttggaaacta ctgcggtgga 180

actttccaag gcttggagtc taagcttgac tatattaagg ggatgggctt tgatgcaatc 240

tggatcaccc ctgttatcgc taatgcgcca ggtggatatc atggatactg ggcgcgggat 300

ctctatagca tcaacgagaa ctatggcacc gctgatgatc tcaaaagcct agttgatgct 360

gctcataaga aggggatcta tgtcatggca gacgtagttg ccaaccacat gggaggcccc 420

atcagcgata acaagccgga gccattgaac caggagagct cctaccattc cacctgcaca 480

atcgactact caagccagga tagtgttgaa aactgccgta tcacagcaga cctacctgat 540

gtgaacacac agagtcccga gatccgtgcc ctcttccaga aatgggtcaa atggctcgtc 600

acggaatacg ggttcgacgg cttgcgcatt gacactgtta agcacgttga aaaggacttc 660

tggtcggcct tctcctccgc tgccggggtt tacaccatcg gcgaagtctg ggacggtgac 720

ccagcttacc ttgccggata tgcacaggac atggacggcc tgctcaacta tgcagtctac 780

tatcccgtga acaactttta ccagcaaaag ggctcttccc aggacatagt tgacatgcat 840

gataaaatcg acaccgcttt ccctgatccc agcgccctgg gcacattcat agacaatcac 900

gacaatgcgc gatggctgag cgtaaagaat gacaagtcgc ttttgaaaaa cgcactcgcc 960

tacgtgattc tcgcccgggg catccccatt gtctactacg gcacggaaca gggctacgcg 1020

ggcggcaacg accccgcaaa ccgcgaggac ctatggcgca gcaaattcag caccgacgcg 1080

gacttgtaca aggccatatc cctgctctca gcggcgagaa acgcttctgg tggcctcgct 1140

gacaacgacc atgtccatct gtacgtcgcg gagtcggcat atgcgtggag cagggcgggt 1200

ggaaacctca tcgtccttac gtccaatggt ggatctgggt ctgaggctaa tcactgcttc 1260

gactctaaga agcctggtgg gacatggaag aataccttg gagaggggac aatcaccgcc 1320

gatgaaggcg gaaaaatttg catctctatc tctaatggtg agcctgcggt gctggttgca 1380

agcacttga 1389

<210> 24
<211> 462
<212> PRT
<213> Unknown

<220>
<223> Obtained from an environmental sample

<220>
<221> SIGNAL
<222> (1)...(20)

<220>
<221> DOMAIN
<222> (33)...(373)
<223> Alpha amylase, catalytic domain

<220>
<221> SITE
<222> (379)...(382)
<223> N-glycosylation site. Prosite id = PS00001

<400> 24

Met Lys Leu Ser His Thr Leu Thr Ala Leu Leu Leu Pro Leu Ile Cys
1 5 10 15

Thr Val Ser Ala Ala Asp Val Lys Ala Trp Lys Ser Arg Asn Ile Tyr

20 25 30

Phe Ala Leu Thr Asp Arg Ile Ala Arg Gly Ser Asp Asp Thr Gly Gly
35 40 45

Asp Ala Cys Gly Asn Leu Gly Asn Tyr Cys Gly Gly Thr Phe Gln Gly
50 55 60

Leu Glu Ser Lys Leu Asp Tyr Ile Lys Gly Met Gly Phe Asp Ala Ile
65 70 75 80

Trp Ile Thr Pro Val Ile Ala Asn Ala Pro Gly Gly Tyr His Gly Tyr
85 90 95

Trp Ala Arg Asp Leu Tyr Ser Ile Asn Glu Asn Tyr Gly Thr Ala Asp
100 105 110

Asp Leu Lys Ser Leu Val Asp Ala Ala His Lys Lys Gly Ile Tyr Val
115 120 125

Met Ala Asp Val Val Ala Asn His Met Gly Gly Pro Ile Ser Asp Asn
130 135 140

Lys Pro Glu Pro Leu Asn Gln Glu Ser Ser Tyr His Ser Thr Cys Thr
145 150 155 160

Ile Asp Tyr Ser Ser Gln Asp Ser Val Glu Asn Cys Arg Ile Thr Ala
165 170 175

Asp Leu Pro Asp Val Asn Thr Gln Ser Pro Glu Ile Arg Ala Leu Phe
180 185 190

Gln Lys Trp Val Lys Trp Leu Val Thr Glu Tyr Gly Phe Asp Gly Leu
195 200 205

Arg Ile Asp Thr Val Lys His Val Glu Lys Asp Phe Trp Ser Ala Phe
210 215 220

Ser Ser Ala Ala Gly Val Tyr Thr Ile Gly Glu Val Trp Asp Gly Asp
225 230 235 240

Pro Ala Tyr Leu Ala Gly Tyr Ala Gln Asp Met Asp Gly Leu Leu Asn
245 250 255

Tyr Ala Val Tyr Tyr Pro Val Asn Asn Phe Tyr Gln Gln Lys Gly Ser
260 265 270

Ser Gln Asp Ile Val Asp Met His Asp Lys Ile Asp Thr Ala Phe Pro
275 280 285

```
Asp Pro Ser Ala Leu Gly Thr Phe Ile Asp Asn His Asp Asn Ala Arg
290                 295                 300

Trp Leu Ser Val Lys Asn Asp Lys Ser Leu Leu Lys Asn Ala Leu Ala
305                 310                 315                 320

Tyr Val Ile Leu Ala Arg Gly Ile Pro Ile Val Tyr Tyr Gly Thr Glu
                325                 330                 335

Gln Gly Tyr Ala Gly Gly Asn Asp Pro Ala Asn Arg Glu Asp Leu Trp
            340                 345                 350

Arg Ser Lys Phe Ser Thr Asp Ala Asp Leu Tyr Lys Ala Ile Ser Leu
        355                 360                 365

Leu Ser Ala Ala Arg Asn Ala Ser Gly Gly Leu Ala Asp Asn Asp His
    370                 375                 380

Val His Leu Tyr Val Ala Glu Ser Ala Tyr Ala Trp Ser Arg Ala Gly
385                 390                 395                 400

Gly Asn Leu Ile Val Leu Thr Ser Asn Gly Gly Ser Gly Ser Glu Ala
                405                 410                 415

Asn His Cys Phe Asp Ser Lys Lys Pro Gly Gly Thr Trp Lys Asn Thr
            420                 425                 430

Phe Gly Glu Gly Thr Ile Thr Ala Asp Glu Gly Gly Lys Ile Cys Ile
        435                 440                 445

Ser Ile Ser Asn Gly Glu Pro Ala Val Leu Val Ala Ser Thr
    450                 455                 460
```

<210> 25
<211> 1923
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 25

atgactattt ctcgcctctc ctccgtgctt tttgcactgg ctctaggtca aagtgcacta 60

gcagcccctc agctgtctcc tcgtgctaca actagcttgg acgcctggct tgccagtgaa 120

actactgtct ctctcaatgg aatcttggac aacatcggtg ccagcggtgc ctatgctcag 180

agtgccaagg ctggtgttgt tattgccagt cccagcacaa gcagcccgga ttattactac 240

acgtggacca gagactctgc ccttacgtta aaagtcctta tcgatctctt ccgtaatggg 300

aacgtagatc tgcagaccgt cattgaggaa tacatcactg cacaagctta tctacagacc 360

gtctccaacc cctccggaga tctctcaagc ggtgctggtc tcgctgagcc caagttcaac 420

gttgacatga gcgcttacac tggcgcttgg ggacgtccgc aacgcgacgg acctgctctc 480

cgtgctattg ccttgatcga ttttggcaac tggttgattg acaatggata ctctagctat 540

gccgttagca acgtctggcc cattgtacgc aacgatctgt cctacgttgc ccagtactgg 600

agccaaagcg gttatgatct ctgggaggag gtcaacagca tgtccttctt caccattgcc 660

aaccagcacc gtgccctcgt cgagggaagc acctttgccg gccgagttgg tgcatcctgc 720

tcgtggtgtg actctcaagc cccccagatc ctctgctaca tgcagaactt ctggaccgga 780

tcttacatca atgccaacac tggcggtggt cgttccggca aggacgccaa cactgttctg 840

gctagtatca gcacttttga tcccgaggct acctgtgacg acgtcacttt ccagccctgc 900

tcctcccgcg ccctggccaa ccacaaagtc tacactgact cgttccgatc tgtctattct 960

ctcgactccg gcattgctga gggcgttgct gtcgcggtag gacgctaccc tgaggattca 1020

tactataacg gcaatccgtg gtttttgacc actctcgctg ctgcggaaca gttgtacgat 1080

gccatatatc aatggaacaa gatcggctcc atcacaatca ccagcacctc tttggctttc 1140

tttaatgacg tctacagctc tgccgctgtt ggcacttacg cctctggcag cactgcctac 1200

acagccattg tcagcgcagt caagacctat gccgacggat atgtcagcat cgtgcaggct 1260

catgccatga ccaacggctc tctctccgag cagtttgaca aggcctccgg tacccagctc 1320

tctgcccgtg atcttacatg gtcgtacgct gccttgctca ctgctaacat gcgccgcaac 1380

ggaattgtgc ctccttcctg gggagctgcc tccgccaact caatccccag ttcttgctct 1440

acgggctctg caactggcac ttacagcact cctaccggaa cctcctggcc cagtacactg 1500

accagcggca ccgcaggtac cactaccact tctgctacta ctaccacctc cacctctgtc 1560

tccaaaacta ccactaccac caccagtact acctcttgca ccaccccgac ctccgtagcc 1620

gtcacctttg acgagattgc aaccacctac tacggcgaga acgtttacat ttctggctcg 1680

atctcgcagc tcggtagctg ggataccagc agtgccattg cgctcagcgc cagccagtac 1740

acttccagca acaacctctg gttcgtgact atcaacctgc ccgctggaac aacctttcag 1800

tacaagtaca ttcgcaagga gtcggatggt tcgattgttt gggagagtga tcctaaccgc 1860

tcgtacactg tgccttccgg ttgtggtgta agcacagcta ctgagagtga tacttggcga 1920

tag 1923

<210> 26
<211> 640
<212> PRT
<213> Unknown

<220>

<223> Obtained from an environmental sample

<220>
<221> SIGNAL
<222> (1)...(21)

<220>
<221> DOMAIN
<222> (42)...(457)
<223> Glycosyl hydrolases family 15

<220>
<221> DOMAIN
<222> (538)...(634)
<223> Starch binding domain

<220>
<221> SITE
<222> (206)...(216)
<223> Glucoamylase active site region signature. Prosite id = PS00820

<220>
<221> SITE
<222> (431)...(434)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (628)...(631)
<223> N-glycosylation site. Prosite id = PS00001

<400> 26

Met Thr Ile Ser Arg Leu Ser Ser Val Leu Phe Ala Leu Ala Leu Gly
1 5 10 15

Gln Ser Ala Leu Ala Ala Pro Gln Leu Ser Pro Arg Ala Thr Thr Ser
20 25 30

Leu Asp Ala Trp Leu Ala Ser Glu Thr Thr Val Ser Leu Asn Gly Ile
35 40 45

Leu Asp Asn Ile Gly Ala Ser Gly Ala Tyr Ala Gln Ser Ala Lys Ala
50 55 60

Gly Val Val Ile Ala Ser Pro Ser Thr Ser Ser Pro Asp Tyr Tyr Tyr
65 70 75 80

Thr Trp Thr Arg Asp Ser Ala Leu Thr Leu Lys Val Leu Ile Asp Leu
85 90 95

Phe Arg Asn Gly Asn Val Asp Leu Gln Thr Val Ile Glu Glu Tyr Ile
100 105 110

Thr Ala Gln Ala Tyr Leu Gln Thr Val Ser Asn Pro Ser Gly Asp Leu
115 120 125

Ser Ser Gly Ala Gly Leu Ala Glu Pro Lys Phe Asn Val Asp Met Ser
130 135 140

```
Ala Tyr Thr Gly Ala Trp Gly Arg Pro Gln Arg Asp Gly Pro Ala Leu
145                 150                 155                 160

Arg Ala Ile Ala Leu Ile Asp Phe Gly Asn Trp Leu Ile Asp Asn Gly
                165                 170                 175

Tyr Ser Ser Tyr Ala Val Ser Asn Val Trp Pro Ile Val Arg Asn Asp
            180                 185                 190

Leu Ser Tyr Val Ala Gln Tyr Trp Ser Gln Ser Gly Tyr Asp Leu Trp
        195                 200                 205

Glu Glu Val Asn Ser Met Ser Phe Phe Thr Ile Ala Asn Gln His Arg
    210                 215                 220

Ala Leu Val Glu Gly Ser Thr Phe Ala Gly Arg Val Gly Ala Ser Cys
225                 230                 235                 240

Ser Trp Cys Asp Ser Gln Ala Pro Gln Ile Leu Cys Tyr Met Gln Asn
                245                 250                 255

Phe Trp Thr Gly Ser Tyr Ile Asn Ala Asn Thr Gly Gly Gly Arg Ser
            260                 265                 270

Gly Lys Asp Ala Asn Thr Val Leu Ala Ser Ile Ser Thr Phe Asp Pro
        275                 280                 285

Glu Ala Thr Cys Asp Asp Val Thr Phe Gln Pro Cys Ser Ser Arg Ala
    290                 295                 300

Leu Ala Asn His Lys Val Tyr Thr Asp Ser Phe Arg Ser Val Tyr Ser
305                 310                 315                 320

Leu Asp Ser Gly Ile Ala Glu Gly Val Ala Val Ala Val Gly Arg Tyr
                325                 330                 335

Pro Glu Asp Ser Tyr Tyr Asn Gly Asn Pro Trp Phe Leu Thr Thr Leu
            340                 345                 350

Ala Ala Ala Glu Gln Leu Tyr Asp Ala Ile Tyr Gln Trp Asn Lys Ile
        355                 360                 365

Gly Ser Ile Thr Ile Thr Ser Thr Ser Leu Ala Phe Phe Asn Asp Val
    370                 375                 380

Tyr Ser Ser Ala Ala Val Gly Thr Tyr Ala Ser Gly Ser Thr Ala Tyr
385                 390                 395                 400

Thr Ala Ile Val Ser Ala Val Lys Thr Tyr Ala Asp Gly Tyr Val Ser
```

405 410 415

Ile Val Gln Ala His Ala Met Thr Asn Gly Ser Leu Ser Glu Gln Phe
420 425 430

Asp Lys Ala Ser Gly Thr Gln Leu Ser Ala Arg Asp Leu Thr Trp Ser
435 440 445

Tyr Ala Ala Leu Leu Thr Ala Asn Met Arg Arg Asn Gly Ile Val Pro
450 455 460

Pro Ser Trp Gly Ala Ala Ser Ala Asn Ser Ile Pro Ser Ser Cys Ser
465 470 475 480

Thr Gly Ser Ala Thr Gly Thr Tyr Ser Thr Pro Thr Gly Thr Ser Trp
485 490 495

Pro Ser Thr Leu Thr Ser Gly Thr Ala Gly Thr Thr Thr Thr Ser Ala
500 505 510

Thr Thr Thr Thr Ser Thr Ser Val Ser Lys Thr Thr Thr Thr Thr Thr
515 520 525

Ser Thr Thr Ser Cys Thr Thr Pro Thr Ser Val Ala Val Thr Phe Asp
530 535 540

Glu Ile Ala Thr Thr Tyr Tyr Gly Glu Asn Val Tyr Ile Ser Gly Ser
545 550 555 560

Ile Ser Gln Leu Gly Ser Trp Asp Thr Ser Ser Ala Ile Ala Leu Ser
565 570 575

Ala Ser Gln Tyr Thr Ser Ser Asn Asn Leu Trp Phe Val Thr Ile Asn
580 585 590

Leu Pro Ala Gly Thr Thr Phe Gln Tyr Lys Tyr Ile Arg Lys Glu Ser
595 600 605

Asp Gly Ser Ile Val Trp Glu Ser Asp Pro Asn Arg Ser Tyr Thr Val
610 615 620

Pro Ser Gly Cys Gly Val Ser Thr Ala Thr Glu Ser Asp Thr Trp Arg
625 630 635 640

<210> 27
<211> 1911
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 27

atgtatatcc tgtcttctgc ttttctcctc ggctccctcg ctcttcagag cgttctgggt 60

agaccggcgg ataagatcca agtgagacaa tctggtatcg aggactttat caagtccgag 120

agcccgattg ccatcgagca gcttctgtgc aacattggtt ccgaagggtg ccatgccaag 180

aatgttcccg ctggcatcgt cattgcttct ccagacaccc aagatcccga ctacttttat 240

acctggactc gagatgctgc tcttgttttc aagtatattg tcgacaggtt cattcaccag 300

tacgatgcta ctctccagaa gcgcattcag gagtacattg cctctcaagc caagctccag 360

ggcgtctcca acccctctgg atcctctcg gatggctctg gccttggtga agccaagtac 420

tacgtcaacc tgagccccta cactggtggc tggggtcgac ctcaacgtga tggccctgcc 480

cttcgagcca ccgccatgat cacttatgcc aactggctga tcgctaacgg ttacacctct 540

acggctaatg acattgtgtg gcccgtgatt cgcaacgatc tcaactacgt tgctcagtat 600

tggactcaaa ctggctttga cttgtgggaa gaggtcaggg gtagctcatt cttcacgact 660

gctgctcaat accgagctct cgtcgaaggt gctgcccttg ctaaggctct tggcaagtct 720

ggcgatacct attctaacat tgcgccccaa acactctgct tcttgcagac ctactgggtc 780

tcgaacggta gatacgtcga ctccaacatt aatgtgaacg atggccgcac cggcaaggac 840

gccaacagca tcctcgcatc tatccacaac tttgacccaa gcattggctg tgatgctgca 900

acattccaac cctgcagtga caaggccctc gccaaccaca aggcagtcac cgacaccttc 960

cgctcctaca acctcaacaa gggcatcgca cagggaactg ctgtggctat cggaagatac 1020

attgaagatg tctactacaa cggcaacccc tggtacctca ccacactcgc cgctgccgag 1080

cagctatatg atgccgtcta cgtctggaag cagaagggat ccatcactgt gactgataca 1140

tctctgtcgt tcttcaagga tcttgtgtcg agtgtttcca ctggcacata tgccagcggc 1200

tcgaccactt tccagcagat tatcgacgcc gtgtcgacct acgccgacgg atacgttgcc 1260

attgttcgca agtacgtggg tccgaacggc gccctggctg agcagttctc caaggacaat 1320

ggcactccca tgtccgctga tgatcttacg tggtcatatg ctgctttcct ctcagccact 1380

gaacgccgag ctggcattgt tcctcctact tggcaaaaga gtgttcctgc agttcccaac 1440

agctgtggat caagcacagt cgttggatca tacacatcgg ccacgcagac ctcgttccct 1500

ccttcgcaaa ccccccagga cggcgtgcca actcccacag gaccaactcc caccgacgga 1560

ggacctactt cctctcctac aagctgtgcc atcgccacat ctgtcgacgt caccttttaat 1620

gaggttgtca agaccgagta cggcgacacc atcaagattg tcggcagcat cgctgccctg 1680

ggcagctggg acaccacgaa ggccatctcc ctgagcgcct ccgactacac ggcctcgaac 1740

cccctgtgga agacgacaat ctccctcacg gctggtcagt cttttgagta caagtatatc 1800

aacatcaaga aggacggctc gctggtgtgg gagcgtgacc ccaaccgttc ttatactgtt 1860

cccaagactt gtgagaccaa ggctaccaag tcggatagtt ggcaaggata a 1911

<210> 28
<211> 636
<212> PRT
<213> Unknown

<220>
<223> Obtained from an environmental sample

<220>
<221> SIGNAL
<222> (1)...(20)

<220>
<221> DOMAIN
<222> (42)...(461)
<223> Glycosyl hydrolases family 15

<220>
<221> DOMAIN
<222> (534)...(630)
<223> Starch binding domain

<220>
<221> SITE
<222> (207)...(217)
<223> Glucoamylase active site region signature. Prosite id = PS00820

<220>
<221> SITE
<222> (624)...(627)
<223> N-glycosylation site. Prosite id = PS00001

<400> 28

```
Met Tyr Ile Leu Ser Ser Ala Phe Leu Leu Gly Ser Leu Ala Leu Gln
1               5                  10                  15

Ser Val Leu Gly Arg Pro Ala Asp Lys Ile Gln Val Arg Gln Ser Gly
            20                  25                  30

Ile Glu Asp Phe Ile Lys Ser Glu Ser Pro Ile Ala Ile Glu Gln Leu
        35                  40                  45

Leu Cys Asn Ile Gly Ser Glu Gly Cys His Ala Lys Asn Val Pro Ala
    50                  55                  60

Gly Ile Val Ile Ala Ser Pro Asp Thr Gln Asp Pro Asp Tyr Phe Tyr
65                  70                  75                  80

Thr Trp Thr Arg Asp Ala Ala Leu Val Phe Lys Tyr Ile Val Asp Arg
                85                  90                  95

Phe Ile His Gln Tyr Asp Ala Thr Leu Gln Lys Arg Ile Gln Glu Tyr
            100                 105                 110

Ile Ala Ser Gln Ala Lys Leu Gln Gly Val Ser Asn Pro Ser Gly Ser
        115                 120                 125
```

```
Leu Ser Asp Gly Ser Gly Leu Gly Glu Ala Lys Tyr Tyr Val Asn Leu
    130                 135                 140

Ser Pro Tyr Thr Gly Gly Trp Gly Arg Pro Gln Arg Asp Gly Pro Ala
145                 150                 155                 160

Leu Arg Ala Thr Ala Met Ile Thr Tyr Ala Asn Trp Leu Ile Ala Asn
                165                 170                 175

Gly Tyr Thr Ser Thr Ala Asn Asp Ile Val Trp Pro Val Ile Arg Asn
            180                 185                 190

Asp Leu Asn Tyr Val Ala Gln Tyr Trp Thr Gln Thr Gly Phe Asp Leu
        195                 200                 205

Trp Glu Glu Val Arg Gly Ser Ser Phe Phe Thr Thr Ala Ala Gln Tyr
    210                 215                 220

Arg Ala Leu Val Glu Gly Ala Ala Leu Ala Lys Ala Leu Gly Lys Ser
225                 230                 235                 240

Gly Asp Thr Tyr Ser Asn Ile Ala Pro Gln Thr Leu Cys Phe Leu Gln
                245                 250                 255

Thr Tyr Trp Val Ser Asn Gly Arg Tyr Val Asp Ser Asn Ile Asn Val
            260                 265                 270

Asn Asp Gly Arg Thr Gly Lys Asp Ala Asn Ser Ile Leu Ala Ser Ile
        275                 280                 285

His Asn Phe Asp Pro Ser Ile Gly Cys Asp Ala Ala Thr Phe Gln Pro
    290                 295                 300

Cys Ser Asp Lys Ala Leu Ala Asn His Lys Ala Val Thr Asp Thr Phe
305                 310                 315                 320

Arg Ser Tyr Asn Leu Asn Lys Gly Ile Ala Gln Gly Thr Ala Val Ala
                325                 330                 335

Ile Gly Arg Tyr Ile Glu Asp Val Tyr Tyr Asn Gly Asn Pro Trp Tyr
            340                 345                 350

Leu Thr Thr Leu Ala Ala Ala Glu Gln Leu Tyr Asp Ala Val Tyr Val
        355                 360                 365

Trp Lys Gln Lys Gly Ser Ile Thr Val Thr Asp Thr Ser Leu Ser Phe
    370                 375                 380
```

```
Phe Lys Asp Leu Val Ser Ser Val Ser Thr Gly Thr Tyr Ala Ser Gly
385                 390                 395                 400

Ser Thr Thr Phe Gln Gln Ile Ile Asp Ala Val Ser Thr Tyr Ala Asp
                405                 410                 415

Gly Tyr Val Ala Ile Val Arg Lys Tyr Val Gly Pro Asn Gly Ala Leu
            420                 425                 430

Ala Glu Gln Phe Ser Lys Asp Asn Gly Thr Pro Met Ser Ala Asp Asp
        435                 440                 445

Leu Thr Trp Ser Tyr Ala Ala Phe Leu Ser Ala Thr Glu Arg Arg Ala
    450                 455                 460

Gly Ile Val Pro Pro Thr Trp Gln Lys Ser Val Pro Ala Val Pro Asn
465                 470                 475                 480

Ser Cys Gly Ser Ser Thr Val Val Gly Ser Tyr Thr Ser Ala Thr Gln
                485                 490                 495

Thr Ser Phe Pro Pro Ser Gln Thr Pro Gln Asp Gly Val Pro Thr Pro
            500                 505                 510

Thr Gly Pro Thr Pro Thr Asp Gly Gly Pro Thr Ser Ser Pro Thr Ser
        515                 520                 525

Cys Ala Ile Ala Thr Ser Val Asp Val Thr Phe Asn Glu Val Val Lys
    530                 535                 540

Thr Glu Tyr Gly Asp Thr Ile Lys Ile Val Gly Ser Ile Ala Ala Leu
545                 550                 555                 560

Gly Ser Trp Asp Thr Thr Lys Ala Ile Ser Leu Ser Ala Ser Asp Tyr
                565                 570                 575

Thr Ala Ser Asn Pro Leu Trp Lys Thr Thr Ile Ser Leu Thr Ala Gly
            580                 585                 590

Gln Ser Phe Glu Tyr Lys Tyr Ile Asn Ile Lys Lys Asp Gly Ser Leu
        595                 600                 605

Val Trp Glu Arg Asp Pro Asn Arg Ser Tyr Thr Val Pro Lys Thr Cys
    610                 615                 620

Glu Thr Lys Ala Thr Lys Ser Asp Ser Trp Gln Gly
625                 630                 635
```

<210> 29
<211> 1767
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 29

atgcttttct cgtcgcttct tcgtgccttg tcagcctcgc ttttggcggg cgctgtccag 60

ggctcaccgg ccaatctcca tgcgcgccaa acctctgtcg accaattcat tcaaacccag 120

gccgaaatat ccatcaaggg ggttctagcg aacatcggtg cagatggatc caaagcccaa 180

ggagcccctg ccgggatcgt cgttgcaagt ccatctcgtt ctaatccaga ctattggtat 240

acctggacca gagacgcagc attgacatac aaggccctca ttgagcgctt tgtcgatggt 300

gatacttctc tccgcacaaa ggtcgacgag tatgtctcag cgcaagcata cctgcaaggc 360

gtgtccaatc catcaggggg gcccgactct ggaggcctag gagagcccaa attcaacgta 420

gaccgcaccg ctttcacagg agcatggggg cgaccacagc gtgatgggcc tccgcttcgt 480

gctacagctc tgatcatcta cgcaaactgg ctggttgcaa acgggcagca gagtcaagca 540

ctcaacactg tgtggccggt cattgcaaag gacttggcgt acaccgttcg atactggaac 600

cagacaggct ttgatctgtg ggaagaagta aacggatcat ctttcttcac gctgtctgcg 660

tcccacagag cgcttgtcga gggcaatgca cttgctcaga aactcggaca aacttgcacc 720

ggatgcgcga acgctgcacc ccaggttctc tgctttgtgc aaagcttttg gacaggcagc 780

tatattgatt ctaatatcaa tgttaacgat gggcgtacgg gcaaggatgc aaactcaatt 840

ctgtcatcta tccatacgtt tgacccggag gctggatgca ctgatgccac attccaacct 900

tgttcttccc gtgctcttgc aaaccacaag gcagtcactg actctttccg atccgtgtat 960

ggcatcaacc gtggaattgc tcaaggcagt gcagtggccg tgggcaggta ctctgaagat 1020

gtgtattaca acggcaaccc atggtatctt accactctcg ccgctgccga gcaactatac 1080

tctgcgatct atcagtggaa taagaaaggc tccatcaccg tagatgcagt atccctcccc 1140

ttcttccgcg atctagtacc atccatcgcg acaggaacat atgccagcag ctcgtcaacc 1200

tacacatcca tcgtctcagc agtaaagacg tacgcagacg gttacatcag cgtgatccag 1260

aaatacacgc cctcgaacgg cgcgctcgcc gagcaattcg aaaagagcaa tggatcacca 1320

ctgtccgccg cagacctcac ctggtcctac gccgcgttcc tcaccgcaac agagcggcgc 1380

gcaggcgtcg taagtcccac ctggggcgaa cccaccaaca acgttccccc atcaacctgc 1440

actggcacac ccgcctgcaa cgcgcgcatc acgttcaacg tgcgcgccac caccacgttc 1500

ggcgacaaca tcttcatcgt ggggcagctg acacagctgg gtaattggga tcctgcgagc 1560

gccgtgccgc tcagtgccag caagtatact agcagcgatc cgttgtggta tgccgatatc 1620

aatctcccgg ccgagacaac gtttgagtac aagtatattc gcaagacgag tgcagggcag 1680

gtggtgtggg agagtgatcc gaatagacgg tatacgacgt ctgcggggtg tggaagtagt 1740

gcgacggtga atgattcttg gaggtag 1767

<210> 30

<211> 588
<212> PRT
<213> Unknown

<220>
<223> Obtained from an environmental sample

<220>
<221> SIGNAL
<222> (1)...(21)

<220>
<221> DOMAIN
<222> (42)...(458)
<223> Glycosyl hydrolases family 15

<220>
<221> DOMAIN
<222> (487)...(583)
<223> Starch binding domain

<220>
<221> SITE
<222> (203)...(206)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (205)...(215)
<223> Glucoamylase active site region signature. Prosite id = PS00820

<220>
<221> SITE
<222> (214)...(217)
<223> N-glycosylation site. Prosite id = PS00001

<400> 30

```
Met Leu Phe Ser Ser Leu Leu Arg Ala Leu Ser Ala Ser Leu Leu Ala
1               5                  10                  15

Gly Ala Val Gln Gly Ser Pro Ala Asn Leu His Ala Arg Gln Thr Ser
            20                  25                  30

Val Asp Gln Phe Ile Gln Thr Gln Ala Glu Ile Ser Ile Lys Gly Val
        35                  40                  45

Leu Ala Asn Ile Gly Ala Asp Gly Ser Lys Ala Gln Gly Ala Pro Ala
    50                  55                  60

Gly Ile Val Val Ala Ser Pro Ser Arg Ser Asn Pro Asp Tyr Trp Tyr
65                  70                  75                  80

Thr Trp Thr Arg Asp Ala Ala Leu Thr Tyr Lys Ala Leu Ile Glu Arg
                85                  90                  95
```

```
Phe Val Asp Gly Asp Thr Ser Leu Arg Thr Lys Val Asp Glu Tyr Val
            100                 105                 110

Ser Ala Gln Ala Tyr Leu Gln Gly Val Ser Asn Pro Ser Gly Gly Pro
        115                 120                 125

Asp Ser Gly Gly Leu Gly Glu Pro Lys Phe Asn Val Asp Arg Thr Ala
    130                 135                 140

Phe Thr Gly Ala Trp Gly Arg Pro Gln Arg Asp Gly Pro Pro Leu Arg
145                 150                 155                 160

Ala Thr Ala Leu Ile Ile Tyr Ala Asn Trp Leu Val Ala Asn Gly Gln
                165                 170                 175

Gln Ser Gln Ala Leu Asn Thr Val Trp Pro Val Ile Ala Lys Asp Leu
            180                 185                 190

Ala Tyr Thr Val Arg Tyr Trp Asn Gln Thr Gly Phe Asp Leu Trp Glu
        195                 200                 205

Glu Val Asn Gly Ser Ser Phe Phe Thr Leu Ser Ala Ser His Arg Ala
    210                 215                 220

Leu Val Glu Gly Asn Ala Leu Ala Gln Lys Leu Gly Gln Thr Cys Thr
225                 230                 235                 240

Gly Cys Ala Asn Ala Ala Pro Gln Val Leu Cys Phe Val Gln Ser Phe
                245                 250                 255

Trp Thr Gly Ser Tyr Ile Asp Ser Asn Ile Asn Val Asn Asp Gly Arg
            260                 265                 270

Thr Gly Lys Asp Ala Asn Ser Ile Leu Ser Ser Ile His Thr Phe Asp
        275                 280                 285

Pro Glu Ala Gly Cys Thr Asp Ala Thr Phe Gln Pro Cys Ser Ser Arg
    290                 295                 300

Ala Leu Ala Asn His Lys Ala Val Thr Asp Ser Phe Arg Ser Val Tyr
305                 310                 315                 320

Gly Ile Asn Arg Gly Ile Ala Gln Gly Ser Ala Val Ala Val Gly Arg
                325                 330                 335

Tyr Ser Glu Asp Val Tyr Tyr Asn Gly Asn Pro Trp Tyr Leu Thr Thr
            340                 345                 350
```

```
Leu Ala Ala Ala Glu Gln Leu Tyr Ser Ala Ile Tyr Gln Trp Asn Lys
        355                 360                 365

Lys Gly Ser Ile Thr Val Asp Ala Val Ser Leu Pro Phe Phe Arg Asp
    370                 375                 380

Leu Val Pro Ser Ile Ala Thr Gly Thr Tyr Ala Ser Ser Ser Ser Thr
385                 390                 395                 400

Tyr Thr Ser Ile Val Ser Ala Val Lys Thr Tyr Ala Asp Gly Tyr Ile
                405                 410                 415

Ser Val Ile Gln Lys Tyr Thr Pro Ser Asn Gly Ala Leu Ala Glu Gln
            420                 425                 430

Phe Glu Lys Ser Asn Gly Ser Pro Leu Ser Ala Ala Asp Leu Thr Trp
        435                 440                 445

Ser Tyr Ala Ala Phe Leu Thr Ala Thr Glu Arg Arg Ala Gly Val Val
    450                 455                 460

Ser Pro Thr Trp Gly Glu Pro Thr Asn Asn Val Pro Pro Ser Thr Cys
465                 470                 475                 480

Thr Gly Thr Pro Ala Cys Asn Ala Arg Ile Thr Phe Asn Val Arg Ala
                485                 490                 495

Thr Thr Thr Phe Gly Asp Asn Ile Phe Ile Val Gly Gln Leu Thr Gln
            500                 505                 510

Leu Gly Asn Trp Asp Pro Ala Ser Ala Val Pro Leu Ser Ala Ser Lys
        515                 520                 525

Tyr Thr Ser Ser Asp Pro Leu Trp Tyr Ala Asp Ile Asn Leu Pro Ala
    530                 535                 540

Glu Thr Thr Phe Glu Tyr Lys Tyr Ile Arg Lys Thr Ser Ala Gly Gln
545                 550                 555                 560

Val Val Trp Glu Ser Asp Pro Asn Arg Arg Tyr Thr Thr Ser Ala Gly
                565                 570                 575

Cys Gly Ser Ser Ala Thr Val Asn Asp Ser Trp Arg
            580                 585
```

<210> 31
<211> 1479
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 31

atggttctag ctcggcttgc ctggcttgcc ggcctggtta gcactgccat tgctgcgacc 60

ccagcggaat ggcgctcgca gtccatctac ttcatgctca cggatcgttt tgcccggacc 120

gatggttcaa ctactgctgc ctgtgatacc gctgacagaa aatactgtgg cggaacctgg 180

cagggtatca ttgacaagct ggactatatc caaggaatgg gctttacggc catttggatc 240

actccggtga ccggtcaatt gagcggggaa accgcgtacg gagatcccta tcacggatac 300

tggcagcagg atatctactc tctcgattcc aactatggaa ccgcagacga tctcaaggcc 360

ctcgctgcgg ctttgcacaa acgcgacatg tatctcatgg tcgatgtcgt agcaaaccac 420

atgggctaca atggcgcagg tgctgacgtg gactacacca aattcaaccc cttcaacgat 480

gcaaagtatt tccactccta ctgcccaatc accgattaca acgacgacac catgtcgcaa 540

aactgctggc ttggcgataa caaggtctcg ctaccggatc tgaatacaca gagcaaggag 600

gtgcaggatc tatggtatga ctgggttgga tctttggtct ccaactactc catcgatgga 660

cttcgcgtcg acacagtcaa acatgtccag aaagatttct ggcccggcta caacaaagcc 720

gcgggcgtct actgcgtagg cgaaatcctt gatggtgacc cagattacac ctatccatac 780

caggaggtaa tggacggagt gctcaactac ccgatttact acccactcct caaggccttc 840

cagtcgagct cgggaagcat gaccgatctg tacaacatga tcaacacggt gaaatcgacc 900

tgcaaggact caacccttct tggaaatttc ttggagaacc acgataaccc acgttttgcc 960

catgccaccg atgacattgc cctcgccaag aacgcagcca catttaccat tatggcagat 1020

ggcattccta ttgtctatgc aggacaggag cagcactaca gtggtggcga ggacccggct 1080

aatcgcgagg ctctgtggtt atccggatac aacaccgaca gcgagctgta caagctcatt 1140

gccaaggcca atggtgctag aaaccaggcc attgctaaga gtaccaatta tactatttac 1200

cagaaccacc caatctacaa agacgagagc gccatcgcca tgcggaaggg cttcgtcggt 1260

ggacagacaa tcactgtcct gacgaatctc ggtgcagggg gtaaagagta ttcagtttca 1320

attcctgata ctggattcaa ggctggtgcg aagttgactg aggttgtgtc ctgcactagt 1380

gttactgttg gtgatagtgg ggaggtgtct gttcctatgg cgagtggagc gccgaggatc 1440

ttgctcccca cgtctttgct cgagggctcg gctctgtgc 1479

<210> 32
<211> 493
<212> PRT
<213> Unknown

<220>
<223> Obtained from an environmental sample

<220>
<221> SIGNAL
<222> (1)...(18)

<220>
<221> DOMAIN
<222> (31)...(387)
<223> Alpha amylase, catalytic domain

<220>
<221> SITE
<222> (197)...(200)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (218)...(221)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (402)...(405)
<223> N-glycosylation site. Prosite id = PS00001

<400> 32

```
Met Val Leu Ala Arg Leu Ala Trp Leu Ala Gly Leu Val Ser Thr Ala
1               5                  10                  15

Ile Ala Ala Thr Pro Ala Glu Trp Arg Ser Gln Ser Ile Tyr Phe Met
            20                  25                  30

Leu Thr Asp Arg Phe Ala Arg Thr Asp Gly Ser Thr Thr Ala Ala Cys
        35                  40                  45

Asp Thr Ala Asp Arg Lys Tyr Cys Gly Gly Thr Trp Gln Gly Ile Ile
    50                  55                  60

Asp Lys Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala Ile Trp Ile
65                  70                  75                  80

Thr Pro Val Thr Gly Gln Leu Ser Gly Glu Thr Ala Tyr Gly Asp Pro
                85                  90                  95

Tyr His Gly Tyr Trp Gln Gln Asp Ile Tyr Ser Leu Asp Ser Asn Tyr
            100                 105                 110

Gly Thr Ala Asp Asp Leu Lys Ala Leu Ala Ala Ala Leu His Lys Arg
        115                 120                 125

Asp Met Tyr Leu Met Val Asp Val Val Ala Asn His Met Gly Tyr Asn
    130                 135                 140

Gly Ala Gly Ala Asp Val Asp Tyr Thr Lys Phe Asn Pro Phe Asn Asp
145                 150                 155                 160

Ala Lys Tyr Phe His Ser Tyr Cys Pro Ile Thr Asp Tyr Asn Asp Asp
                165                 170                 175
```

```
Thr Met Ser Gln Asn Cys Trp Leu Gly Asp Asn Lys Val Ser Leu Pro
            180                 185                 190

Asp Leu Asn Thr Gln Ser Lys Glu Val Gln Asp Leu Trp Tyr Asp Trp
        195                 200                 205

Val Gly Ser Leu Val Ser Asn Tyr Ser Ile Asp Gly Leu Arg Val Asp
    210                 215                 220

Thr Val Lys His Val Gln Lys Asp Phe Trp Pro Gly Tyr Asn Lys Ala
225                 230                 235                 240

Ala Gly Val Tyr Cys Val Gly Glu Ile Leu Asp Gly Asp Pro Asp Tyr
                245                 250                 255

Thr Tyr Pro Tyr Gln Glu Val Met Asp Gly Val Leu Asn Tyr Pro Ile
            260                 265                 270

Tyr Tyr Pro Leu Leu Lys Ala Phe Gln Ser Ser Ser Gly Ser Met Thr
        275                 280                 285

Asp Leu Tyr Asn Met Ile Asn Thr Val Lys Ser Thr Cys Lys Asp Ser
    290                 295                 300

Thr Leu Leu Gly Asn Phe Leu Glu Asn His Asp Asn Pro Arg Phe Ala
305                 310                 315                 320

His Ala Thr Asp Asp Ile Ala Leu Ala Lys Asn Ala Ala Thr Phe Thr
                325                 330                 335

Ile Met Ala Asp Gly Ile Pro Ile Val Tyr Ala Gly Gln Glu Gln His
            340                 345                 350

Tyr Ser Gly Gly Glu Asp Pro Ala Asn Arg Glu Ala Leu Trp Leu Ser
        355                 360                 365

Gly Tyr Asn Thr Asp Ser Glu Leu Tyr Lys Leu Ile Ala Lys Ala Asn
    370                 375                 380

Gly Ala Arg Asn Gln Ala Ile Ala Lys Ser Thr Asn Tyr Thr Ile Tyr
385                 390                 395                 400

Gln Asn His Pro Ile Tyr Lys Asp Glu Ser Ala Ile Ala Met Arg Lys
                405                 410                 415

Gly Phe Val Gly Gly Gln Thr Ile Thr Val Leu Thr Asn Leu Gly Ala
            420                 425                 430
```

Gly Gly Lys Glu Tyr Ser Val Ser Ile Pro Asp Thr Gly Phe Lys Ala
435 440 445

Gly Ala Lys Leu Thr Glu Val Val Ser Cys Thr Ser Val Thr Val Gly
450 455 460

Asp Ser Gly Glu Val Ser Val Pro Met Ala Ser Gly Ala Pro Arg Ile
465 470 475 480

Leu Leu Pro Thr Ser Leu Leu Glu Gly Ser Ala Leu Cys
485 490

<210> 33
<211> 1485
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 33

atggtaggct tcaatatctt gactctcgcc ttgctggccc cagctgcact ctcatctgct 60

gtccaccctc accggcgcca gtctggactt gatgccttta ttcagtccga gtcatcggtt 120

tcactccaag gcattctgaa caatatcgga gccaatggct ctgctgtttc aggcgcatcc 180

gccggtgtag ttgttgcgtc accatccaag tcagaccccg attattttta cacttggact 240

cgcgatgcgg ccctgacact cgcggtactg attgaccaat tcatcgcggg agaaagctcc 300

ctagagacgg tcatccagca gtacatctcc gcccaagcaa aactgcagac agtgtccaac 360

ccatcaggag acctgtccga cggttcaggt ctggcagagc ccaaattcca aaccgatctc 420

agcgccttca ctagatcctg gggtcgacct caacgcgatg gacctgctct ccgtgcgaca 480

gcgctcatcg tgtacggaaa tcacctcctc tcttccggaa agcagtccgt cgtcaaatcc 540

aacatctggc cgatcgtgca gaacgatctc aactacgtgg cacagtactg gaaccagact 600

ggtttcgatc tgtgggaaga ggtccagggc tcgtcctttt tcaccatcgc agcgcaacac 660

cgtgccctgg tggagggagc tgcattcgcc aaatcgctcg gagaggcctg tgatggctgt 720

gattcacagg ctccgcaggt tctttgcttc ctccaggact tctggaatgg aaatgctgtt 780

gtttctaatc tggcaaatga tggtcgctca gggttggatg ccaattctat cctcagctcg 840

atccagacct tcgatcctag tgccacttgc gatgatagca ctttccagcc atgctctggt 900

cgtgcactgc taaaccacaa ggcggtggtt gattctttcc gatccatcta caacattaac 960

agtggtaaag aagctggcaa agccgtggct gttggtcgct atgccgaaga cacctaccag 1020

ggaggcaacc cttggtatct cgctactgct gctgccgccg agcagctgta tgatgctctc 1080

taccagtgga agaaacaggg atcgctggcg attacccaga cgagtcttcc cttcttccag 1140

gatttggact cgactgctcg tgttggcaat tactccagct cttcctcaac ctacacctct 1200

ttgactggcg ctgtcaagac ctatgcggat ggtttcctat cgattgttca gcagtatacg 1260

cccagcaatg gagctctggc cgagcagttc acgcgagaca atggcacccc cgtctctgca 1320

cacgacctga cctggtctta tgcatcattt ttgactgctg ccgaccgtcg caatggaatt 1380

gtccctgcta gctggggtgc atctaaggcc aaccaagtgc cgacgcagtg ccagggcagt 1440

tcagcgacgg ggtcttatac tacgccaact gtggggtcct ggtga 1485

<210> 34
<211> 494
<212> PRT
<213> Unknown

<220>
<223> Obtained from an environmental sample

<220>
<221> DOMAIN
<222> (39)...(455)

<223> Glycosyl hydrolases family 15

<220>
<221> SITE
<222> (52)...(55)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (201)...(204)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (203)...(213)
<223> Glucoamylase active site region signature. Prosite id = PS00820

<400> 34

```
Met Val Gly Phe Asn Ile Leu Thr Leu Ala Leu Leu Ala Pro Ala Ala
1               5                  10                  15

Leu Ser Ser Ala Val His Pro His Arg Arg Gln Ser Gly Leu Asp Ala
            20                  25                  30

Phe Ile Gln Ser Glu Ser Ser Val Ser Leu Gln Gly Ile Leu Asn Asn
        35                  40                  45

Ile Gly Ala Asn Gly Ser Ala Val Ser Gly Ala Ser Ala Gly Val Val
    50                  55                  60

Val Ala Ser Pro Ser Lys Ser Asp Pro Asp Tyr Phe Tyr Thr Trp Thr
65                  70                  75                  80

Arg Asp Ala Ala Leu Thr Leu Ala Val Leu Ile Asp Gln Phe Ile Ala
                85                  90                  95

Gly Glu Ser Ser Leu Glu Thr Val Ile Gln Gln Tyr Ile Ser Ala Gln
            100                 105                 110
```

```
Ala Lys Leu Gln Thr Val Ser Asn Pro Ser Gly Asp Leu Ser Asp Gly
        115                 120                 125

Ser Gly Leu Ala Glu Pro Lys Phe Gln Thr Asp Leu Ser Ala Phe Thr
    130                 135                 140

Arg Ser Trp Gly Arg Pro Gln Arg Asp Gly Pro Ala Leu Arg Ala Thr
145                 150                 155                 160

Ala Leu Ile Val Tyr Gly Asn His Leu Leu Ser Ser Gly Lys Gln Ser
                165                 170                 175

Val Val Lys Ser Asn Ile Trp Pro Ile Val Gln Asn Asp Leu Asn Tyr
            180                 185                 190

Val Ala Gln Tyr Trp Asn Gln Thr Gly Phe Asp Leu Trp Glu Glu Val
        195                 200                 205

Gln Gly Ser Ser Phe Phe Thr Ile Ala Ala Gln His Arg Ala Leu Val
    210                 215                 220

Glu Gly Ala Ala Phe Ala Lys Ser Leu Gly Glu Ala Cys Asp Gly Cys
225                 230                 235                 240

Asp Ser Gln Ala Pro Gln Val Leu Cys Phe Leu Gln Asp Phe Trp Asn
                245                 250                 255

Gly Asn Ala Val Val Ser Asn Leu Ala Asn Asp Gly Arg Ser Gly Leu
            260                 265                 270

Asp Ala Asn Ser Ile Leu Ser Ser Ile Gln Thr Phe Asp Pro Ser Ala
        275                 280                 285

Thr Cys Asp Asp Ser Thr Phe Gln Pro Cys Ser Gly Arg Ala Leu Leu
    290                 295                 300

Asn His Lys Ala Val Val Asp Ser Phe Arg Ser Ile Tyr Asn Ile Asn
305                 310                 315                 320

Ser Gly Lys Glu Ala Gly Lys Ala Val Ala Val Gly Arg Tyr Ala Glu
                325                 330                 335

Asp Thr Tyr Gln Gly Gly Asn Pro Trp Tyr Leu Ala Thr Ala Ala Ala
            340                 345                 350

Ala Glu Gln Leu Tyr Asp Ala Leu Tyr Gln Trp Lys Lys Gln Gly Ser
        355                 360                 365
```

```
Leu Ala Ile Thr Gln Thr Ser Leu Pro Phe Phe Gln Asp Leu Asp Ser
    370                 375                 380

Thr Ala Arg Val Gly Asn Tyr Ser Ser Ser Ser Ser Thr Tyr Thr Ser
385                 390                 395                 400

Leu Thr Gly Ala Val Lys Thr Tyr Ala Asp Gly Phe Leu Ser Ile Val
                405                 410                 415

Gln Gln Tyr Thr Pro Ser Asn Gly Ala Leu Ala Glu Gln Phe Thr Arg
            420                 425                 430

Asp Asn Gly Thr Pro Val Ser Ala His Asp Leu Thr Trp Ser Tyr Ala
        435                 440                 445

Ser Phe Leu Thr Ala Ala Asp Arg Arg Asn Gly Ile Val Pro Ala Ser
    450                 455                 460

Trp Gly Ala Ser Lys Ala Asn Gln Val Pro Thr Gln Cys Gln Gly Ser
465                 470                 475                 480

Ser Ala Thr Gly Ser Tyr Thr Thr Pro Thr Val Gly Ser Trp
                485                 490
```

<210> 35
<211> 1914
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 35

atggctcctc gtttttggac tacgctgtgt gcgctgactc ttggttcagc tgcgctggcc 60

gcgccacagt tggcaccgcg tgcaactggt agcttggaca cttggctggc atccgagaaa 120

gctgttgcaa gggagggtat tctcgataat atcgggtcgg atggggcgta tgctaagagc 180

tcgaagtcgg ggattgtcat tgccagtccg agtacagaca gtcccgatta ttactacact 240

tggactcgtg actcggccct ggtcatgaag accttggtgg acctgttcaa gaatggcgat 300

accgaccttc tcaccgttat cgaggagtac atcagctccc aggcctatat ccagaccgtt 360

tccaacccat ctggaagtct ttcgagtggt ggactcggtg aacccaagtt caatgttgat 420

gaaacatcgt ataccggatc ttggggtcga cctcagcgcg atggtcctgc actccgagct 480

actgcactgg ttgccttcgg acagtggttg attgacaacg gatatacatc taccgccacc 540

gatattgtgt ggccaattgt tcgcaatgat ctttcctacg tcgcacaata ctggaacagc 600

tctggatacg acctatggga agaggtcaac ggtgaatcat tcttcaccac cgccgttcaa 660

caccgtgctt tggtcgaggg cagcaagttc gccagccagg tgggatcatc ttgctcctac 720

tgcgactctc aggcacccca ggttctctgc ttccttcaat catactggac cggctcgtac 780

actctagcca atttcggtag cagccgcaca ggcaaagacg cgaacaccct cctgggcagc 840

attcacacat tcgaccccga ggcaggatgc gatgacacga ccttccagcc ttgctcggcc 900

cgcgcgctgg caaatcacaa ggtcgtcact gactcgttcc gctctattta taccgtcaat 960

tcaggcaaga gtgcgggaca agcagtggca gttggtcgat accccgagga ttcttactac 1020

aatggtaacc catggtactt gtgcactatg gctgctgccg agcttctcta cgatgctttg 1080

taccaatgga ataaagctgg ctctttgacc atcagcagcg tctcgctgag cttcttcacc 1140

gacctgtaca gctcagctgc aactggtacc tattcatcat ccagttctac gttctcatcg 1200

atagtcagcg cggtgaagac ctacgcggat ggctacatga gcattgccga gcaatatgcc 1260

tatacgaacg gctctatgtc tgaacaattc tccaagtctg atggaactcc agagtctgcc 1320

cgtgacctga cttggtcata tgcagccctg cttaccgcaa acatgcgtcg caactcggtt 1380

gttcccccaa cctggggcga gacatctgca agcagcgtcc cgggaacatg ctccgcgacg 1440

tcggcgacgg gaacctacag cactgcaacc aacacgaact ggcccgtcac attgactggt 1500

ggatcgggct ctactactac tggtggaaca acgaccagca aaaccagcac caccacaact 1560

tccaagacga gcactaccac tacatcatgc acgactccaa ctagtgtggc agttacattt 1620

gatgttatcg cgaccacttc atacggcgag aacatcaagt tggctgggtc aattgctgcc 1680

cttggtagct gggacaccag tagcgctatt gcactaagtg cagataaata tactagctcg 1740

aacaacctat ggtatgtaac tgtgaatctg gctgctggtc aggtcattca gtacaagtat 1800

atccgggttg aaagtgatag tacgattgag tgggagagtg atccgaaccg ctcttatact 1860

gtgccagcag cctgtgccac aactgccgtg acaatcagcg acacttggcg gtaa 1914

<210> 36
<211> 637
<212> PRT
<213> Unknown

<220>
<223> Obtained from an environmental sample

<220>
<221> SIGNAL
<222> (1)...(20)

<220>
<221> DOMAIN
<222> (41)...(455)
<223> Glycosyl hydrolases family 15

<220>
<221> DOMAIN
<222> (535)...(631)
<223> Starch binding domain

<220>
<221> SITE
<222> (202)...(205)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (204)...(214)
<223> Glucoamylase active site region signature. Prosite id = PS00820

<220>
<221> SITE
<222> (429)...(432)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (625)...(628)
<223> N-glycosylation site. Prosite id = PS00001

<400> 36

```
Met Ala Pro Arg Phe Trp Thr Thr Leu Cys Ala Leu Thr Leu Gly Ser
1               5                  10                  15

Ala Ala Leu Ala Ala Pro Gln Leu Ala Pro Arg Ala Thr Gly Ser Leu
            20                  25                  30

Asp Thr Trp Leu Ala Ser Glu Lys Ala Val Ala Arg Glu Gly Ile Leu
        35                  40                  45

Asp Asn Ile Gly Ser Asp Gly Ala Tyr Ala Lys Ser Ser Lys Ser Gly
    50                  55                  60

Ile Val Ile Ala Ser Pro Ser Thr Asp Ser Pro Asp Tyr Tyr Tyr Thr
65                  70                  75                  80

Trp Thr Arg Asp Ser Ala Leu Val Met Lys Thr Leu Val Asp Leu Phe
                85                  90                  95

Lys Asn Gly Asp Thr Asp Leu Leu Thr Val Ile Glu Glu Tyr Ile Ser
            100                 105                 110

Ser Gln Ala Tyr Ile Gln Thr Val Ser Asn Pro Ser Gly Ser Leu Ser
        115                 120                 125

Ser Gly Gly Leu Gly Glu Pro Lys Phe Asn Val Asp Glu Thr Ser Tyr
    130                 135                 140

Thr Gly Ser Trp Gly Arg Pro Gln Arg Asp Gly Pro Ala Leu Arg Ala
145                 150                 155                 160

Thr Ala Leu Val Ala Phe Gly Gln Trp Leu Ile Asp Asn Gly Tyr Thr
                165                 170                 175

Ser Thr Ala Thr Asp Ile Val Trp Pro Ile Val Arg Asn Asp Leu Ser
            180                 185                 190
```

```
Tyr Val Ala Gln Tyr Trp Asn Ser Ser Gly Tyr Asp Leu Trp Glu Glu
        195                 200                 205

Val Asn Gly Glu Ser Phe Phe Thr Thr Ala Val Gln His Arg Ala Leu
    210                 215                 220

Val Glu Gly Ser Lys Phe Ala Ser Gln Val Gly Ser Ser Cys Ser Tyr
225                 230                 235                 240

Cys Asp Ser Gln Ala Pro Gln Val Leu Cys Phe Leu Gln Ser Tyr Trp
                245                 250                 255

Thr Gly Ser Tyr Thr Leu Ala Asn Phe Gly Ser Ser Arg Thr Gly Lys
            260                 265                 270

Asp Ala Asn Thr Leu Leu Gly Ser Ile His Thr Phe Asp Pro Glu Ala
        275                 280                 285

Gly Cys Asp Asp Thr Thr Phe Gln Pro Cys Ser Ala Arg Ala Leu Ala
    290                 295                 300

Asn His Lys Val Val Thr Asp Ser Phe Arg Ser Ile Tyr Thr Val Asn
305                 310                 315                 320

Ser Gly Lys Ser Ala Gly Gln Ala Val Ala Val Gly Arg Tyr Pro Glu
                325                 330                 335

Asp Ser Tyr Tyr Asn Gly Asn Pro Trp Tyr Leu Cys Thr Met Ala Ala
            340                 345                 350

Ala Glu Leu Leu Tyr Asp Ala Leu Tyr Gln Trp Asn Lys Ala Gly Ser
        355                 360                 365

Leu Thr Ile Ser Ser Val Ser Leu Ser Phe Phe Thr Asp Leu Tyr Ser
    370                 375                 380

Ser Ala Ala Thr Gly Thr Tyr Ser Ser Ser Ser Ser Thr Phe Ser Ser
385                 390                 395                 400

Ile Val Ser Ala Val Lys Thr Tyr Ala Asp Gly Tyr Met Ser Ile Ala
                405                 410                 415

Glu Gln Tyr Ala Tyr Thr Asn Gly Ser Met Ser Glu Gln Phe Ser Lys
            420                 425                 430

Ser Asp Gly Thr Pro Glu Ser Ala Arg Asp Leu Thr Trp Ser Tyr Ala
        435                 440                 445
```

```
Ala Leu Leu Thr Ala Asn Met Arg Arg Asn Ser Val Val Pro Pro Thr
 450                 455                 460

Trp Gly Glu Thr Ser Ala Ser Ser Val Pro Gly Thr Cys Ser Ala Thr
465                 470                 475                 480

Ser Ala Thr Gly Thr Tyr Ser Thr Ala Thr Asn Thr Asn Trp Pro Val
                485                 490                 495

Thr Leu Thr Gly Gly Ser Gly Ser Thr Thr Thr Gly Gly Thr Thr Thr
            500                 505                 510

Ser Lys Thr Ser Thr Thr Thr Thr Ser Lys Thr Ser Thr Thr Thr Thr
        515                 520                 525

Ser Cys Thr Thr Pro Thr Ser Val Ala Val Thr Phe Asp Val Ile Ala
 530                 535                 540

Thr Thr Ser Tyr Gly Glu Asn Ile Lys Leu Ala Gly Ser Ile Ala Ala
545                 550                 555                 560

Leu Gly Ser Trp Asp Thr Ser Ser Ala Ile Ala Leu Ser Ala Asp Lys
                565                 570                 575

Tyr Thr Ser Ser Asn Asn Leu Trp Tyr Val Thr Val Asn Leu Ala Ala
            580                 585                 590

Gly Gln Val Ile Gln Tyr Lys Tyr Ile Arg Val Glu Ser Asp Ser Thr
        595                 600                 605

Ile Glu Trp Glu Ser Asp Pro Asn Arg Ser Tyr Thr Val Pro Ala Ala
 610                 615                 620

Cys Ala Thr Thr Ala Val Thr Ile Ser Asp Thr Trp Arg
625                 630                 635
```

<210> 37
<211> 1923
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 37

atggctcctc gattctggat tgcgctttgg gcgcttactt tcggtcaggc gatcgctgcg 60

ccgcagatcg ccttggcacc tcgcgcgaca ggtagcttgg acacctggtt ggcatcggag 120

accacggtgg ccagacaagg tatcctgaac aatatcggtt ccgctggcgc gtattctgcg 180

agcgcgaagc cgggaatcct gattgctagt cctagcactt ccagtcccga ttattactac 240

acatggaccc gtgactctgc tttggtcttc aaggccttgg tggatatgtt caagaatggt 300

gacactgccc ttctcactgt gatcgaagaa tatatcagcg cacaggccta tatccagaca 360

gtctccaacc cttctggtgg tctttccagc ggaggactcg gagagcccaa attcaatgtt 420

gatgagactg ccttcaccgg gtcttggggc cgcccccagc gtgatggtcc ggctctccgc 480

gcaactgcct tgatctcatt cggacagtgg ctccttgaca acggatactc cacatacgcg 540

accaacattg tttggccggt tgtacgaaac gatctctctt acgttgctca atactggaac 600

caaactggat acgatctctg ggaagaggtt tccggctcct ccttcttcac gattgccgcc 660

cagcaccgcg ccttggtgga aggtagcact ttcgccacca gggtcggtgc ttcgtgctca 720

tactgtgatt ctcaggcacc ccaggtgctg tgcttcctcc aatccttctg gactggctca 780

tacacactgg ccaactttgg cggtggccgc tctggcaaag acgcgaatac tcttctcgga 840

agcattcaca catttgaccc cgaggccggc tgcgatgaca ccaccttcca accatgctcg 900

gctcgagcat tggccaacca caaggtggtg actgactctt tccgatcggt ttacacaatc 960

aactctggga ttgcggcagg caaggctgtc tctgttggcc gttactcgga ggattcttac 1020

tataatggaa acccttggta tctgtgtacc ctggctgcag cggagcagtt atatgacgct 1080

ctttacacgt ggaatcgggt tggctctttg actatcactt ctgtctcgct gagtttcttc 1140

aaagatttgt acagttctgc tgctactggt acctactcat cgtccagtgc tacatactct 1200

tcgatcgtca gcgcagtcaa gacttacgcc gatggatatg tcagtattgt ggagaactac 1260

gctctgacca atggctctat gtccgagcaa ttttcaaagt ccgatggttc tcaactgtcc 1320

gctcgtgact tgacttggtc atacgcagct cttctcactg ccaatgagcg ccgaaatgcc 1380

gtcgtcccgg cgccatgggg cgagacggcg gccagcagtg tacctgccca atgcagctcc 1440

acttctgcca ctggtacttt cagtacggcc accaacaccg catggccatc gaccctcact 1500

agcggaaccg gaagtggaac caccacgacc ggcactggga cgaccacaaa ggcaaccacc 1560

acaacttcta ccaagactac ctccacgacc acatcatgta ccacaccgac gtcggtggcg 1620

gtgacctttg atgtgattgc caccactgtg tatggagaga atatcaagct tgccggctct 1680

atttctcaac ttggctcgtg ggatacaagc agtgcgattg ctctcagcgc ttcttcttac 1740

acttcaagca accatctttg gtatgtgacg gtcacactgc ccgctggttc taccttcacc 1800

tataagtata ttcgggtcga gagtgatggc tcaatccagt gggagagtga tccaaacttg 1860

tcatacaccg tccctcaagc ttgcggcact tcagctgtca caattagcga tacttggagg 1920

tga 1923

<210> 38
<211> 640
<212> PRT
<213> Unknown

<220>
<223> Obtained from an environmental sample

<220>
<221> SIGNAL
<222> (1)...(19)

<220>
<221> DOMAIN
<222> (42)...(456)
<223> Glycosyl hydrolases family 15

<220>
<221> DOMAIN
<222> (538)...(634)
<223> Starch binding domain

<220>
<221> SITE
<222> (203)...(206)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (205)...(215)
<223> Glucoamylase active site region signature. Prosite id = PS00820

<220>
<221> SITE
<222> (430)...(433)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (628)...(631)
<223> N-glycosylation site. Prosite id = PS00001

<400> 38

```
Met Ala Pro Arg Phe Trp Ile Ala Leu Trp Ala Leu Thr Phe Gly Gln
1               5                  10                  15

Ala Ile Ala Ala Pro Gln Ile Ala Leu Ala Pro Arg Ala Thr Gly Ser
            20                  25                  30

Leu Asp Thr Trp Leu Ala Ser Glu Thr Thr Val Ala Arg Gln Gly Ile
        35                  40                  45

Leu Asn Asn Ile Gly Ser Ala Gly Ala Tyr Ser Ala Ser Ala Lys Pro
    50                  55                  60

Gly Ile Leu Ile Ala Ser Pro Ser Thr Ser Ser Pro Asp Tyr Tyr Tyr
65                  70                  75                  80

Thr Trp Thr Arg Asp Ser Ala Leu Val Phe Lys Ala Leu Val Asp Met
                85                  90                  95

Phe Lys Asn Gly Asp Thr Ala Leu Leu Thr Val Ile Glu Glu Tyr Ile
            100                 105                 110
```

Ser Ala Gln Ala Tyr Ile Gln Thr Val Ser Asn Pro Ser Gly Gly Leu
115 120 125

Ser Ser Gly Gly Leu Gly Glu Pro Lys Phe Asn Val Asp Glu Thr Ala
130 135 140

Phe Thr Gly Ser Trp Gly Arg Pro Gln Arg Asp Gly Pro Ala Leu Arg
145 150 155 160

Ala Thr Ala Leu Ile Ser Phe Gly Gln Trp Leu Leu Asp Asn Gly Tyr
165 170 175

Ser Thr Tyr Ala Thr Asn Ile Val Trp Pro Val Val Arg Asn Asp Leu
180 185 190

Ser Tyr Val Ala Gln Tyr Trp Asn Gln Thr Gly Tyr Asp Leu Trp Glu
195 200 205

Glu Val Ser Gly Ser Ser Phe Phe Thr Ile Ala Ala Gln His Arg Ala
210 215 220

Leu Val Glu Gly Ser Thr Phe Ala Thr Arg Val Gly Ala Ser Cys Ser
225 230 235 240

Tyr Cys Asp Ser Gln Ala Pro Gln Val Leu Cys Phe Leu Gln Ser Phe
245 250 255

Trp Thr Gly Ser Tyr Thr Leu Ala Asn Phe Gly Gly Gly Arg Ser Gly
260 265 270

Lys Asp Ala Asn Thr Leu Leu Gly Ser Ile His Thr Phe Asp Pro Glu
275 280 285

Ala Gly Cys Asp Asp Thr Thr Phe Gln Pro Cys Ser Ala Arg Ala Leu
290 295 300

Ala Asn His Lys Val Val Thr Asp Ser Phe Arg Ser Val Tyr Thr Ile
305 310 315 320

Asn Ser Gly Ile Ala Ala Gly Lys Ala Val Ser Val Gly Arg Tyr Ser
325 330 335

Glu Asp Ser Tyr Tyr Asn Gly Asn Pro Trp Tyr Leu Cys Thr Leu Ala
340 345 350

Ala Ala Glu Gln Leu Tyr Asp Ala Leu Tyr Thr Trp Asn Arg Val Gly
355 360 365

Ser Leu Thr Ile Thr Ser Val Ser Leu Ser Phe Phe Lys Asp Leu Tyr

370 375 380

Ser Ser Ala Ala Thr Gly Thr Tyr Ser Ser Ser Ser Ala Thr Tyr Ser
385 390 395 400

Ser Ile Val Ser Ala Val Lys Thr Tyr Ala Asp Gly Tyr Val Ser Ile
405 410 415

Val Glu Asn Tyr Ala Leu Thr Asn Gly Ser Met Ser Glu Gln Phe Ser
420 425 430

Lys Ser Asp Gly Ser Gln Leu Ser Ala Arg Asp Leu Thr Trp Ser Tyr
435 440 445

Ala Ala Leu Leu Thr Ala Asn Glu Arg Arg Asn Ala Val Val Pro Ala
450 455 460

Pro Trp Gly Glu Thr Ala Ala Ser Ser Val Pro Ala Gln Cys Ser Ser
465 470 475 480

Thr Ser Ala Thr Gly Thr Phe Ser Thr Ala Thr Asn Thr Ala Trp Pro
485 490 495

Ser Thr Leu Thr Ser Gly Thr Gly Ser Gly Thr Thr Thr Thr Gly Thr
500 505 510

Gly Thr Thr Thr Lys Ala Thr Thr Thr Thr Ser Thr Lys Thr Thr Ser
515 520 525

Thr Thr Thr Ser Cys Thr Thr Pro Thr Ser Val Ala Val Thr Phe Asp
530 535 540

Val Ile Ala Thr Thr Val Tyr Gly Glu Asn Ile Lys Leu Ala Gly Ser
545 550 555 560

Ile Ser Gln Leu Gly Ser Trp Asp Thr Ser Ser Ala Ile Ala Leu Ser
565 570 575

Ala Ser Ser Tyr Thr Ser Ser Asn His Leu Trp Tyr Val Thr Val Thr
580 585 590

Leu Pro Ala Gly Ser Thr Phe Thr Tyr Lys Tyr Ile Arg Val Glu Ser
595 600 605

Asp Gly Ser Ile Gln Trp Glu Ser Asp Pro Asn Leu Ser Tyr Thr Val
610 615 620

Pro Gln Ala Cys Gly Thr Ser Ala Val Thr Ile Ser Asp Thr Trp Arg
625 630 635 640

<210> 39
<211> 1896
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 39

atggctcctc gtttttggac tgcgctttgg gcgcttacgc ttggccatgc cgttgtggcc 60

acgccacaga tattggctcc tcgtgcgact ggcagtttgg atacctggtt ggcgtctgag 120

actgtggtgg caagacaggg tatcttggac aacatcggat cggctggtgc ccatgctgcc 180

aatgcaaagc cgggcgttgt actcgccagc ccgagtacct ccgaccctga ttattattat 240

acttggactc gtgactcggc cctagtgttc aaaaatctgg tcgacatgtt caggagcggc 300

gacagcgctt tgttggaggt tatcgaagaa tacatcagct cgcaggccta tatccagacg 360

gtgtcaaatc catctggagg tctttccggc ggcggtggat tgggagagcc caagttcaac 420

gccgatgaga cagctttcac tggctcttgg ggacgccctc agcgtgatgg accagccttg 480

cgagccactg ccttgatctc atttggccaa tggcttattg acaatgggta caccacctac 540

gcgaccgaca ttgtctggcc tgtcgtgcgc aatgatctct cctatgtttc ccagtactgg 600

aatcagactg gatttgatct ctgggaagaa gtttctggct catcattctt caccgtcgcg 660

gctcagcacc gcgctttggt ggagggaagc acattcgcaa gtcaggtcgg ttcttcatgt 720

ttgtactgtg actcccaggc tccgcaggtt ctgtgcttcc tacagtcctt ctggactgga 780

tcttacattc tggccaactt tggtggtggc cgctctggga aggacgccaa tacactgctc 840

ggcagcattc acacattcga cccagaggca ggatgcgatg acacgacgtt ccagccttgc 900

tcagcacgag cactcgcaaa ccacaaagtt gtaactgact cgttccgatc gatctactct 960

gtcaactctg gtatcgctgc gggtaaggct gtttctgttg gtcgataccc agaggactca 1020

tactacaatg gtaacccttg gtatctgtgc actttggctg cagctgagca gttgtatgat 1080

gctatctaca catggaaccg tattggttct ttgaccatta cctctgtctc tttgagcttc 1140

ttcaaagacc tatacagctc tgctgcgacc ggcacctact cctcatccag tgatacatac 1200

tcctcgatcg tggccgctgt aaaggaatat gcagatggat atgttagcat cgtggaaaaa 1260

tacgctgcat caagtggttc cttgtccgag caattctcca agtcagatgg ctcgcagctg 1320

tcagctcgtg acctgacttg gtcctacgca gccctactta ctgccaatga gcgtcgaaac 1380

gccatagtcc ctgcaccatg gggggagaca tctgctagca gtgtccctgg gcagtgtcaa 1440

tatacttcgg ccattggtac ttacagcagc gcaacaaaca ccgcctggcc taccactttg 1500

actagtggat cgggcagtgt aactaccacc aagacgacta ccactacctc gaagccgaca 1560

acaacatcat gcactactcc gaccactgtt gcagtgacgt tcaatgtgat tgctactact 1620

gaatatggcc agaacatcaa actcgctgga tctatctctc aacttggctc atggtcgcca 1680

agcagtgctg tcgcattgag cgcttccaaa tacaccacga gcaaccacct gtggtttgtg 1740

accgtgacac ttccagtagg cactagcttc agctacaagt acatccaggt ggcgagcgat 1800

ggcactatca agtgggaaag tgacccaaac cagtcgtata ccgttcccgc cacgtgtggt 1860

actactgctg tcaccgtcag tgatacatgg aggtag 1896

<210> 40
<211> 631
<212> PRT
<213> Unknown

<220>
<223> Obtained from an environmental sample

<220>
<221> SIGNAL
<222> (1)...(20)

<220>
<221> DOMAIN
<222> (42)...(457)
<223> Glycosyl hydrolases family 15

<220>
<221> DOMAIN
<222> (529)...(625)
<223> Starch binding domain

<220>
<221> SITE
<222> (204)...(207)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (206)...(216)
<223> Glucoamylase active site region signature. Prosite id = PS00820

<220>
<221> SITE
<222> (619)...(622)
<223> N-glycosylation site. Prosite id = PS00001

<400> 40

Met Ala Pro Arg Phe Trp Thr Ala Leu Trp Ala Leu Thr Leu Gly His
1 5 10 15

Ala Val Val Ala Thr Pro Gln Ile Leu Ala Pro Arg Ala Thr Gly Ser
20 25 30

Leu Asp Thr Trp Leu Ala Ser Glu Thr Val Val Ala Arg Gln Gly Ile
35 40 45

Leu Asp Asn Ile Gly Ser Ala Gly Ala His Ala Ala Asn Ala Lys Pro
50 55 60

Gly Val Val Leu Ala Ser Pro Ser Thr Ser Asp Pro Asp Tyr Tyr Tyr

65 70 75 80

Thr Trp Thr Arg Asp Ser Ala Leu Val Phe Lys Asn Leu Val Asp Met
85 90 95

Phe Arg Ser Gly Asp Ser Ala Leu Leu Glu Val Ile Glu Glu Tyr Ile
100 105 110

Ser Ser Gln Ala Tyr Ile Gln Thr Val Ser Asn Pro Ser Gly Gly Leu
115 120 125

Ser Gly Gly Gly Gly Leu Gly Glu Pro Lys Phe Asn Ala Asp Glu Thr
130 135 140

Ala Phe Thr Gly Ser Trp Gly Arg Pro Gln Arg Asp Gly Pro Ala Leu
145 150 155 160

Arg Ala Thr Ala Leu Ile Ser Phe Gly Gln Trp Leu Ile Asp Asn Gly
165 170 175

Tyr Thr Thr Tyr Ala Thr Asp Ile Val Trp Pro Val Val Arg Asn Asp
180 185 190

Leu Ser Tyr Val Ser Gln Tyr Trp Asn Gln Thr Gly Phe Asp Leu Trp
195 200 205

Glu Glu Val Ser Gly Ser Ser Phe Phe Thr Val Ala Ala Gln His Arg
210 215 220

Ala Leu Val Glu Gly Ser Thr Phe Ala Ser Gln Val Gly Ser Ser Cys
225 230 235 240

Leu Tyr Cys Asp Ser Gln Ala Pro Gln Val Leu Cys Phe Leu Gln Ser
245 250 255

Phe Trp Thr Gly Ser Tyr Ile Leu Ala Asn Phe Gly Gly Gly Arg Ser
260 265 270

Gly Lys Asp Ala Asn Thr Leu Leu Gly Ser Ile His Thr Phe Asp Pro
275 280 285

Glu Ala Gly Cys Asp Asp Thr Thr Phe Gln Pro Cys Ser Ala Arg Ala
290 295 300

Leu Ala Asn His Lys Val Val Thr Asp Ser Phe Arg Ser Ile Tyr Ser
305 310 315 320

Val Asn Ser Gly Ile Ala Ala Gly Lys Ala Val Ser Val Gly Arg Tyr
325 330 335

```
Pro Glu Asp Ser Tyr Tyr Asn Gly Asn Pro Trp Tyr Leu Cys Thr Leu
            340                 345                 350

Ala Ala Ala Glu Gln Leu Tyr Asp Ala Ile Tyr Thr Trp Asn Arg Ile
        355                 360                 365

Gly Ser Leu Thr Ile Thr Ser Val Ser Leu Ser Phe Phe Lys Asp Leu
    370                 375                 380

Tyr Ser Ser Ala Ala Thr Gly Thr Tyr Ser Ser Ser Ser Asp Thr Tyr
385                 390                 395                 400

Ser Ser Ile Val Ala Ala Val Lys Glu Tyr Ala Asp Gly Tyr Val Ser
                405                 410                 415

Ile Val Glu Lys Tyr Ala Ala Ser Ser Gly Ser Leu Ser Glu Gln Phe
            420                 425                 430

Ser Lys Ser Asp Gly Ser Gln Leu Ser Ala Arg Asp Leu Thr Trp Ser
        435                 440                 445

Tyr Ala Ala Leu Leu Thr Ala Asn Glu Arg Arg Asn Ala Ile Val Pro
    450                 455                 460

Ala Pro Trp Gly Glu Thr Ser Ala Ser Ser Val Pro Gly Gln Cys Gln
465                 470                 475                 480

Tyr Thr Ser Ala Ile Gly Thr Tyr Ser Ser Ala Thr Asn Thr Ala Trp
                485                 490                 495

Pro Thr Thr Leu Thr Ser Gly Ser Gly Ser Val Thr Thr Thr Lys Thr
            500                 505                 510

Thr Thr Thr Thr Ser Lys Pro Thr Thr Thr Ser Cys Thr Thr Pro Thr
        515                 520                 525

Thr Val Ala Val Thr Phe Asn Val Ile Ala Thr Thr Glu Tyr Gly Gln
    530                 535                 540

Asn Ile Lys Leu Ala Gly Ser Ile Ser Gln Leu Gly Ser Trp Ser Pro
545                 550                 555                 560

Ser Ser Ala Val Ala Leu Ser Ala Ser Lys Tyr Thr Thr Ser Asn His
                565                 570                 575

Leu Trp Phe Val Thr Val Thr Leu Pro Val Gly Thr Ser Phe Ser Tyr
            580                 585                 590
```

Lys Tyr Ile Gln Val Ala Ser Asp Gly Thr Ile Lys Trp Glu Ser Asp
595 600 605

Pro Asn Gln Ser Tyr Thr Val Pro Ala Thr Cys Gly Thr Thr Ala Val
610 615 620

Thr Val Ser Asp Thr Trp Arg
625 630

<210> 41
<211> 1563
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 41

atgctggttc ctgtggtcat tctcacacat tttctccact atgcttgcgc tttctcgata 60

ccccatttgc acttgaaatt tgcgagtgcc agtgacgcac aaataccaca gaagccgctg 120

cgggatacgc tggaagtctg gctcgaagct gaagagcgca tagctctaaa taaattactc 180

gccaacgtag ctccaggcgg tagcaatgtc gagggcaaag gcgtggcacc cggaactgtc 240

attgcgagtc cgagtcagga cgggccagat tattggtatc agtgggttcg agatgccgct 300

atcaccatga atacgctcgt agatttgtat gccgagaacc cttcatccgc cttatcatct 360

cgtctttcga cgaccttaga tgcatacgct tccctacaac gtgatctgca gcatacatca 420

aatccctcgg gctcatttga tgattcgtcc ggccttggtg aaccgaaatt tgaagtcgac 480

ggaacgccat ttacaggctc atggggaagg ccacaacgcg atggaccggc gttaagagcg 540

ctgactctta tgcggtatct tcgagaatac aacgctagcc atccttcgct ttggtcttca 600

aatgaggcta ccgacttcta tagtctttat tacgaagcag agatgccacc gcggagcgtg 660

atcaaggcag accttgaata cgtgagccat ttctggaacg aatccagttt tgatctttgg 720

gaagaaacag aaggtcttca cttctttaac ttgatggtca gcgcacgaag tctaagggag 780

ggcagtgagc tagcaagagc atttggagat attggtgcag cagaatggta tatcgaacaa 840

gctggctaca tcgaaaagct tctgagcaaa ttctggaacc cgaacaaagg ccatctcgtg 900

gagaccttgt ggagtaagcg ctcaggtcta gattgtggtc ttcttctcgg gtctctacat 960

gctcttcctg ctcagggctt ggaagaagag gcagttttcc cgccctggtc cgacgagatc 1020

ctcgtgagct tgcttgctct gacagaagat caaagagacc gcttcccaat caacagcaac 1080

ccatatgagg atcaagatgg atcccatcca gcgttcgagg gtacaggagt tggacgttat 1140

cccgaagacg tctacgacgg ctatggcacc agtaatcgcg gaggcaaccc atggttccta 1200

tgcacatcaa gcgcagccga aatactgtac cgcagtgctt cttacttcta tacagcctcc 1260

aacctcacca tctccaccgc atctcttccg ttctacacct ccctgcttgc aacttcaagc 1320

ctcgatgtcc aagtcggaac atttggaccc tcagatacgc ttttccactc tgtcatcgag 1380

catctcaagt ccgcaggcga ttcgttcctt gaagttgtga aaacgcatgt agatgatgag 1440

ggacgaatga gcgagcaatt tgacagagtg acgggttata tgcgcggtgc gcgggatttg 1500

acctggagtt acggtgcatt tctacaggcg gtgaaggcgc gaaggagtat tcaggaggtt 1560

tga 1563

<210> 42
<211> 520
<212> PRT
<213> Unknown

<220>
<223> Obtained from an environmental sample

<220>
<221> DOMAIN
<222> (53)...(512)
<223> Glycosyl hydrolases family 15

<220>
<221> SITE
<222> (193)...(195)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (236)...(239)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (427)...(430)
<223> N-glycosylation site. Prosite id = PS00001

<400> 42

Met Leu Val Pro Val Val Ile Leu Thr His Phe Leu His Tyr Ala Cys
1 5 10 15

Ala Phe Ser Ile Pro His Leu His Leu Lys Phe Ala Ser Ala Ser Asp
20 25 30

Ala Gln Ile Pro Gln Lys Pro Leu Arg Asp Thr Leu Glu Val Trp Leu
35 40 45

Glu Ala Glu Glu Arg Ile Ala Leu Asn Lys Leu Leu Ala Asn Val Ala
50 55 60

Pro Gly Gly Ser Asn Val Glu Gly Lys Gly Val Ala Pro Gly Thr Val
65 70 75 80

Ile Ala Ser Pro Ser Gln Asp Gly Pro Asp Tyr Trp Tyr Gln Trp Val
85 90 95

Arg Asp Ala Ala Ile Thr Met Asn Thr Leu Val Asp Leu Tyr Ala Glu

100 105 110

Asn Pro Ser Ser Ala Leu Ser Ser Arg Leu Ser Thr Thr Leu Asp Ala
115 120 125

Tyr Ala Ser Leu Gln Arg Asp Leu Gln His Thr Ser Asn Pro Ser Gly
130 135 140

Ser Phe Asp Asp Ser Ser Gly Leu Gly Glu Pro Lys Phe Glu Val Asp
145 150 155 160

Gly Thr Pro Phe Thr Gly Ser Trp Gly Arg Pro Gln Arg Asp Gly Pro
165 170 175

Ala Leu Arg Ala Leu Thr Leu Met Arg Tyr Leu Arg Glu Tyr Asn Ala
180 185 190

Ser His Pro Ser Leu Trp Ser Ser Asn Glu Ala Thr Asp Phe Tyr Ser
195 200 205

Leu Tyr Tyr Glu Ala Glu Met Pro Pro Arg Ser Val Ile Lys Ala Asp
210 215 220

Leu Glu Tyr Val Ser His Phe Trp Asn Glu Ser Ser Phe Asp Leu Trp
225 230 235 240

Glu Glu Thr Glu Gly Leu His Phe Phe Asn Leu Met Val Ser Ala Arg
245 250 255

Ser Leu Arg Glu Gly Ser Glu Leu Ala Arg Ala Phe Gly Asp Ile Gly
260 265 270

Ala Ala Glu Trp Tyr Ile Glu Gln Ala Gly Tyr Ile Glu Lys Leu Leu
275 280 285

Ser Lys Phe Trp Asn Pro Asn Lys Gly His Leu Val Glu Thr Leu Trp
290 295 300

Ser Lys Arg Ser Gly Leu Asp Cys Gly Leu Leu Leu Gly Ser Leu His
305 310 315 320

Ala Leu Pro Ala Gln Gly Leu Glu Glu Glu Ala Val Phe Pro Pro Trp
325 330 335

Ser Asp Glu Ile Leu Val Ser Leu Leu Ala Leu Thr Glu Asp Gln Arg
340 345 350

Asp Arg Phe Pro Ile Asn Ser Asn Pro Tyr Glu Asp Gln Asp Gly Ser
355 360 365

```
His Pro Ala Phe Glu Gly Thr Gly Val Gly Arg Tyr Pro Glu Asp Val
370                 375                 380

Tyr Asp Gly Tyr Gly Thr Ser Asn Arg Gly Gly Asn Pro Trp Phe Leu
385                 390                 395                 400

Cys Thr Ser Ser Ala Ala Glu Ile Leu Tyr Arg Ser Ala Ser Tyr Phe
                405                 410                 415

Tyr Thr Ala Ser Asn Leu Thr Ile Ser Thr Ala Ser Leu Pro Phe Tyr
            420                 425                 430

Thr Ser Leu Leu Ala Thr Ser Ser Leu Asp Val Gln Val Gly Thr Phe
        435                 440                 445

Gly Pro Ser Asp Thr Leu Phe His Ser Val Ile Glu His Leu Lys Ser
    450                 455                 460

Ala Gly Asp Ser Phe Leu Glu Val Val Lys Thr His Val Asp Asp Glu
465                 470                 475                 480

Gly Arg Met Ser Glu Gln Phe Asp Arg Val Thr Gly Tyr Met Arg Gly
                485                 490                 495

Ala Arg Asp Leu Thr Trp Ser Tyr Gly Ala Phe Leu Gln Ala Val Lys
            500                 505                 510

Ala Arg Arg Ser Ile Gln Glu Val
        515                 520
```

<210> 43
<211> 3030
<212> DNA
<213> Cochliobolus heterostrophus ATCC 48332

<400> 43

atgacgcaca caagtttcgt ccaggcttct actgtgctgt cctctcttct cgcactcaca 60

gctggccaag cgcccaccac ttcttcaaat ggtggatgga gtactacgct cgctggcacg 120

cccacctact tcaatcccat ctttaccatc cctccatctg cggatgaggg cgttcaacag 180

attcccaaca tctatgaccc acaagccgtg gatgcgcaag acgtttgccc tggttacaca 240

gcatcaaacc ctcagcagag tgatcgtggc ctgaccgcca cgttgaccct ggctggaaag 300

ccatgcaatg tttacggcac cgatatccag gaactagact tgaaagtcga gtaccaggcc 360

aagggaagac tgtcagtcaa cattgtacca aagtacactg gtgcaagcaa ccagtcgcat 420

tggattgttc ccgaagatct gattccgcgt cctcaggtcg aggaatcatc cgagcagacc 480

gatctcaagt tcaactgggg aaatgagcca tcttttggt tcaatgtcga gcgctcctcg 540

actggagaca tcatcttcac aactcaaggc acccatctca tttacgaaaa tcaatttgtt 600

gaatttgtaa acagcctgcc agaggactac aacctttacg gtctgggtga acgaattcac 660

ggtcttcgtc ttaacaacaa cttcactgcg accatctacg ctgctgacgt aggtgatcct 720

atcgaccgca acctgtacgg tagccaccct ttttacctag agacccggta ctttgagaag 780

ggcgaaaact gtagcaccaa gcccttgacg cagtccgaga tcggccagaa gaaggatacc 840

aaaacaaatg gttcgcccta cgagtcgcgt tcccacggcg tttattaccg caacactcat 900

ggtatggacg tggtgctgaa acctgatcac ctaacttgga ggaccctggg aggtgctatt 960

gatctgtact tcttcgatgg accttcacag ccggacgtta ctaaagcgta tcaaaaggcg 1020

gctattggcc tgccagcaat gcagcaatac tggacctttg gctttcatca atgccgttgg 1080

ggctaccgca actggaccga gactcgagaa attgttgaga ccatgagagc tttcaacatc 1140

ccaatggaaa ccatctggct cgacattgac tacatggacc agtaccgtga ctttacgctg 1200

gatcccgtgg ctttccctcc ctctgaagtg gccgacttct tcggatggct gcatggaaac 1260

aaccagcatt ttgtacccat cgttgatgca gctatttaca tccccaaccc gcaaaacgca 1320

agcgatgcat atgacacata tacgcgcggc aatgaatctg gtgtgttctt gtccaaccca 1380

gacggcagcc aatatatcgg tgctgtctgg cctgggtaca ccgttttccc tgattggttg 1440

tcgcccaacg gtgtatcttg gtgggtaaag gagatggtcg agtggtacaa agaggtacca 1500

tatagcggtt tctgggttga catgactgaa gtctcttctt tctgcgttgg atcctgtggt 1560

actggcaacg tcaccctgaa cccggctcac ccgcccttct cgcttcccgg tgaggtgaac 1620

aacgtcatct atgattaccc agagagcttc aacatcacaa atggtaccga ggcggcgtcc 1680

gcttctgcag cagcttccgc ccaagcatcc aggaaggcaa ctgcgacggc gacggtgacc 1740

gacgaagtga cttctacttc gacaagctac ttccgctcga cccctaccgc tggagagcga 1800

aacatcaact atcctcctta tgtcatcaac cacgtacaag atggcgctga tcttgctgtt 1860

catgctgtca gccccaatgc aactcatgca aatggtgtgg aggaatacga tgtccacaac 1920

ctctttggtc accagatcat taacgctact tatcacggtc tcctttcggt gttccctgga 1980

aagcggccct ttatcattgg acgctccaca tttgctggca gcggcaagtg ggctggtcat 2040

tggggtggcg acaacgcttc taaatgggcc tacatgttct tctcgattcc acaagcactt 2100

tccttttcac tatttggtat tccgatgttt ggtgtcgata cctgcggatt caacggaaac 2160

actgacatgg agctttgctc tcgatggatg cagctttcgg cctttttccc cttttaccgc 2220

aaccacaacg tactctccgc cattccccaa gagccctacc gatgggaggc cgtggcttct 2280

gcttcgagga ccgcaatgca catccgatac tctctgttgc catacatgta cacgctattc 2340

aatgacgctc acaagactgg gtcaactgtg atgcgggcgc tggcatggga attccccaac 2400

gagccccaac tcgctggtgt agatacacaa ttcttgcttg ggccaaacat cctagtcacg 2460

cctgtcctcg agcctcaagt tgatactgtc aagggagtgt tccctggtat tgtcgacggc 2520

gagacatggt ttgattggta ttctggcgag cgtgtccaag ccgaggctgg cgtaaacacg 2580

accatctcag caccttttggg ccacatcccg gtatacattc gcggaggttc ggtcctacct 2640

attcaggagc ctggatacac gactaccgag tcccgccgga acccctgggg tctcattgtc 2700

gcactctcca gtgaaggcac tgcatcgggt cacctatacg ttgatgatgg tgagtccatc 2760

gagccagact cgtgcctgaa tgttgcattt gctgctacga gcggaaagtt ggaggttgat 2820

gttcagggcg agttcaagga cacgaacgca cttgccaacg tgacagtttt gggagctcct 2880

gcggtccaaa acgtcaagct gaatggtgag gcgattgatg ctagcaatgt tgattacaac 2940

aagaccagta gcgttttgaa gctgacggga ctcaatgaac tgacaagttc tggagcttgg 3000

cagggtagct ggacgctgac ttgggaataa 3030

<210> 44
<211> 1009
<212> PRT
<213> Cochliobolus heterostrophus ATCC 48332

<220>
<221> DOMAIN
<222> (323)...(879)
<223> Glycosyl hydrolases family 31

<220>
<221> SITE
<222> (139)...(142)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (230)...(233)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (267)...(270)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (369)...(372)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (445)...(448)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (457)...(460)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (531)...(534)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (559)...(562)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (635)...(638)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (657)...(660)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (695)...(698)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (872)...(875)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (967)...(970)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (995) ... (998)
<223> N-glycosylation site. Prosite id = PS00001

<400> 44

```
Met Thr His Thr Ser Phe Val Gln Ala Ser Thr Val Leu Ser Ser Leu
1               5                  10                  15

Leu Ala Leu Thr Ala Gly Gln Ala Pro Thr Thr Ser Ser Asn Gly Gly
            20                  25                  30

Trp Ser Thr Thr Leu Ala Gly Thr Pro Thr Tyr Phe Asn Pro Ile Phe
        35                  40                  45

Thr Ile Pro Pro Ser Ala Asp Glu Gly Val Gln Gln Ile Pro Asn Ile
    50                  55                  60

Tyr Asp Pro Gln Ala Val Asp Ala Gln Asp Val Cys Pro Gly Tyr Thr
65                  70                  75                  80

Ala Ser Asn Pro Gln Gln Ser Asp Arg Gly Leu Thr Ala Thr Leu Thr
                85                  90                  95

Leu Ala Gly Lys Pro Cys Asn Val Tyr Gly Thr Asp Ile Gln Glu Leu
            100                 105                 110

Asp Leu Lys Val Glu Tyr Gln Ala Lys Gly Arg Leu Ser Val Asn Ile
```

115 120 125

Val Pro Lys Tyr Thr Gly Ala Ser Asn Gln Ser His Trp Ile Val Pro
130 135 140

Glu Asp Leu Ile Pro Arg Pro Gln Val Glu Glu Ser Ser Glu Gln Thr
145 150 155 160

Asp Leu Lys Phe Asn Trp Gly Asn Glu Pro Ser Phe Trp Phe Asn Val
165 170 175

Glu Arg Ser Ser Thr Gly Asp Ile Ile Phe Thr Thr Gln Gly Thr His
180 185 190

Leu Ile Tyr Glu Asn Gln Phe Val Glu Phe Val Asn Ser Leu Pro Glu
195 200 205

Asp Tyr Asn Leu Tyr Gly Leu Gly Glu Arg Ile His Gly Leu Arg Leu
210 215 220

Asn Asn Asn Phe Thr Ala Thr Ile Tyr Ala Ala Asp Val Gly Asp Pro
225 230 235 240

Ile Asp Arg Asn Leu Tyr Gly Ser His Pro Phe Tyr Leu Glu Thr Arg
245 250 255

Tyr Phe Glu Lys Gly Glu Asn Cys Ser Thr Lys Pro Leu Thr Gln Ser
260 265 270

Glu Ile Gly Gln Lys Lys Asp Thr Lys Thr Asn Gly Ser Pro Tyr Glu
275 280 285

Ser Arg Ser His Gly Val Tyr Tyr Arg Asn Thr His Gly Met Asp Val
290 295 300

Val Leu Lys Pro Asp His Leu Thr Trp Arg Thr Leu Gly Gly Ala Ile
305 310 315 320

Asp Leu Tyr Phe Phe Asp Gly Pro Ser Gln Pro Asp Val Thr Lys Ala
325 330 335

Tyr Gln Lys Ala Ala Ile Gly Leu Pro Ala Met Gln Gln Tyr Trp Thr
340 345 350

Phe Gly Phe His Gln Cys Arg Trp Gly Tyr Arg Asn Trp Thr Glu Thr
355 360 365

Arg Glu Ile Val Glu Thr Met Arg Ala Phe Asn Ile Pro Met Glu Thr
370 375 380

Ile Trp Leu Asp Ile Asp Tyr Met Asp Gln Tyr Arg Asp Phe Thr Leu
385 390 395 400

Asp Pro Val Ala Phe Pro Pro Ser Glu Val Ala Asp Phe Phe Gly Trp
405 410 415

Leu His Gly Asn Asn Gln His Phe Val Pro Ile Val Asp Ala Ala Ile
420 425 430

Tyr Ile Pro Asn Pro Gln Asn Ala Ser Asp Ala Tyr Asp Thr Tyr Thr
435 440 445

Arg Gly Asn Glu Ser Gly Val Phe Leu Ser Asn Pro Asp Gly Ser Gln
450 455 460

Tyr Ile Gly Ala Val Trp Pro Gly Tyr Thr Val Phe Pro Asp Trp Leu
465 470 475 480

Ser Pro Asn Gly Val Ser Trp Trp Val Lys Glu Met Val Glu Trp Tyr
485 490 495

Lys Glu Val Pro Tyr Ser Gly Phe Trp Val Asp Met Thr Glu Val Ser
500 505 510

Ser Phe Cys Val Gly Ser Cys Gly Thr Gly Asn Val Thr Leu Asn Pro
515 520 525

Ala His Pro Pro Phe Ser Leu Pro Gly Glu Val Asn Asn Val Ile Tyr
530 535 540

Asp Tyr Pro Glu Ser Phe Asn Ile Thr Asn Gly Thr Glu Ala Ala Ser
545 550 555 560

Ala Ser Ala Ala Ala Ser Ala Gln Ala Ser Arg Lys Ala Thr Ala Thr
565 570 575

Ala Thr Val Thr Asp Glu Val Thr Ser Thr Ser Thr Ser Tyr Phe Arg
580 585 590

Ser Thr Pro Thr Ala Gly Glu Arg Asn Ile Asn Tyr Pro Pro Tyr Val
595 600 605

Ile Asn His Val Gln Asp Gly Ala Asp Leu Ala Val His Ala Val Ser
610 615 620

Pro Asn Ala Thr His Ala Asn Gly Val Glu Glu Tyr Asp Val His Asn
625 630 635 640

Leu Phe Gly His Gln Ile Ile Asn Ala Thr Tyr His Gly Leu Leu Ser
645 650 655

Val Phe Pro Gly Lys Arg Pro Phe Ile Ile Gly Arg Ser Thr Phe Ala
660 665 670

Gly Ser Gly Lys Trp Ala Gly His Trp Gly Gly Asp Asn Ala Ser Lys
675 680 685

Trp Ala Tyr Met Phe Phe Ser Ile Pro Gln Ala Leu Ser Phe Ser Leu
690 695 700

Phe Gly Ile Pro Met Phe Gly Val Asp Thr Cys Gly Phe Asn Gly Asn
705 710 715 720

Thr Asp Met Glu Leu Cys Ser Arg Trp Met Gln Leu Ser Ala Phe Phe
725 730 735

Pro Phe Tyr Arg Asn His Asn Val Leu Ser Ala Ile Pro Gln Glu Pro
740 745 750

Tyr Arg Trp Glu Ala Val Ala Ser Ala Ser Arg Thr Ala Met His Ile
755 760 765

Arg Tyr Ser Leu Leu Pro Tyr Met Tyr Thr Leu Phe Asn Asp Ala His
770 775 780

Lys Thr Gly Ser Thr Val Met Arg Ala Leu Ala Trp Glu Phe Pro Asn
785 790 795 800

Glu Pro Gln Leu Ala Gly Val Asp Thr Gln Phe Leu Leu Gly Pro Asn
805 810 815

Ile Leu Val Thr Pro Val Leu Glu Pro Gln Val Asp Thr Val Lys Gly
820 825 830

Val Phe Pro Gly Ile Val Asp Gly Glu Thr Trp Phe Asp Trp Tyr Ser
835 840 845

Gly Glu Arg Val Gln Ala Glu Ala Gly Val Asn Thr Thr Ile Ser Ala
850 855 860

Pro Leu Gly His Ile Pro Val Tyr Ile Arg Gly Gly Ser Val Leu Pro
865 870 875 880

Ile Gln Glu Pro Gly Tyr Thr Thr Thr Glu Ser Arg Arg Asn Pro Trp
885 890 895

Gly Leu Ile Val Ala Leu Ser Ser Glu Gly Thr Ala Ser Gly His Leu
900 905 910

Tyr Val Asp Asp Gly Glu Ser Ile Glu Pro Asp Ser Cys Leu Asn Val
915 920 925

Ala Phe Ala Ala Thr Ser Gly Lys Leu Glu Val Asp Val Gln Gly Glu
930 935 940

Phe Lys Asp Thr Asn Ala Leu Ala Asn Val Thr Val Leu Gly Ala Pro
945 950 955 960

Ala Val Gln Asn Val Lys Leu Asn Gly Glu Ala Ile Asp Ala Ser Asn
965 970 975

Val Asp Tyr Asn Lys Thr Ser Ser Val Leu Lys Leu Thr Gly Leu Asn
980 985 990

Glu Leu Thr Ser Ser Gly Ala Trp Gln Gly Ser Trp Thr Leu Thr Trp
995 1000 1005

Glu

<210> 45
<211> 1380
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 45

atgaagcttc ttcaactcgc cgccctggtg gcgtccctca gccccttcac caacgctgcc 60

gacgcaaacg cctggaagtc gcgaaacatt tactttgcgc ttacagaccg cgttgcgcgc 120

agcggtagcg ataacggtgg caatgcctgt ggcaatcttg gaaattattg tggtggaacg 180

ttcaagggtc ttgaggctaa gcttgattac atcaagggca tgggctttga tgctatctgg 240

attactcctg ttgttgagaa caccgatgga ggataccatg gatactgggc taaagacctg 300

tacgcggtta attccaagta tggtaccaag gatgacttga agaatcttgt caaggctgcc 360

cacggcaaga acatgtacgt catggccgac gtcgtcgcaa accacatggg caagggcatc 420

caaaaccaca agccggagcc cctcaaccag caaagctcct accacagctc ctgcgccatc 480

gactacaaca accaaaacag catcgagcag tgcgaaatcg ccggtctgcc cgatctcaac 540

accggcaagg cagaagtcaa gaaggtcctc aacgactgga tcaagtggct cgtctccgag 600

tacagcttcg acggtatccg catcgacaca gtcaagcacg tcgagaagag cttctggcct 660

gatttccaga aggcagctgg cgttttcgcc atcggtgagg tttgggatgg aagccctgat 720

taccttgctg gttactccaa ggtcatgcct ggtctgctga actatgctat gtactatccc 780

atgaaccgat tctatcagca gaagggtgat ccttctgctg tggtggatat gtacaacgag 840

atcagccaga agttcgatga tcctacgcag cttggtacct tcatcgacaa ccacgacaat 900

gcgcgctggt tgagccaaaa gaacgacaag gccctcctca agaacgccct cgcattcacc 960

atcctcgccc gcggtattcc catcgtgtac tacggcaccg aacaaggcta cgcaggaggc 1020

aacgaccccg ccaaccgcga agatctctgg cgcagcaact tcagcaccga ctccgacctg 1080

taccaaacca tttccaagct cggcaaggct cgctccgccg tcggtggtct tgccggcaac 1140

gaccagaaat tcctcaagtc caatgacagc gcactcatct ggagccgcgc agacggcgat 1200

ctgatcgttg ttacgcttaa ccgtggaaag ggatattctg gagagtactg cttcaacact 1260

ggcaagaaca acaagacttg ggatcgtgtt ttgggatctg gaagtgttaa gtctgatggt 1320

agtggtaagc tttgtgttag ctacactaat ggtgagcctg aggttcttgt tgctgcttag 1380

<210> 46
<211> 459
<212> PRT
<213> Unknown

<220>
<223> Obtained from an environmental sample

<220>
<221> SIGNAL
<222> (1)...(19)

<220>
<221> DOMAIN

<222> (32)...(371)
<223> Alpha amylase, catalytic domain

<220>
<221> SITE
<222> (358)...(361)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (393)... (396)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (430)...(433)
<223> N-glycosylation site. Prosite id = PS00001

<400> 46

Met Lys Leu Leu Gln Leu Ala Ala Leu Val Ala Ser Leu Ser Pro Phe
1 5 10 15

Thr Asn Ala Ala Asp Ala Asn Ala Trp Lys Ser Arg Asn Ile Tyr Phe
20 25 30

Ala Leu Thr Asp Arg Val Ala Arg Ser Gly Ser Asp Asn Gly Gly Asn
35 40 45

```
Ala Cys Gly Asn Leu Gly Asn Tyr Cys Gly Gly Thr Phe Lys Gly Leu
     50                  55                  60

Glu Ala Lys Leu Asp Tyr Ile Lys Gly Met Gly Phe Asp Ala Ile Trp
65                  70                  75                  80

Ile Thr Pro Val Val Glu Asn Thr Asp Gly Gly Tyr His Gly Tyr Trp
                 85                  90                  95

Ala Lys Asp Leu Tyr Ala Val Asn Ser Lys Tyr Gly Thr Lys Asp Asp
            100                 105                 110

Leu Lys Asn Leu Val Lys Ala Ala His Gly Lys Asn Met Tyr Val Met
        115                 120                 125

Ala Asp Val Val Ala Asn His Met Gly Lys Gly Ile Gln Asn His Lys
    130                 135                 140

Pro Glu Pro Leu Asn Gln Gln Ser Ser Tyr His Ser Ser Cys Ala Ile
145                 150                 155                 160

Asp Tyr Asn Asn Gln Asn Ser Ile Glu Gln Cys Glu Ile Ala Gly Leu
                165                 170                 175

Pro Asp Leu Asn Thr Gly Lys Ala Glu Val Lys Lys Val Leu Asn Asp
            180                 185                 190

Trp Ile Lys Trp Leu Val Ser Glu Tyr Ser Phe Asp Gly Ile Arg Ile
        195                 200                 205

Asp Thr Val Lys His Val Glu Lys Ser Phe Trp Pro Asp Phe Gln Lys
    210                 215                 220

Ala Ala Gly Val Phe Ala Ile Gly Glu Val Trp Asp Gly Ser Pro Asp
225                 230                 235                 240

Tyr Leu Ala Gly Tyr Ser Lys Val Met Pro Gly Leu Leu Asn Tyr Ala
                245                 250                 255

Met Tyr Tyr Pro Met Asn Arg Phe Tyr Gln Gln Lys Gly Asp Pro Ser
            260                 265                 270

Ala Val Val Asp Met Tyr Asn Glu Ile Ser Gln Lys Phe Asp Asp Pro
        275                 280                 285

Thr Gln Leu Gly Thr Phe Ile Asp Asn His Asp Asn Ala Arg Trp Leu
    290                 295                 300
```

Ser Gln Lys Asn Asp Lys Ala Leu Leu Lys Asn Ala Leu Ala Phe Thr
305 310 315 320

Ile Leu Ala Arg Gly Ile Pro Ile Val Tyr Tyr Gly Thr Glu Gln Gly
325 330 335

Tyr Ala Gly Gly Asn Asp Pro Ala Asn Arg Glu Asp Leu Trp Arg Ser
340 345 350

Asn Phe Ser Thr Asp Ser Asp Leu Tyr Gln Thr Ile Ser Lys Leu Gly
355 360 365

Lys Ala Arg Ser Ala Val Gly Gly Leu Ala Gly Asn Asp Gln Lys Phe
370 375 380

Leu Lys Ser Asn Asp Ser Ala Leu Ile Trp Ser Arg Ala Asp Gly Asp
385 390 395 400

Leu Ile Val Val Thr Leu Asn Arg Gly Lys Gly Tyr Ser Gly Glu Tyr
405 410 415

Cys Phe Asn Thr Gly Lys Asn Asn Lys Thr Trp Asp Arg Val Leu Gly
420 425 430

Ser Gly Ser Val Lys Ser Asp Gly Ser Gly Lys Leu Cys Val Ser Tyr
435 440 445

Thr Asn Gly Glu Pro Glu Val Leu Val Ala Ala
450 455

<210> 47
<211> 1905
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 47

atgacacgca ttctcaccct cgcccttcat gggctggctc ttgtccaaag tgttgttggg 60

gctccccaat tggcccccag agcgacaacc agtctggatg catggttggc atcggagacg 120

accgttgcgc tggatgggat ccttgacaac gtgggttcta gtggagccta cgccaaaagt 180

gcgaagtccg gtatcgtgat tgccagtcca agcaccgaca acccagacta ctactacacg 240

tggactcgcg atgctgcgtt gaccgtcaag gccctgatcg atcttttccg caatggcgag 300

acaagccttc agaccgtgat catggagtac attagctctc aggcgtacct ccagaccgta 360

tccaacccct cggggtcctt gtccaccggt ggtctggcag aaccaaaata ttatgtcgat 420

gagactgcct acacgggcag ctggggtcgt ccccagcggg atggtcctgc cctgagagcg 480

acggcgatga tcgactttgg taactggctc attgacaacg gatattcaac ctacgcctct 540

aacattgtgt ggccgatcgt ccgcaacgat ctgtcgtacg ttgcccaata ttggaaccaa 600

accggatatg acctctggga agaagtgaac ggatcctcct tcttcaccat tgccgtgcag 660

caccgggctc tggtggaagg cagcaccttc gcctccaaag ttggcgcctc atgctcgtgg 720

tgcgactcgc aggcgccgca ggtgctttgc ttcctgcaga gattctggac aggctcgtac 780

atcatggcca attttggcgg cgggcgatcg ggcaaagatg ccaacaccgt cctgggaagc 840

atccatacct tcgacccgaa tgccggttgc gacgacacca cgttccagcc atgctctccg 900

cgagcgctgg cgaaccacaa ggtctacacc gattcgttcc gttctatcta ctctatcaac 960

tcgggaatta gccagggcaa ggctgttgcc gtgggtcgct accccgagga ctcttactac 1020

aatggaaacc cgtggttcct tacgacgttg gctgccgcag agcagttgta cgatgccatt 1080

taccagtggc agaagattgg gtctattacc atcacggacg tctcgctggc cttcttcaag 1140

gatctttaca gctctgcggc cgttgggacg tacgcctctt cgagctcggc cttcacctcc 1200

atcgtgaatg ccgtgaagac gtacgctgat ggatatatga gtattgtgca aacccatgcg 1260

atgacgaatg gctccctttc tgagcagttc ggcaagtccg acggcttctc cctgtccgcc 1320

cgcgatctca cttggtcgta tgcagccctc ctcacagcca acttgagaag aaactcggtc 1380

gttcccccat cctggggtga gacgaccgca actagcgtac cctccgtctg ctccgcgacc 1440

tctgccactg gcacctacag taccgccacc aacaccgcgt ggcccagcac tctgaccagc 1500

gggactggcg ctacaacgac cacaagcaag gcgacttcta ctactactac ctcgtcggcc 1560

tcgacgacca cagctggatg tgtcgttccg accgcggtgg cagtcacctt tgatgagatt 1620

gctaccacaa cctatggcga gaatgtctac gtggtgggtt ccatctcgca actgggcagc 1680

tgggacacca gcaaggccgt ggccctgagt gccagcaaat acacctccag caacaatctc 1740

tggtatgcca cggtcaccct tcccgctggg acgaccttcc agtacaagtt catccgggtt 1800

tcgagcagcg ggactgttac atgggagagt gacccgaacc gttcatacac ggttccgtcc 1860

gcttgtggga cgtctactgc ggtggtgaac accacttggc gctag 1905

<210> 48
<211> 634
<212> PRT
<213> Unknown

<220>
<223> Obtained from an environmental sample

<220>
<221> SIGNAL
<222> (1)...(20)

<220>

<221> DOMAIN
<222> (41)...(455)
<223> Glycosyl hydrolases family 15

<220>
<221> DOMAIN
<222> (532)...(628)
<223> Starch binding domain

<220>
<221> SITE
<222> (202)...(205)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (204)...(214)
<223> Glucoamylase active site region signature. Prosite id = PS00820

<220>
<221> SITE
<222> (213)...(216)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (429)...(432)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (622)...(625)
<223> N-glycosylation site. Prosite id = PS00001

<400> 48

```
Met Thr Arg Ile Leu Thr Leu Ala Leu His Gly Leu Ala Leu Val Gln
1               5                  10                  15

Ser Val Val Gly Ala Pro Gln Leu Ala Pro Arg Ala Thr Thr Ser Leu
            20                  25                  30

Asp Ala Trp Leu Ala Ser Glu Thr Thr Val Ala Leu Asp Gly Ile Leu
        35                  40                  45

Asp Asn Val Gly Ser Ser Gly Ala Tyr Ala Lys Ser Ala Lys Ser Gly
    50                  55                  60

Ile Val Ile Ala Ser Pro Ser Thr Asp Asn Pro Asp Tyr Tyr Tyr Thr
65                  70                  75                  80

Trp Thr Arg Asp Ala Ala Leu Thr Val Lys Ala Leu Ile Asp Leu Phe
                85                  90                  95

Arg Asn Gly Glu Thr Ser Leu Gln Thr Val Ile Met Glu Tyr Ile Ser
            100                 105                 110

Ser Gln Ala Tyr Leu Gln Thr Val Ser Asn Pro Ser Gly Ser Leu Ser
        115                 120                 125

Thr Gly Gly Leu Ala Glu Pro Lys Tyr Tyr Val Asp Glu Thr Ala Tyr
```

130 135 140

Thr Gly Ser Trp Gly Arg Pro Gln Arg Asp Gly Pro Ala Leu Arg Ala
145 150 155 160

Thr Ala Met Ile Asp Phe Gly Asn Trp Leu Ile Asp Asn Gly Tyr Ser
165 170 175

Thr Tyr Ala Ser Asn Ile Val Trp Pro Ile Val Arg Asn Asp Leu Ser
180 185 190

Tyr Val Ala Gln Tyr Trp Asn Gln Thr Gly Tyr Asp Leu Trp Glu Glu
195 200 205

Val Asn Gly Ser Ser Phe Phe Thr Ile Ala Val Gln His Arg Ala Leu
210 215 220

Val Glu Gly Ser Thr Phe Ala Ser Lys Val Gly Ala Ser Cys Ser Trp
225 230 235 240

Cys Asp Ser Gln Ala Pro Gln Val Leu Cys Phe Leu Gln Arg Phe Trp
245 250 255

Thr Gly Ser Tyr Ile Met Ala Asn Phe Gly Gly Gly Arg Ser Gly Lys
260 265 270

Asp Ala Asn Thr Val Leu Gly Ser Ile His Thr Phe Asp Pro Asn Ala
275 280 285

Gly Cys Asp Asp Thr Thr Phe Gln Pro Cys Ser Pro Arg Ala Leu Ala
290 295 300

Asn His Lys Val Tyr Thr Asp Ser Phe Arg Ser Ile Tyr Ser Ile Asn
305 310 315 320

Ser Gly Ile Ser Gln Gly Lys Ala Val Ala Val Gly Arg Tyr Pro Glu
325 330 335

Asp Ser Tyr Tyr Asn Gly Asn Pro Trp Phe Leu Thr Thr Leu Ala Ala
340 345 350

Ala Glu Gln Leu Tyr Asp Ala Ile Tyr Gln Trp Gln Lys Ile Gly Ser
355 360 365

Ile Thr Ile Thr Asp Val Ser Leu Ala Phe Phe Lys Asp Leu Tyr Ser
370 375 380

Ser Ala Ala Val Gly Thr Tyr Ala Ser Ser Ser Ser Ala Phe Thr Ser
385 390 395 400

Ile Val Asn Ala Val Lys Thr Tyr Ala Asp Gly Tyr Met Ser Ile Val
405 410 415

Gln Thr His Ala Met Thr Asn Gly Ser Leu Ser Glu Gln Phe Gly Lys
420 425 430

Ser Asp Gly Phe Ser Leu Ser Ala Arg Asp Leu Thr Trp Ser Tyr Ala
435 440 445

Ala Leu Leu Thr Ala Asn Leu Arg Arg Asn Ser Val Val Pro Pro Ser
450 455 460

Trp Gly Glu Thr Thr Ala Thr Ser Val Pro Ser Val Cys Ser Ala Thr
465 470 475 480

Ser Ala Thr Gly Thr Tyr Ser Thr Ala Thr Asn Thr Ala Trp Pro Ser
485 490 495

Thr Leu Thr Ser Gly Thr Gly Ala Thr Thr Thr Ser Lys Ala Thr
500 505 510

Ser Thr Thr Thr Thr Ser Ser Ala Ser Thr Thr Thr Ala Gly Cys Val
515 520 525

Val Pro Thr Ala Val Ala Val Thr Phe Asp Glu Ile Ala Thr Thr Thr
530 535 540

Tyr Gly Glu Asn Val Tyr Val Val Gly Ser Ile Ser Gln Leu Gly Ser
545 550 555 560

Trp Asp Thr Ser Lys Ala Val Ala Leu Ser Ala Ser Lys Tyr Thr Ser
565 570 575

Ser Asn Asn Leu Trp Tyr Ala Thr Val Thr Leu Pro Ala Gly Thr Thr
580 585 590

Phe Gln Tyr Lys Phe Ile Arg Val Ser Ser Ser Gly Thr Val Thr Trp
595 600 605

Glu Ser Asp Pro Asn Arg Ser Tyr Thr Val Pro Ser Ala Cys Gly Thr
610 615 620

Ser Thr Ala Val Val Asn Thr Thr Trp Arg
625 630

<210> 49
<211> 1620

<212> DNA
<213> Aspergillus terreus

<400> 49

atgtcctttt tcctgtcctg cctctatctg agcctgtgtg gctcagcact ggcagccaat 60
ctcacgtcat ggaaatccag atccatctac cagacgatga cggacaggtt cgcgcggacc 120
gacggctcga ccaccgccgc gtgcaacacc accgccggtt tatactgcgg agggacgtgg 180
cgcggcacga tcaatcatct cgactacatc caggggatgg gcttcgatgc ggtcatgatc 240
tcccccatca tcgagaatat cgatggccgc gtctcctacg gtgaagccta ccatgggtac 300
tggccgctgg acctggagtc tttgaacaca cgcttcggca cgaaacagga cctcctcgat 360
ttgagtaatg cgctccactc ccgcgggatg tacctgatga tggataccgt gattaataac 420
atggcatata tcacgcgtgg gcaggaccct gcgacggcaa ttgactactc ggtgttcacg 480
ccgttcaaca acgcggatta ttttcatccc tactgcaaga tcacggattg gaacaacctc 540
accgacgcta gtctgtgtca gacgggtgat ctggaggtcg cattgcccga cctgttcaca 600
gagcatacgg acgtgcagga tcgtctcata agctgggcca aggaaatgat ccaaacatat 660
tccatcgacg gacttcgcat tgacgccgcc aaacacgtcg atcctgagtt tctcgccaga 720
ttcgtgaacg aagtcgatgt attcaccacc ggagaggtcc tccagcgcga agtcgacatc 780
atctgcgact accacaacaa atacatcacc agcatgccca attacccgat ctacttctcc 840
atgctggatg ctttcacaga gggcaacacg tcgtcattga tgagccaagt ggaagcaatg 900
aagggtcctt gtcacgacgt taccgccctt gtgtcttttt ccgagaacca tgaccagccg 960
cggattccca gcatgaacaa agacatcgcg ctcgccaaaa atgttctcac tttcaccatt 1020
ctttttgatg gcattcccat ggtctatcaa ggtcaagagc aacacttgga cggatctggg 1080
acaccgaaaa accgtgaagc cctgtggttg tccaagtatg atacccaagc cgaattatac 1140
caactgctcg ccaaactcaa cgcgatccgc aaacatgcca cctccctggg cagcgactac 1200
ctctatgccc aaaccagacc tatctaccgg ggcggaagtg agctcgcatt ctacaaaggc 1260
attgagggcc gacaggtgat tacggttcta tcatcgcaag gcgctcaggg aaatccatac 1320
gatctgtatc tgcccgtgtc atataatccg ggaacagcgg tgatggaggt cctcaactgt 1380
gtgaactcca cggtgggtga cgacggtcag ctcaaggtgc ccatggagaa gggagagccg 1440
cgggtgttct tcccgattga gctgatgggc ggaagcggac tgtgtggata ttccaaggat 1500
aatgttacgg tttctcagtt gaaaacaggg cacgactcga catcccgggg aagcaagatg 1560
acgggcagtg ctgcacttct tatgatgctg tcgttgggag cgagcctggt gttatggtga 1620

<210> 50
<211> 539
<212> PRT

<213> Aspergillus terreus

<220>
<221> SIGNAL
<222> (1)...(18)

<220>
<221> DOMAIN
<222> (31)...(390)
<223> Alpha amylase, catalytic domain

<220>
<221> SITE
<222> (20)...(23)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (49)... (52)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (181)...(184)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (293)...(296)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (469)...(472)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (508)...(511)
<223> N-glycosylation site. Prosite id = PS00001

<400> 50

```
Met Ser Phe Phe Leu Ser Cys Leu Tyr Leu Ser Leu Cys Gly Ser Ala
1               5                  10                  15

Leu Ala Ala Asn Leu Thr Ser Trp Lys Ser Arg Ser Ile Tyr Gln Thr
            20                  25                  30

Met Thr Asp Arg Phe Ala Arg Thr Asp Gly Ser Thr Thr Ala Ala Cys
        35                  40                  45

Asn Thr Thr Ala Gly Leu Tyr Cys Gly Gly Thr Trp Arg Gly Thr Ile
    50                  55                  60

Asn His Leu Asp Tyr Ile Gln Gly Met Gly Phe Asp Ala Val Met Ile
65                  70                  75                  80

Ser Pro Ile Ile Glu Asn Ile Asp Gly Arg Val Ser Tyr Gly Glu Ala
                85                  90                  95

Tyr His Gly Tyr Trp Pro Leu Asp Leu Glu Ser Leu Asn Thr Arg Phe
            100                 105                 110
```

Gly Thr Lys Gln Asp Leu Leu Asp Leu Ser Asn Ala Leu His Ser Arg
115 120 125

Gly Met Tyr Leu Met Met Asp Thr Val Ile Asn Asn Met Ala Tyr Ile
130 135 140

Thr Arg Gly Gln Asp Pro Ala Thr Ala Ile Asp Tyr Ser Val Phe Thr
145 150 155 160

Pro Phe Asn Asn Ala Asp Tyr Phe His Pro Tyr Cys Lys Ile Thr Asp
165 170 175

Trp Asn Asn Leu Thr Asp Ala Ser Leu Cys Gln Thr Gly Asp Leu Glu
180 185 190

Val Ala Leu Pro Asp Leu Phe Thr Glu His Thr Asp Val Gln Asp Arg
195 200 205

Leu Ile Ser Trp Ala Lys Glu Met Ile Gln Thr Tyr Ser Ile Asp Gly
210 215 220

Leu Arg Ile Asp Ala Ala Lys His Val Asp Pro Glu Phe Leu Ala Arg
225 230 235 240

Phe Val Asn Glu Val Asp Val Phe Thr Thr Gly Glu Val Leu Gln Arg
245 250 255

Glu Val Asp Ile Ile Cys Asp Tyr His Asn Lys Tyr Ile Thr Ser Met
260 265 270

Pro Asn Tyr Pro Ile Tyr Phe Ser Met Leu Asp Ala Phe Thr Glu Gly
275 280 285

Asn Thr Ser Ser Leu Met Ser Gln Val Glu Ala Met Lys Gly Pro Cys
290 295 300

His Asp Val Thr Ala Leu Val Ser Phe Ser Glu Asn His Asp Gln Pro
305 310 315 320

Arg Ile Pro Ser Met Asn Lys Asp Ile Ala Leu Ala Lys Asn Val Leu
325 330 335

Thr Phe Thr Ile Leu Phe Asp Gly Ile Pro Met Val Tyr Gln Gly Gln
340 345 350

Glu Gln His Leu Asp Gly Ser Gly Thr Pro Lys Asn Arg Glu Ala Leu
355 360 365

Trp Leu Ser Lys Tyr Asp Thr Gln Ala Glu Leu Tyr Gln Leu Leu Ala

```
     370                 375                 380

Lys Leu Asn Ala Ile Arg Lys His Ala Thr Ser Leu Gly Ser Asp Tyr
385                 390                 395                 400

Leu Tyr Ala Gln Thr Arg Pro Ile Tyr Arg Gly Gly Ser Glu Leu Ala
                405                 410                 415

Phe Tyr Lys Gly Ile Glu Gly Arg Gln Val Ile Thr Val Leu Ser Ser
            420                 425                 430

Gln Gly Ala Gln Gly Asn Pro Tyr Asp Leu Tyr Leu Pro Val Ser Tyr
        435                 440                 445

Asn Pro Gly Thr Ala Val Met Glu Val Leu Asn Cys Val Asn Ser Thr
    450                 455                 460

Val Gly Asp Asp Gly Gln Leu Lys Val Pro Met Glu Lys Gly Glu Pro
465                 470                 475                 480

Arg Val Phe Phe Pro Ile Glu Leu Met Gly Gly Ser Gly Leu Cys Gly
                485                 490                 495

Tyr Ser Lys Asp Asn Val Thr Val Ser Gln Leu Lys Thr Gly His Asp
            500                 505                 510

Ser Thr Ser Arg Gly Ser Lys Met Thr Gly Ser Ala Ala Leu Leu Met
        515                 520                 525

Met Leu Ser Leu Gly Ala Ser Leu Val Leu Trp
    530                 535
```

<210> 51
<211> 1824
<212> DNA
<213> Aspergillus terreus

<400> 51

atgaagtgga ccttctcgct cctcctctta ctgtccgtgt tcggtcaggc tactcatgcc 60

ctgaccccag cagaatggcg cagccagtca atctacttcc tgttgaccga tcgctttggt 120

cgaacagaca attccacaac tgctgcctgt gacaccaccg acagagtata ctgcggtggt 180

agctggcagg gaatcatcaa ccatctcgat tacatccaag ggatgggatt cactgccatc 240

tggatcaccc cggtcactgg acagttctac gaaaacacgg gcgacggcac ctcctaccat 300

ggatactggc agcaggacat ctacgacctc aactacaact acggaacggc ccaagacctc 360

aagaacctag ccagtgcttt gcacgagcgc ggcatgtatt tgatggttga tgtggttgcc 420

aatcacatgg gctatgacgg agcgggaaac accgtggact acagtgtttt caacccccttc 480

tcctcctcca gctactttca cccatactgc ctcatctcca attacgacaa ccagaccaat 540

gttgaagact gctggctggg tgataccacc gtttcgctgc cagatctcga cacgacaagc 600

acagccgtgc gggacatctg gtacgactgg gtggcagact tggtcgccaa ctattccatc 660

gacggtctgc gtgtcgacac tgtaaaacac gtcgaaaaag acttttggcc cgactacaac 720

agcgcagcag gcgtctactg tgtcggcgag gtcttttcag gcgatcctgc atacacatgc 780

ccgtaccaga actacatgga cggcgtgctc aactatccaa tctactacca gcttctctat 840

gcgtttgagt cgtccagcgg cagcatcagc gatctctata acatgatcag ctccgttgcc 900

tccagctgca aggatcccac actcctgggt aatttcatcg agaaccacga taacccccgc 960

tttgcttcct acacgagcga ctactcgcag gctaagaacg tgatcacctt catcttcctg 1020

agcgacggta ttcccatcgt ctacgccgga caggaacagc actacagcgg aggcagcgac 1080

ccagctaacc gcgaggccac ctggctgtct ggatactcca cgagcgccac gctgtacacc 1140

tggatcgcct ctacaaacca gatccgcagc ctggcgatct ccaaggacgc gggatacgtg 1200

caggccaaga acaacccctt ctactccgat tccaacacca tcgccatgcg caagggcacg 1260

acagccggcg cgcaagtcat caccgtcctc agcaacaagg gcgcctccgg cagctcctac 1320

accctctctt tgagcggcac aggctactcc gccggcgcga ccctggtcga gacgtacacc 1380

tgcactacgg tgactgtaga ctcgagcggc aacctgcccg tcccaatgac atccggcttg 1440

ccgcgagtgt ttgtcccgtc gtcctgggtg aatgggagcg cgctttgcaa cacagaatgc 1500

acggccgcca cgtccctccc ggttctcttc gaggaactgg ttacgacgac ctacggggag 1560

aacatttatc tgagcggctc gatcagccag ctgggcagtt ggaatacggc ctcggctgtt 1620

gctctgtcgg cgagtcaata tacctcgtcc aacccgaaat ggtatgtgag tgtgacgttg 1680

cctgtgggca cgtcgttcca gtacaagttt atcaagaagg ggtcggacgg gagtgttgtc 1740

tgggagagtg atccgaaccg gtcgtatacc gttccggctg ggtgcgaggg cgcgacggtg 1800

acagttgctg atacttggag gtga 1824

<210> 52
<211> 607
<212> PRT
<213> Aspergillus terreus

<220>
<221> SIGNAL
<222> (1)...(20)

<220>
<221> DOMAIN
<222> (33)...(389)
<223> Alpha amylase, catalytic domain

<220>
<221> DOMAIN
<222> (505)...(602)
<223> Starch binding domain

<220>
<221> SITE
<222> (44)...(47)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (179) ... (182)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (220)...(223)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (498)...(501)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (595)...(598)
<223> N-glycosylation site. Prosite id = PS00001

<400> 52

```
Met Lys Trp Thr Phe Ser Leu Leu Leu Leu Leu Ser Val Phe Gly Gln
1               5                  10                  15

Ala Thr His Ala Leu Thr Pro Ala Glu Trp Arg Ser Gln Ser Ile Tyr
            20                  25                  30

Phe Leu Leu Thr Asp Arg Phe Gly Arg Thr Asp Asn Ser Thr Thr Ala
        35                  40                  45

Ala Cys Asp Thr Thr Asp Arg Val Tyr Cys Gly Gly Ser Trp Gln Gly
    50                  55                  60

Ile Ile Asn His Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala Ile
65                  70                  75                  80

Trp Ile Thr Pro Val Thr Gly Gln Phe Tyr Glu Asn Thr Gly Asp Gly
                85                  90                  95

Thr Ser Tyr His Gly Tyr Trp Gln Gln Asp Ile Tyr Asp Leu Asn Tyr
            100                 105                 110

Asn Tyr Gly Thr Ala Gln Asp Leu Lys Asn Leu Ala Ser Ala Leu His
        115                 120                 125

Glu Arg Gly Met Tyr Leu Met Val Asp Val Val Ala Asn His Met Gly
    130                 135                 140

Tyr Asp Gly Ala Gly Asn Thr Val Asp Tyr Ser Val Phe Asn Pro Phe
145                 150                 155                 160
```

```
Ser Ser Ser Ser Tyr Phe His Pro Tyr Cys Leu Ile Ser Asn Tyr Asp
                165                 170                 175

Asn Gln Thr Asn Val Glu Asp Cys Trp Leu Gly Asp Thr Thr Val Ser
            180                 185                 190

Leu Pro Asp Leu Asp Thr Thr Ser Thr Ala Val Arg Asp Ile Trp Tyr
        195                 200                 205

Asp Trp Val Ala Asp Leu Val Ala Asn Tyr Ser Ile Asp Gly Leu Arg
    210                 215                 220

Val Asp Thr Val Lys His Val Glu Lys Asp Phe Trp Pro Asp Tyr Asn
225                 230                 235                 240

Ser Ala Ala Gly Val Tyr Cys Val Gly Glu Val Phe Ser Gly Asp Pro
                245                 250                 255

Ala Tyr Thr Cys Pro Tyr Gln Asn Tyr Met Asp Gly Val Leu Asn Tyr
            260                 265                 270

Pro Ile Tyr Tyr Gln Leu Leu Tyr Ala Phe Glu Ser Ser Ser Gly Ser
        275                 280                 285

Ile Ser Asp Leu Tyr Asn Met Ile Ser Ser Val Ala Ser Ser Cys Lys
    290                 295                 300

Asp Pro Thr Leu Leu Gly Asn Phe Ile Glu Asn His Asp Asn Pro Arg
305                 310                 315                 320

Phe Ala Ser Tyr Thr Ser Asp Tyr Ser Gln Ala Lys Asn Val Ile Thr
                325                 330                 335

Phe Ile Phe Leu Ser Asp Gly Ile Pro Ile Val Tyr Ala Gly Gln Glu
            340                 345                 350

Gln His Tyr Ser Gly Gly Ser Asp Pro Ala Asn Arg Glu Ala Thr Trp
        355                 360                 365

Leu Ser Gly Tyr Ser Thr Ser Ala Thr Leu Tyr Thr Trp Ile Ala Ser
    370                 375                 380

Thr Asn Gln Ile Arg Ser Leu Ala Ile Ser Lys Asp Ala Gly Tyr Val
385                 390                 395                 400

Gln Ala Lys Asn Asn Pro Phe Tyr Ser Asp Ser Asn Thr Ile Ala Met
                405                 410                 415
```

Arg Lys Gly Thr Thr Ala Gly Ala Gln Val Ile Thr Val Leu Ser Asn
420 425 430

Lys Gly Ala Ser Gly Ser Ser Tyr Thr Leu Ser Leu Ser Gly Thr Gly
435 440 445

Tyr Ser Ala Gly Ala Thr Leu Val Glu Thr Tyr Thr Cys Thr Thr Val
450 455 460

Thr Val Asp Ser Ser Gly Asn Leu Pro Val Pro Met Thr Ser Gly Leu
465 470 475 480

Pro Arg Val Phe Val Pro Ser Ser Trp Val Asn Gly Ser Ala Leu Cys
485 490 495

Asn Thr Glu Cys Thr Ala Ala Thr Ser Leu Pro Val Leu Phe Glu Glu
500 505 510

Leu Val Thr Thr Thr Tyr Gly Glu Asn Ile Tyr Leu Ser Gly Ser Ile
515 520 525

Ser Gln Leu Gly Ser Trp Asn Thr Ala Ser Ala Val Ala Leu Ser Ala
530 535 540

Ser Gln Tyr Thr Ser Ser Asn Pro Lys Trp Tyr Val Ser Val Thr Leu
545 550 555 560

Pro Val Gly Thr Ser Phe Gln Tyr Lys Phe Ile Lys Lys Gly Ser Asp
565 570 575

Gly Ser Val Val Trp Glu Ser Asp Pro Asn Arg Ser Tyr Thr Val Pro
580 585 590

Ala Gly Cys Glu Gly Ala Thr Val Thr Val Ala Asp Thr Trp Arg
595 600 605

<210> 53
<211> 1389
<212> DNA
<213> Aspergillus terreus

<400> 53

atgaagctat cccgcgccct gacagtcttc cttctccacc ttacatccac tgcattggca 60

gcggatgtca atgcgtggaa gtctcgcaat atttactttg ctttgacgga tcgcattgcc 120

cgcagtagcg atgatactgg tggaagtgct tgcggtaacc tgggagacta ctgcggcgga 180

accttccaag gcttgcagtc caagcttgac tacatcaagg ggatgggctt tgatgctatc 240

tggatcactc ctgtggtagc taatgctccg ggtggttatc atggttattg ggcacaggat 300

ctctatagta tcaactccaa ctatggcacc gctgatgacc tcaaaagcct ggtcaatgct 360

gctcatgaga agggaatgta tgttatggcg gacgtcgtgg ccaaccacat gggaagtccc 420

atcagcgata acaagcccga gccaatgaac caggagagct cttaccattc agcctgcaca 480

attgactact cagaccagag tagcattgaa gattgccgta tcgcatcaga cctgcccgat 540

gtcaacacag aaagttccga aattcggacc ctcttccagg agtggatcag ctggctggtg 600

aaggaatacc agtttgacgg gctgcgtatc gacacggtca agcatgttga aaaagacttc 660

tggccgggct tctgctctgc cgccggcgtc tacaccatcg gcgaagtctg ggacggcgac 720

ccaaactacc ttgctggata cgcaaacagt atggacgctg tgctcaacta cgcaatctac 780

tatcccatga accgattcta ccagcaacag gggtcctcct ccgacatcgt cagcatgcac 840

gatcaaatca gttccctgtt ccccaaccca accgccctcg gcacgttcct ggacaaccac 900

gacaacgccc gttggctgag ccagaagaac gacgcctctc tgctgaaaaa cgccctcgcc 960

tatgtcattc tcgcccgcgg catccccatc gtgtactacg gcacggagca gggctacgcc 1020

ggcggcaatg accccgcaaa ccgcgaggac ctatggcgca gcaacttcga caccgacgcc 1080

gacttgtacc aggccatatc ccggctctcg gcggcgaggg catcatttgg cgggctcgct 1140

gatgacgacc atgtccatct gtatgtcgct gatacggcgt acgcgtggag cagggccggt 1200

ggggacctga ttgtcctcac gtctaatagt ggatctggct ctgagtctaa gtactgcttc 1260

gattcaaaga agcctggtgg atcatggaac aacacctttg gaacgggaac atatactgct 1320

gatggtgacg ggcaactctg tgtcaccacc tcaaatggtg aacccgtggt gctggttgca 1380

gatgtgtag 1389

<210> 54
<211> 462
<212> PRT
<213> Aspergillus terreus

<220>
<221> SIGNAL
<222> (1)...(20)

<220>
<221> DOMAIN
<222> (33)...(373)

<223> Alpha amylase, catalytic domain

<220>
<221> SITE
<222> (436)...(439)
<223> N-glycosylation site. Prosite id = PS00001

<400> 54

Met Lys Leu Ser Arg Ala Leu Thr Val Phe Leu Leu His Leu Thr Ser
1 5 10 15

Thr Ala Leu Ala Ala Asp Val Asn Ala Trp Lys Ser Arg Asn Ile Tyr
20 25 30

```
Phe Ala Leu Thr Asp Arg Ile Ala Arg Ser Ser Asp Asp Thr Gly Gly
        35                  40                  45

Ser Ala Cys Gly Asn Leu Gly Asp Tyr Cys Gly Gly Thr Phe Gln Gly
    50                  55                  60

Leu Gln Ser Lys Leu Asp Tyr Ile Lys Gly Met Gly Phe Asp Ala Ile
65                  70                  75                  80

Trp Ile Thr Pro Val Val Ala Asn Ala Pro Gly Gly Tyr His Gly Tyr
                85                  90                  95

Trp Ala Gln Asp Leu Tyr Ser Ile Asn Ser Asn Tyr Gly Thr Ala Asp
            100                 105                 110

Asp Leu Lys Ser Leu Val Asn Ala Ala His Glu Lys Gly Met Tyr Val
        115                 120                 125

Met Ala Asp Val Val Ala Asn His Met Gly Ser Pro Ile Ser Asp Asn
    130                 135                 140

Lys Pro Glu Pro Met Asn Gln Glu Ser Ser Tyr His Ser Ala Cys Thr
145                 150                 155                 160

Ile Asp Tyr Ser Asp Gln Ser Ser Ile Glu Asp Cys Arg Ile Ala Ser
                165                 170                 175

Asp Leu Pro Asp Val Asn Thr Glu Ser Ser Glu Ile Arg Thr Leu Phe
            180                 185                 190

Gln Glu Trp Ile Ser Trp Leu Val Lys Glu Tyr Gln Phe Asp Gly Leu
        195                 200                 205

Arg Ile Asp Thr Val Lys His Val Glu Lys Asp Phe Trp Pro Gly Phe
    210                 215                 220

Cys Ser Ala Ala Gly Val Tyr Thr Ile Gly Glu Val Trp Asp Gly Asp
225                 230                 235                 240

Pro Asn Tyr Leu Ala Gly Tyr Ala Asn Ser Met Asp Ala Val Leu Asn
                245                 250                 255

Tyr Ala Ile Tyr Tyr Pro Met Asn Arg Phe Tyr Gln Gln Gln Gly Ser
            260                 265                 270

Ser Ser Asp Ile Val Ser Met His Asp Gln Ile Ser Ser Leu Phe Pro
        275                 280                 285

Asn Pro Thr Ala Leu Gly Thr Phe Leu Asp Asn His Asp Asn Ala Arg
```

290 295 300

Trp Leu Ser Gln Lys Asn Asp Ala Ser Leu Leu Lys Asn Ala Leu Ala
305 310 315 320

Tyr Val Ile Leu Ala Arg Gly Ile Pro Ile Val Tyr Tyr Gly Thr Glu
325 330 335

Gln Gly Tyr Ala Gly Gly Asn Asp Pro Ala Asn Arg Glu Asp Leu Trp
340 345 350

Arg Ser Asn Phe Asp Thr Asp Ala Asp Leu Tyr Gln Ala Ile Ser Arg
355 360 365

Leu Ser Ala Ala Arg Ala Ser Phe Gly Gly Leu Ala Asp Asp Asp His
370 375 380

Val His Leu Tyr Val Ala Asp Thr Ala Tyr Ala Trp Ser Arg Ala Gly
385 390 395 400

Gly Asp Leu Ile Val Leu Thr Ser Asn Ser Gly Ser Gly Ser Glu Ser
405 410 415

Lys Tyr Cys Phe Asp Ser Lys Lys Pro Gly Gly Ser Trp Asn Asn Thr
420 425 430

Phe Gly Thr Gly Thr Tyr Thr Ala Asp Gly Asp Gly Gln Leu Cys Val
435 440 445

Thr Thr Ser Asn Gly Glu Pro Val Val Leu Val Ala Asp Val
450 455 460

<210> 55
<211> 1269
<212> DNA
<213> Unknown

<220>
<223> Obtained from environmental sample

<400> 55

atgttgaagc agttcacgaa gcgcctgatc accctgacga gcctgctggc gctcgtcctc 60

gtcgcaccgt tggccagcgc gggcccgctg gatggcaaca gcagcgacgt catgttgcag 120

ggcttccatt ggtactcgta ccagtcgttt ccgtggtggg gcgtcatcaa gaacaacgcg 180

gcgagcatca aggccgacgg cttcaccatg gtgtggctgc cgccgcccag cgacgcggcc 240

tccaacgagg gctacctgcc gcgccggctc gagctgctgg acagcaagta tggcacccgg 300

acggacctgg tcaacgccct gtccgcgctg aatgccaatg gtgtgaagcc cattgcggac 360

atcgtcatca accaccgcgt gggcaccacg ggctgggcgg acttcacgct gcctccgtgg 420

ggctcgaacg cggtgtgccg cggcgacgag tggagcgggg ccacgggcaa cgcggatacg 480

ggcgatggct tcaacgccgg gcgcgacatc gatcacacgc agaccttcgt gcaggacggc 540

atcgtcacct ggatgaacaa ctcgctgaag agcgtcgggt tcgcgggttg gcggtatgac 600

tacgtgaagg gctacagcgg ctcctacgtc ggctcgtaca acacccgcac gacgccgtac 660

ttctccgtgg gcgagctgtg gacggacctg gacctgaaca accccaaccc ccaccgccag 720

ctgatcatga attggatcga cgcgacgggt ggccggtccg cggcgttcga cttcacgacc 780

aagggcctgc tgcagcaggc ggtgcagtac aacgagttct ggcggctgaa ggatgcggcg 840

ggcgcgccag cgggtgccat tggttggtgg gcagcgaagt ccgtgacctt catcgacaat 900

cacgacacgg gcccgagcta tccgagcggc ggccagaacc actggccgtt ccctggtgac 960

aagatcctcc aggggtacgc ctacatcctg actcactctg gcatcccctg cgtgtactgg 1020

gtgcactaca aggactgggg ccaggcgaac acggacgcca tcaagaagct gatcagcatc 1080

cgcaagtcca agggcatcac cagcacctcc tcggtgagca tccaggccgc ggacagctcg 1140

aagtacgccg ccatcatcac cggcaacaac ggcaaggtgg ccgtgaagat cggcttcggc 1200

gcctggtctc cgccgggcac ctggacgctg gccacctccg gcaacaacta cgccgtctgg 1260

acgcagtaa 1269

<210> 56
<211> 422
<212> PRT
<213> Unknown

<220>
<223> Obtained from environmental sample

<220>
<221> SIGNAL
<222> (1)...(27)

<220>
<221> DOMAIN
<222> (36)...(361)
<223> Alpha amylase, catalytic domain

<220>
<221> DOMAIN
<222> (362)...(422)
<223> Alpha-amylase C-terminal beta-sheet domain

<220>
<221> SITE
<222> (33)...(36)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (188)...(191)
<223> N-glycosylation site. Prosite id = PS00001

<400> 56

Met Leu Lys Gln Phe Thr Lys Arg Leu Ile Thr Leu Thr Ser Leu Leu

```
1               5                  10                  15

Ala Leu Val Leu Val Ala Pro Leu Ala Ser Ala Gly Pro Leu Asp Gly
            20                  25                  30

Asn Ser Ser Asp Val Met Leu Gln Gly Phe His Trp Tyr Ser Tyr Gln
        35                  40                  45

Ser Phe Pro Trp Trp Gly Val Ile Lys Asn Asn Ala Ala Ser Ile Lys
    50                  55                  60

Ala Asp Gly Phe Thr Met Val Trp Leu Pro Pro Pro Ser Asp Ala Ala
65                  70                  75                  80

Ser Asn Glu Gly Tyr Leu Pro Arg Arg Leu Glu Leu Leu Asp Ser Lys
                85                  90                  95

Tyr Gly Thr Arg Thr Asp Leu Val Asn Ala Leu Ser Ala Leu Asn Ala
            100                 105                 110

Asn Gly Val Lys Pro Ile Ala Asp Ile Val Ile Asn His Arg Val Gly
        115                 120                 125

Thr Thr Gly Trp Ala Asp Phe Thr Leu Pro Pro Trp Gly Ser Asn Ala
    130                 135                 140

Val Cys Arg Gly Asp Glu Trp Ser Gly Ala Thr Gly Asn Ala Asp Thr
145                 150                 155                 160

Gly Asp Gly Phe Asn Ala Gly Arg Asp Ile Asp His Thr Gln Thr Phe
                165                 170                 175

Val Gln Asp Gly Ile Val Thr Trp Met Asn Asn Ser Leu Lys Ser Val
            180                 185                 190

Gly Phe Ala Gly Trp Arg Tyr Asp Tyr Val Lys Gly Tyr Ser Gly Ser
        195                 200                 205

Tyr Val Gly Ser Tyr Asn Thr Arg Thr Thr Pro Tyr Phe Ser Val Gly
    210                 215                 220

Glu Leu Trp Thr Asp Leu Asp Leu Asn Asn Pro Asn Pro His Arg Gln
225                 230                 235                 240

Leu Ile Met Asn Trp Ile Asp Ala Thr Gly Gly Arg Ser Ala Ala Phe
                245                 250                 255

Asp Phe Thr Thr Lys Gly Leu Leu Gln Gln Ala Val Gln Tyr Asn Glu
            260                 265                 270
```

Phe Trp Arg Leu Lys Asp Ala Ala Gly Ala Pro Ala Gly Ala Ile Gly
275 280 285

Trp Trp Ala Ala Lys Ser Val Thr Phe Ile Asp Asn His Asp Thr Gly
290 295 300

Pro Ser Tyr Pro Ser Gly Gly Gln Asn His Trp Pro Phe Pro Gly Asp
305 310 315 320

Lys Ile Leu Gln Gly Tyr Ala Tyr Ile Leu Thr His Ser Gly Ile Pro
325 330 335

Cys Val Tyr Trp Val His Tyr Lys Asp Trp Gly Gln Ala Asn Thr Asp
340 345 350

Ala Ile Lys Lys Leu Ile Ser Ile Arg Lys Ser Lys Gly Ile Thr Ser
355 360 365

Thr Ser Ser Val Ser Ile Gln Ala Ala Asp Ser Ser Lys Tyr Ala Ala
370 375 380

Ile Ile Thr Gly Asn Asn Gly Lys Val Ala Val Lys Ile Gly Phe Gly
385 390 395 400

Ala Trp Ser Pro Pro Gly Thr Trp Thr Leu Ala Thr Ser Gly Asn Asn
405 410 415

Tyr Ala Val Trp Thr Gln
420

<210> 57
<211> 1581
<212> DNA
<213> Unknown

<220>
<223> Obtained from environmental sample

<400> 57

atgccaaact ggttaaaaga cgctgttttc tacgagattt accctcagtc cttcaaggac 60

accaactgcg acggtatcgg cgacatcaat ggaatcattg agaagctgga ttatgtgaag 120

gagctgggat gcaatgccct ttggatcaat ccttgtttcg attcaccatt taaggatgcc 180

ggctacgacg tcagagacta caagaaggta gcacccagat atggcacaaa tgctgacctt 240

tacagattat tcggcgaagc ccacaacaga ggcattcacg tactccttga cctggttccg 300

ggccacactt ccgaggaaca tgcgtggttc caggagagca aaaaggctga aaagaatgaa 360

tacacagacc gctatgtatg gaccaactgc tggatccatg gcattgctgg tcatccctat 420

attggcggcg aggctgacag agacggctgc tacatgctga acttctttaa gtgccagcct 480

gcccttaact acggattcct taaccgcacc gatgattggc agtctgcacc tgacgcacct 540

gagtgcatcg caacaagaga ggccctcaag gacatcatgc gtttctggct tgaccacggc 600

tgcgatggtt tccgtgttga tatggcagat tctctggtaa aagaggatga tgagaacaag 660

tccgctaccg gcgcaatctg gagaaatatt agagaaatgc ttgataagga ctatcctgaa 720

gctgccattg tttcagagtg gagcaatcct cagcaggccc ttaagagcgg cttccacgct 780

gacttctatc ttgatcacca cggaaacggc tataataccc ttatccgcga caatgagacc 840

ccgggcggcg accacagttt ccttaagaag gatggaaacg gcgatatcat gcgtttcctc 900

ctggattacc tgcccaagta tgacagcacc aagaatgatg gctacataag ctttatcacc 960

tgcaatcacg acactccaag agcccgcaga acactgggct acgatgagct taagattgca 1020

tgggctcttt tcctaacact tcccggagtt ccgttcatct actacggtga tgagattgga 1080

atgagatatc tggacattcc tacgaaggaa ggcggataca caagaaccgg caccagaact 1140

ccaatgcagt gggacaactc caagaaccac ggtttctcag atgcaggtgc tgatgtcctc 1200

tatcttccac aggatccgtc tggcgatgct cctacagtcg aagatcagga aaaagatcct 1260

tcttcactcc ttaacgtgac aaaagagctg acagctcttc gccataaata cactgacctg 1320

caggccgatg gctcttttga cgtcatctat gcagaaaaag agcagttccc atttatctac 1380

aaacgcggaa atctgctcct tgcgatcaac ccatcagaaa gcaagtccag tgctgccctt 1440

ccggatgagg catatataaa gaaggatgat acaaaagccg gcaagcttac acctgtatac 1500

tcaatcggtg agttcaagca ggaggatcac agcctcacgc ttcagggcca gtccttcgtt 1560

gtcttctcac tcgaatcata a 1581

<210> 58
<211> 526
<212> PRT
<213> Unknown

<220>
<223> Obtained from environmental sample

<220>
<221> DOMAIN
<222> (12)...(434)
<223> Alpha amylase, catalytic domain

<220>
<221> SITE
<222> (170)...(173)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (222)...(225)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (431)...(434)
<223> N-glycosylation site. Prosite id = PS00001

<400> 58

```
Met Pro Asn Trp Leu Lys Asp Ala Val Phe Tyr Glu Ile Tyr Pro Gln
1               5                  10                  15

Ser Phe Lys Asp Thr Asn Cys Asp Gly Ile Gly Asp Ile Asn Gly Ile
            20                  25                  30

Ile Glu Lys Leu Asp Tyr Val Lys Glu Leu Gly Cys Asn Ala Leu Trp
        35                  40                  45

Ile Asn Pro Cys Phe Asp Ser Pro Phe Lys Asp Ala Gly Tyr Asp Val
    50                  55                  60

Arg Asp Tyr Lys Lys Val Ala Pro Arg Tyr Gly Thr Asn Ala Asp Leu
65                  70                  75                  80

Tyr Arg Leu Phe Gly Glu Ala His Asn Arg Gly Ile His Val Leu Leu
                85                  90                  95

Asp Leu Val Pro Gly His Thr Ser Glu Glu His Ala Trp Phe Gln Glu
            100                 105                 110

Ser Lys Lys Ala Glu Lys Asn Glu Tyr Thr Asp Arg Tyr Val Trp Thr
        115                 120                 125

Asn Cys Trp Ile His Gly Ile Ala Gly His Pro Tyr Ile Gly Gly Glu
    130                 135                 140

Ala Asp Arg Asp Gly Cys Tyr Met Leu Asn Phe Phe Lys Cys Gln Pro
145                 150                 155                 160

Ala Leu Asn Tyr Gly Phe Leu Asn Arg Thr Asp Asp Trp Gln Ser Ala
                165                 170                 175

Pro Asp Ala Pro Glu Cys Ile Ala Thr Arg Glu Ala Leu Lys Asp Ile
            180                 185                 190

Met Arg Phe Trp Leu Asp His Gly Cys Asp Gly Phe Arg Val Asp Met
        195                 200                 205

Ala Asp Ser Leu Val Lys Glu Asp Asp Glu Asn Lys Ser Ala Thr Gly
    210                 215                 220

Ala Ile Trp Arg Asn Ile Arg Glu Met Leu Asp Lys Asp Tyr Pro Glu
225                 230                 235                 240

Ala Ala Ile Val Ser Glu Trp Ser Asn Pro Gln Gln Ala Leu Lys Ser
```

245 250 255

Gly Phe His Ala Asp Phe Tyr Leu Asp His His Gly Asn Gly Tyr Asn
260 265 270

Thr Leu Ile Arg Asp Asn Glu Thr Pro Gly Gly Asp His Ser Phe Leu
275 280 285

Lys Lys Asp Gly Asn Gly Asp Ile Met Arg Phe Leu Leu Asp Tyr Leu
290 295 300

Pro Lys Tyr Asp Ser Thr Lys Asn Asp Gly Tyr Ile Ser Phe Ile Thr
305 310 315 320

Cys Asn His Asp Thr Pro Arg Ala Arg Arg Thr Leu Gly Tyr Asp Glu
325 330 335

Leu Lys Ile Ala Trp Ala Leu Phe Leu Thr Leu Pro Gly Val Pro Phe
340 345 350

Ile Tyr Tyr Gly Asp Glu Ile Gly Met Arg Tyr Leu Asp Ile Pro Thr
355 360 365

Lys Glu Gly Gly Tyr Thr Arg Thr Gly Thr Arg Thr Pro Met Gln Trp
370 375 380

Asp Asn Ser Lys Asn His Gly Phe Ser Asp Ala Gly Ala Asp Val Leu
385 390 395 400

Tyr Leu Pro Gln Asp Pro Ser Gly Asp Ala Pro Thr Val Glu Asp Gln
405 410 415

Glu Lys Asp Pro Ser Ser Leu Leu Asn Val Thr Lys Glu Leu Thr Ala
420 425 430

Leu Arg His Lys Tyr Thr Asp Leu Gln Ala Asp Gly Ser Phe Asp Val
435 440 445

Ile Tyr Ala Glu Lys Glu Gln Phe Pro Phe Ile Tyr Lys Arg Gly Asn
450 455 460

Leu Leu Leu Ala Ile Asn Pro Ser Glu Ser Lys Ser Ser Ala Ala Leu
465 470 475 480

Pro Asp Glu Ala Tyr Ile Lys Lys Asp Asp Thr Lys Ala Gly Lys Leu
485 490 495

Thr Pro Val Tyr Ser Ile Gly Glu Phe Lys Gln Glu Asp His Ser Leu
500 505 510

Thr Leu Gln Gly Gln Ser Phe Val Val Phe Ser Leu Glu Ser
515 520 525

<210> 59
<211> 1572
<212> DNA
<213> Unknown

<220>
<223> Obtained from environmental sample

<400> 59

atgcccggat ggttgaagga tgcagtattt tacgaaatct acccccagtc ctttgccgat 60
tcaaacggcg acgggatcgg tgatctgcag gggatcatcg gaaagcttga ctatgtgaag 120
gagcttggat gcaacgcgct ctggatcaat ccctgttacg attcgccctt taaggatgcc 180
ggctacgacg tccgcgacta caaaaaggtg gccgaaaggt acggcacaaa cgacgatctg 240
aaggagctct tttcaaaggc acatgagaag ggcatccacg tccttctgga cctggtcccc 300
ggccacacct ccgaggaaca cgcctggttt aaggagagtt caaaagccga aaagaacgag 360
ttttccggcc gctatatctg gaccggacac tggctcgagg gcgtaccagg tcatccctgg 420
atcgcgggag aatgcgaaag accggcctgc tatatgctca acttcttcaa atgccagccg 480
gccctgaatt acggtttttt acatcccgaa aagccctggc agtcctccac ggattccgct 540
gaggcgagag cgacacatga agccatgaag gatgtcatgc gcttctggct ggacgccggc 600
tgcgacggct tccgcgtgga catggcagac agccttgtaa aggatgatga cgagaagaaa 660
tcctgtacct gcgcgctgtg gcgcgaggtg aggcagatgc tcgatgacga ttatccggag 720
gccgccatca tctccgagtg gtccaatccc gaattggcca ttaattccgc acattttcac 780
atggacttct gtctcgacca cgcgagcaac ggttatcata cacttctcag ggattactgg 840
ggtgacgacg gcgacaacag ctttttcaaa gccggcggtc acggcgatgt gatgcgtttc 900
cttgatgact atctgcccag atatgaggcg acaaaggata acggctttat aagccttata 960
tcctgcaatc acgacaccag aaggcctgct tattctctct cagagcgcga gatcaaggtg 1020
gcctatgcct ttatctatac gatgcccggc gtccgctttg tctactacgg ggacgagata 1080
gcgatgagat atctggatct tcccaccaaa gaaggcggat atgacaggac cggcagcagg 1140
acgccgatgc agtgggatgg cggcgccaac ttcggtttct cctccgctcc cgcggacaaa 1200
ctctacctgc cgcaggatcc ctcaaaggat gctcccacgg cagagaaggc catggccgat 1260
cccgcctctg tatatcacgt gataaaagcc cttctgacca taagacatga gcacaaagca 1320
ttgcaggcgg acggcggatt ggaagtacta tacgccgaaa aagaaaagct tccctttgtt 1380
tacgcaagaa gaagcggcga cgacggcgtg ctggtggcgc tcaatccgtc attaaatgag 1440
gtctcagccc gggtgaacgc cgcagtggat gacgagctcc tctccctcgg tgagacctcc 1500

gtatcctgtg aaggcagcga gagcaccata accatggggc ctcagtcatt catcatattt 1560

aagctggcct ga 1572

<210> 60
<211> 523
<212> PRT
<213> Unknown

<220>
<223> Obtained from environmental sample

<220>
<221> DOMAIN
<222> (12)...(435)
<223> Alpha amylase, catalytic domain

<400> 60

```
Met Pro Gly Trp Leu Lys Asp Ala Val Phe Tyr Glu Ile Tyr Pro Gln
1               5                  10                  15

Ser Phe Ala Asp Ser Asn Gly Asp Gly Ile Gly Asp Leu Gln Gly Ile
            20                  25                  30

Ile Gly Lys Leu Asp Tyr Val Lys Glu Leu Gly Cys Asn Ala Leu Trp
        35                  40                  45

Ile Asn Pro Cys Tyr Asp Ser Pro Phe Lys Asp Ala Gly Tyr Asp Val
    50                  55                  60

Arg Asp Tyr Lys Lys Val Ala Glu Arg Tyr Gly Thr Asn Asp Asp Leu
65                  70                  75                  80

Lys Glu Leu Phe Ser Lys Ala His Glu Lys Gly Ile His Val Leu Leu
                85                  90                  95

Asp Leu Val Pro Gly His Thr Ser Glu Glu His Ala Trp Phe Lys Glu
            100                 105                 110

Ser Ser Lys Ala Glu Lys Asn Glu Phe Ser Gly Arg Tyr Ile Trp Thr
        115                 120                 125

Gly His Trp Leu Glu Gly Val Pro Gly His Pro Trp Ile Ala Gly Glu
    130                 135                 140

Cys Glu Arg Pro Ala Cys Tyr Met Leu Asn Phe Phe Lys Cys Gln Pro
145                 150                 155                 160

Ala Leu Asn Tyr Gly Phe Leu His Pro Glu Lys Pro Trp Gln Ser Ser
                165                 170                 175

Thr Asp Ser Pro Glu Ala Arg Ala Thr His Glu Ala Met Lys Asp Val
            180                 185                 190
```

```
Met Arg Phe Trp Leu Asp Ala Gly Cys Asp Gly Phe Arg Val Asp Met
        195                 200                 205

Ala Asp Ser Leu Val Lys Asp Asp Asp Glu Lys Lys Ser Cys Thr Cys
    210                 215                 220

Ala Leu Trp Arg Glu Val Arg Gln Met Leu Asp Asp Asp Tyr Pro Glu
225                 230                 235                 240

Ala Ala Ile Ile Ser Glu Trp Ser Asn Pro Glu Leu Ala Ile Asn Ser
                245                 250                 255

Ala His Phe His Met Asp Phe Cys Leu Asp His Ala Ser Asn Gly Tyr
            260                 265                 270

His Thr Leu Leu Arg Asp Tyr Trp Gly Asp Asp Gly Asp Asn Ser Phe
        275                 280                 285

Phe Lys Ala Gly Gly His Gly Asp Val Met Arg Phe Leu Asp Asp Tyr
    290                 295                 300

Leu Pro Arg Tyr Glu Ala Thr Lys Asp Asn Gly Phe Ile Ser Leu Ile
305                 310                 315                 320

Ser Cys Asn His Asp Thr Arg Arg Pro Ala Tyr Ser Leu Ser Glu Arg
                325                 330                 335

Glu Ile Lys Val Ala Tyr Ala Phe Ile Tyr Thr Met Pro Gly Val Pro
            340                 345                 350

Phe Val Tyr Tyr Gly Asp Glu Ile Ala Met Arg Tyr Leu Asp Leu Pro
        355                 360                 365

Thr Lys Glu Gly Gly Tyr Asp Arg Thr Gly Ser Arg Thr Pro Met Gln
    370                 375                 380

Trp Asp Gly Gly Ala Asn Phe Gly Phe Ser Ser Ala Pro Ala Asp Lys
385                 390                 395                 400

Leu Tyr Leu Pro Gln Asp Pro Ser Lys Asp Ala Pro Thr Ala Glu Lys
                405                 410                 415

Ala Met Ala Asp Pro Ala Ser Val Tyr His Val Ile Lys Ala Leu Leu
            420                 425                 430

Thr Ile Arg His Glu His Lys Ala Leu Gln Ala Asp Gly Gly Leu Glu
        435                 440                 445
```

Val Leu Tyr Ala Glu Lys Glu Lys Leu Pro Phe Val Tyr Ala Arg Arg
450 455 460

Ser Gly Asp Asp Gly Val Leu Val Ala Leu Asn Pro Ser Leu Asn Glu
465 470 475 480

Val Ser Ala Arg Val Asn Ala Ala Val Asp Asp Glu Leu Leu Ser Leu
485 490 495

Gly Glu Thr Ser Val Ser Cys Glu Gly Ser Glu Ser Thr Ile Thr Met
500 505 510

Gly Pro Gln Ser Phe Ile Ile Phe Lys Leu Ala
515 520

<210> 61
<211> 2061
<212> DNA
<213> Unknown

<220>
<223> Obtained from environmental sample

<400> 61

atggtagcag gctttgggct ctacggagct gccttgctca ccccaatggc ggcacaggcc 60

gaaacgggca gagaagtcat gctgcagggc tttaactgga attccacctc gcaagcgggt 120

gggcactata atgagctggc aaatcgtgct ggtgaaatcg ctggggctgg tatcgacatt 180

gtttggttcc cacctccatc cagagccgca gaccgggttg gatacctgcc caacgagtgg 240

tataatatga actccaatta tggaaatcgg acgactctac aggcggctat cgggaatctt 300

cgcaacaacg gtgtcaaaac cgtcgcggat attgtcgtta accaccgtgt gggcacaacg 360

aattgggcgg acttcacgaa tccttctttc ggtgataaca accgtgccat cacacgcgat 420

gatgaatggc atcagtcgtc gggtaactgg gataccggtg aggcgtatag tgccgcccgt 480

gacttggacc acacctacgg ccccgtgcaa aatgagatta aaaactggct gaattggttg 540

aagagtgaca tcggctttga tggttggcgc tatgatatgg tcaagggatt cagcgggtac 600

tatgtgggcg agtacaacac agcaactagc ccctacattt ccgttggtga gttcttcgac 660

tatgatcgcc agaaggtggt cggctggatc aacgccacca atgctcgctc tcgcgccttc 720

gattttccaa cacgcaacct cctctatgtg gctgtcaccc aaaacaacta cggagttctt 780

cgtgatggtg aaggtaaggc caatggactc atcggttggt ggccacaacg ggctatcaca 840

ttcattgaga accatgatac ggaagaagcc cgcaacggtg agtacacccc cgccttcccc 900

caatgggcta ccatgcaggg ttatgcctac attttgacgc accccggcat tccatgcgtt 960

ttctggaatg attggcgttg ggacttccgt tcggaaatca atcaactgat cgccatccgc 1020

agggcccaag gcatcaacga tggcagcagc ctcagcatcc aggttgccga tggcagccgc 1080

tacggggcga tcatcaatgg aaacacggcg gtcaagattg gtcctggaaa ctggagccca 1140

agtggtagtt ggacgctggc tgccgccgga accaattacg ccgtctggac aaacggtggc 1200

ggaacaccaa cgccaacacc cacaccaacc ccaactcagg gccccacaac ggttacttgg 1260

aatccatcca ctccgacagc cggacaaaat gtgacgatca cttatccttc cggccgctcc 1320

cttgccagtt cctccaacgt caacctctac tggggtgtca atggatggac caatgtccaa 1380

accaaagcga tgaccaagaa cagttccaat gactggacaa ccacgatcac acttccctcc 1440

aacacaacac gtttgaactt tgtgttcaat aacggctcca gttgggacaa caacagcagt 1500

caggattgga atgtcaacgt gaccgctgta acgcccacac caacaccgac accgactccc 1560

acacctacgg caactccgac accgacaccc accccgacgc ccactgccac acccacccca 1620

actccgactc cttcgatcat ttggtatcgc atcgaagccc gtcacagtgg taaagttcta 1680

gacgttgcca gtgcatcaac ttcaaacggt ggcaatgtgc atcagtggtc ttatgctggt 1740

ggacagaacc aacaatggcg tgttgtcgac gctgggaacg ggtttgttta catcctaaac 1800

cggaacagtg gaaaagcact tgaagtgggc aacttctcca ccagtaacgg cgggaacgtc 1860

cagcaatggg attatgccgg tggttccagt caacagtgga agctcattga gacgaccaac 1920

ggatatgtgc aaatccagaa tcggaacagc ggcaaagcca ttgatgtttc cgctgcttcg 1980

actaccaatg gtgccaacat ccatcagtgg acctacggcg gcggcaacaa tcagcagtgg 2040

aagctgatcc caatcaatta a 2061

<210> 62
<211> 686
<212> PRT
<213> Unknown

<220>
<223> Obtained from environmental sample

<220>
<221> SIGNAL
<222> (1)...(20)

<220>
<221> DOMAIN
<222> (25)...(340)
<223> Alpha amylase, catalytic domain

<220>
<221> DOMAIN
<222> (341)...(398)
<223> Alpha-amylase C-terminal beta-sheet domain

<220>
<221> DOMAIN
<222> (547)...(680)
<223> Ricin-type beta-trefoil lectin domain

<220>
<221> SITE
<222> (34)...(37)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (90)...(93)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (234)...(237)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (436)...(439)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (474)...(477)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (488)...(491)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (498)...(501)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (504)...(507)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (513)...(516)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (620)...(623)
<223> N-glycosylation site. Prosite id = PS00001

<400> 62

```
Met Val Ala Gly Phe Gly Leu Tyr Gly Ala Ala Leu Leu Thr Pro Met
1               5                  10                  15

Ala Ala Gln Ala Glu Thr Gly Arg Glu Val Met Leu Gln Gly Phe Asn
            20                  25                  30

Trp Asn Ser Thr Ser Gln Ala Gly Gly His Tyr Asn Glu Leu Ala Asn
        35                  40                  45

Arg Ala Gly Glu Ile Ala Gly Ala Gly Ile Asp Ile Val Trp Phe Pro
    50                  55                  60

Pro Pro Ser Arg Ala Ala Asp Arg Val Gly Tyr Leu Pro Asn Glu Trp
65                  70                  75                  80
```

```
Tyr Asn Met Asn Ser Asn Tyr Gly Asn Arg Thr Thr Leu Gln Ala Ala
                85                  90                  95

Ile Gly Asn Leu Arg Asn Asn Gly Val Lys Thr Val Ala Asp Ile Val
            100                 105                 110

Val Asn His Arg Val Gly Thr Thr Asn Trp Ala Asp Phe Thr Asn Pro
        115                 120                 125

Ser Phe Gly Asp Asn Asn Arg Ala Ile Thr Arg Asp Asp Glu Trp His
    130                 135                 140

Gln Ser Ser Gly Asn Trp Asp Thr Gly Glu Ala Tyr Ser Ala Ala Arg
145                 150                 155                 160

Asp Leu Asp His Thr Tyr Gly Pro Val Gln Asn Glu Ile Lys Asn Trp
                165                 170                 175

Leu Asn Trp Leu Lys Ser Asp Ile Gly Phe Asp Gly Trp Arg Tyr Asp
            180                 185                 190

Met Val Lys Gly Phe Ser Gly Tyr Tyr Val Gly Glu Tyr Asn Thr Ala
        195                 200                 205

Thr Ser Pro Tyr Ile Ser Val Gly Glu Phe Phe Asp Tyr Asp Arg Gln
    210                 215                 220

Lys Val Val Gly Trp Ile Asn Ala Thr Asn Ala Arg Ser Arg Ala Phe
225                 230                 235                 240

Asp Phe Pro Thr Arg Asn Leu Leu Tyr Val Ala Val Thr Gln Asn Asn
                245                 250                 255

Tyr Gly Val Leu Arg Asp Gly Glu Gly Lys Ala Asn Gly Leu Ile Gly
            260                 265                 270

Trp Trp Pro Gln Arg Ala Ile Thr Phe Ile Glu Asn His Asp Thr Glu
        275                 280                 285

Glu Ala Arg Asn Gly Glu Tyr Thr Pro Ala Phe Pro Gln Trp Ala Thr
    290                 295                 300

Met Gln Gly Tyr Ala Tyr Ile Leu Thr His Pro Gly Ile Pro Cys Val
305                 310                 315                 320

Phe Trp Asn Asp Trp Arg Trp Asp Phe Arg Ser Glu Ile Asn Gln Leu
                325                 330                 335
```

```
Ile Ala Ile Arg Arg Ala Gln Gly Ile Asn Asp Gly Ser Ser Leu Ser
            340                 345                 350

Ile Gln Val Ala Asp Gly Ser Arg Tyr Gly Ala Ile Ile Asn Gly Asn
        355                 360                 365

Thr Ala Val Lys Ile Gly Pro Gly Asn Trp Ser Pro Ser Gly Ser Trp
    370                 375                 380

Thr Leu Ala Ala Ala Gly Thr Asn Tyr Ala Val Trp Thr Asn Gly Gly
385                 390                 395                 400

Gly Thr Pro Thr Pro Thr Pro Thr Pro Thr Pro Thr Pro Thr Gln Gly Pro Thr
                405                 410                 415

Thr Val Thr Trp Asn Pro Ser Thr Pro Thr Ala Gly Gln Asn Val Thr
            420                 425                 430

Ile Thr Tyr Pro Ser Gly Arg Ser Leu Ala Ser Ser Ser Asn Val Asn
        435                 440                 445

Leu Tyr Trp Gly Val Asn Gly Trp Thr Asn Val Gln Thr Lys Ala Met
    450                 455                 460

Thr Lys Asn Ser Ser Asn Asp Trp Thr Thr Thr Ile Thr Leu Pro Ser
465                 470                 475                 480

Asn Thr Thr Arg Leu Asn Phe Val Phe Asn Asn Gly Ser Ser Trp Asp
                485                 490                 495

Asn Asn Ser Ser Gln Asp Trp Asn Val Asn Val Thr Ala Val Thr Pro
            500                 505                 510

Thr Pro Thr Pro Thr Pro Thr Pro Thr Pro Thr Ala Thr Pro Thr Pro
        515                 520                 525

Thr Pro Thr Pro Thr Pro Thr Ala Thr Pro Thr Pro Thr Pro Thr Pro
    530                 535                 540

Ser Ile Ile Trp Tyr Arg Ile Glu Ala Arg His Ser Gly Lys Val Leu
545                 550                 555                 560

Asp Val Ala Ser Ala Ser Thr Ser Asn Gly Gly Asn Val His Gln Trp
                565                 570                 575

Ser Tyr Ala Gly Gly Gln Asn Gln Gln Trp Arg Val Val Asp Ala Gly
            580                 585                 590
```

Asn Gly Phe Val Tyr Ile Leu Asn Arg Asn Ser Gly Lys Ala Leu Glu
595 600 605

Val Gly Asn Phe Ser Thr Ser Asn Gly Gly Asn Val Gln Gln Trp Asp
610 615 620

Tyr Ala Gly Gly Ser Ser Gln Gln Trp Lys Leu Ile Glu Thr Thr Asn
625 630 635 640

Gly Tyr Val Gln Ile Gln Asn Arg Asn Ser Gly Lys Ala Ile Asp Val
645 650 655

Ser Ala Ala Ser Thr Thr Asn Gly Ala Asn Ile His Gln Trp Thr Tyr
660 665 670

Gly Gly Gly Asn Asn Gln Gln Trp Lys Leu Ile Pro Ile Asn
675 680 685

<210> 63
<211> 1611
<212> DNA
<213> Unknown

<220>
<223> Obtained from environmental sample

<400> 63

atgacagagt ggtggcgtgg tgcagtgacc tatcaagtct atccaaggtc gtttcaggac 60

agcaacggcg acggcatcgg cgacctgccc ggcatcaccg cccggcttga gtatctggcc 120

gatcttggcg tggacgcggt ctggctgtca ccgttcttca aaagcccgat gaaggacatg 180

ggctatgacg tcagcgacta ttgcgatgtc gatccggtct tcggcaccct cgccgatttt 240

gacgccctgc tggcccgcgc gcatgagctg gggctcaagg tgatcatcga ccaggtcctt 300

agccacagtt ccgacctgca ccctgccttt gtgaccagtc gcagcgaccg cgtgaacccg 360

aaggcggact ggtatgtctg ggccgatccc aagcccgacg gcagcccgcc caacaactgg 420

ctgtcggtgt tcggtggctc ggcatgggcc tgggacgcgc gcagaaaaca gtattacctg 480

cacaatttcc tgaccagcca gccggacctg aactaccaca acccgaaggt gcaggactgg 540

gcgctggaca acatgcgttt ctggctggac cggggcgtgg acgggttccg ctttgacacc 600

gtcaactact tcttccacga tcccttgttg cgcagcaacc ctgccgatca ccgcaacaag 660

cctgaggctg acggcaatcc ctacggcatg cagtaccacc tgcatgacaa gaaccagccc 720

gagaacctga tctggatgga gcggatacgg gtgcttctgg accaatacgg tgccgcaagc 780

gtcggcgaga tgggcgaaag tcaccacgcc atccggatga tgggcgacta caccgctccg 840

gggcggctgc atcaatgcta cagctttgaa ttcatggggt atgaatacac cgcaaacctg 900

ttccgggacc ggatagaaag ctttttcaag ggtgccccta aaggctggcc gatgtgggcg 960

ttttcaaacc acgatgtcgt ccgccatgtc agtcgctggg caaaacatgg cctcaccccc 1020

gaggcggttg ccaagcagac aggtgcgttg cttctgtcgc ttgagggctc gatctgcctg 1080

tgggagggcg aggagctggg ccagaccgat accgaactgg ccttggatga gttgaccgat 1140

ccgcagggca tcgtcttttg gcccgaaccg atcggccgcg acaatactcg gacgccaatg 1200

gtttgggacg catcgccgca tggcgggttt tcgaccgtca caccctggct gccggtgaaa 1260

ccggaacagg ccgcgcgtca tgtggccggg caaaccggtg atgccgcctc ggtgctggaa 1320

agctaccggg cgatgctggc cttccggcgc gctgaaccgg cccttaggac cgggcggacg 1380

cggtttctgg atctggccga accggttctg ggctttgtgc gcggcgaagg ggagggtgcg 1440

atcctgtgcc tgttcaatct gtcgcctgtt gcgcgggggg ttgcggtcga aggcgtgggc 1500

ccgccgatcg gcccgggcca gcaggctatc ctttcgggcg gacggctagg ccttggcccg 1560

aacggcgccg ccttcctgcg ggtgaccgga acagtccgcg ttctggacta a 1611

<210> 64
<211> 536
<212> PRT
<213> Unknown

<220>
<223> Obtained from environmental sample

<220>
<221> DOMAIN
<222> (12)...(449)
<223> Alpha amylase, catalytic domain

<400> 64

```
Met Thr Glu Trp Trp Arg Gly Ala Val Thr Tyr Gln Val Tyr Pro Arg
1               5                  10                  15

Ser Phe Gln Asp Ser Asn Gly Asp Gly Ile Gly Asp Leu Pro Gly Ile
            20                  25                  30

Thr Ala Arg Leu Glu Tyr Leu Ala Asp Leu Gly Val Asp Ala Val Trp
        35                  40                  45

Leu Ser Pro Phe Phe Lys Ser Pro Met Lys Asp Met Gly Tyr Asp Val
    50                  55                  60

Ser Asp Tyr Cys Asp Val Asp Pro Val Phe Gly Thr Leu Ala Asp Phe
65                  70                  75                  80

Asp Ala Leu Leu Ala Arg Ala His Glu Leu Gly Leu Lys Val Ile Ile
                85                  90                  95

Asp Gln Val Leu Ser His Ser Ser Asp Leu His Pro Ala Phe Val Thr
            100                 105                 110

Ser Arg Ser Asp Arg Val Asn Pro Lys Ala Asp Trp Tyr Val Trp Ala
```

115 120 125

Asp Pro Lys Pro Asp Gly Ser Pro Pro Asn Asn Trp Leu Ser Val Phe
130 135 140

Gly Gly Ser Ala Trp Ala Trp Asp Ala Arg Arg Lys Gln Tyr Tyr Leu
145 150 155 160

His Asn Phe Leu Thr Ser Gln Pro Asp Leu Asn Tyr His Asn Pro Lys
165 170 175

Val Gln Asp Trp Ala Leu Asp Asn Met Arg Phe Trp Leu Asp Arg Gly
180 185 190

Val Asp Gly Phe Arg Phe Asp Thr Val Asn Tyr Phe Phe His Asp Pro
195 200 205

Leu Leu Arg Ser Asn Pro Ala Asp His Arg Asn Lys Pro Glu Ala Asp
210 215 220

Gly Asn Pro Tyr Gly Met Gln Tyr His Leu His Asp Lys Asn Gln Pro
225 230 235 240

Glu Asn Leu Ile Trp Met Glu Arg Ile Arg Val Leu Leu Asp Gln Tyr
245 250 255

Gly Ala Ala Ser Val Gly Glu Met Gly Glu Ser His His Ala Ile Arg
260 265 270

Met Met Gly Asp Tyr Thr Ala Pro Gly Arg Leu His Gln Cys Tyr Ser
275 280 285

Phe Glu Phe Met Gly Tyr Glu Tyr Thr Ala Asn Leu Phe Arg Asp Arg
290 295 300

Ile Glu Ser Phe Phe Lys Gly Ala Pro Lys Gly Trp Pro Met Trp Ala
305 310 315 320

Phe Ser Asn His Asp Val Val Arg His Val Ser Arg Trp Ala Lys His
325 330 335

Gly Leu Thr Pro Glu Ala Val Ala Lys Gln Thr Gly Ala Leu Leu Leu
340 345 350

Ser Leu Glu Gly Ser Ile Cys Leu Trp Glu Gly Glu Glu Leu Gly Gln
355 360 365

Thr Asp Thr Glu Leu Ala Leu Asp Glu Leu Thr Asp Pro Gln Gly Ile
370 375 380

```
Val Phe Trp Pro Glu Pro Ile Gly Arg Asp Asn Thr Arg Thr Pro Met
385                 390                 395                 400

Val Trp Asp Ala Ser Pro His Gly Gly Phe Ser Thr Val Thr Pro Trp
                405                 410                 415

Leu Pro Val Lys Pro Glu Gln Ala Ala Arg His Val Ala Gly Gln Thr
            420                 425                 430

Gly Asp Ala Ala Ser Val Leu Glu Ser Tyr Arg Ala Met Leu Ala Phe
        435                 440                 445

Arg Arg Ala Glu Pro Ala Leu Arg Thr Gly Arg Thr Arg Phe Leu Asp
    450                 455                 460

Leu Ala Glu Pro Val Leu Gly Phe Val Arg Gly Glu Gly Glu Gly Ala
465                 470                 475                 480

Ile Leu Cys Leu Phe Asn Leu Ser Pro Val Ala Arg Gly Val Ala Val
                485                 490                 495

Glu Gly Val Gly Pro Pro Ile Gly Pro Gly Gln Gln Ala Ile Leu Ser
            500                 505                 510

Gly Gly Arg Leu Gly Leu Gly Pro Asn Gly Ala Ala Phe Leu Arg Val
        515                 520                 525

Thr Gly Thr Val Arg Val Leu Asp
    530                 535
```

<210> 65
<211> 1386
<212> DNA
<213> Unknown

<220>
<223> Obtained from environmental sample

<400> 65

atgaagttga agtaccttgc cttagttttg ttggctgtgg cttcgatagg cctactctcg 60

actccagtgg gtgctgccaa gtactccgaa ctcgaagagg gcggtgttat aatgcaggcc 120

ttctactggg atgttcccgg agggggaatc tggtgggaca ccataagaca gaaaatcccg 180

gagtggtacg acgctggaat ctcggcgata tggattcctc cagctagcaa agggatgggc 240

ggtggttatt ccatgggcta cgatccctac gatttctttg acctcggcga gtactatcag 300

aagggaacag ttgagacgcg cttcggctca aaggaggaac tggtgaacat gataaacacc 360

gcacactcct atggcataaa ggtgatagcg gacatagtca taaaccaccg cgccggtgga 420

gaccttgagt ggaacccctt tgtaaacaac tatacttgga cagacttctc caaggtcgcc 480

tccggtaaat acacggccaa ctaccttgac ttccacccaa acgaggtcaa gtgctgcgat 540

gagggtacat ttggtgactt tccggacatc gcccacgaga agagctggga tcagtactgg 600

ctctgggcaa gcaatgagag ctacgccgca tatctccgga gcatagggat cgatgcatgg 660

cgtttcgact acgtcaaagg ttacggagcg tgggttgtta atgactggct cagctggtgg 720

ggaggctggg ccgttggaga gtactgggac acgaacgttg atgcactcct taactgggca 780

tacgacagcg gtgccaaggt ctttgacttc ccgctctact acaagatgga cgaagccttt 840

gacaacacca acatccccgc tttggtttac gccctccaga acggaggaac agtcgtttcc 900

cgcgatccct tcaaggcagt aactttcgtt gccaaccacg atacagatat aatctggaac 960

aagtatccgg cttatgcgtt catccttacc tatgagggac agcctgttat attttaccgc 1020

gactacgagg agtggctcaa caaggataag cttaacaacc ttatctggat acacgagcac 1080

cttgccggag gaagtaccaa gatcctctac tacgataacg atgagctaat attcatgagg 1140

gagggctacg ggagcaagcc gggcctcata acctacataa acctcggaaa cgactgggcc 1200

gagcgctggg tgaacgtcgg ctcaaagttt gccggctaca caatccatga atacacaggc 1260

aatctcggtg gctgggttga caggtgggtt cagtacgatg gatgggttaa actgacggca 1320

cctcctcatg atccagccaa cggatattac ggctactcag tctggagcta cgcaggcgtc 1380

ggatga 1386

<210> 66
<211> 461
<212> PRT
<213> Unknown

<220>
<223> Obtained from environmental sample

<220>
<221> SIGNAL
<222> (1)...(25)

<220>
<221> DOMAIN
<222> (35)...(368)
<223> Alpha amylase, catalytic domain

<220>
<221> SITE
<222> (152)...(155)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (208)...(211)
<223> N-glycosylation site. Prosite id = PS00001

<400> 66

Met Lys Leu Lys Tyr Leu Ala Leu Val Leu Leu Ala Val Ala Ser Ile
1 5 10 15

```
Gly Leu Leu Ser Thr Pro Val Gly Ala Ala Lys Tyr Ser Glu Leu Glu
            20                  25                  30

Glu Gly Gly Val Ile Met Gln Ala Phe Tyr Trp Asp Val Pro Gly Gly
        35                  40                  45

Gly Ile Trp Trp Asp Thr Ile Arg Gln Lys Ile Pro Glu Trp Tyr Asp
    50                  55                  60

Ala Gly Ile Ser Ala Ile Trp Ile Pro Pro Ala Ser Lys Gly Met Gly
65                  70                  75                  80

Gly Gly Tyr Ser Met Gly Tyr Asp Pro Tyr Asp Phe Phe Asp Leu Gly
                85                  90                  95

Glu Tyr Tyr Gln Lys Gly Thr Val Glu Thr Arg Phe Gly Ser Lys Glu
            100                 105                 110

Glu Leu Val Asn Met Ile Asn Thr Ala His Ser Tyr Gly Ile Lys Val
        115                 120                 125

Ile Ala Asp Ile Val Ile Asn His Arg Ala Gly Gly Asp Leu Glu Trp
    130                 135                 140

Asn Pro Phe Val Asn Asn Tyr Thr Trp Thr Asp Phe Ser Lys Val Ala
145                 150                 155                 160

Ser Gly Lys Tyr Thr Ala Asn Tyr Leu Asp Phe His Pro Asn Glu Val
                165                 170                 175

Lys Cys Cys Asp Glu Gly Thr Phe Gly Asp Phe Pro Asp Ile Ala His
            180                 185                 190

Glu Lys Ser Trp Asp Gln Tyr Trp Leu Trp Ala Ser Asn Glu Ser Tyr
        195                 200                 205

Ala Ala Tyr Leu Arg Ser Ile Gly Ile Asp Ala Trp Arg Phe Asp Tyr
    210                 215                 220

Val Lys Gly Tyr Gly Ala Trp Val Val Asn Asp Trp Leu Ser Trp Trp
225                 230                 235                 240

Gly Gly Trp Ala Val Gly Glu Tyr Trp Asp Thr Asn Val Asp Ala Leu
                245                 250                 255

Leu Asn Trp Ala Tyr Asp Ser Gly Ala Lys Val Phe Asp Phe Pro Leu
            260                 265                 270
```

Tyr Tyr Lys Met Asp Glu Ala Phe Asp Asn Thr Asn Ile Pro Ala Leu
275 280 285

Val Tyr Ala Leu Gln Asn Gly Gly Thr Val Val Ser Arg Asp Pro Phe
290 295 300

Lys Ala Val Thr Phe Val Ala Asn His Asp Thr Asp Ile Ile Trp Asn
305 310 315 320

Lys Tyr Pro Ala Tyr Ala Phe Ile Leu Thr Tyr Glu Gly Gln Pro Val
325 330 335

Ile Phe Tyr Arg Asp Tyr Glu Glu Trp Leu Asn Lys Asp Lys Leu Asn
340 345 350

Asn Leu Ile Trp Ile His Glu His Leu Ala Gly Gly Ser Thr Lys Ile
355 360 365

Leu Tyr Tyr Asp Asn Asp Glu Leu Ile Phe Met Arg Glu Gly Tyr Gly
370 375 380

Ser Lys Pro Gly Leu Ile Thr Tyr Ile Asn Leu Gly Asn Asp Trp Ala
385 390 395 400

Glu Arg Trp Val Asn Val Gly Ser Lys Phe Ala Gly Tyr Thr Ile His
405 410 415

Glu Tyr Thr Gly Asn Leu Gly Gly Trp Val Asp Arg Trp Val Gln Tyr
420 425 430

Asp Gly Trp Val Lys Leu Thr Ala Pro Pro His Asp Pro Ala Asn Gly
435 440 445

Tyr Tyr Gly Tyr Ser Val Trp Ser Tyr Ala Gly Val Gly
450 455 460

<210> 67
<211> 2022
<212> DNA
<213> Unknown

<220>
<223> Obtained from environmental sample

<400> 67

ttgaaaaaaa acaccattag cgccctggtc gcaggtatgg tattaggctt tgcatccaac 60

gcaatggcgg ttcctagaac cgcttttgta cacctctttg aatggaaatg ggaagatgtt 120

gcacaggagt gtgaaacatt tctcggacct aaaggctttg ccgcagtgca agtctctccg 180

ccaactaaat ctcacaacac ggatgcatgg tggggccgtt atcaacccgt tagttatgct 240

tttgaaggac gcagcggtaa tcgcagccaa tttaaaaata tggtgcaacg ttgtaaagct 300

gtaggcgtcg atatatacgt agatgcagtg attaaccaca tggcagccta cgacagaaat 360

ttccctgatg taccctatag cagtaatgac tttaactcct gtacaggaga tattgactat 420

aataaccgtt ggcaaacaca gcattgtgat ttagtcggtc ttaatgatct aaaaacagga 480

tctgactacg tccgccaaaa aatagcggat tatatgaacg acgcaatcag tatgggtgta 540

gctggtttcc gtattgatgc agccaaacat ataccagcag gtgatatagc tgccattaaa 600

ggtaaattaa atggtaatcc atacatcttc caagaggtaa ttggtgcatc cggcgaacct 660

gttcgaccga ctgaatacac ctttatcggt ggtgtcacgg aatttcaatt tgctcgaaaa 720

ttgggtccag ccttccgcaa tagtaatatt gcttggttaa aagacattgg cagtcaaatg 780

gaattatcca gtgctgatgc cgtaacattt gtaacgaatc atgatgaaga gcgtcataac 840

ccgaatggtc ctatttggca cggcgttcaa ggtaatggtt atgcattagc aaatattttc 900

accttagctt acccttacgg ctatccaaaa atcatgtcag gatacttctt ccacggtgac 960

tttaacgcag ctccaccaag cagtggtata cacacaggaa atgcgtgtgg ttttgatggc 1020

ggagactggg tatgcgaaca caaatggcgc ggtattgcta acatggttgc cttccgcaac 1080

tatacagcaa gcgaatggcg tatcagtaat tggtggcaaa acagtaacga ccaaattgct 1140

tttggtcgcg gtggtttagg ttttgttgtt attaataaac gtgctaatgg tagcattaat 1200

caaagttttg atacgggaat gcctgatggc caatactgta acataataga agctaacttt 1260

gatgaaagca ccggccaatg tagtgcagct acagattcca acggtcaagc cgttattacc 1320

gtcagtggtg ggcaagctaa ctttaatgta gcaggcgatc atgctgctgc aattcatgtt 1380

ggcgcaaaaa ttggtgatca atgtagtggt gatgattgcc catgtacagg atccgattgt 1440

aataatgatc ctaaacctga ttttgcagta ccagcaacat caatttgtac atcagaaaat 1500

ttacctacgc tatattactg gggagcacag cctacagata gcttagcgaa tgcagcttgg 1560

ccaggtgtcg caatgcaaac aaatggcgac tttaagtgtc atgatttagg tgtcgaacta 1620

accaaaatta acgccatctt tagtgacaat ggtgcaaata aaacagctga tctaactgtt 1680

actggtgcag gttgttataa agacgggact tggagcacct tacaaaattg tggctttgaa 1740

attaccggtg cacaaaccaa tccagtcggt ggcgacgaag tctggtactt ccgaggtact 1800

gctaatgact ggggtaaagc acaattagat tatgacgcaa ctagcggttt gtattacaca 1860

atacaaagct ttaatggtga agaagcacct gcgcgtttta aaattgataa tggtagttgg 1920

actgaagctt atccaacagc tgattaccaa gttacagata acaattcata ccgcattaac 1980

tttaatagcg atagcaaagc gattacagta aacgcacaat aa 2022

<210> 68
<211> 673

<212> PRT
<213> Unknown

<220>
<223> Obtained from environmental sample

<220>
<221> SIGNAL
<222> (1) ... (23)

<220>
<221> DOMAIN
<222> (27) ... (359)
<223> Alpha amylase, catalytic domain

<220>
<221> DOMAIN
<222> (372)...(463)
<223> Alpha amylase, C-terminal all-beta domain

<220>
<221> SITE
<222> (88)...(91)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (365)...(368)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (402)...(405)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (406)...(409)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (561)...(564)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (646)...(649)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (664)...(667)
<223> N-glycosylation site. Prosite id = PS00001

<400> 68

```
Met Lys Lys Asn Thr Ile Ser Ala Leu Val Ala Gly Met Val Leu Gly
1               5                  10                  15

Phe Ala Ser Asn Ala Met Ala Val Pro Arg Thr Ala Phe Val His Leu
            20                  25                  30

Phe Glu Trp Lys Trp Glu Asp Val Ala Gln Glu Cys Glu Thr Phe Leu
        35                  40                  45
```

```
Gly Pro Lys Gly Phe Ala Ala Val Gln Val Ser Pro Pro Thr Lys Ser
    50                  55                  60

His Asn Thr Asp Ala Trp Trp Gly Arg Tyr Gln Pro Val Ser Tyr Ala
65                  70                  75                  80

Phe Glu Gly Arg Ser Gly Asn Arg Ser Gln Phe Lys Asn Met Val Gln
                85                  90                  95

Arg Cys Lys Ala Val Gly Val Asp Ile Tyr Val Asp Ala Val Ile Asn
            100                 105                 110

His Met Ala Ala Tyr Asp Arg Asn Phe Pro Asp Val Pro Tyr Ser Ser
        115                 120                 125

Asn Asp Phe Asn Ser Cys Thr Gly Asp Ile Asp Tyr Asn Asn Arg Trp
    130                 135                 140

Gln Thr Gln His Cys Asp Leu Val Gly Leu Asn Asp Leu Lys Thr Gly
145                 150                 155                 160

Ser Asp Tyr Val Arg Gln Lys Ile Ala Asp Tyr Met Asn Asp Ala Ile
                165                 170                 175

Ser Met Gly Val Ala Gly Phe Arg Ile Asp Ala Ala Lys His Ile Pro
            180                 185                 190

Ala Gly Asp Ile Ala Ala Ile Lys Gly Lys Leu Asn Gly Asn Pro Tyr
        195                 200                 205

Ile Phe Gln Glu Val Ile Gly Ala Ser Gly Glu Pro Val Arg Pro Thr
    210                 215                 220

Glu Tyr Thr Phe Ile Gly Gly Val Thr Glu Phe Gln Phe Ala Arg Lys
225                 230                 235                 240

Leu Gly Pro Ala Phe Arg Asn Ser Asn Ile Ala Trp Leu Lys Asp Ile
                245                 250                 255

Gly Ser Gln Met Glu Leu Ser Ser Ala Asp Ala Val Thr Phe Val Thr
            260                 265                 270

Asn His Asp Glu Glu Arg His Asn Pro Asn Gly Pro Ile Trp His Gly
        275                 280                 285

Val Gln Gly Asn Gly Tyr Ala Leu Ala Asn Ile Phe Thr Leu Ala Tyr
    290                 295                 300

Pro Tyr Gly Tyr Pro Lys Ile Met Ser Gly Tyr Phe Phe His Gly Asp
```

305 310 315 320

Phe Asn Ala Ala Pro Pro Ser Ser Gly Ile His Thr Gly Asn Ala Cys
325 330 335

Gly Phe Asp Gly Gly Asp Trp Val Cys Glu His Lys Trp Arg Gly Ile
340 345 350

Ala Asn Met Val Ala Phe Arg Asn Tyr Thr Ala Ser Glu Trp Arg Ile
355 360 365

Ser Asn Trp Trp Gln Asn Ser Asn Asp Gln Ile Ala Phe Gly Arg Gly
370 375 380

Gly Leu Gly Phe Val Val Ile Asn Lys Arg Ala Asn Gly Ser Ile Asn
385 390 395 400

Gln Ser Phe Asp Thr Gly Met Pro Asp Gly Gln Tyr Cys Asn Ile Ile
405 410 415

Glu Ala Asn Phe Asp Glu Ser Thr Gly Gln Cys Ser Ala Ala Thr Asp
420 425 430

Ser Asn Gly Gln Ala Val Ile Thr Val Ser Gly Gly Gln Ala Asn Phe
435 440 445

Asn Val Ala Gly Asp His Ala Ala Ala Ile His Val Gly Ala Lys Ile
450 455 460

Gly Asp Gln Cys Ser Gly Asp Asp Cys Pro Cys Thr Gly Ser Asp Cys
465 470 475 480

Asn Asn Asp Pro Lys Pro Asp Phe Ala Val Pro Ala Thr Ser Ile Cys
485 490 495

Thr Ser Glu Asn Leu Pro Thr Leu Tyr Tyr Trp Gly Ala Gln Pro Thr
500 505 510

Asp Ser Leu Ala Asn Ala Ala Trp Pro Gly Val Ala Met Gln Thr Asn
515 520 525

Gly Asp Phe Lys Cys His Asp Leu Gly Val Glu Leu Thr Lys Ile Asn
530 535 540

Ala Ile Phe Ser Asp Asn Gly Ala Asn Lys Thr Ala Asp Leu Thr Val
545 550 555 560

Thr Gly Ala Gly Cys Tyr Lys Asp Gly Thr Trp Ser Thr Leu Gln Asn
565 570 575

Cys Gly Phe Glu Ile Thr Gly Ala Gln Thr Asn Pro Val Gly Gly Asp
580 585 590

Glu Val Trp Tyr Phe Arg Gly Thr Ala Asn Asp Trp Gly Lys Ala Gln
595 600 605

Leu Asp Tyr Asp Ala Thr Ser Gly Leu Tyr Tyr Thr Ile Gln Ser Phe
610 615 620

Asn Gly Glu Glu Ala Pro Ala Arg Phe Lys Ile Asp Asn Gly Ser Trp
625 630 635 640

Thr Glu Ala Tyr Pro Thr Ala Asp Tyr Gln Val Thr Asp Asn Asn Ser
645 650 655

Tyr Arg Ile Asn Phe Asn Ser Asp Ser Lys Ala Ile Thr Val Asn Ala
660 665 670

Gln

<210> 69
<211> 1455
<212> DNA
<213> Unknown

<220>
<223> Obtained from environmental sample

<400> 69

atgaaccgtc caggcacggg cgcctcgggg cgcccacaat ctcgctccgc cacgtcgtgg 60

caatcccgca acggcggctg gctgctcgcc tcgctgctgg ccgtgtgttt cgcaacggcg 120

cccgtgcgcg ccgatgtcat cctgcatgca ttcaactggc cgtatgcgac ggtcgaagcc 180

cgcgccaacg agctgcgaga cctgggctat cgcgccgtac tggtcgcacc gccggtgaag 240

tccgaaggca atgcgtggtg ggcgcgctac cagccgcagg actaccgggt catcgagcat 300

ccgctcggca atcgcgagtc gttcgtgcgc atgtccagtg ctctgcgcgc ccgcggcatt 360

cgcgtctacg ccgacatcgt gctgaatcac atggccaatg aagccccgca gcgaccggat 420

ctgaactacc ccggtcaacg ggtcctggat cagtatgcag gcaataccgc gtacttcgcg 480

cagcaacgac tctacggcga tctccgctac aacttcatgt cggcctggga ctttggcccc 540

gcccactgca tcggcaacta ccacgacgtc tggcaggtgc agaactggcg gctgtgcagc 600

ggcgctggcg atgccggcct gcccgacctg ctggccagcg attacatcgt cggacagcag 660

cgcacctatc tccaggcact gaagaacctc ggcgtctccg ggttgcgcat cgacgcggcc 720

aagcacatgc cgctcagcca catcaaccgc gtactgactg ccgacctcaa ggccggcatg 780

catgtgttcg gcgaagtcat cacccacggt ggcgtcggtg atcccgaata cgatctgttt 840

ctccggccct atctggatgg caccgatcac ggtgcctacg acttcccgct cttcgaggcg 900

atccgccgcg cattcggctt cggtggcagc atgagcaccc tggtcgatcc tggggccgtc 960

ggactggccc tgcccaatgc ccggtcgatc accttcaccg tcacccacga catcccgaac 1020

aatggtgtgt tccggcatct gctgctggat gccggcgacg aaaccctggc gtacgcctac 1080

atcctgggtc gtgacggcgg cagtccactg ctgtactccg atcacaacga gagcggcgac 1140

aaccgctggg ttcatgccta tcggcgcaac gacctcgcgg ccatgatccg ctttcacaat 1200

gccaatcatg gcaatgacat gcaggtgctc gcacacggga actgccatct gctctttcgc 1260

cgcggcaatc gcggcatcgt ggcgatcaac aagtgcgggc atacggtcaa cgccacggtg 1320

aacatgaaca acagtgtgct gtggtggcat accccgtatc gcgatgtcct tgatgccggc 1380

agcgtggtgc agattggcag cgccagccac acgttcagcc ttccgccgcg gcgcgctcgg 1440

atgtggctgc gctga 1455

<210> 70
<211> 484
<212> PRT
<213> Unknown

<220>
<223> Obtained from environmental sample

<220>
<221> SIGNAL

<222> (1)...(44)

<220>
<221> DOMAIN
<222> (45)...(399)
<223> Alpha amylase, catalytic domain

<220>
<221> SITE
<222> (381)...(384)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (443)...(446)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (449)...(452)
<223> N-glycosylation site. Prosite id = PS00001

<400> 70

Met Asn Arg Pro Gly Thr Gly Ala Ser Gly Arg Pro Gln Ser Arg Ser
1 5 10 15

Ala Thr Ser Trp Gln Ser Arg Asn Gly Gly Trp Leu Leu Ala Ser Leu
20 25 30

Leu Ala Val Cys Phe Ala Thr Ala Pro Val Arg Ala Asp Val Ile Leu
35 40 45

His Ala Phe Asn Trp Pro Tyr Ala Thr Val Glu Ala Arg Ala Asn Glu
50 55 60

Leu Arg Asp Leu Gly Tyr Arg Ala Val Leu Val Ala Pro Pro Val Lys
65 70 75 80

Ser Glu Gly Asn Ala Trp Trp Ala Arg Tyr Gln Pro Gln Asp Tyr Arg
85 90 95

Val Ile Glu His Pro Leu Gly Asn Arg Glu Ser Phe Val Arg Met Ser
100 105 110

Ser Ala Leu Arg Ala Arg Gly Ile Arg Val Tyr Ala Asp Ile Val Leu
115 120 125

Asn His Met Ala Asn Glu Ala Pro Gln Arg Pro Asp Leu Asn Tyr Pro
130 135 140

Gly Gln Arg Val Leu Asp Gln Tyr Ala Gly Asn Thr Ala Tyr Phe Ala
145 150 155 160

Gln Gln Arg Leu Tyr Gly Asp Leu Arg Tyr Asn Phe Met Ser Ala Trp
165 170 175

Asp Phe Gly Pro Ala His Cys Ile Gly Asn Tyr His Asp Val Trp Gln
180 185 190

Val Gln Asn Trp Arg Leu Cys Ser Gly Ala Gly Asp Ala Gly Leu Pro
195 200 205

Asp Leu Leu Ala Ser Asp Tyr Ile Val Gly Gln Gln Arg Thr Tyr Leu
210 215 220

Gln Ala Leu Lys Asn Leu Gly Val Ser Gly Leu Arg Ile Asp Ala Ala
225 230 235 240

Lys His Met Pro Leu Ser His Ile Asn Arg Val Leu Thr Ala Asp Leu
245 250 255

Lys Ala Gly Met His Val Phe Gly Glu Val Ile Thr His Gly Gly Val
260 265 270

Gly Asp Pro Glu Tyr Asp Leu Phe Leu Arg Pro Tyr Leu Asp Gly Thr
275 280 285

Asp His Gly Ala Tyr Asp Phe Pro Leu Phe Glu Ala Ile Arg Arg Ala

290 295 300

Phe Gly Phe Gly Gly Ser Met Ser Thr Leu Val Asp Pro Gly Ala Val
305 310 315 320

Gly Leu Ala Leu Pro Asn Ala Arg Ser Ile Thr Phe Thr Val Thr His
325 330 335

Asp Ile Pro Asn Asn Gly Val Phe Arg His Leu Leu Leu Asp Ala Gly
340 345 350

Asp Glu Thr Leu Ala Tyr Ala Tyr Ile Leu Gly Arg Asp Gly Gly Ser
355 360 365

Pro Leu Leu Tyr Ser Asp His Asn Glu Ser Gly Asp Asn Arg Trp Val
370 375 380

His Ala Tyr Arg Arg Asn Asp Leu Ala Ala Met Ile Arg Phe His Asn
385 390 395 400

Ala Asn His Gly Asn Asp Met Gln Val Leu Ala His Gly Asn Cys His
405 410 415

Leu Leu Phe Arg Arg Gly Asn Arg Gly Ile Val Ala Ile Asn Lys Cys
420 425 430

Gly His Thr Val Asn Ala Thr Val Asn Met Asn Asn Ser Val Leu Trp
435 440 445

Trp His Thr Pro Tyr Arg Asp Val Leu Asp Ala Gly Ser Val Val Gln
450 455 460

Ile Gly Ser Ala Ser His Thr Phe Ser Leu Pro Pro Arg Arg Ala Arg
465 470 475 480

Met Trp Leu Arg

<210> 71
<211> 1719
<212> DNA
<213> Unknown

<220>
<223> Obtained from environmental sample

<400> 71

gtgcccgaac tgaaatggtg gcagacagct atattttatc aaatctaccc gcgctctttc 60

gccgacggca acggcgatgg catcggcgat ttcaaaggca tcatcggcaa actcgattat 120

ctacaaaatc ttggcataga tgcgctctgg ctctcgcctc acttcccctc ccccaactgg 180

gattgcggct acgatatcag cgattaccgc aacgttgcgc cggaatacgg cacgctggac 240

gatttcaaaa ccttcctgag cgaatcgcac aaacgcggta tccgcgtcat tctcgacctc 300

gtgctgaatc acacctccga tgaacatccg tggttcatcg aatcgaaatc cagccgcgat 360

aatcccaaat ccgattggta tgtgtgggtc gatacgccgc ccaacaattg gcagtcctgc 420

ttcgatggcg atgcctggac atacgtccct gaacgcggcc aatattatta tcactacttc 480

atgaaacagc agcccgatct caactggcat aatccgcagg tcaaacaggc catgtgggag 540

gcggtgcgct tctggctcga tctcggcgtg gacggcttcc gcctggacgc catcggcacg 600

atctacgaag acccaaatct cacgccgcat aatgtcccga tgaatttggc tgagctgcgt 660

cacttcacag atgtcgccaa aacgccggaa gagatcaagc tcaaagaaaa atactggcac 720

gacatgttca agcatcaatg gggtcagccc ggcgttcatg acctgatgaa agaactgcgc 780

gccatcctcg atgaatatga tggcgaccgc atgctggtcg gcgaagatga caacatcgat 840

tacatgggca acggagacga cgaattgcag ctggtcttca acttcccgtt gatgcgcgcc 900

gatcgtctca cccccgacca tattcggcgc aaccaaaaag agcgtttgac tcgtctgaat 960

gctttacccg ttaaaggctg ggcttgcaac acgctcggca accatgatag ttcacgcgtc 1020

tacaccaaat tcggtgaccg gatccacggc gcggaccatg cacgtctcaa cctggcgctt 1080

ttgctcaccc tgcacggcac gccgttctta tacaacggcg aagagatcgg catgaccgac 1140

cacatcatta ccgatcccac caaactgcgc gacaccatgg caacctggta ttacaacagc 1200

cttgtcaacg aaatgaaggt cgagccagcg gaggccgccc ttcgcgccgg acagatgacg 1260

cgcgacaaaa accgtacccc catgcaatgg gacaataagc ccaatgccgg tttttgccca 1320

gataaagccg aaccctggtt gccagtcaac cccaattacc gcgcaggcat taacgtccgc 1380

gagcaaacat cgaacccgaa ctcgctgctc aattactata aacgtctcat ccacttgcgg 1440

cgggaaacgc ctgccttgat cgctggagat tacgttccgc ttcaccagac atccaaagat 1500

catctggcct tcctgcgcaa aacagattca caaacgatcc tggtcgtttt gaattactcc 1560

cccaataaat tggaattgga tttctcgcgc accgtcgaaa tgaaaggccg cccgctgatc 1620

gcaattttct ccagcgcaga tgaccgcccg caggcggcac aaagcccaaa gaaagtatcg 1680

gtcggcgctt acggagttct gctggcagaa gtaaaatag 1719

<210> 72
<211> 572
<212> PRT
<213> Unknown

<220>
<223> Obtained from environmental sample

<220>
<221> DOMAIN
<222> (14)...(405)
<223> Alpha amylase, catalytic domain

<220>
<221> SITE
<222> (104)...(107)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (525)...(528)
<223> N-glycosylation site. Prosite id = PS00001

<400> 72

```
Met Pro Glu Leu Lys Trp Trp Gln Thr Ala Ile Phe Tyr Gln Ile Tyr
1               5                  10                  15

Pro Arg Ser Phe Ala Asp Gly Asn Gly Asp Gly Ile Gly Asp Phe Lys
            20                  25                  30

Gly Ile Ile Gly Lys Leu Asp Tyr Leu Gln Asn Leu Gly Ile Asp Ala
        35                  40                  45

Leu Trp Leu Ser Pro His Phe Pro Ser Pro Asn Trp Asp Cys Gly Tyr
    50                  55                  60

Asp Ile Ser Asp Tyr Arg Asn Val Ala Pro Glu Tyr Gly Thr Leu Asp
65                  70                  75                  80

Asp Phe Lys Thr Phe Leu Ser Glu Ser His Lys Arg Gly Ile Arg Val
                85                  90                  95

Ile Leu Asp Leu Val Leu Asn His Thr Ser Asp Glu His Pro Trp Phe
            100                 105                 110

Ile Glu Ser Lys Ser Ser Arg Asp Asn Pro Lys Ser Asp Trp Tyr Val
        115                 120                 125

Trp Val Asp Thr Pro Pro Asn Asn Trp Gln Ser Cys Phe Asp Gly Asp
    130                 135                 140

Ala Trp Thr Tyr Val Pro Glu Arg Gly Gln Tyr Tyr Tyr His Tyr Phe
145                 150                 155                 160

Met Lys Gln Gln Pro Asp Leu Asn Trp His Asn Pro Gln Val Lys Gln
                165                 170                 175

Ala Met Trp Glu Ala Val Arg Phe Trp Leu Asp Leu Gly Val Asp Gly
            180                 185                 190

Phe Arg Leu Asp Ala Ile Gly Thr Ile Tyr Glu Asp Pro Asn Leu Thr
        195                 200                 205

Pro His Asn Val Pro Met Asn Leu Ala Glu Leu Arg His Phe Thr Asp
```

210 215 220

Val Ala Lys Thr Pro Glu Glu Ile Lys Leu Lys Glu Lys Tyr Trp His
225 230 235 240

Asp Met Phe Lys His Gln Trp Gly Gln Pro Gly Val His Asp Leu Met
245 250 255

Lys Glu Leu Arg Ala Ile Leu Asp Glu Tyr Asp Gly Asp Arg Met Leu
260 265 270

Val Gly Glu Asp Asp Asn Ile Asp Tyr Met Gly Asn Gly Asp Asp Glu
275 280 285

Leu Gln Leu Val Phe Asn Phe Pro Leu Met Arg Ala Asp Arg Leu Thr
290 295 300

Pro Asp His Ile Arg Arg Asn Gln Lys Glu Arg Leu Thr Arg Leu Asn
305 310 315 320

Ala Leu Pro Val Lys Gly Trp Ala Cys Asn Thr Leu Gly Asn His Asp
325 330 335

Ser Ser Arg Val Tyr Thr Lys Phe Gly Asp Arg Ile His Gly Ala Asp
340 345 350

His Ala Arg Leu Asn Leu Ala Leu Leu Leu Thr Leu His Gly Thr Pro
355 360 365

Phe Leu Tyr Asn Gly Glu Glu Ile Gly Met Thr Asp His Ile Ile Thr
370 375 380

Asp Pro Thr Lys Leu Arg Asp Thr Met Ala Thr Trp Tyr Tyr Asn Ser
385 390 395 400

Leu Val Asn Glu Met Lys Val Glu Pro Ala Glu Ala Ala Leu Arg Ala
405 410 415

Gly Gln Met Thr Arg Asp Lys Asn Arg Thr Pro Met Gln Trp Asp Asn
420 425 430

Lys Pro Asn Ala Gly Phe Cys Pro Asp Lys Ala Glu Pro Trp Leu Pro
435 440 445

Val Asn Pro Asn Tyr Arg Ala Gly Ile Asn Val Arg Glu Gln Thr Ser
450 455 460

Asn Pro Asn Ser Leu Leu Asn Tyr Tyr Lys Arg Leu Ile His Leu Arg
465 470 475 480

```
Arg Glu Thr Pro Ala Leu Ile Ala Gly Asp Tyr Val Pro Leu His Gln
                485                 490                 495

Thr Ser Lys Asp His Leu Ala Phe Leu Arg Lys Thr Asp Ser Gln Thr
            500                 505                 510

Ile Leu Val Val Leu Asn Tyr Ser Pro Asn Lys Leu Glu Leu Asp Phe
        515                 520                 525

Ser Arg Thr Val Glu Met Lys Gly Arg Pro Leu Ile Ala Ile Phe Ser
    530                 535                 540

Ser Ala Asp Asp Arg Pro Gln Ala Ala Gln Ser Pro Lys Lys Val Ser
545                 550                 555                 560

Val Gly Ala Tyr Gly Val Leu Leu Ala Glu Val Lys
                565                 570
```

<210> 73
<211> 1836
<212> DNA
<213> Thermomyces lanuginosus ATCC 200065

<400> 73

ttgtgcgcgg cccttggact cgccgccttg atcgtccaag gcggagaagc cagacctgaa 60

acaaccgtcc cacatgcaac gggctcgctc gacgacttcc tcgccgcaca gagtccgatt 120

gctttccaag gcatcctgaa caatatcggg cctagcggag cgtactcgga aggtgtcaat 180

ccgggtgtgg tcattgcgag tccaagtaaa caagatcccg actactttta cacctgggtg 240

cgcgacgctg ctctcactgt ccaatatctg gtggaggagc tggttgcagg aaatgccagt 300

cttcagttcc tcattcagga ctacatcagc tcccaggcac gactgcagac ggtggaaaat 360

ccatccggct ccctctcgtc gggtggtcta ggagagccca agtttcatgt cgacgagacc 420

gcctttacgg actcctgggg ccgaccacag cgggacggcc cgcctctccg cgccattgcc 480

atgatttcgt ttgccaatta cctgattgac aacggtcatc aatcgactgt ggaggacatc 540

atctggccga ttgttcgcaa tgacttgtcc tatgtctcgc agcattggaa cgaaacaact 600

tttgacatct gggaggaagt ccatagctca tcgtttttca ccacggctgt ccagtaccgt 660

gctctggtcc aaggcagtgc cttggctagc aagctcggcc atacctgcga caactgcggg 720

tcccaagcac cgcagatcct ttgcttcctg cagtcgtatt ggaccgggtc gcacatctta 780

gccaacaccg gtggcggccg ctcgggaaag gacgtcagca cgatcctcgg cgtcattggc 840

tcgtttgatc cgaacgccga ctgtgatgac gttaccttcc agccctgctc ggcccgggct 900

cttgcaaatc acaagcaggt cgttgacagc ttccgcagta tctatgccat caacgctggc 960

atcccgtcag ggtcggctgt tgcggttgga cgttatcccg aggatgtcta tcagggtgga 1020

caccctggt acctaacaac ggctgcggcg gcggagcagc tttacgacgc catttaccag 1080

tggaaccatg tagggcacat cgacatcaat gctgtcaatc tggacttctt caagagcatt 1140

tatccgtcag ccgccgaggg cacatacaca tcagactctt caacatttca agacattata 1200

tctgctgtac ggacctatgc ggacgggttt ctcagcgtaa ttgagaaata cactccgccg 1260

gataacttgc ttgccgagca gttccaccgg gagacgggca ttccactatc ggcagcttct 1320

ctgacatggt cctacgccgc gctcaacacg gccgcgcagc ggcgagcgtc aatcgtgccc 1380

tcaccgtgga actctaacag cacagatctc ccggacaaat gctcggcaac ctcggcaaca 1440

gggccgtatg ccacgcccac aaacacggca tggccaacca ctacgcagcc accggagcgg 1500

ccggcatgca caccgccgtc ggaagtaaca ctcaccttca acgcgctcgt cgacaccgcg 1560

tttggccaga atatttatct cgtgggctcc attccggagc tcggatcgtg ggatccggcc 1620

aacgccctct tgatgagcgc aaagagctgg actagcggaa atccggtctg gacgctatcc 1680

atttcccttc cagcaggaac ctcttttgag tacaagttca ttcgaaagga tgatggttcc 1740

tcggatgttg tctgggaaag tgacccgaat cgttcgtaca acgtgccgaa ggattgcggt 1800

gccaacacgg ccaccgtgaa ttcttggtgg cgatga 1836

<210> 74
<211> 611
<212> PRT
<213> Thermomyces lanuginosus ATCC 200065

<220>
<221> DOMAIN
<222> (39)...(453)
<223> Glycosyl hydrolases family 15

<220>
<221> DOMAIN
<222> (508)...(605)
<223> Starch binding domain

<220>
<221> SITE
<222> (99)...(102)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (200)...(203)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (473)...(476)
<223> N-glycosylation site. Prosite id = PS00001

<220>
<221> SITE
<222> (599)...(602)
<223> N-glycosylation site. Prosite id = PS00001

<400> 74

```
Met Cys Ala Ala Leu Gly Leu Ala Ala Leu Ile Val Gln Gly Gly Glu
1               5                  10                  15

Ala Arg Pro Glu Thr Thr Val Pro His Ala Thr Gly Ser Leu Asp Asp
            20                  25                  30

Phe Leu Ala Ala Gln Ser Pro Ile Ala Phe Gln Gly Ile Leu Asn Asn
        35                  40                  45

Ile Gly Pro Ser Gly Ala Tyr Ser Glu Gly Val Asn Pro Gly Val Val
    50                  55                  60

Ile Ala Ser Pro Ser Lys Gln Asp Pro Asp Tyr Phe Tyr Thr Trp Val
65                  70                  75                  80

Arg Asp Ala Ala Leu Thr Val Gln Tyr Leu Val Glu Glu Leu Val Ala
                85                  90                  95

Gly Asn Ala Ser Leu Gln Phe Leu Ile Gln Asp Tyr Ile Ser Ser Gln
            100                 105                 110

Ala Arg Leu Gln Thr Val Glu Asn Pro Ser Gly Ser Leu Ser Ser Gly
        115                 120                 125

Gly Leu Gly Glu Pro Lys Phe His Val Asp Glu Thr Ala Phe Thr Asp
    130                 135                 140

Ser Trp Gly Arg Pro Gln Arg Asp Gly Pro Pro Leu Arg Ala Ile Ala
145                 150                 155                 160

Met Ile Ser Phe Ala Asn Tyr Leu Ile Asp Asn Gly His Gln Ser Thr
                165                 170                 175

Val Glu Asp Ile Ile Trp Pro Ile Val Arg Asn Asp Leu Ser Tyr Val
            180                 185                 190

Ser Gln His Trp Asn Glu Thr Thr Phe Asp Ile Trp Glu Glu Val His
        195                 200                 205

Ser Ser Ser Phe Phe Thr Thr Ala Val Gln Tyr Arg Ala Leu Val Gln
    210                 215                 220

Gly Ser Ala Leu Ala Ser Lys Leu Gly His Thr Cys Asp Asn Cys Gly
225                 230                 235                 240

Ser Gln Ala Pro Gln Ile Leu Cys Phe Leu Gln Ser Tyr Trp Thr Gly
                245                 250                 255

Ser His Ile Leu Ala Asn Thr Gly Gly Gly Arg Ser Gly Lys Asp Val
```

260 265 270

Ser Thr Ile Leu Gly Val Ile Gly Ser Phe Asp Pro Asn Ala Asp Cys
275 280 285

Asp Asp Val Thr Phe Gln Pro Cys Ser Ala Arg Ala Leu Ala Asn His
290 295 300

Lys Gln Val Val Asp Ser Phe Arg Ser Ile Tyr Ala Ile Asn Ala Gly
305 310 315 320

Ile Pro Ser Gly Ser Ala Val Ala Val Gly Arg Tyr Pro Glu Asp Val
325 330 335

Tyr Gln Gly Gly His Pro Trp Tyr Leu Thr Thr Ala Ala Ala Ala Glu
340 345 350

Gln Leu Tyr Asp Ala Ile Tyr Gln Trp Asn His Val Gly His Ile Asp
355 360 365

Ile Asn Ala Val Asn Leu Asp Phe Phe Lys Ser Ile Tyr Pro Ser Ala
370 375 380

Ala Glu Gly Thr Tyr Thr Ser Asp Ser Ser Thr Phe Gln Asp Ile Ile
385 390 395 400

Ser Ala Val Arg Thr Tyr Ala Asp Gly Phe Leu Ser Val Ile Glu Lys
405 410 415

Tyr Thr Pro Pro Asp Asn Leu Leu Ala Glu Gln Phe His Arg Glu Thr
420 425 430

Gly Ile Pro Leu Ser Ala Ala Ser Leu Thr Trp Ser Tyr Ala Ala Leu
435 440 445

Asn Thr Ala Ala Gln Arg Arg Ala Ser Ile Val Pro Ser Pro Trp Asn
450 455 460

Ser Asn Ser Thr Asp Leu Pro Asp Lys Cys Ser Ala Thr Ser Ala Thr
465 470 475 480

Gly Pro Tyr Ala Thr Pro Thr Asn Thr Ala Trp Pro Thr Thr Thr Gln
485 490 495

Pro Pro Glu Arg Pro Ala Cys Thr Pro Pro Ser Glu Val Thr Leu Thr
500 505 510

Phe Asn Ala Leu Val Asp Thr Ala Phe Gly Gln Asn Ile Tyr Leu Val
515 520 525

Gly Ser Ile Pro Glu Leu Gly Ser Trp Asp Pro Ala Asn Ala Leu Leu
530 535 540

Met Ser Ala Lys Ser Trp Thr Ser Gly Asn Pro Val Trp Thr Leu Ser
545 550 555 560

Ile Ser Leu Pro Ala Gly Thr Ser Phe Glu Tyr Lys Phe Ile Arg Lys
565 570 575

Asp Asp Gly Ser Ser Asp Val Val Trp Glu Ser Asp Pro Asn Arg Ser
580 585 590

Tyr Asn Val Pro Lys Asp Cys Gly Ala Asn Thr Ala Thr Val Asn Ser
595 600 605

Trp Trp Arg
610

<210> 75
<211> 1344
<212> DNA
<213> Unknown

<220>
<223> Obtained from an environmental sample

<400> 75

atgaaccggg ttaagagact ttcgattttg gtcgttcttt tccttaccgc cttcattccg 60

actgtttttg ccggcgagca gcctctcgcc atctttcacg cctttaacga ccccttcact 120

cttgttgaat cctatgtctg cgaactcgcc gggcagggat actcacatgt ccagatatct 180

ccagcgcaga agtcgaaccc tgctcgagcc tggtatgccc ggtatcaacc cgtagatttt 240

actgtcatcg aagggatggg cactgagagc gatctgagga agctcacgga taaggcccac 300

gcgtgtggaa taaaggtgat cgccgatgtg gtcttcaacc acatgtcgag catggacgag 360

tacaaggggc ttgacaagtt tccgggactt gctcctgctg atttccaccg gcagtgcggc 420

atcgattatt caaaacgaga ttcggtgcgg aactgttggc tcggaggcga cttgcccgat 480

ctggaccagt cccggccgag ggtacaggat gttcagagag cccacataag gaagctcctt 540

tccctcggca tagacggctt ccgcttcgat gcggctaaac acatcgaccc cattgttgtg 600

aaagactaca tcgatctcat cgacagggag agcaacggca ggacctggaa ctacctcgag 660

gtcatcgagg atgacggcac tcaggccacg gactacaact ggatagcggc agtgaccgat 720

ttcgtcctct acaaggagtc gttgaggaag gccttcagtc tcggcgggga cctgcgatcg 780

ctcaagatgc ctgtggctgt caatgattcg cggagtatcg tcttcgggag aaatcacgac 840

accgtgccgg agaataacca gaactgcatc gtcggctgct acgacagccg ggaggactcc 900

tatcttgcca cggcatacgt cctggcccgc gaatcgggag tcccgctggt cctcaactgg 960

gacaactacg acgcgcccta catcagcacc ggcgtgaagt tccgccagat catgacgcag 1020

cgaggacgat cggccatgaa cgtgaaggag aatgtgctgg gcgtcatcga cagtcctgtc 1080

gtcatgatga tggagcgcgg gagtgaaggc tttttcgtcc tcaacaagag cgccgaccgg 1140

ttcgatatcc cagttctgga tctgacactg accaatctcg agggatgtta tcgggagctg 1200

agaagaaaat tcaccgtcgc catcgagaga aagtacggta agaaatttgt cacccggtgg 1260

ggacgatggg accgggggg cctcgaaatc tacggccgcg acgctctcta cttcatacgg 1320

gaaccctggg agcagtgcag gtaa 1344

<210> 76
<211> 447
<212> PRT
<213> Unknown

<220>
<223> Obtained from an environmental sample

<220>
<221> SIGNAL
<222> (1)...(24)

<400> 76

```
Met Asn Arg Val Lys Arg Leu Ser Ile Leu Val Val Leu Phe Leu Thr
 1               5                  10                  15

Ala Phe Ile Pro Thr Val Phe Ala Gly Glu Gln Pro Leu Ala Ile Phe
            20                  25                  30

His Ala Phe Asn Asp Pro Phe Thr Leu Val Glu Ser Tyr Val Cys Glu
        35                  40                  45

Leu Ala Gly Gln Gly Tyr Ser His Val Gln Ile Ser Pro Ala Gln Lys
    50                  55                  60

Ser Asn Pro Ala Arg Ala Trp Tyr Ala Arg Tyr Gln Pro Val Asp Phe
65                  70                  75                  80

Thr Val Ile Glu Gly Met Gly Thr Glu Ser Asp Leu Arg Lys Leu Thr
                85                  90                  95

Asp Lys Ala His Ala Cys Gly Ile Lys Val Ile Ala Asp Val Val Phe
            100                 105                 110

Asn His Met Ser Ser Met Asp Glu Tyr Lys Gly Leu Asp Lys Phe Pro
        115                 120                 125

Gly Leu Ala Pro Ala Asp Phe His Arg Gln Cys Gly Ile Asp Tyr Ser
    130                 135                 140

Lys Arg Asp Ser Val Arg Asn Cys Trp Leu Gly Gly Asp Leu Pro Asp
```

145 150 155 160

Leu Asp Gln Ser Arg Pro Arg Val Gln Asp Val Gln Arg Ala His Ile
165 170 175

Arg Lys Leu Leu Ser Leu Gly Ile Asp Gly Phe Arg Phe Asp Ala Ala
180 185 190

Lys His Ile Asp Pro Ile Val Val Lys Asp Tyr Ile Asp Leu Ile Asp
195 200 205

Arg Glu Ser Asn Gly Arg Thr Trp Asn Tyr Leu Glu Val Ile Glu Asp
210 215 220

Asp Gly Thr Gln Ala Thr Asp Tyr Asn Trp Ile Ala Ala Val Thr Asp
225 230 235 240

Phe Val Leu Tyr Lys Glu Ser Leu Arg Lys Ala Phe Ser Leu Gly Gly
245 250 255

Asp Leu Arg Ser Leu Lys Met Pro Val Ala Val Asn Asp Ser Arg Ser
260 265 270

Ile Val Phe Gly Arg Asn His Asp Thr Val Pro Glu Asn Asn Gln Asn
275 280 285

Cys Ile Val Gly Cys Tyr Asp Ser Arg Glu Asp Ser Tyr Leu Ala Thr
290 295 300

Ala Tyr Val Leu Ala Arg Glu Ser Gly Val Pro Leu Val Leu Asn Trp
305 310 315 320

Asp Asn Tyr Asp Ala Pro Tyr Ile Ser Thr Gly Val Lys Phe Arg Gln
325 330 335

Ile Met Thr Gln Arg Gly Arg Ser Ala Met Asn Val Lys Glu Asn Val
340 345 350

Leu Gly Val Ile Asp Ser Pro Val Val Met Met Met Glu Arg Gly Ser
355 360 365

Glu Gly Phe Phe Val Leu Asn Lys Ser Ala Asp Arg Phe Asp Ile Pro
370 375 380

Val Leu Asp Leu Thr Leu Thr Asn Leu Glu Gly Cys Tyr Arg Glu Leu
385 390 395 400

Arg Arg Lys Phe Thr Val Ala Ile Glu Arg Lys Tyr Gly Lys Lys Phe
405 410 415

Val Thr Arg Trp Gly Arg Trp Asp Arg Gly Gly Leu Glu Ile Tyr Gly
420 425 430

Arg Asp Ala Leu Tyr Phe Ile Arg Glu Pro Trp Glu Gln Cys Arg
435 440 445

<210> 77
<211> 1311
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetically generated polynucleotide

<400> 77

atggccaagt acctggagct cgaagagggc ggggtcataa tgcaggcgtt ctactgggac 60
gtgccttcag gaggaatatg gtgggacaca atacggcaga agataccgga gtggtacgat 120
gccggaatct ccgcaatatg gattcctccc gcgagcaagg gtatgagcgg cggctattcg 180
atgggctacg acccctacga ttattttgac ctcggtgagt actaccagaa gggaacggtg 240
gaaacgaggt tcggctcaaa gcaggagctc ataaacatga taaacacggc ccatgcctac 300
ggcataaagg tcatagcgga catcgtcata aaccaccgcg caggcggaga cctcgagtgg 360
aacccgttcg ttggggacta cacctggacg gacttctcaa aggtggcctc gggcaaatat 420
actgccaact acctcgactt ccacccgaac gagctccatg cgggcgattc cggaacattt 480
ggaggctatc ccgacatatg ccacgacaag agctgggacc agtactggct ctgggccagc 540
caggagagct acgcggcata tctcaggagc atcggcatcg atgcctggcg ctttgactac 600
gtgaagggct acggagcgtg ggtcgtcaag gactggctca actggtgggg cggctgggcc 660
gttggcgagt actgggacac caacgttgat gcactcctca actgggccta ctcgagcggc 720
gccaaggtct tcgacttccc gctctactac aagatggacg cggcctttga caacaagaac 780
attcccgcac tcgtcgaggc cctcaagaac gggggcacag tcgtcagccg cgacccgttt 840
aaggccgtaa ccttcgttgc aaaccacgac accgatataa tctggaacaa gtatccagcc 900
tacgcgttca tcctcaccta cgagggccag ccgacaatat tctaccgcga ctacgaggag 960
tggctcaaca aggataagct caagaacctc atctggatac atgacaacct cgccggagga 1020
agcacgagca tagtttacta cgacagcgac gagatgatct tcgtgaggaa cggctatgga 1080
agcaagcctg gccttataac ttacatcaac ctcggctcga gcaaggttgg aaggtgggtt 1140
tatgtgccga agttcgcggg cgcgtgcatc cacgagtata ctggtaacct cggaggctgg 1200
gtagacaagt acgtctactc aagcggctgg gtctatctcg aagctccagc ttacgaccct 1260
gccaacgggc agtatggcta ctccgtgtgg agctactgcg gtgttgggtg a 1311

<210> 78
<211> 436
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetically generated polypeptide

<400> 78

```
Met Ala Lys Tyr Leu Glu Leu Glu Glu Gly Gly Val Ile Met Gln Ala
 1               5                  10                  15
Phe Tyr Trp Asp Val Pro Ser Gly Gly Ile Trp Trp Asp Thr Ile Arg
            20                  25                  30
Gln Lys Ile Pro Glu Trp Tyr Asp Ala Gly Ile Ser Ala Ile Trp Ile
        35                  40                  45
Pro Pro Ala Ser Lys Gly Met Ser Gly Gly Tyr Ser Met Gly Tyr Asp
    50                  55                  60
Pro Tyr Asp Tyr Phe Asp Leu Gly Glu Tyr Tyr Gln Lys Gly Thr Val
65                  70                  75                  80
Glu Thr Arg Phe Gly Ser Lys Gln Glu Leu Ile Asn Met Ile Asn Thr
                85                  90                  95
Ala His Ala Tyr Gly Ile Lys Val Ile Ala Asp Ile Val Ile Asn His
            100                 105                 110

Arg Ala Gly Gly Asp Leu Glu Trp Asn Pro Phe Val Gly Asp Tyr Thr
        115                 120                 125
Trp Thr Asp Phe Ser Lys Val Ala Ser Gly Lys Tyr Thr Ala Asn Tyr
    130                 135                 140
Leu Asp Phe His Pro Asn Glu Leu His Ala Gly Asp Ser Gly Thr Phe
145                 150                 155                 160
Gly Gly Tyr Pro Asp Ile Cys His Asp Lys Ser Trp Asp Gln Tyr Trp
                165                 170                 175
Leu Trp Ala Ser Gln Glu Ser Tyr Ala Ala Tyr Leu Arg Ser Ile Gly
            180                 185                 190
Ile Asp Ala Trp Arg Phe Asp Tyr Val Lys Gly Tyr Gly Ala Trp Val
        195                 200                 205
Val Lys Asp Trp Leu Asn Trp Trp Gly Gly Trp Ala Val Gly Glu Tyr
    210                 215                 220
Trp Asp Thr Asn Val Asp Ala Leu Leu Asn Trp Ala Tyr Ser Ser Gly
225                 230                 235                 240
Ala Lys Val Phe Asp Phe Pro Leu Tyr Tyr Lys Met Asp Ala Ala Phe
                245                 250                 255
Asp Asn Lys Asn Ile Pro Ala Leu Val Glu Ala Leu Lys Asn Gly Gly
            260                 265                 270
Thr Val Val Ser Arg Asp Pro Phe Lys Ala Val Thr Phe Val Ala Asn
        275                 280                 285
His Asp Thr Asp Ile Ile Trp Asn Lys Tyr Pro Ala Tyr Ala Phe Ile
    290                 295                 300
Leu Thr Tyr Glu Gly Gln Pro Thr Ile Phe Tyr Arg Asp Tyr Glu Glu
305                 310                 315                 320
Trp Leu Asn Lys Asp Lys Leu Lys Asn Leu Ile Trp Ile His Asp Asn
                325                 330                 335
Leu Ala Gly Gly Ser Thr Ser Ile Val Tyr Tyr Asp Ser Asp Glu Met
            340                 345                 350
Ile Phe Val Arg Asn Gly Tyr Gly Ser Lys Pro Gly Leu Ile Thr Tyr
        355                 360                 365
Ile Asn Leu Gly Ser Ser Lys Val Gly Arg Trp Val Tyr Val Pro Lys
    370                 375                 380
Phe Ala Gly Ala Cys Ile His Glu Tyr Thr Gly Asn Leu Gly Gly Trp
385                 390                 395                 400
Val Asp Lys Tyr Val Tyr Ser Ser Gly Trp Val Tyr Leu Glu Ala Pro
                405                 410                 415
Ala Tyr Asp Pro Ala Asn Gly Gln Tyr Gly Tyr Ser Val Trp Ser Tyr
            420                 425                 430
Cys Gly Val Gly
        435
```

<210> 79
<211> 1764
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetically generated polynucleotide

<400> 79

cttactccag ctgagtggag gtctcagagc atctacttcc tcctcactga tcgcttcggc 60

aggaccgata actctactac cgctgcttgc gataccactg atagggttta ctgcggcggc 120

tcttggcagg gcatcatcaa ccacctggat tacatccagg gcatgggctt cactgctatc 180

tggatcactc cagtgactgg ccagttctac gagaacactg gcgatggcac ttcataccac 240

ggctactggc agcaggatat ctacgacctg aactacaact acggcactgc tcaggatctt 300

aagaacctgg cttctgctct tcacgagagg ggcatgtacc tgatggtgga tgtggtggct 360

aaccacatgg gctacgatgg cgctggcaac actgtggatt actccgtgtt caacccattc 420

agcagcagct cttacttcca cccgtactgc ctgatcagca actacgacaa ccagaccaac 480

gttgaggatt gctggcttgg cgatacaacc gtgtctctgc cagacctgga taccacttct 540

actgctgtga gggatatctg gtatgattgg gtggccgatc ttgtggctaa ctactctatc 600

gatggcctga gggtggacac tgttaagcac gtggagaagg atttctggcc agactacaac 660

tctgctgctg gcgtttactg tgttggggag gttttctctg gcgatccagc ttacacttgc 720

ccgtaccaga actacatgga tggcgtgctg aactacccaa tctactacca gctcctgtac 780

gctttcgagt caagctctgg ctctatcagc gacctgtaca acatgatcag cagcgtggct 840

tcttcttgca aggacccaac ccttcttggc aactttatcg agaaccacga caacccacgc 900

ttcgctagct acaccagcga ttactctcag gccaagaacg tgatcacctt catcttcctc 960

tcagacggca tcccaatcgt ttacgctggc caggagcagc attactctgg cggctctgat 1020

ccagctaacc gcgaggctac ttggctttct ggctactcta cctctgctac cctgtacact 1080

tggatcgcta gcactaacca gatccgctca ctggctatct ctaaggatgc tggctacgtc 1140

caggctaaga acaacccatt ctactccgac tctaacacca tcgctatgag gaagggcact 1200

actgctggcg ctcaggtgat cactgtgctg tctaacaagg gcgcttctgg ctcttcttac 1260

acccttttctc tgtctggcac tggctactct gctggcgcta ctcttgtgga gacctacacc 1320

tgcaccactg tgaccgttga ttcttctggc aacctgccag tgccaatgac ttctggcctt 1380

ccaagggtgt tcgttccaag ctcttgggtt aacggctctg ctctgtgcaa cactgagtgc 1440

actgctgcta cttctctgcc agtgctgttc gaggagctgg tgactactac ttacggcgag 1500

aacatctacc ttagcggctc tatctctcag cttggctctt ggaacactgc ttctgctgtg 1560

gctctttctg ctagccagta caccagctct aacccaaagt ggtacgtgtc tgtgactctt 1620

ccagtgggca ctagcttcca gtacaagttt atcaagaagg gctctgacgg ctctgttgtt 1680

tgggagagcg acccaaacag gtcttacact gttccagctg gctgcgaggg cgctactgtt 1740

actgttgccg atacctggcg ctga 1764

<210> 80
<211> 1710
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetically generated polynucleotide

<400> 80

gccgatacaa acgcttggaa gagccgctct atctacttcg tgctgaccga taggatcgct 60

aggaactctt ctgatactgg cggctctgct tgctctgatc ttggcaacta ctgcggcggc 120

actttccagg gccttgagag caagctggat tacatcaagg gccttggctt cgatgctatc 180

tggatcactc cagtggtgtc taacaaggct gctggctacc acggctactg ggctgaggat 240

ctgtacgctg tgaactctaa ctacggcact gctgctgatc tgaagtctct ggtggctgct 300

gctcacgcta agggcatcta catgatggtg gacgtggtgg ctaaccacat gggcccaggc 360

gctatcacta acaacaggcc agagccactt aaccaggctt caagctacca tccaccgtgc 420

aacatcgact acaacaacca gacttctgtg gaggtttgcc agatcgctgg ccttccagat 480

atctacacca ccaagagcga gatcaggact ctgctgaaca cttgggtgaa ctggcttgtg 540

aacgagtact ctttcgatgg cgtcaggatc gataccgtta agcacgtgga gaaggatttc 600

tggccaggct tctctgctgc tactggcgtt tacaacatcg gcgaggtgtt cgatggcgat 660

ccagcttacc ttgctccata cgccaagctg atgccaggcc ttcttaacta cgccgtgtac 720

tacccgatga acaacttcta ccagcagact ggctcttctc aggctctggt ggacatgatg 780

aacaccgtgt ccaacacttt cccagatccc tctgctcttg gcaccttcct ggacaaccac 840

gataacaagc ggtggctgaa cgtgaagaac gatcagaccc tgctgaagaa cgctctggct 900

tacgttatcc ttgctagggg catcccaatc ctttactacg gcacagagca gggctacgct 960

ggcggcgatg atccagctaa ccgcgaggat ctttggcgct ctggcttcaa cactaacgcc 1020

aacctgtacc aggctatcaa gaagctgact gctgctaggc aggctgctgg cggccttgct 1080

ggcaacgatc acgtccacct gtacgtggct gatactgctt acgcttggtc tagggctaac 1140

ggcaacctga tcgtgcttac tactaacgct ggcggcaact ctaacactca gcactgcttc 1200

aacacccaga aggctaacgg ccgctggact aacgtttacg gcaacggcgc tactgtttct 1260

gctgatagca acggccaaat ctgcgtgtct gttactaacg gcgagccagt ggttcttctt 1320

gctggctctg ctactccaac tactggcacc accctgtcta ctaggactgc taccgctact 1380

gctacaccaa ctgcttgccc aaccgctgtg tctgtgtctt tcacccacag ggtgacaact 1440

gttccaggcg acacgatcaa gatcactggc aacactgccc agcttggcaa ctggactcca 1500

gctaacggcc ttgctctttc tgctgctagc tacaccagct ctaacccaat ctggaccatc 1560

actgtgccac ttgctgctgg ctctagcatc agctacaagt tcgtgaagat cgattctggc 1620

ggcactgtta cttgggagag cgacccaaac aggtcttaca ctgctccatc ttgccaggct 1680

tcagctagcg ttaacagcag ctggcagtaa 1710

<210> 81
<211> 1851
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetically generated polynucleotide

<400> 81

gaggctgctc cacagcttgc tccaagggct actacctctc tggatgcttg gcttgcttct 60

gagaccactg ttgctctgga tgggattctt gataatgttg gctcttctgg cgcttacgct 120

aagtctgcta agtcaggcat cgtgatcgct tctccatcta ccgacaaccc ggactactac 180

tacacttgga ccagggatgc tgctcttact gtgaaggccc tgatcgatct tttcaggaac 240

ggcgagacct ctcttcagac tgtgatcatg gagtacatca gctctcaggc ttacctccag 300

actgtgtcta acccatctgg ctcactttct actggcggcc ttgccgagcc aaagtactac 360

gtggatgaga ctgcttacac tggctcttgg ggcaggccac agagggatgg cccagctctt 420

agggctactg ccatgatcga tttcggcaac tggcttatcg ataacggcta ctctacctac 480

gctagcaaca ttgtgtggcc aattgtgagg aacgatctgt cttacgtggc tcagtactgg 540

aaccagactg gctacgatct ttgggaggag gttaacggct ctagcttctt cactatcgcc 600

gttcagcaca gggctcttgt tgagggctct accttcgctt ctaaggttgg ggcttcttgc 660

tcttggtgcg attctcaggc tccacaggtt ctgtgcttcc ttcagaggtt ctggactggc 720

tcttacatca tggctaactt cggcggcggc cgctctggca aggacgctaa cactgtgctg 780

ggctctatcc acactttcga tccaaacgct ggctgcgacg atacaacttt ccagccatgc 840

tctccaaggg ctctggctaa ccacaaggtg tacaccgata gcttccgctc tatctactct 900

atcaacagcg gcatctctca gggcaaggct gttgctgttg gccgctaccc agaggattct 960

tactacaacg gcaacccgtg gttccttact actcttgctg ctgccgagca gctttacgat 1020

gctatctacc agtggcagaa gatcggctct atcaccatca ctgacgtgtc tctggccttc 1080

ttcaaggacc tgtactcttc tgctgctgtt ggcacctacg cttctagctc ttctgccttc 1140

acctctatcg tgaacgccgt taagacttac gctgacggct acatgtctat cgttcagacc 1200

cacgctatga ctaacggctc tctgtctgag cagttcggca agtctgatgg cttctctctg 1260

tctgctaggg atctgacttg gtcttacgct gctctgctga ctgctaacct taggcgcaac 1320

tctgttgttc caccatcttg gggcgagact actgctactt ctgtgccatc tgtgtgctct 1380

gctacttcag ctaccggcac ctactctacc gctactaaca ctgcttggcc atctactctg 1440

acatctggca ctggcgctac cactactacc tctaaggcta cctctaccac cactaccagc 1500

tctgcttcta ctactactgc tggctgcgtt gttccaactg ctgtggctgt gaccttcgac 1560

gagatcgcta ctactactta cggcgagaac gtgtacgttg tgggctctat ctctcagctt 1620

ggcagctggg atacttctaa ggctgtggcc ctgtctgctt ctaagtacac cagcagcaac 1680

aacctttggt acgctaccgt tactcttcca gctggcacca ctttccagta caagtttatc 1740

agggtgtcat cttcaggcac tgtgacctgg gagtctgatc caaacaggtc ttacaccgtg 1800

ccatctgctt gcggcacttc tactgctgtg gtgaacacca cttggcgcta a 1851

<210> 82
<211> 1860
<212> DNA
<213> Artificial sequence

<220>

<223> Synthetically generated polynucleotide

<400> 82

gccccacagc tttctccaag ggctaccacc tctcttgatg cttggctggc ttctgagacc 60

actgttagcc tgaacgggat tcttgataat atcggcgctt caggcgctta cgctcagtct 120

gctaaggctg gcgttgttat cgctagccca agcacctcta gcccagacta ctactacacc 180

tggaccaggg attctgctct gacccttaag gtgctgatcg acctttttcag gaacggcaac 240

gttgatcttc agaccgtgat cgaggagtac atcactgctc aggcttacct tcagactgtg 300

agcaacccat ctggcgatct ttcttcaggc gctggccttg ccgagccaaa gttcaacgtg 360

gacatgtctg cttacactgg cgcttggggc aggccacaga gggatggccc agctcttagg 420

gctatcgccc ttatcgattt cggcaactgg cttatcgata acggctactc tagctacgct 480

gtgtctaatg tgtggccaat tgtgaggaac gatctgtctt acgtggctca gtactggtct 540

cagtctggct acgatctttg ggaggaggtg aacagcatgt ctttcttcac catcgctaac 600

cagcacaggg ctcttgttga gggctctact ttcgctggca gagttggcgc ttcttgctct 660

tggtgcgatt ctcaggctcc acagatcctt tgctacatgc agaatttctg gaccggctct 720

tacatcaacg ctaacactgg cggcggccgc tctggcaagg atgctaacac cgtgctggct 780

agcatctcta ctttcgaccc agaggctact tgcgatgatg tgaccttcca gccatgctct 840

tctagggctc tggctaacca caaggtgtac accgattctt tccgctctgt gtactctctt 900

gactctggga tcgctgaggg cgttgctgtt gctgttggcc gctacccaga ggattcttac 960

tacaacggca acccgtggtt ccttactact cttgctgctg ccgagcagct ttacgacgct 1020

atctaccagt ggaacaagat cggctctatc accatcacct ctacctcact ggctttcttc 1080

aacgacgtgt actcttctgc tgctgttggc acttacgctt caggctctac cgcttacact 1140

gctatcgtgt ccgctgttaa gacttacgct gacggctacg tgtctatcgt tcaggctcac 1200

gctatgacta acggctctct gtctgagcag ttcgataagg cttctggcac tcagctttct 1260

gctagggatc tgacctggtc ttacgctgct ctgctgactg ctaacatgag gcgcaacggc 1320

atcgttccac catcttgggg cgctgcttca gctaactcta tcccaagctc ttgctctact 1380

ggctctgcta ctggcactta ctctacccca actggcactt cttggccatc tactctgact 1440

tcaggcactg ctggcaccac tactacctct gctaccacca ccacctctac tagcgtgtct 1500

aagaccacca ccactaccac ttctactacc agctgcacca ctccaacttc agtggctgtg 1560

accttcgatg agatcgctac cacttactac ggcgagaacg tgtacatcag cggctctatc 1620

tctcagcttg gcagctggga tacttcttct gccatcgccc tttctgcttc tcagtacacc 1680

agcagcaaca acctgtggtt cgtgaccatc aaccttccag ctggcactac tttccagtac 1740

aagtatatca ggaaggagtc tgacggctct atcgtttggg agagcgaccc aaacaggtct 1800

tacactgtgc catctggctg cggcgtttct actgctaccg agagcgatac ttggcgctaa 1860

Claims

1. An isolated, synthetic or recombinant nucleic acid consisting of:

   (a) a nucleic acid sequence having at least 98%, 99% or 100% sequence identity to SEQ ID NO:47, wherein the nucleic acid encodes at least one polypeptide having a glucoamylase activity;
   (b) a nucleic acid sequence encoding a polypeptide having the sequence of SEQ ID NO:48;
   (c) a nucleic acid sequence fully complementary to (a), or (b); or,
   (d) a nucleic acid sequence as set forth in SEQ ID NO:47.

2. The isolated, synthetic or recombinant nucleic acid of claim 1,
   wherein glucoamylase activity comprises hydrolyzing glucosidic bonds, and optionally hydrolyzing glucosidic bonds in a polysaccharide, an oligosaccharide, or a starch, or optionally the glucosidic bonds comprise an α-1,4-glucosidic bond and/or an α-1,6-glucosidic bond, or wherein the glucoamylase activity comprises cleaving a maltose or a D-glucose unit from non-reducing end of a polysaccharide, an oligosaccharide, or a starch.

3. A vector, an expression cassette or a cloning vehicle comprising a nucleic acid comprising the nucleic acid sequence of claim 1;
   wherein optionally the nucleic acid is operably linked to a promoter;
   wherein optionally the promoter is a plant promoter or a viral promoter;
   wherein optionally the promoter is an inducible promoter or a tissue-specific promoter; or
   wherein optionally the tissue-specific promoter is optionally seed-specific, an endosperm-specific promoter, a leaf-specific, a root-specific, a stem-specific or an abscission-induced promoter.

4. A transformed cell comprising a nucleic acid comprising the nucleic acid sequence of claim 1, or a vector, expression cassette, or cloning vehicle as set forth in claim 3 wherein the cell is optionally a bacterial cell, a mammalian cell, a fungal cell, a yeast cell, an insect cell or a plant cell, wherein the plant cell is optionally a potato, rice, corn, wheat, tobacco or barley cell.

5. An isolated, synthetic or recombinant polypeptide having glucoamylase activity comprising:

   (i) an amino acid sequence having at least 99% or complete sequence identity to the sequence of SEQ ID NO:48 over the full length of the polypeptide;
   (ii) an amino acid sequence encoded by the nucleic acid of claim 1;
   (iii) the polypeptide of any of (i) to (ii) wherein the polypeptide has a sequence as set forth in SEQ ID NO:48 or enzymatically active fragments thereof having glucoamylase activity.

6. The isolated, synthetic or recombinant polypeptide of claim 5 wherein the glucoamylase activity comprises hydrolyzing glucosidic bonds, and optionally the glucosidic bonds comprise an α-1,4-glucosidic bond or an α-1,6-glucosidic bond, and/or wherein optionally the glucoamylase activity comprises hydrolyzing glucosidic bonds in a polysaccharide, oligosaccharide or starch, or and/or the glucoamylase activity comprises cleaving a maltose or a D-glucose unit from non-reducing end of a polysaccharide, oligosaccharide or starch.

7. A method of producing a recombinant polypeptide comprising the steps of: (a) providing a nucleic acid operably linked to a promoter, wherein the nucleic acid comprises the nucleic acid sequence of claim 1; and (b) expressing the nucleic acid of step (a) under conditions that allow expression of the polypeptide, thereby producing a recombinant polypeptide, further comprising transforming a host cell with the nucleic acid of step (a) followed by expressing the nucleic acid of step (a), thereby producing a recombinant polypeptide in a transformed cell.

8. A method for hydrolyzing a polysaccharide, oligosaccharide or starch comprising the following steps:

   (a) providing the polypeptide of claim 5;
   (b) providing a composition comprising a polysaccharide, oligosaccharide or starch; wherein the composition optionally comprises an α-1,4-glucosidic bond or an α-1,6-glucosidic bond and
   (c) contacting the polypeptide of step (a) with the composition of step (b) under conditions wherein the polypeptide hydrolyzes the polysaccharide, oligosaccharide or starch.

9. A method for liquefying or removing a polysaccharide, oligosaccharide or starch comprising the following steps:

(a) providing the polypeptide of claim 5;
(b) providing a composition comprising a polysaccharide, oligosaccharide or starch; and
(c) contacting the polypeptide of step (a) with the composition of step (b) under conditions wherein the polypeptide liquefies or removes the polysaccharide, oligosaccharide or starch.

10. A method for washing an object comprising the following steps:

(a) providing a composition comprising the polypeptide of claim 5;
(b) providing an object; and
(c) contacting the polypeptide of step (a) and the object of step (b) under conditions wherein the composition can wash the object.

11. A method for hydrolyzing a polysaccharide, oligosaccharide or starch in a feed or a food prior to consumption by an animal comprising the following steps:

(a) obtaining a feed material comprising a starch, where polysaccharide, oligosaccharide or starch in the polysaccharide, oligosaccharide or starch can be hydrolyzed by the polypeptide of claim 5; and
(b) adding the polypeptide of step (a) to the feed or food material in an amount sufficient for a sufficient time period to cause hydrolysis of the polysaccharide, oligosaccharide or starch and formation of a treated food or feed, thereby hydrolyzing the polysaccharide, oligosaccharide or starch in the food or the feed prior to consumption by the animal, wherein the food or feed optionally comprises rice, corn, barley, wheat, legumes, or potato.

12. A method for textile desizing comprising the following steps:

(a) providing the polypeptide of claim 5;
(b) providing a fabric; and
(c) contacting the polypeptide of step (a) and the fabric of step (b) under conditions wherein the glucoamylase can desize the fabric.

13. A method for treatment of lignocellulosic fibers comprising the following steps:

(a) providing the polypeptide of claim 5;
(b) providing a lignocellulosic fiber; and
(c) contacting the polypeptide of step (a) and the fiber of step (b) under conditions wherein the polypeptide can treat the fiber thereby improving the fiber properties.

14. A method for producing a high-maltose or a high-glucose syrup comprising the following steps:

(a) providing the polypeptide of claim 5;
(b) providing a composition comprising a polysaccharide, oligosaccharide or starch; optionally wherein the polysaccharide, oligosaccharide or starch is from rice, corn, barley, wheat, legumes, potato, or sweet potato and
(c) contacting the polypeptide of step (a) and the composition of step (b) under conditions wherein the polypeptide of step (a) can hydrolyze the composition of step (b), thereby producing a high-maltose or a high-glucose syrup.

15. A method for improving the flow of the polysaccharide, oligosaccharide or starch-containing production fluids, comprising the following steps:

(a) providing the polypeptide of claim 5;
(b) providing production fluid comprising a polysaccharide, oligosaccharide or starch; optionally wherein the production fluid is from a subterranean formation; and
(c) contacting the polypeptide of step (a) and the production fluid of step (b) under conditions wherein the polypeptide can hydrolyze the polysaccharide, oligosaccharide or starch in the production fluid, thereby improving its flow by decreasing its density.

16. A method for changing the viscosity of a composition, comprising the following steps:

(i) (a) providing a composition and the polypeptide of claim 5; and (b) treating the composition with the polypeptide

of claim 5; or
(ii) the method of (i), wherein the composition comprises a soil or a drilling mud.

17. A method for using a glucoamylase in brewing or alcohol production comprising the following steps:

(a) providing the polypeptide of claim 5;
(b) providing a composition used for brewing or in alcohol production comprising a polysaccharide, oligosaccharide or starch; wherein the composition comprising a polysaccharide, oligosaccharide or starch is optionally a beer;
(c) combining the polypeptide of step (a) with the composition of the step (b) under conditions wherein the polypeptide can hydrolyze the polysaccharide, oligosaccharide or starch in the composition used for brewing or alcohol production.

18. A method for bio-bleaching a composition comprising:

(i) providing a composition wherein the composition is a paper or a pulp product, and the polypeptide of claim 5;
(ii) treating the composition with the polypeptide of claim 5.

19. A method for deinking of paper and fibers comprising the following steps:

(a) providing the polypeptide as set forth in claim 5;
(b) providing a composition comprising paper or fiber;
(c) contacting the polypeptide of step (a) and the composition of step (b) under

conditions wherein the polypeptide can deink the paper or fiber.

20. A method for making a fuel comprising:

(i) (a) providing the polypeptide of claim 5; (b) providing a composition comprising a polysaccharide, oligosaccharide or starch; and (c) contacting the polypeptide of (a) with the composition of (b) under conditions wherein the polypeptide hydrolyzes the polysaccharide, oligosaccharide or starch; or
(ii) the method of (i) wherein the polypeptide is a thermostable enzyme;
(iii) the method of (i) wherein the fuel is ethanol-based.

21. A method for processing a biomass material comprising lignocellulose comprising

(i) (a) providing a composition comprising the polypeptide of claim 5, and, providing a biomass material; and (b) contacting the composition with the biomass material;
(ii) the method of (i), wherein the biomass material comprises or is derived from an agricultural crop, or is a byproduct of a food or a feed production, or is a lignocellulosic waste product, or is a plant residue or a waste paper or waste paper product,
and optionally the biomass material comprises potatoes, soybean, barley, rye, corn, oats, wheat, beets or sugar cane;
(iii) the method of (i) or (ii), wherein the polypeptide has activity comprising a glucoamylase activity;
(iv) the method of any of (ii), wherein the plant residue comprise stems, leaves, hulls, husks, cobs, wood, wood chips, wood pulp and sawdust, or, the paper waste comprises discarded or used photocopy paper, computer printer paper, notebook paper, notepad paper, typewriter paper, newspapers, magazines, cardboard and paper-based packaging materials; or
(v) the method of (i) to (iv), wherein the processing of the biomass material generates a bioethanol, biopropanol, biobutanol, or a biodiesel;
(vi) the method of (i) to (v), wherein the biomass material comprises a lignocellulose.

22. A method for making bioethanol, biopropanol, biobutanol, or a biodiesel, comprising

(i)

(a) providing the polypeptide of claim 5, and, providing a composition comprising a polysaccharide or oligosaccharide; and

(b) contacting the composition comprising a polysaccharide or oligosaccharide with the polypeptide of claim 5;

(ii) the method of (i), wherein the composition comprising a polysaccharide or oligosaccharide comprises a plant, plant product or plant derivative;
(iii) the method of (ii), wherein the plant or plant product comprises cane sugar plants or plant products, beets or sugarbeets, wheat, corn, soybeans, potato, rice or barley;
(iv) the method of any of (i) to (iii), wherein the polypeptide has activity comprising a glucoamylase activity; or
(v) the method of any of (i) to (iv), wherein the polysaccharide or oligosaccharide comprises a fermentable sugar.

23. A method for making a fuel comprising

(i)

(a) providing the polypeptide of claim 5, and, providing a composition comprising a fermentable sugar; and
(b) contacting the composition comprising a fermentable sugar with the polypeptide of claim 5;

(ii) the method of (i), wherein the composition comprising a fermentable sugar comprises a plant, plant product or plant derivative;
(iii) the method of (ii), wherein the plant or plant product comprises cane sugar plants or plant products, beets or sugarbeets, wheat, corn, soybeans, potato, rice or barley;
(iv) the method of any of (i) to (iii), wherein the polypeptide has activity comprising a glucoamylase activity; or
(v) the method of any of (i) to (iv), wherein the fuel comprises a bioethanol or a gasoline-ethanol mix, or comprises a bioethanol, biopropanol, biobutanol, or a biodiesel.

24. A method for producing a sugar, the method comprising:

(i)

(a) providing the polypeptide of claim 5;
(b) providing a composition comprising a polysaccharide or an oligosaccharide; and
(c) contacting the composition of step (b) with the polypeptide of step (a), thereby generating sugars;

(ii) the method of (i), wherein the composition comprising a polysaccharide comprises a starch;
(iii) the method of any of (i) or (ii), wherein the sugar is a fermentable sugar.

25. A composition comprising the polypeptide of claim 5,
wherein the composition is selected from: a detergent, a feed or a food, a textile or fabric, a paper or paper product or paper pulp, a high-maltose or a high-glucose liquid or syrup, an anti-staling composition, an alcoholic beverage, a beer, a pharmaceutical composition, an oral care product, a toothpaste, a dental cream, a gel or a tooth powder, an odontic, a mouth wash, a pre- or post brushing rinse formulation, a chewing gum, a lozenge or a candy, an oil well drilling fluid, a bio-bleaching solution, a disinfectant, a biodefense or bio-detoxifying agent, a dairy product, or a biomass material.

26. A delayed release or controlled release composition comprising the polypeptide of claim 5, comprising

(a) a desired ingredient coated by a latex polymer coating;
(b) the delayed release or controlled release composition of (a), wherein the desired ingredient comprises an enzyme;
(c) the delayed release or controlled release composition of (a) or (b), wherein the desired ingredient comprises a small molecule, a drug, a polysaccharide, a lipid, a nucleic acid, a vitamin, an antibiotics or an insecticide;
(d) the delayed release or controlled release composition of (a), (b) or (c),
wherein the desired ingredient comprises a pellet or a matrix;
(e) the delayed release or controlled release composition of any of (a) to (d), wherein the pellet or matrix comprises edible material;
(f) the delayed release composition or controlled release of any of (a) to (e), wherein the latex polymer coating comprises a latex paint; or
(g) the delayed release or controlled release composition of any of (a) to (f), wherein the latex polymer coating

comprises a (meth)acrylate, a vinyl acetate, a styrene, an ethylene, a vinyl chloride, a butadiene, a vinylidene chloride, a vinyl versatate, a vinyl propionate, a t-butyl acrylate, an acrylonitrile, a neoprene, a maleate, a fumarate or a combination thereof or a derivative thereof.

Patentansprüche

1. Isolierte, synthetische oder rekombinante Nukleinsäure, bestehend aus:

   (a) einer Nukleinsäure-Sequenz mit mindestens 98 %, 99 % oder 100 % Sequenz-Identität zu SEQ ID NR.: 47, wobei die Nukleinsäure für mindestens ein Polypeptid mit einer Glucoamylase-Aktivität kodiert;
   (b) einer Nukleinsäure-Sequenz, die für ein Polypeptid mit der Sequenz von SEQ ID NR.: 48 kodiert;
   (c) einer Nukleinsäure-Sequenz vollständig komplementär zu (a) oder (b); oder
   (d) einer Nukleinsäure-Sequenz wie in SEQ ID NR.: 47 angeführt.

2. Isolierte, synthetische oder rekombinante Nukleinsäure nach Anspruch 1, wobei die Glucoamylase-Aktivität Hydrolysieren glucosidischer Bindungen, und gegebenenfalls Hydrolysieren glucosidischer Bindungen in einem Polysaccharid, einem Oligosaccharid oder einer Stärke umfasst, oder gegebenenfalls die glucosidischen Bindungen eine α-1,4-glucosidische Bindung und/oder eine α-1,6-glucosidische Bindung umfassen, oder wobei die Glucoamylase-Aktivität Spalten einer Maltose- oder einer D-Glucose-Einheit von dem nicht-reduzierenden Ende von einem Polysaccharid, einem Oligosaccharid oder einer Stärke umfasst.

3. Vektor, eine Expressions-Kassette oder ein Klonierungs-Vehikulum, umfassend eine Nukleinsäure, die die Nukleinsäure-Sequenz nach Anspruch 1 umfasst;
   wobei die Nukleinsäure gegebenenfalls mit einem Promotor funktionsfähig verbunden ist;
   wobei der Promotor gegebenenfalls ein Pflanzen-Promotor oder ein viraler Promotor ist;
   wobei der Promotor gegebenenfalls ein induzierbarer Promotor oder ein Gewebe-spezifischer Promotor ist; oder
   wobei gegebenenfalls der Gewebe-spezifische Promotor gegebenenfalls ein Samen-spezifischer, ein Endosperm-spezifischer Promotor, ein Blatt-spezifischer, ein Wurzelspezifischer, ein Stamm-spezifischer oder ein Abzissions-induzierter Promotor ist.

4. Transformierte Zelle, umfassend eine Nukleinsäure, die die Nukleinsäure-Sequenz nach Anspruch 1 umfasst, oder einen Vektor, eine Expressions-Kassette oder ein Klonierungs-Vehikulum, wie in Anspruch 3 angeführt, wobei die Zelle gegebenenfalls eine bakterielle Zelle, eine Säuger-Zelle, eine Pilz-Zelle, eine Hefe-Zelle, eine Insekten-Zelle oder eine Pflanzen-Zelle ist, wobei die Pflanzen-Zelle gegebenenfalls eine Kartoffel-, Reis-, Mais-, Weizen-, Tabak- oder Gersten-Zelle ist.

5. Isoliertes, synthetisches oder rekombinantes Polypeptid mit Glucoamylase-Aktivität, umfassend:

   (i) eine Aminosäure-Sequenz mit mindestens 99 % oder kompletter Sequenz-Identität zu der Sequenz von SEQ ID NR.: 48 über die volle Länge von dem Polypeptid;
   (ii) eine Aminosäure-Sequenz, kodiert durch die Nukleinsäure nach Anspruch 1;
   (iii) das Polypeptid nach einem beliebigen von (i) bis (ii), wobei das Polypeptid eine Sequenz, wie in SEQ ID NR.: 48 angeführt oder enzymatisch aktive Fragmente davon mit Glucoamylase-Aktivität aufweist.

6. Isoliertes, synthetisches oder rekombinantes Polypeptid nach Anspruch 5, wobei die Glucoamylase-Aktivität Hydrolysieren glucosidischer Bindungen umfasst, und die glucosidischen Bindungen gegebenenfalls eine α-1,4-glucosidische Bindung oder eine α-1,6-glucosidische Bindung umfassen, und/oder wobei gegebenenfalls die Glucoamylase-Aktivität Hydrolysieren glucosidischer Bindungen in einem Polysaccharid, einem Oligosaccharid oder einer Stärke umfasst, oder und/oder die Glucoamylase-Aktivität Spalten einer Maltose- oder einer D-Glucose-Einheit von dem nicht-reduzierenden Ende von einem Polysaccharid, einem Oligosaccharid oder einer Stärke umfasst.

7. Verfahren zum Erzeugen eines rekombinanten Polypeptids, umfassend die Schritte von: (a) Bereitstellen einer Nukleinsäure, die mit einem Promotor funktionsfähig verbunden ist, wobei die Nukleinsäure die Nukleinsäure-Sequenz nach Anspruch 1 umfasst; und (b) Exprimieren der Nukleinsäure von Schritt (a) unter Bedingungen, die Expression von dem Polypeptid erlauben, wodurch ein rekombinantes Polypeptid erzeugt wird, weiterhin umfassend Transformieren einer Wirts-Zelle mit der Nukleinsäure von Schritt (a), gefolgt von Exprimieren der Nukleinsäure von Schritt (a), wodurch ein rekombinantes Polypeptid in einer transformierten Zelle erzeugt wird.

8. Verfahren zum Hydrolysieren eines Polysaccharids, eines Oligosaccharids oder einer Stärke, umfassend die nachstehenden Schritte:

(a) Bereitstellen des Polypeptids nach Anspruch 5;
(b) Bereitstellen einer Zusammensetzung, umfassend ein Polysaccharid, ein Oligosaccharid oder eine Stärke; wobei die Zusammensetzung gegebenenfalls eine α-1,4-glucosidische Bindung oder eine α-1,6-glucosidische Bindung umfasst und
(c) In-Kontakt-Bringen des Polypeptids von Schritt (a) mit der Zusammensetzung von Schritt (b) unter Bedingungen, unter denen das Polypeptid das Polysaccharid, das Oligosaccharid oder die Stärke hydrolysiert.

9. Verfahren zum Verflüssigen oder Entfernen eines Polysaccharids, eines Oligosaccharids oder einer Stärke, umfassend die nachstehenden Schritte:

(a) Bereitstellen des Polypeptids nach Anspruch 5;
(b) Bereitstellen einer Zusammensetzung, umfassend ein Polysaccharid, ein Oligosaccharid oder eine Stärke; und
(c) In-Kontakt-Bringen des Polypeptids von Schritt (a) mit der Zusammensetzung von Schritt (b) unter Bedingungen, unter denen das Polypeptid das Polysaccharid, das Oligosaccharid oder die Stärke verflüssigt oder entfernt wird.

10. Verfahren zum Waschen eines Gegenstands, umfassend die nachstehenden Schritte:

(a) Bereitstellen einer Zusammensetzung, umfassend das Polypeptid nach Anspruch 5;
(b) Bereitstellen eines Gegenstands; und
(c) In-Kontakt-Bringen des Polypeptids von Schritt (a) und des Gegenstands von Schritt (b) unter Bedingungen, unter denen die Zusammensetzung den Gegenstand waschen kann.

11. Verfahren zum Hydrolysieren eines Polysaccharids, eines Oligosaccharids oder einer Stärke in einem Futter oder einem Lebensmittel vor dem Verbrauch durch ein Lebewesen, umfassend die nachstehenden Schritte:

(a) Gewinnen eines Futter-Materials, umfassend eine Stärke, wobei Polysaccharid, Oligosaccharid oder Stärke in dem Polysaccharid, dem Oligosaccharid oder der Stärke durch das Polypeptid nach Anspruch 5 hydrolysiert werden können; und
(b) Zusetzen des Polypeptids von Schritt (a) zu dem Futter- oder Lebensmittel-Material in einer ausreichenden Menge für einen ausreichenden Zeitraum, um Hydrolyse von dem Polysaccharid, dem Oligosaccharid oder der Stärke zu veranlassen und Bildung von einem behandelten Lebensmittel oder Futter, wodurch das Polysaccharid, das Oligosaccharid oder die Stärke in dem Lebensmittel oder dem Futter vor dem Verbrauch durch das Lebewesen hydrolysiert werden, wobei das Lebensmittel oder Futter gegebenenfalls Reis, Mais, Gerste, Weizen, Leguminosen oder Kartoffel umfasst.

12. Verfahren zum Textil-Entschlichten, umfassend die nachstehenden Schritte:

(a) Bereitstellen des Polypeptids nach Anspruch 5;
(b) Bereitstellen eines Gewebes bzw. Stoffs; und
(c) In-Kontakt-Bringen des Polypeptids von Schritt (a) und des Gewebes bzw. Stoffs von Schritt (b) unter Bedingungen, unter denen die Glucoamylase das Gewebe bzw. den Stoff entschlichten kann.

13. Verfahren zur Behandlung von Lignozellulose-Fasern, umfassend die nachstehenden Schritte:

(a) Bereitstellen des Polypeptids nach Anspruch 5;
(b) Bereitstellen einer Lignozellulose-Faser; und
(c) In-Kontakt-Bringen des Polypeptids von Schritt (a) und der Faser von Schritt (b) unter Bedingungen, unter denen das Polypeptid die Faser behandeln kann, wodurch die Faser-Eigenschaften verbessert werden.

14. Verfahren zur Herstellung eines Sirups mit hohem Maltose-Gehalt oder mit hohem Glucose-Gehalt, umfassend die nachstehenden Schritte:

(a) Bereitstellen des Polypeptids nach Anspruch 5;

(b) Bereitstellen einer Zusammensetzung, umfassend ein Polysaccharid, ein Oligosaccharid oder eine Stärke; wobei das Polysaccharid, das Oligosaccharid oder die Stärke gegebenenfalls von Reis, Mais, Gerste, Weizen, Leguminosen, Kartoffel oder Süßkartoffel ist und
(c) In-Kontakt-Bringen des Polypeptids von Schritt (a) und der Zusammensetzung von Schritt (b) unter Bedingungen, unter denen das Polypeptid von Schritt (a) die Zusammensetzung von Schritt (b) hydrolysieren kann, wodurch ein Sirup mit hohem Maltose-Gehalt oder mit hohem Glucose-Gehalt erzeugt wird.

15. Verfahren zum Verbessern des Flusses der Polysaccharid-, Oligosaccharid- oder Stärke-enthaltenden Förderungs-Fluide, umfassend die nachstehenden Schritte:

(a) Bereitstellen des Polypeptids nach Anspruch 5;
(b) Bereitstellen des Förderungs-Fluids, umfassend ein Polysaccharid, ein Oligosaccharid oder eine Stärke; wobei das Förderungs-Fluid gegebenenfalls von einer unterirdischen Formation ist; und
(c) In-Kontakt-Bringen des Polypeptids von Schritt (a) und des Förderungs-Fluids von Schritt (b) unter Bedingungen, unter denen das Polypeptid das Polysaccharid, das Oligosaccharid oder die Stärke in dem Förderungs-Fluid hydrolysieren kann, wodurch sein Fluss durch Senken seiner Dichte verbessert wird.

16. Verfahren zum Ändern der Viskosität einer Zusammensetzung, umfassend die nachstehenden Schritte:

(i)

(a) Bereitstellen einer Zusammensetzung und des Polypeptids nach Anspruch 5; und
(b) Behandeln der Zusammensetzung mit dem Polypeptid nach Anspruch 5; oder

(ii) das Verfahren von (i), wobei die Zusammensetzung einen Boden oder einen Bohr-Schlamm umfasst.

17. Verfahren zur Verwendung einer Glucoamylase beim Brauen oder der Alkohol-Produktion, umfassend die nachstehenden Schritte:

(a) Bereitstellen des Polypeptids nach Anspruch 5;
(b) Bereitstellen einer Zusammensetzung, verwendet zum Brauen oder in der Alkohol-Produktion, umfassend ein Polysaccharid, ein Oligosaccharid oder eine Stärke; wobei die Zusammensetzung, die ein Polysaccharid, ein Oligosaccharid oder eine Stärke umfasst, gegebenenfalls ein Bier ist;
(c) Kombinieren des Polypeptids von Schritt (a) mit der Zusammensetzung von dem Schritt (b) unter Bedingungen, unter denen das Polypeptid das Polysaccharid, das Oligosaccharid oder die Stärke in der zum Brauen oder Alkohol-Produktion verwendeten Zusammensetzung hydrolysieren kann.

18. Verfahren zum Bio-Bleichen einer Zusammensetzung, umfassend:

(i) Bereitstellen einer Zusammensetzung, wobei die Zusammensetzung ein Papier oder ein Zellstoff-Produkt ist und des Polypeptids nach Anspruch 5;
(ii) Behandeln der Zusammensetzung mit dem Polypeptid nach Anspruch 5.

19. Verfahren zum Deinken von Papier und Fasern, umfassend die nachstehenden Schritte:

(a) Bereitstellen des Polypeptids wie in Anspruch 5 angeführt;
(b) Bereitstellen einer Zusammensetzung, umfassend Papier oder Faser;
(c) In-Kontakt-Bringen des Polypeptids von Schritt (a) und der Zusammensetzung von Schritt (b) unter Bedingungen, unter denen das Polypeptid das Papier oder die Faser deinken kann.

20. Verfahren zur Erzeugung eines Kraftstoffs, umfassend:

(i) (a) Bereitstellen des Polypeptids nach Anspruch 5; (b) Bereitstellen einer Zusammensetzung, umfassend ein Polysaccharid, ein Oligosaccharid oder eine Stärke; und (c) In-Kontakt-Bringen des Polypeptids von (a) mit der Zusammensetzung von (b) unter Bedingungen, unter denen das Polypeptid das Polysaccharid, das Oligosaccharid oder die Stärke hydrolysiert; oder
(ii) das Verfahren von (i), wobei das Polypeptid ein thermostabiles Enzym ist;
(iii) das Verfahren von (i), wobei der Kraftstoff Ethanol-basiert ist.

21. Verfahren zum Verarbeiten eines Biomasse-Materials, das Lignozellulose umfasst, umfassend

(i) (a) Bereitstellen einer Zusammensetzung, umfassend das Polypeptid nach Anspruch 5, und Bereitstellen eines Biomasse-Materials; und (b) In-Kontakt-Bringen der Zusammensetzung mit dem Biomasse-Material;
(ii) das Verfahren von (i), wobei das Biomasse-Material eine landwirtschaftliche Kultur umfasst oder davon abgeleitet ist, oder ein NebenProdukt von einer Lebensmittel- oder einer Futter-Produktion ist oder ein Lignozellulose-Abfall-Produkt ist oder ein Pflanzen-Rückstand oder ein Altpapier oder Altpapier-Produkt ist, und das Biomasse-Material gegebenenfalls Kartoffeln, Sojabohne, Gerste, Roggen, Mais, Hafer, Weizen, Rüben oder Zucker-Rohr umfasst;
(iii) das Verfahren von (i) oder (ii), wobei das Polypeptid Aktivität aufweist, umfassend eine Glucoamylase-Aktivität;
(iv) das Verfahren nach einem beliebigen von (ii), wobei der Pflanzen-Rückstand Stämme, Blätter, Hülsen, Spreu, Kolben, Holz, Sägespäne, Holzzellstoff und Sägemehl umfasst oder das Altpapier weggeworfenes oder verbrauchtes Photokopier-Papier, Computer-Drucker-Papier, Notebook-Papier, Notizblock-Papier, Schreibmaschinen-Papier, Zeitungen, Magazine, Karton und Papierbasierte Verpackungs-Materialien umfasst; oder
(v) das Verfahren von (i) bis (iv), wobei das Verarbeiten des Biomasse-Materials ein Bioethanol, Biopropanol, Biobutanol oder einen Biodiesel erzeugt;
(vi) das Verfahren von (i) bis (v), wobei das Biomasse-Material eine Lignozellulose umfasst.

22. Verfahren zur Herstellung von Bioethanol, Biopropanol, Biobutanol oder einem Biodiesel, umfassend

(i)

(a) Bereitstellen des Polypeptids nach Anspruch 5 und Bereitstellen einer Zusammensetzung, umfassend ein Polysaccharid oder Oligosaccharid; und
(b) In-Kontakt-Bringen der Zusammensetzung, umfassend ein Polysaccharid oder Oligosaccharid mit dem Polypeptid nach Anspruch 5;

(ii) das Verfahren von (i), wobei die Zusammensetzung, umfassend ein Polysaccharid oder Oligosaccharid, eine Pflanze, ein Pflanzen-Produkt oder Pflanzen-Derivat umfasst;
(iii) das Verfahren von (ii), wobei die Pflanze oder das Pflanzen-Produkt Rohr-Zucker-Pflanzen oder Pflanzen-Produkte, Rüben oder Zucker-Rüben, Weizen, Mais, Sojabohnen, Kartoffel, Reis oder Gerste umfasst;
(iv) das Verfahren nach einem beliebigen von (i) bis (iii), wobei das Polypeptid Aktivität aufweist, umfassend eine Glucoamylase-Aktivität; oder
(v) das Verfahren nach einem beliebigen von (i) bis (iv), wobei das Polysaccharid oder Oligosaccharid einen fermentierbaren Zucker umfasst.

23. Verfahren zur Herstellung eines Kraftstoffs, umfassend

(i)

(a) Bereitstellen des Polypeptids nach Anspruch 5 und Bereitstellen einer Zusammensetzung, umfassend einen fermentierbaren Zucker; und
(b) In-Kontakt-Bringen der Zusammensetzung, umfassend einen fermentierbaren Zucker, mit dem Polypeptid nach Anspruch 5;

(ii) das Verfahren von (i), wobei die Zusammensetzung, umfassend einen fermentierbaren Zucker, eine Pflanze, ein Pflanzen-Produkt oder Pflanzen-Derivat umfasst;
(iii) das Verfahren von (ii), wobei die Pflanze oder das Pflanzen-Produkt Rohr-Zucker-Pflanzen oder Pflanzen-Produkte, Rüben oder Zucker-Rüben, Weizen, Mais, Sojabohnen, Kartoffel, Reis oder Gerste umfasst;
(iv) das Verfahren nach einem beliebigen von (i) bis (iii), wobei das Polypeptid Aktivität aufweist, umfassend eine Glucoamylase-Aktivität; oder
(v) das Verfahren nach einem beliebigen von (i) bis (iv), wobei der Kraftstoff ein Bioethanol oder ein Treibstoff-Ethanol-Gemisch umfasst, oder ein Bioethanol, Biopropanol, Biobutanol oder einen Biodiesel umfasst.

24. Verfahren zur Herstellung eines Zuckers, wobei das Verfahren umfasst:

(i)

(a) Bereitstellen des Polypeptids nach Anspruch 5;
(b) Bereitstellen einer Zusammensetzung, umfassend ein Polysaccharid oder ein Oligosaccharid; und
(c) In-Kontakt-Bringen der Zusammensetzung von Schritt (b) mit dem Polypeptid von Schritt (a), wodurch Zucker erzeugt werden;

(ii) das Verfahren von (i), wobei die Zusammensetzung, umfassend ein Polysaccharid, eine Stärke umfasst;
(iii) das Verfahren nach einem beliebigen von (i) oder (ii), wobei der Zucker ein fermentierbarer Zucker ist.

25. Zusammensetzung, umfassend das Polypeptid nach Anspruch 5, wobei die Zusammensetzung ausgewählt ist aus: einem Waschmittel, einem Futter oder einem Lebensmittel, einem Textil oder Gewebe bzw. Stoff, einem Papier- oder Papier-Produkt oder Papier-Zellstoff, einer Flüssigkeit oder einem Sirup mit hohem Maltose-Gehalt oder hohem Glucose-Gehalt, einer Zusammensetzung zum Verhindern von Altbackenwerden bzw. Schalwerden, einem alkoholischen Getränk, einem Bier, einer pharmazeutischen Zusammensetzung, einem Mund-Pflege-Produkt, einer Zahnpaste, einer Zahncreme, einem Gel oder einem Zahn-Pulver, einem odontischen Mittel, einer Mund-Wäsche, einer Vor- oder Nach-Bürsten-Spülungs-Formulierung, einem Kaugummi, einer Pastille oder einem Bonbon, einer Öl-Sonden-Bohr-Spülung, einer Bio-Bleich-Lösung, einem Desinfektionsmittel, einem Bio-Schutz- oder Bio-Entgiftungs-Mittel, einem Milch-Produkt oder einem Biomasse-Material.

26. Zusammensetzung zur verzögerten Freisetzung oder gesteuerten Freisetzung, umfassend das Polypeptid nach Anspruch 5, umfassend

(a) einen gewünschten Bestandteil, beschichtet mit einer Latex-Polymer-Beschichtung;
(b) die Zusammensetzung zur verzögerten Freisetzung oder gesteuerten Freisetzung von (a), wobei der gewünschte Bestandteil ein Enzym umfasst;
(c) die Zusammensetzung zur verzögerten Freisetzung oder gesteuerten Freisetzung von (a) oder (b), wobei der gewünschte Bestandteil ein kleines Molekül, einen Arzneistoff, ein Polysaccharid, ein Lipid, eine Nukleinsäure, ein Vitamin, ein Antibiotikum oder ein Insektizid umfasst;
(d) die Zusammensetzung zur verzögerten Freisetzung oder gesteuerten Freisetzung von (a), (b) oder (c), wobei der gewünschte Bestandteil ein Pellet oder eine Mastrix umfasst;
(e) die Zusammensetzung zur verzögerten Freisetzung oder gesteuerten Freisetzung nach einem beliebigen von (a) bis (d), wobei das Pellet oder die Matrix essbares Material umfasst;
(f) die Zusammensetzung zur verzögerten Freisetzung oder gesteuerten Freisetzung nach einem beliebigen von (a) bis (e), wobei die Latex-Polymer-Beschichtung einen Latex-Anstrichstoff umfasst; oder
(g) die Zusammensetzung zur verzögerten Freisetzung oder gesteuerten Freisetzung nach einem beliebigen von (a) bis (f), wobei die Latex-Polymer-Beschichtung ein (Meth)acrylat, ein Vinylacetat, ein Styrol, ein Ethylen, ein Vinylchlorid, ein Butadien, ein Vinylidenchlorid, ein Vinylversatat, ein Vinylpropionat, ein t-Butylacrylat, ein Acrylnitril, ein Neopren, ein Maleat, ein Fumarat oder eine Kombination davon oder ein Derivat davon umfasst.

Revendications

1. Acide nucléique isolé, synthétique ou recombinant constitué de :

(a) une séquence d'acide nucléique ayant au moins 98 %, 99 % ou 100 % d'identité de séquence avec SEQ ID NO: 47, où l'acide nucléique code pour au moins un polypeptide ayant une activité glucoamylase ;
(b) une séquence d'acide nucléique codant pour un polypeptide ayant la séquence de SEQ ID NO:48 ;
(c) une séquence d'acide nucléique totalement complémentaire de (a), ou (b) ; ou,
(d) une séquence d'acide nucléique telle que décrite dans SEQ ID NO: 47.

2. Acide nucléique isolé, synthétique ou recombinant de la revendication 1, où l'activité glucoamylase comprend l'hydrolyse de liaisons glucosidiques et, facultativement, l'hydrolyse de liaisons glucosidiques dans un polysaccharide, un oligosaccharidee ou un amidon, ou, facultativement, les liaisons glucosidiques comprennent une liaison α-1,4-glucosidique et/ou une liaison α-1,6-glucosidique, ou où l'activité glucoamylase comprend le clivage d'un motif de maltose ou de D-glucose depuis l'extrémité non réductrice d'un polysaccharide, un oligosaccharide ou d'un amidon.

3. Vecteur, cassette d'expression ou véhicule de clonage comprenant un acide nucléique comprenant la séquence d'acide nucléique de la revendication 1 ; où, facultativement, l'acide nucléique est fonctionnellement lié à un

promoteur ;
où, facultativement, le promoteur est un promoteur de plante ou un promoteur viral ;
où, facultativement, le promoteur est un promoteur inductible ou un promoteur tissu-spécifique ; ou
où, facultativement, le promoteur tissu-spécifique est facultativement spécifique de graine, un promoteur spécifique de l'endosperme, un promoteur spécifique de feuille, spécifique de racine, spécifique de tige ou induit par abscission.

4. Cellule transformée comprenant un acide nucléique comprenant la séquence d'acide nucléique de la revendication 1, ou un vecteur, une cassette d'expression ou un véhicule de clonage tels que décrits dans la revendication 3 où la cellule est facultativement une cellule bactérienne, une cellule de mammifère, une cellule fongique, une cellule de levure, une cellule d'insecte ou une cellule de plante, la cellule de plante étant facultativement une cellule de pomme de terre, de riz, de maïs, de blé, de tabac ou d'orge.

5. Polypeptide isolé, synthétique ou recombinant ayant une activité glucoamylase comprenant :

(i) une séquence d'acides aminés ayant au moins une identité de séquence de 99 % ou totale avec la séquence de SEQ ID NO: 48 sur la pleine longueur du polypeptide ;
(ii) une séquence d'acides aminés codée par l'acide nucléique de la revendication 1 ;
(iii) le polypeptide de l'un quelconque de (i) à (ii) où le polypeptide possède une séquence telle que décrite dans SEQ ID NO: 48 ou des fragments enzymatiquement actifs de celui-ci ayant une activité glucoamylase.

6. Polypeptide isolé, synthétique ou recombinant de la revendication 5 où l'activité glucoamylase comprend l'hydrolyse de liaisons glucosidiques, et facultativement les liaisons glucosidiques comprennent une liaison α-1,4-glucosidique ou une liaison α-1,6-glucosidique, et/ou facultativement, l'activité glucoamylase comprend l'hydrolyse de liaisons glucosidiques dans un polysaccharide, un oligosaccharide ou un amidon, ou et/ou l'activité glucoamylase comprend le clivage d'un motif de maltose ou de D-glucose depuis l'extrémité non réductrice d'un polysaccharide, d'un oligosaccharide ou d'un amidon.

7. Procédé de production d'un polypeptide recombinant comprenant les étapes de : (a) fourniture d'un acide nucléique fonctionnellement lié à un promoteur, où l'acide nucléique comprend la séquence d'acide nucléique de la revendication 1 ; et (b) expression de l'acide nucléique de l'étape (a) dans des conditions qui permettent l'expression du polypeptide, de manière à produire un polypeptide recombinant, comprenant en outre la transformation d'une cellule hôte avec l'acide nucléique de l'étape (a) suivie par l'expression de l'acide nucléique de l'étape (a), de manière à produire un polypeptide recombinant dans une cellule transformée.

8. Procédé d'hydrolyse d'un polysaccharide, d'un oligosaccharide ou d'un amidon comprenant les étapes suivantes :

(a) fourniture du polypeptide de la revendication 5 ;
(b) fourniture d'une composition comprenant un polysaccharide, un oligosaccharide ou un amidon ; où la composition comprend facultativement une liaison α-1,4-glucosidique ou une liaison α-1,6-glucosidique et
(c) mise en contact du polypeptide de l'étape (a) avec la composition de l'étape (b) dans des conditions dans lesquelles le polypeptide hydrolyse le polysaccharide, l'oligosaccharide ou l'amidon.

9. Procédé de liquéfaction ou d'extraction d'un polysaccharide, d'un oligosaccharide ou d'un amidon comprenant les étapes suivantes :

(a) fourniture du polypeptide de la revendication 5 ;
(b) fourniture d'une composition comprenant un polysaccharide, un oligosaccharide ou un amidon ; et
(c) mise en contact du polypeptide de l'étape (a) avec la composition de l'étape (b) dans des conditions dans lesquelles le polypeptide liquéfie ou extrait le polysaccharide, l'oligosaccharide ou l'amidon.

10. Procédé de lavage d'un objet comprenant les étapes suivantes :

(a) fourniture d'une composition comprenant le polypeptide de la revendication 5 ;
(b) fourniture d'un objet ; et
(c) mise en contact du polypeptide de l'étape (a) et de l'objet de l'étape (b) dans des conditions dans lesquelles la composition peut laver l'objet.

11. Procédé d'hydrolyse d'un polysaccharide, d'un oligosaccharide ou d'un amidon dans un aliment pour animaux ou

un aliment pour humains avant consommation par un animal comprenant les étapes suivantes :

(a) obtention d'un matériau d'aliment pour animaux comprenant un amidon, où le polysaccharide, l'oligosaccharide ou l'amidon dans le polysaccharide, l'oligosaccharide ou l'amidon peut être hydrolyse par le polypeptide de la revendication 5 ; et
(b) ajout du polypeptide de l'étape (a) au matériau d'aliment pour animaux ou d'aliment pour humains en une quantité suffisante pendant une durée suffisante pour causer l'hydrolyse du polysaccharide, de l'oligosaccharide ou de l'amidon et la formation pour humains ou aliment pour animaux traités, de manière à hydrolyser le polysaccharide, l'oligosaccharide ou l'amidon dans l'aliment pour humains ou l'aliment pour animaux avant consommation par l'animal, où l'aliment pour humains ou l'aliment pour animaux comprend facultativement du riz, du maïs, de l'orge, du blé, des légumineuses ou de la pomme de terre.

12. Procédé de désencollage de textile comprenant les étapes suivantes :

(a) fourniture du polypeptide de la revendication 5 ;
(b) fourniture d'un tissu ; et
(c) mise en contact du polypeptide de l'étape (a) et du tissu de l'étape (b) dans des conditions dans lesquelles la glucoamylase peut désencoller le tissu.

13. Procédé de traitement de fibres lignocellulosiques comprenant les étapes suivantes :

(a) fourniture du polypeptide de la revendication 5 ;
(b) fourniture d'une fibre lignocellulosique ; et
(c) mise en contact du polypeptide de l'étape (a) et de la fibre de l'étape (b) dans des conditions dans lesquelles le polypeptide peut traiter la fibre de manière à améliorer les propriétés de la fibre.

14. Procédé de production d'un sirop riche en maltose ou riche en glucose comprenant les étapes suivantes :

(a) fourniture du polypeptide de la revendication 5 ;
(b) fourniture d'une composition comprenant un polysaccharide, un oligosaccharide ou un amidon ; facultativement dans lequel le polysaccharide, l'oligosaccharide ou l'amidon provient de riz, de maïs, d'orge, de blé, de légumineuses, de pommes de terre ou de patate douce et
(c) mise en contact du polypeptide de l'étape (a) et du composition de l'étape (b) dans des conditions dans lesquelles le polypeptide de l'étape (a) peut hydrolyser la composition de l'étape (b), de manière à produire un sirop riche en maltose ou riche en glucose.

15. Procédé d'amélioration de l'écoulement des fluides de production contenant un polysaccharide, un oligosaccharide ou un amidon, comprenant les étapes suivantes :

(a) fourniture du polypeptide de la revendication 5 ;
(b) fourniture d'un fluide de production comprenant un polysaccharide, un oligosaccharide ou un amidon ; facultativement où le fluide de production provient d'une formation souterraine ; et
(c) mise en contact du polypeptide de l'étape (a) et du fluide de production de l'étape (b) dans des conditions dans lesquelles le polypeptide peut hydrolyser le polysaccharide, l'oligosaccharide ou l'amidon dans le fluide de production, de manière à améliorer son écoulement en diminuant sa masse volumique.

16. Procédé de modification de la viscosité d'une composition, comprenant les étapes suivantes :

(i)

(a) fourniture d'une composition et du polypeptide de la revendication 5 ; et
(b) traitement de la composition avec le polypeptide de la revendication 5 ; ou

(ii) le procédé de (i), dans lequel la composition comprend un sol ou une boue de forage.

17. Procédé d'utilisation d'une glucoamylase en brasserie ou dans la production d'alcool comprenant les étapes suivantes :

(a) fourniture du polypeptide de la revendication 5 ;
(b) fourniture d'une composition utilisée pour la brasserie ou dans la production d'alcool comprenant un polysaccharide, un oligosaccharide ou un amidon ; où la composition comprenant un polysaccharide, un oligosaccharide ou un amidon est facultativement une bière ;
(c) combinaison du polypeptide de l'étape (a) avec la composition de l'étape (b) dans des conditions dans lesquelles le polypeptide peut hydrolyser le polysaccharide, l'oligosaccharide ou l'amidon dans la composition utilisée en brasserie ou dans la production d'alcool.

18. Procédé de bio-blanchiment d'une composition comprenant :

(i) la fourniture d'une composition où la composition est un produit de papier ou un produit de pâte à papier, et le polypeptide de la revendication 5 ;
(ii) le traitement de la composition avec le polypeptide de la revendication 5.

19. Procédé de désencrage de papier et de fibres comprenant les étapes suivantes :

(a) fourniture du polypeptide tel que décrit dans la revendication 5 ;
(b) fourniture d'une composition comprenant du papier ou une fibre ;
(c) mise en contact du polypeptide de l'étape (a) et de la composition de l'étape (b)

dans des conditions dans lesquelles le polypeptide peut désencrer le papier ou une fibre.

20. Procédé de fabrication d'un combustible comprenant :

(i) (a) fourniture du polypeptide de la revendication 5 ; (b) fourniture d'une composition comprenant un polysaccharide, un oligosaccharide ou un amidon ; et (c) mise en contact du polypeptide de (a) avec la composition de (b) dans des conditions dans lesquelles le polypeptide hydrolyse le polysaccharide, l'oligosaccharide ou l'amidon ; ou
(ii) le procédé de (i) dans lequel le polypeptide est une enzyme thermostable ;
(iii) le procédé de (i) dans lequel le combustible est à base d'éthanol.

21. Procédé de traitement d'un matériau de biomasse comprenant de la lignocellulose comprenant

(i) (a) la fourniture d'une composition comprenant le polypeptide de la revendication 5, et, la fourniture d'un matériau de biomasse ; et (b) la mise en contact de la composition avec le matériau de biomasse ;
(ii) le procédé de (i), dans lequel le matériau de biomasse comprend ou est dérivé d'une culture agricole, ou est un sous-produit d'une production d'aliment pour humains ou d'aliment pour animaux, ou est un produit de déchets lignocellulosique, ou est un résidu de plante ou un papier usagé ou un produit de papier usagé,
et facultativement le matériau de biomasse comprend des pommes de terre, du soja, de l'orge, du seigle, du maïs, de l'avoine, du blé, des betteraves ou de la canne à sucre ; (iii) le procédé de (i) ou (ii), où le polypeptide a une activité comprenant une activité glucoamylase ;
(iv) le procédé de l'un quelconque de (ii), dans lequel le résidu de plante comprend des tiges, des feuilles, des coques, des balles, des rafles, du bois, des copeaux de bois, de la pâte de bois et de la sciure, ou, le déchet de papier comprend du papier pour photocopie, du papier pour imprimante informatique, du papier de cahier, du papier de bloc-notes, du papier pour machine à écrire, des journaux, des magazines, du carton et des matériaux d'emballage à base de papier rejetés ou usagés ; ou
(v) le procédé de (i) à (iv), dans lequel le traitement du matériau de biomasse génère un bioéthanol, un biopropanol, un biobutanol ou un biodiesel ;
(vi) le procédé de (i) à (v), dans lequel le matériau de biomasse comprend une lignocellulose.

22. Procédé de fabrication de bioéthanol, de biopropanol, de biobutanol, ou d'un biodiesel, comprenant

(i)

(a) la fourniture du polypeptide de la revendication 5, et, la fourniture d'une composition comprenant un polysaccharide ou un oligosaccharide ; et
(b) la mise en contact de la composition comprenant un polysaccharide ou un oligosaccharide avec le polypeptide de la revendication 5 ;

(ii) le procédé de (i), dans lequel la composition comprenant un polysaccharide ou un oligosaccharide comprend une plante, un produit de plante ou un dérivé de plante ;
(iii) le procédé de (ii), dans lequel la plante ou le produit de plante comprend des plantes ou des produits de plante de canne à sucre, des betteraves ou des betteraves sucrières, du blé, du maïs, du soja, de la pomme de terre, du riz ou de l'orge ;
(iv) le procédé de l'un quelconque de (i) à (iii), dans lequel le polypeptide a une activité comprenant une activité glucoamylase ; ou
(v) le procédé de l'un quelconque de (i) à (iv), dans lequel le polysaccharide ou l'oligosaccharide comprend un sucre fermentable.

23. Procédé de fabrication d'un combustible comprenant

(i)

(a) la fourniture du polypeptide de la revendication 5, et, la fourniture d'une composition comprenant un sucre fermentable ; et
(b) la mise en contact de la composition comprenant un sucre fermentable avec le polypeptide de la revendication 5 ;

(ii) le procédé de (i), dans lequel la composition comprenant un sucre fermentable comprend une plante, un produit de plante ou un dérivé de plante ;
(iii) le procédé de (ii), dans lequel la plante ou le produit de plante comprend des plantes ou des produits de plante de canne à sucre, des betteraves ou des betteraves sucrières, du blé, du maïs, du soja, de la pomme de terre, du riz ou de l'orge ;
(iv) le procédé de l'un quelconque de (i) à (iii), dans lequel le polypeptide a une activité comprenant une activité glucoamylase ; ou
(v) le procédé de l'un quelconque de (i) à (iv), dans lequel le combustible comprend un bioéthanol ou un mélange essence-éthanol, ou comprend un bioéthanol, un biopropanol, un biobutanol ou un biodiesel.

24. Procédé de production d'un sucre, le procédé comprenant :

(i)

(a) fourniture du polypeptide de la revendication 5 ;
(b) fourniture d'une composition comprenant un polysaccharide ou un oligosaccharide ; et
(c) mise en contact de la composition de l'étape (b) avec le polypeptide de l'étape (a), de manière à générer des sucres ;

(ii) le procédé de (i), dans lequel la composition comprenant un polysaccharide comprend un amidon ;
(iii) le procédé de l'un quelconque de (i) ou (ii), dans lequel le sucre est un sucre fermentable.

25. Composition comprenant le polypeptide de la revendication 5,
où la composition est choisie parmi : à détergent, un aliment pour animaux ou un aliment pour humains, un textile ou un tissu, un papier ou un produit de papier ou une pâte à papier, un liquide ou sirop riche en maltose ou riches en glucose, une composition anti-rassissement, une boisson alcoolisée, une bière, une composition pharmaceutique, un produit de soin buccal, une pâte dentifrice, une crème dentaire, un gel ou une poudre dentaire, un produit odontique, un bain de bouche, une formulation de rinçage pré- ou post-brossage, une gomme à mâcher, une tablette ou un bonbon, un fluide de forage de puits de pétrole, une solution de bio-blanchiment, un désinfectant, un agent de biodéfense ou bio-détoxifiant, un produit laitier ou un matériau de biomasse.

26. Composition à libération retardée ou libération contrôlée comprenant le polypeptide de la revendication 5, comprenant

(a) un composant souhaité enrobé par un revêtement de polymère de latex ;
(b) la composition à libération retardée ou libération contrôlée de (a), dans laquelle le composant souhaité comprend une enzyme ;
(c) la composition à libération retardée ou libération contrôlée de (a) ou (b), dans laquelle le composant souhaité comprend une petite molécule, un médicament, un polysaccharide, un lipide, un acide nucléique, une vitamine,

un antibiotique ou un insecticide ;
(d) la composition à libération retardée ou libération contrôlée de (a), (b) ou (c), dans laquelle le composant souhaité comprend un granule ou une matrice ;
(e) la composition à libération retardée ou libération contrôlée de l'un quelconque de (a) à (d), dans laquelle le granule ou la matrice comprend un matériau comestible ;
(f) la composition à libération retardée ou libération contrôlée de l'un quelconque de (a) à (e), dans laquelle l'enrobage de polymère de latex comprend une peinture au latex ; ou
(g) la composition à libération retardée ou libération contrôlée de l'un quelconque de (a) à (f), dans laquelle l'enrobage de polymère de latex comprend un (méth)acrylate, un acétate de vinyle, un styrène, un éthylène, un chlorure de vinyle, un butadiène, un chlorure de vinylidène, un versatate de vinyle, un propionate de vinyle, un acrylate de t-butyle, un acrylonitrile, un néoprène, un maléate, un fumarate ou une combinaison de ceux-ci ou un dérivé de ceux-ci.

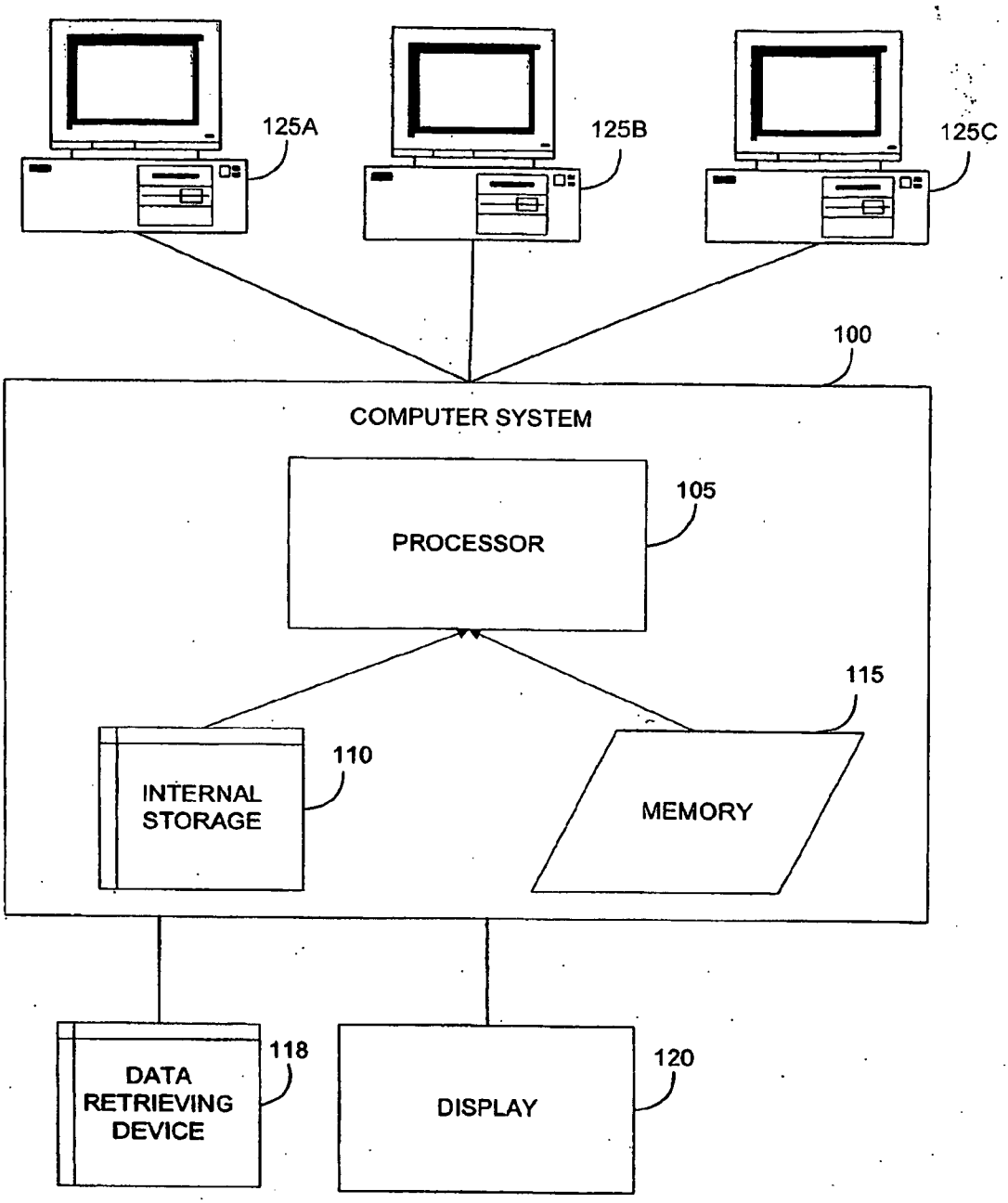

125A
125B
125C
100
COMPUTER SYSTEM
PROCESSOR
105
115
INTERNAL STORAGE
110
MEMORY
DATA RETRIEVING DEVICE
118
DISPLAY
120

FIGURE 1

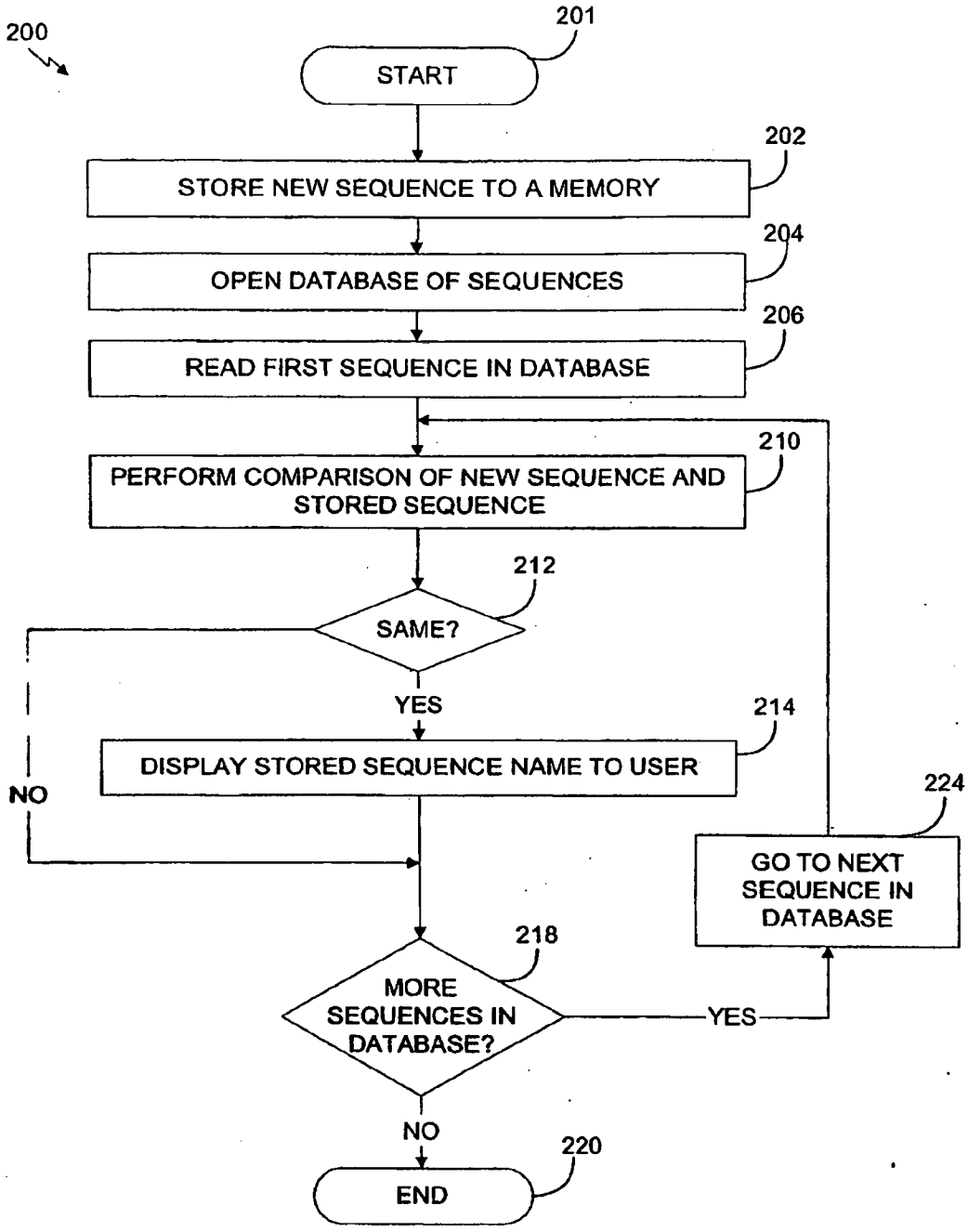

200
201
START
202
STORE NEW SEQUENCE TO A MEMORY
204
OPEN DATABASE OF SEQUENCES
206
READ FIRST SEQUENCE IN DATABASE
210
PERFORM COMPARISON OF NEW SEQUENCE AND STORED SEQUENCE
212
SAME?
YES
214
DISPLAY STORED SEQUENCE NAME TO USER
NO
224
GO TO NEXT SEQUENCE IN DATABASE
218
MORE SEQUENCES IN DATABASE?
YES
NO
220
END

FIGURE 2

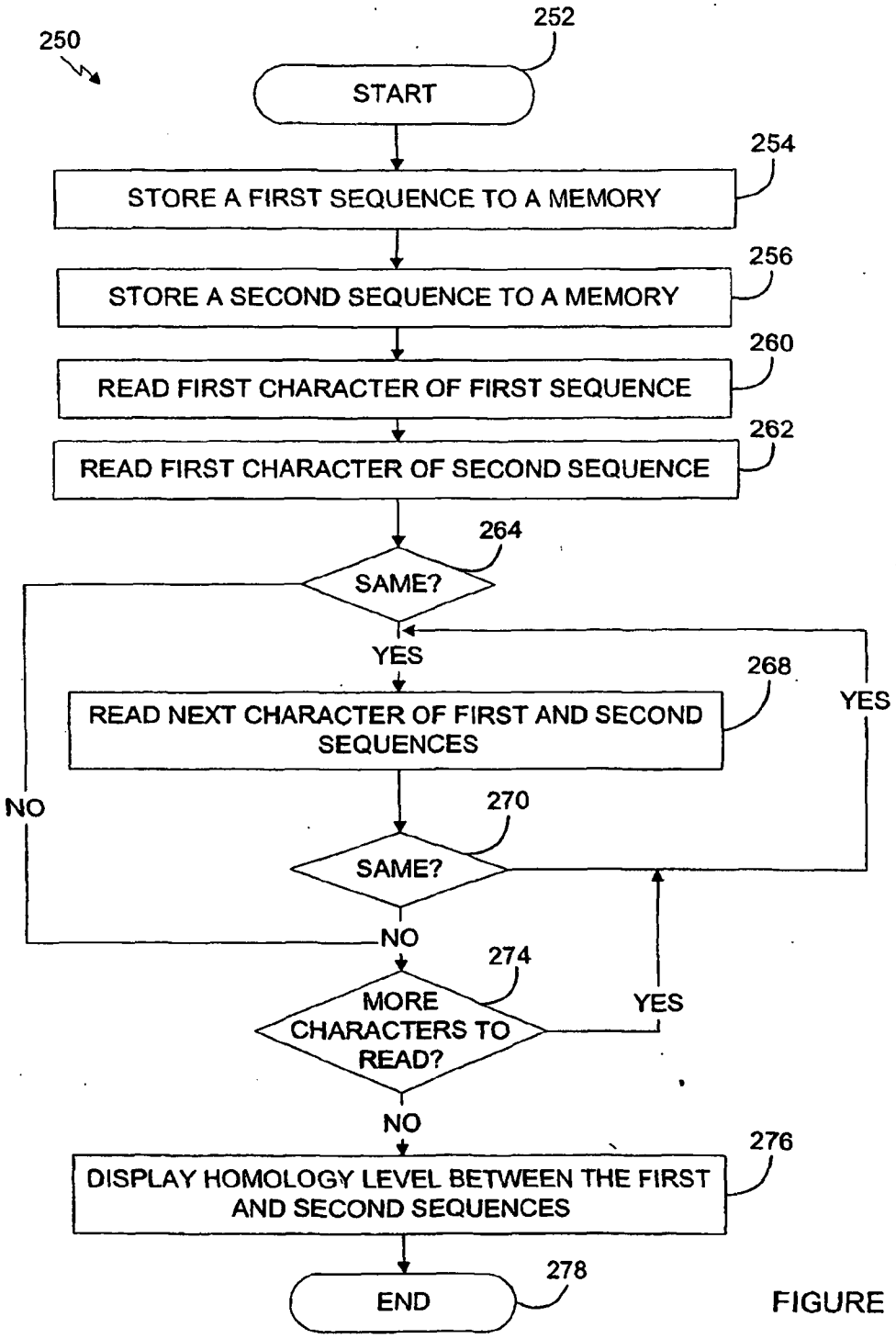

250
252
START
254
STORE A FIRST SEQUENCE TO A MEMORY
256
STORE A SECOND SEQUENCE TO A MEMORY
260
READ FIRST CHARACTER OF FIRST SEQUENCE
262
READ FIRST CHARACTER OF SECOND SEQUENCE
264
SAME?
YES
268
READ NEXT CHARACTER OF FIRST AND SECOND SEQUENCES
YES
NO
270
SAME?
NO
274
MORE CHARACTERS TO READ?
YES
NO
276
DISPLAY HOMOLOGY LEVEL BETWEEN THE FIRST AND SECOND SEQUENCES
278
END

FIGURE 3

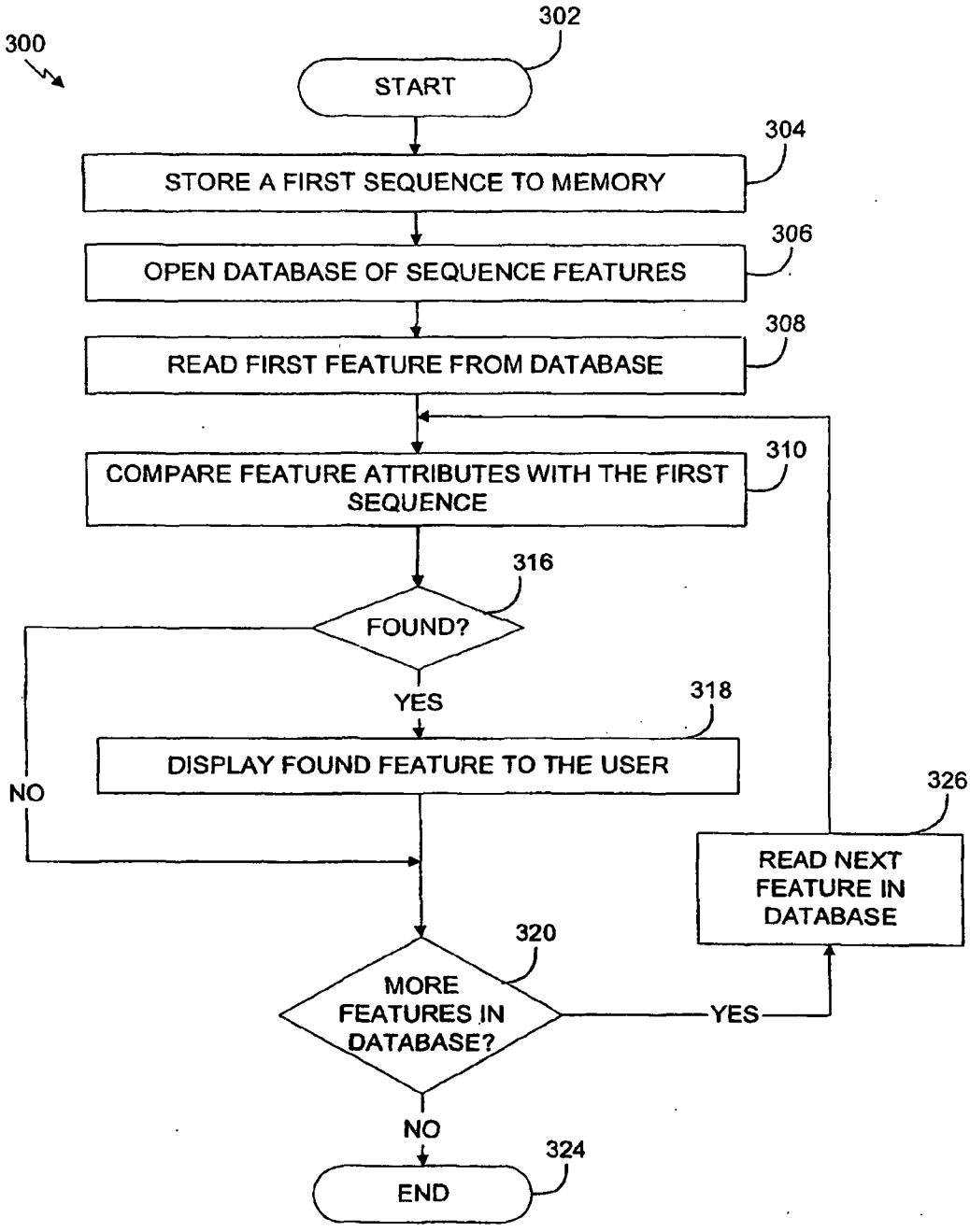

300
302
START
304
STORE A FIRST SEQUENCE TO MEMORY
306
OPEN DATABASE OF SEQUENCE FEATURES
308
READ FIRST FEATURE FROM DATABASE
310
COMPARE FEATURE ATTRIBUTES WITH THE FIRST SEQUENCE
316
FOUND?
YES
318
DISPLAY FOUND FEATURE TO THE USER
NO
326
READ NEXT FEATURE IN DATABASE
320
MORE FEATURES IN DATABASE?
YES
NO
324
END

FIGURE 4

Figure 5

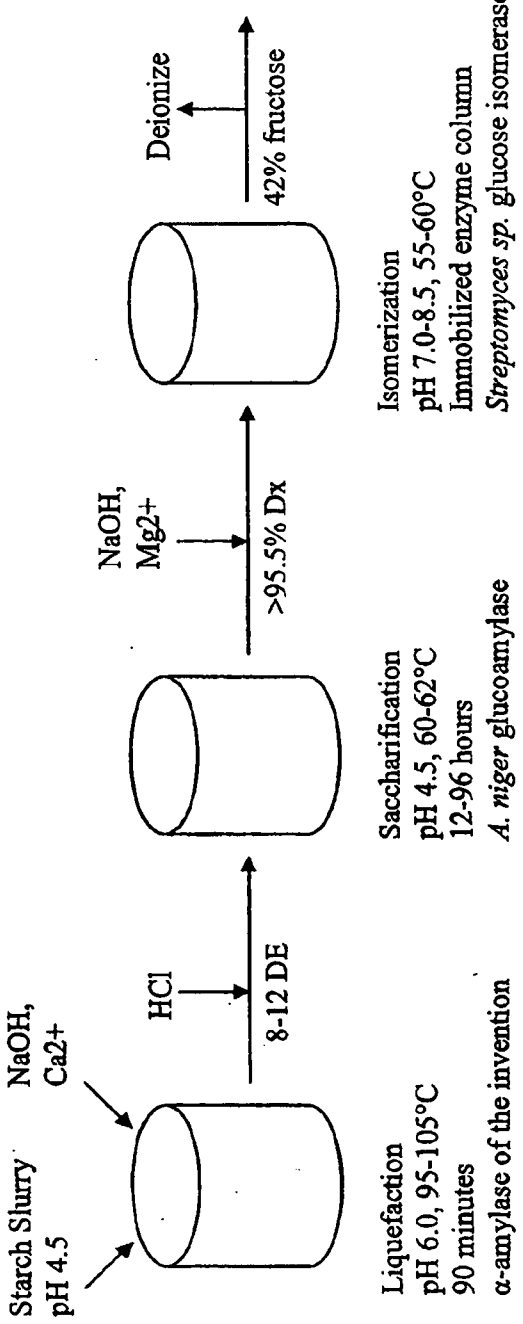

Starch Slurry
pH 4.5
NaOH,
Ca2+
HCl
8-12 DE
NaOH,
Mg2+
>95.5% Dx
Deionize
42% fructose
Liquefaction
pH 6.0, 95-105°C
90 minutes
α-amylase of the invention
Saccharification
pH 4.5, 60-62°C
12-96 hours
A. niger glucoamylase
Isomerization
pH 7.0-8.5, 55-60°C
Immobilized enzyme column
Streptomyces sp. glucose isomerase

FIGURE 6

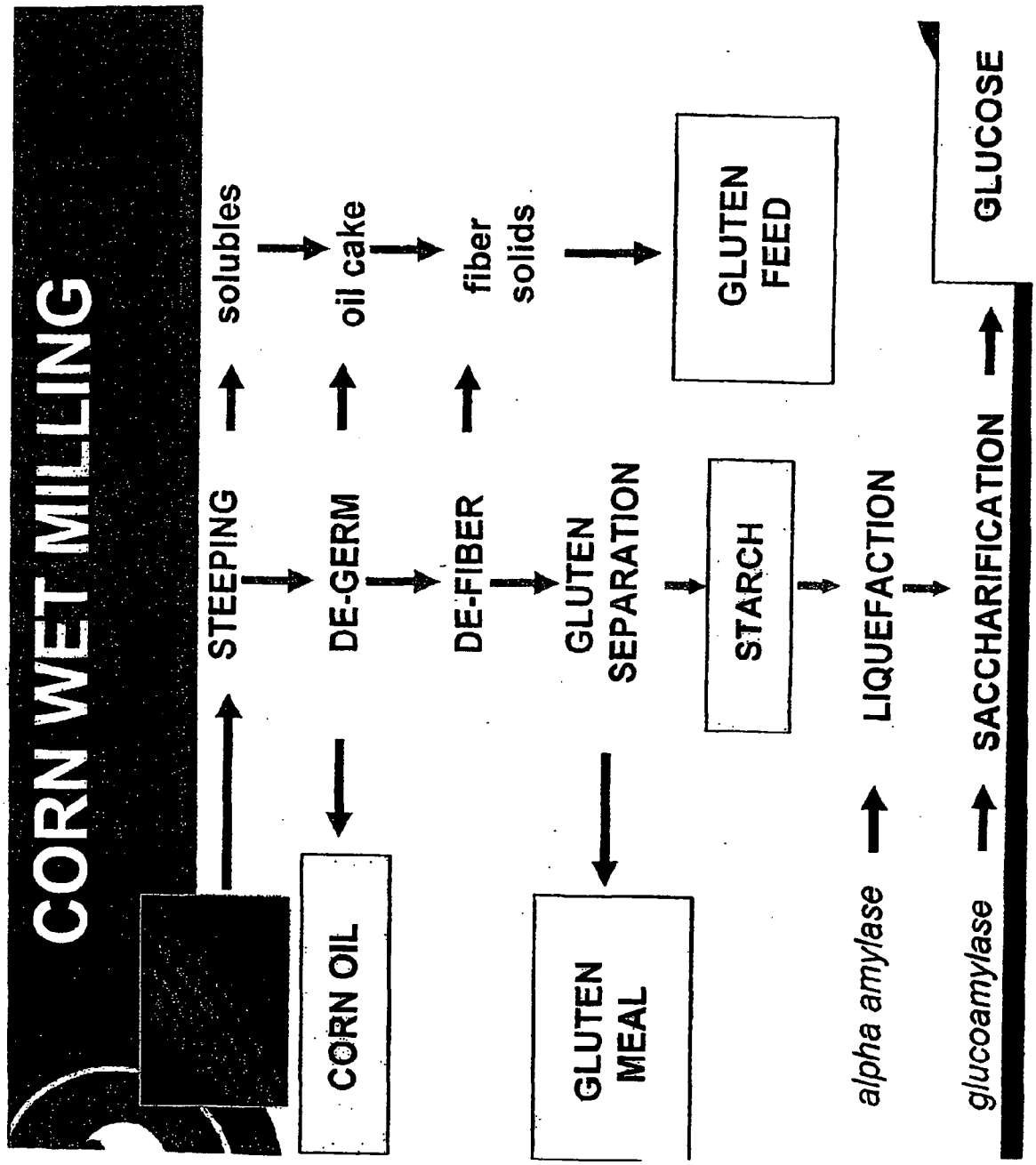

CORN WET MILLING
STEEPING
DE-GERM
DE-FIBER
GLUTEN SEPARATION
STARCH
LIQUEFACTION
SACCHARIFICATION
GLUCOSE
solubles
oil cake
fiber solids
GLUTEN FEED
CORN OIL
GLUTEN MEAL
alpha amylase
glucoamylase

FIGURE 7

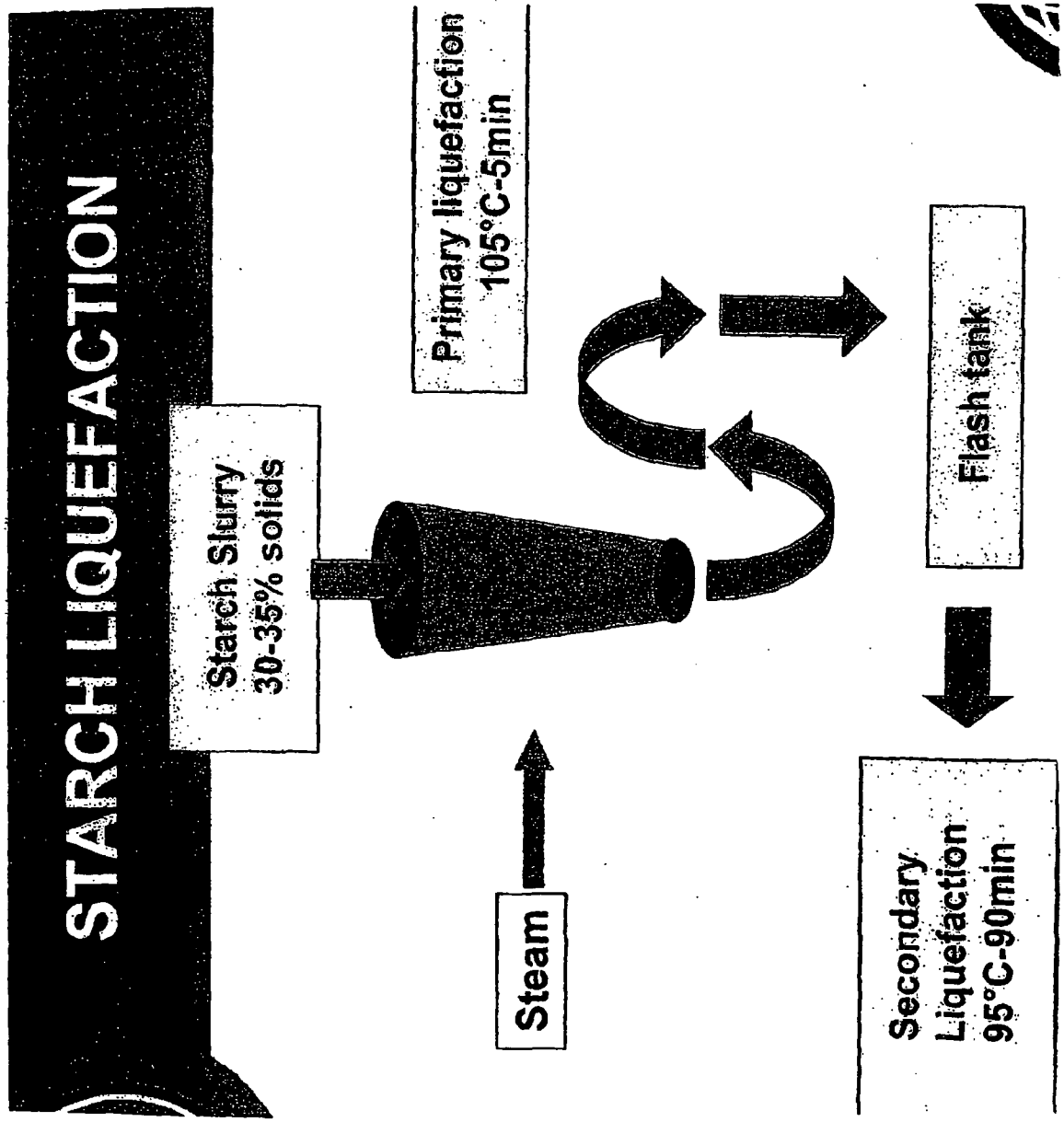

STARCH LIQUEFACTION
Starch Slurry
30-35% solids
Primary liquefaction
105°C-5min
Steam
Flash tank
Secondary
Liquefaction
95°C-90min

FIGURE 8

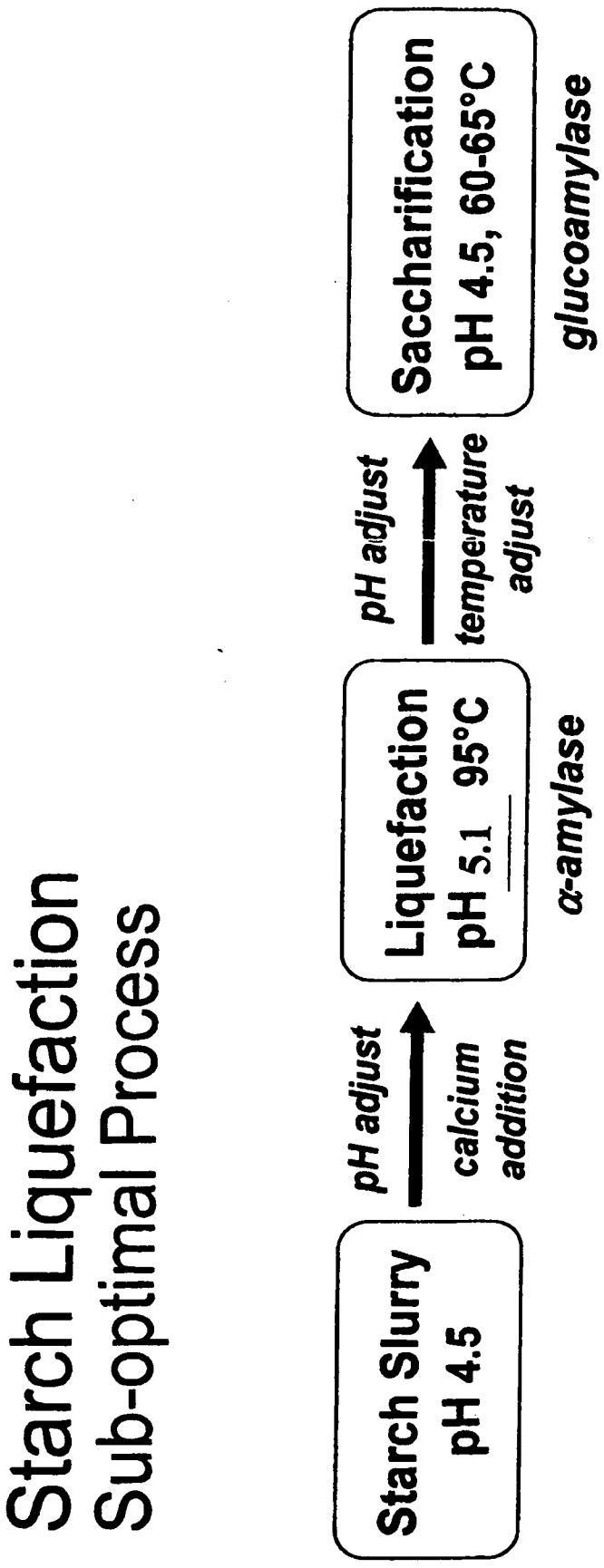

Starch Liquefaction
Sub-optimal Process
Starch Slurry
pH 4.5
pH adjust
calcium
addition
Liquefaction
pH 5.1 95°C
α-amylase
pH adjust
temperature
adjust
Saccharification
pH 4.5, 60-65°C
glucoamylase

FIGURE 9

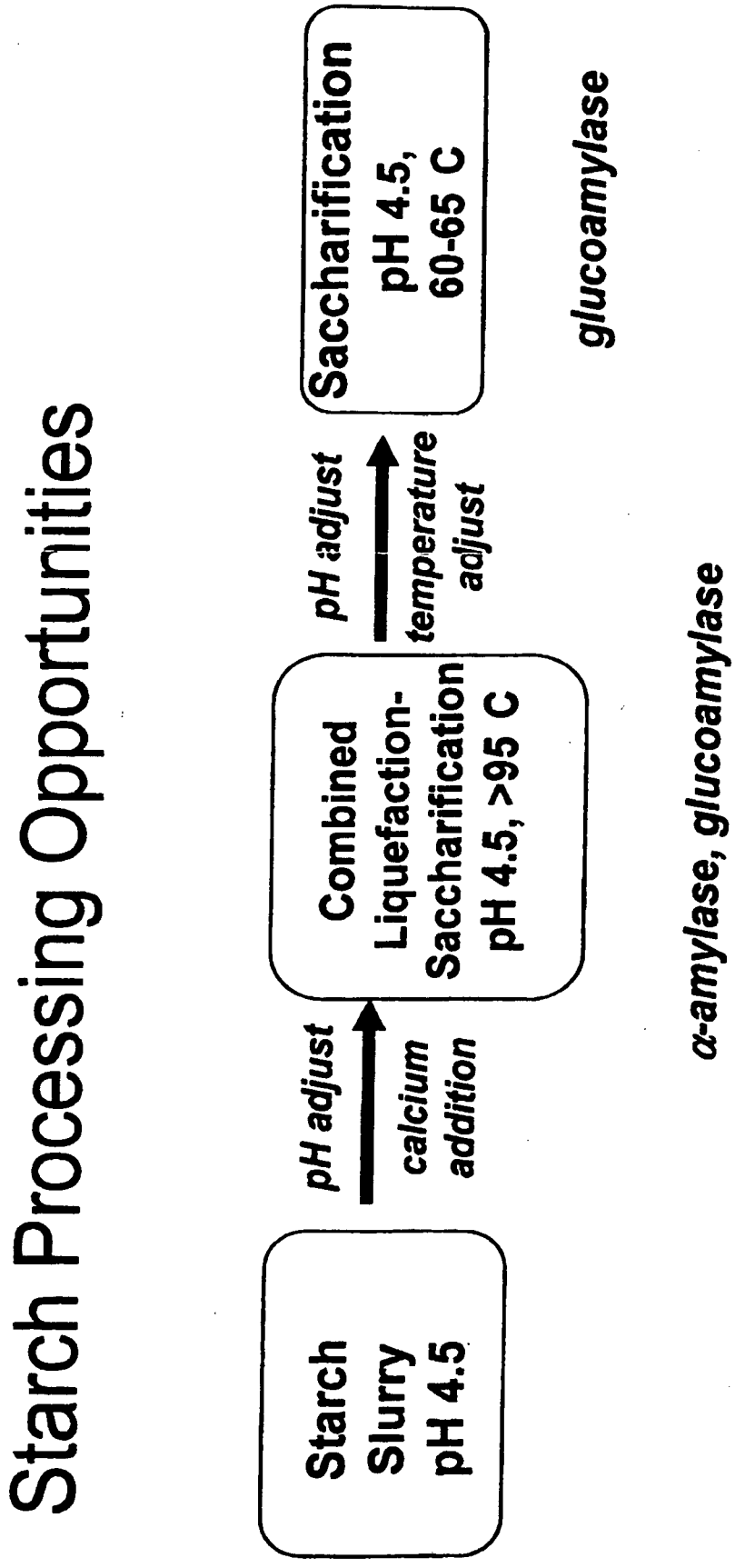

Starch Processing Opportunities
Starch Slurry pH 4.5
pH adjust
calcium addition
Combined Liquefaction-Saccharification pH 4.5, >95 C
pH adjust
temperature adjust
Saccharification pH 4.5, 60-65 C
α-amylase, glucoamylase
glucoamylase

Figure 10

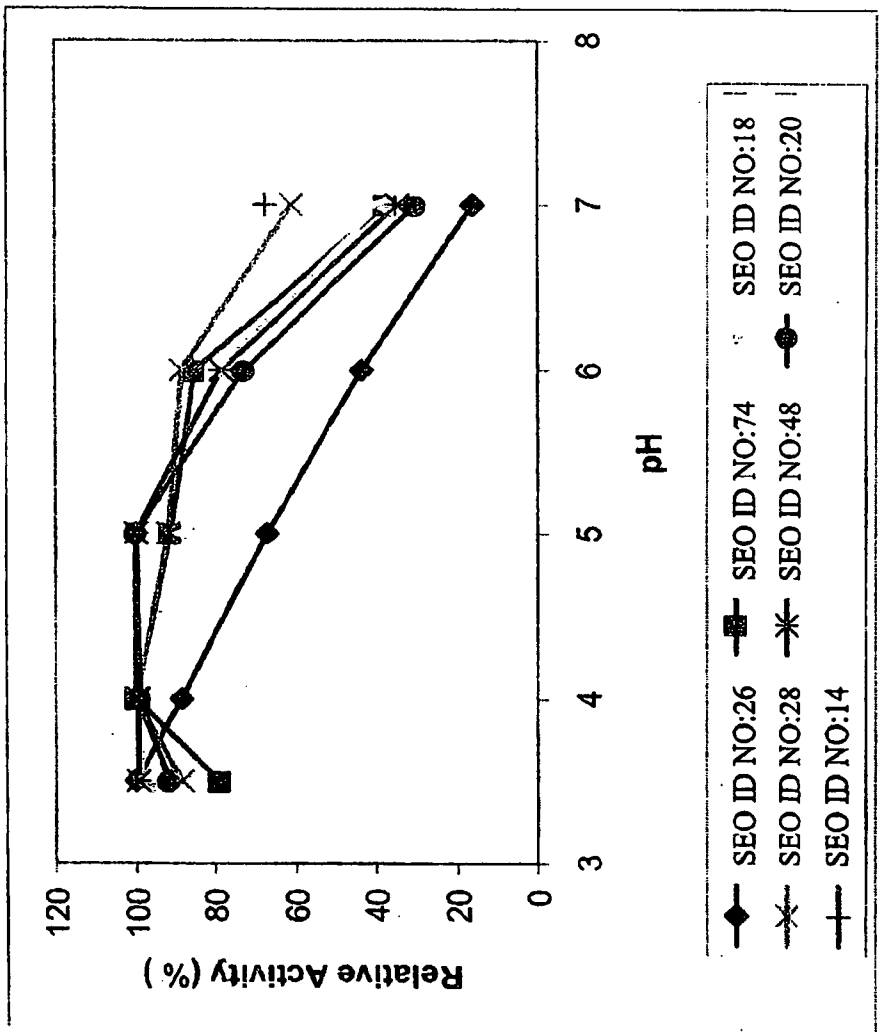

Relative Activity (%)
120
100
80
60
40
20
0
3
4
5
6
7
8
pH
SEQ ID NO:26
SEQ ID NO:74
SEQ ID NO:18
SEQ ID NO:28
SEQ ID NO:48
SEQ ID NO:20
SEQ ID NO:14

Figure 11

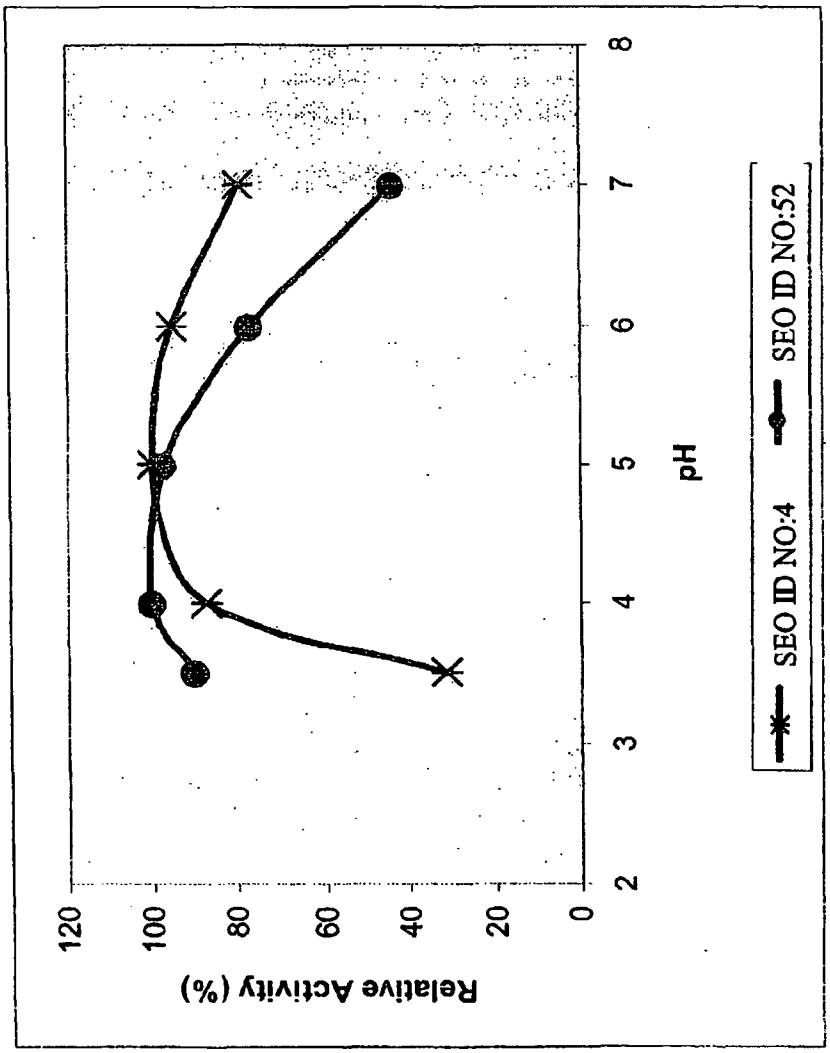

Relative Activity (%)
120
100
80
60
40
20
0
2
3
4
5
6
7
8
pH
SEO ID NO:4
SEO ID NO:52

Figure 12

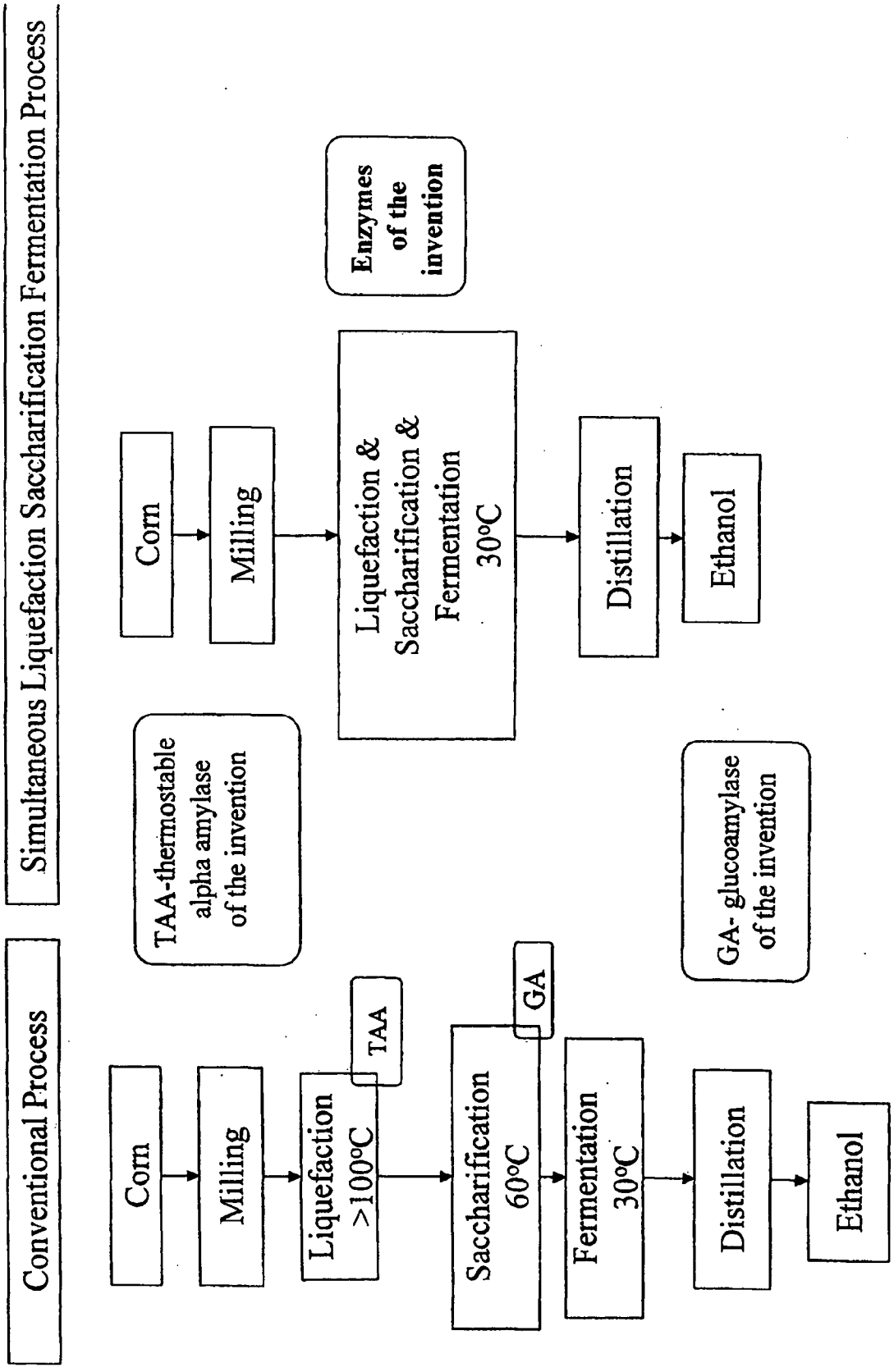

Conventional Process
Corn
Milling
Liquefaction >100°C
TAA
Saccharification 60°C
GA
Fermentation 30°C
Distillation
Ethanol
Simultaneous Liquefaction Saccharification Fermentation Process
TAA-thermostable alpha amylase of the invention
GA- glucoamylase of the invention
Corn
Milling
Liquefaction & Saccharification & Fermentation 30°C
Enzymes of the invention
Distillation
Ethanol

Figure 13

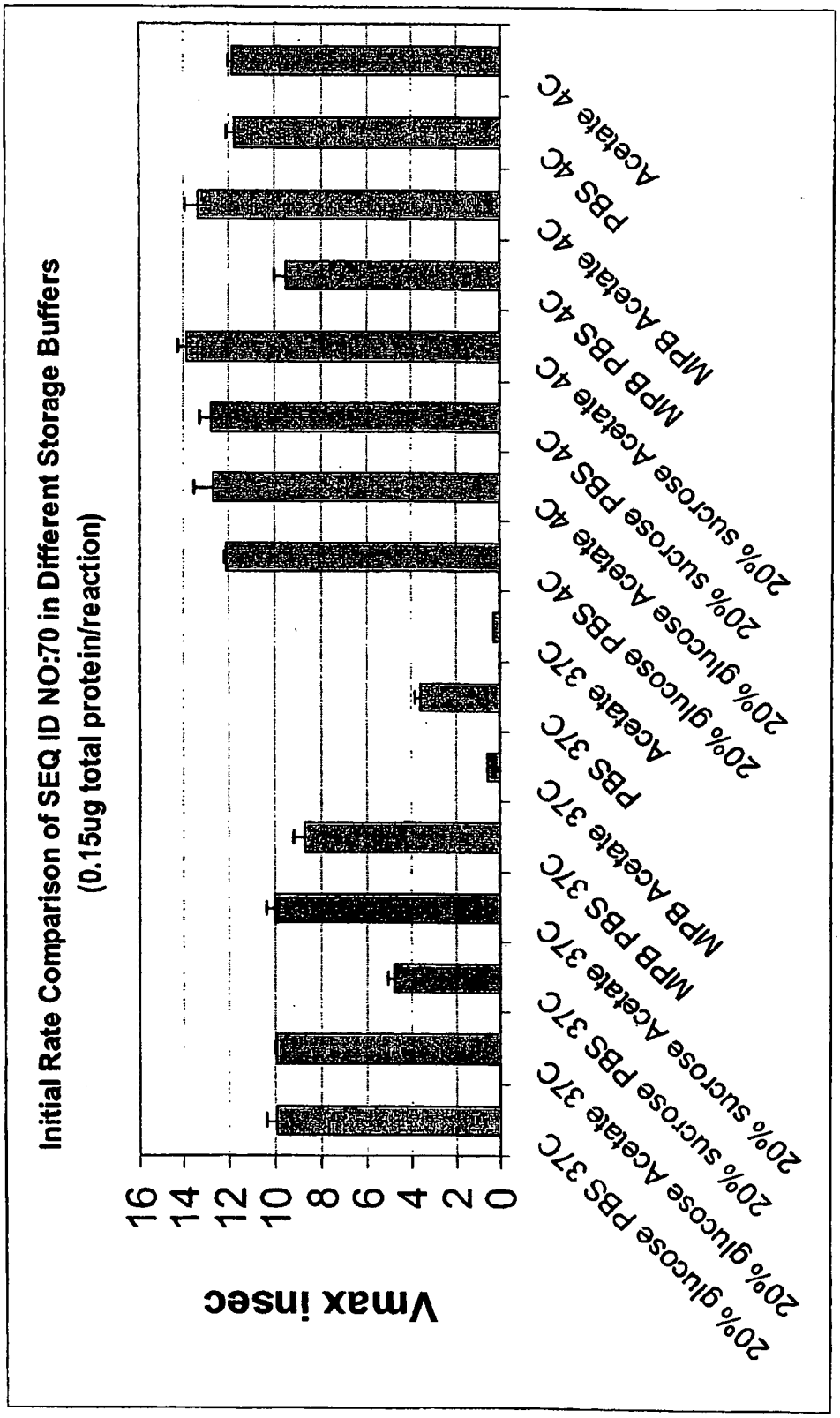

Initial Rate Comparison of SEQ ID NO:70 in Different Storage Buffers
(0.15ug total protein/reaction)
Vmax inSec
0
2
4
6
8
10
12
14
16
20% glucose PBS 37C
20% glucose Acetate 37C
20% sucrose PBS 37C
20% sucrose Acetate 37C
MPB PBS 37C
MPB Acetate 37C
PBS 37C
Acetate 37C
20% glucose PBS 4C
20% glucose Acetate 4C
20% sucrose PBS 4C
20% sucrose Acetate 4C
MPB PBS 4C
MPB Acetate 4C
PBS 4C
Acetate 4C

Figure 14

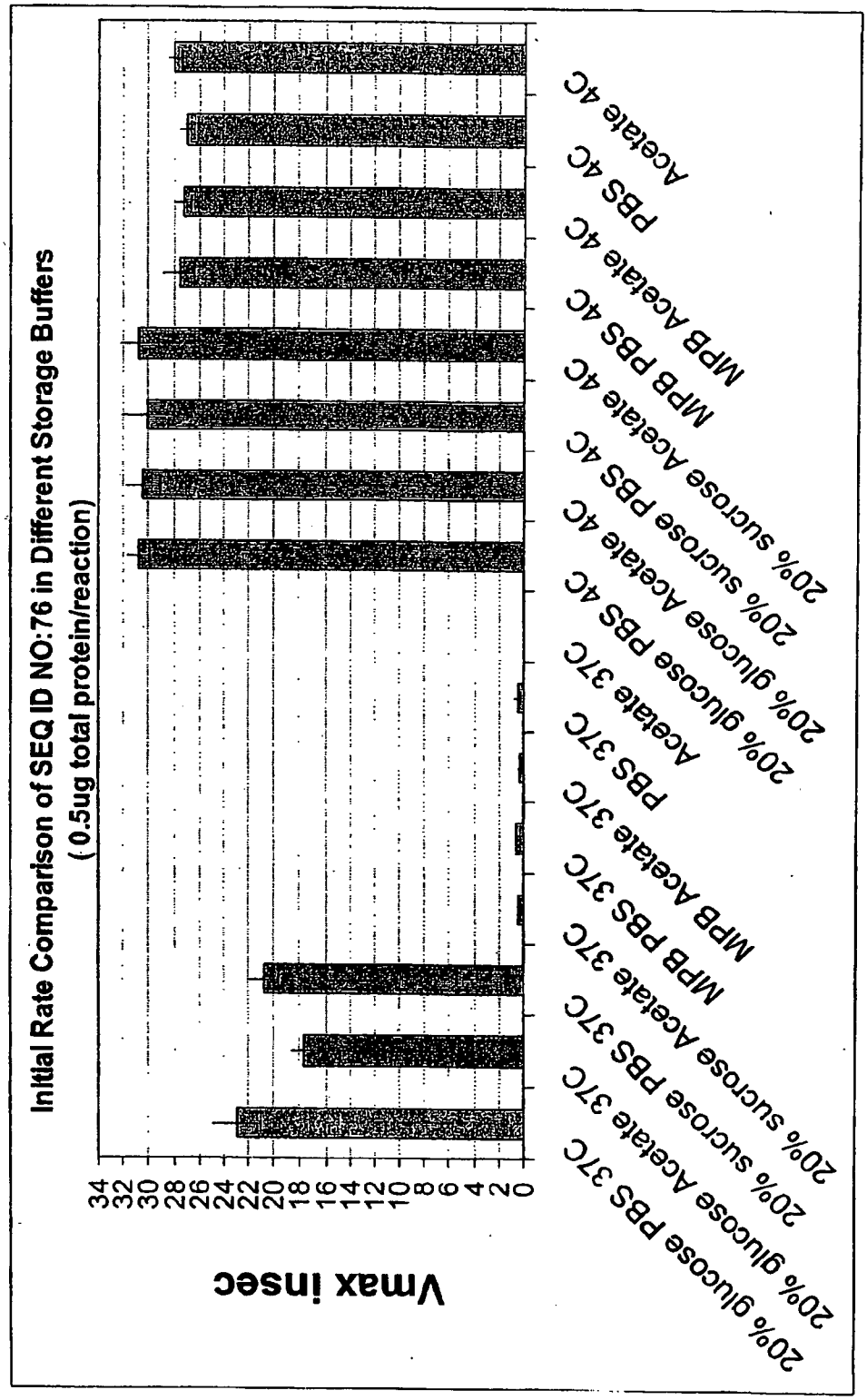

Initial Rate Comparison of SEQ ID NO:76 in Different Storage Buffers
(0.5ug total protein/reaction)
Vmax inSec
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
20% glucose PBS 37C
20% glucose Acetate 37C
20% sucrose PBS 37C
20% sucrose Acetate 37C
MPB PBS 37C
MPB Acetate 37C
PBS 37C
Acetate 37C
20% glucose PBS 4C
20% glucose Acetate 4C
20% sucrose PBS 4C
20% sucrose Acetate 4C
MPB PBS 4C
MPB Acetate 4C
PBS 4C
Acetate 4C

Figure 15

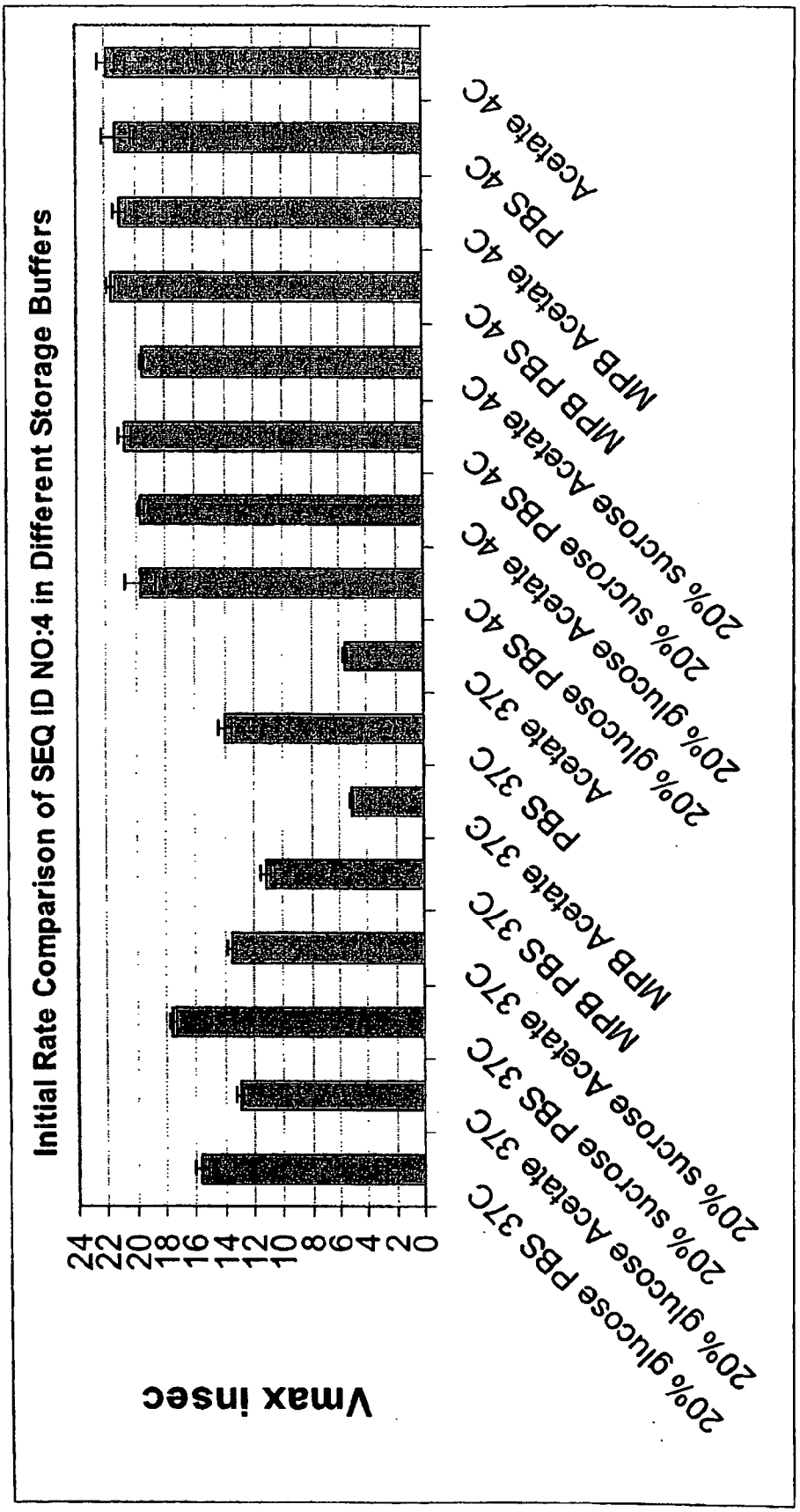

Initial Rate Comparison of SEQ ID NO:4 in Different Storage Buffers
Vmax inSec
24
22
20
18
16
14
12
10
8
6
4
2
0
Acetate 4C
PBS 4C
MPB Acetate 4C
MPB PBS 4C
20% sucrose Acetate 4C
20% sucrose PBS 4C
20% glucose Acetate 4C
20% glucose PBS 4C
Acetate 37C
PBS 37C
MPB Acetate 37C
MPB PBS 37C
20% sucrose Acetate 37C
20% sucrose PBS 37C
20% glucose Acetate 37C
20% glucose PBS 37C

Figure 16

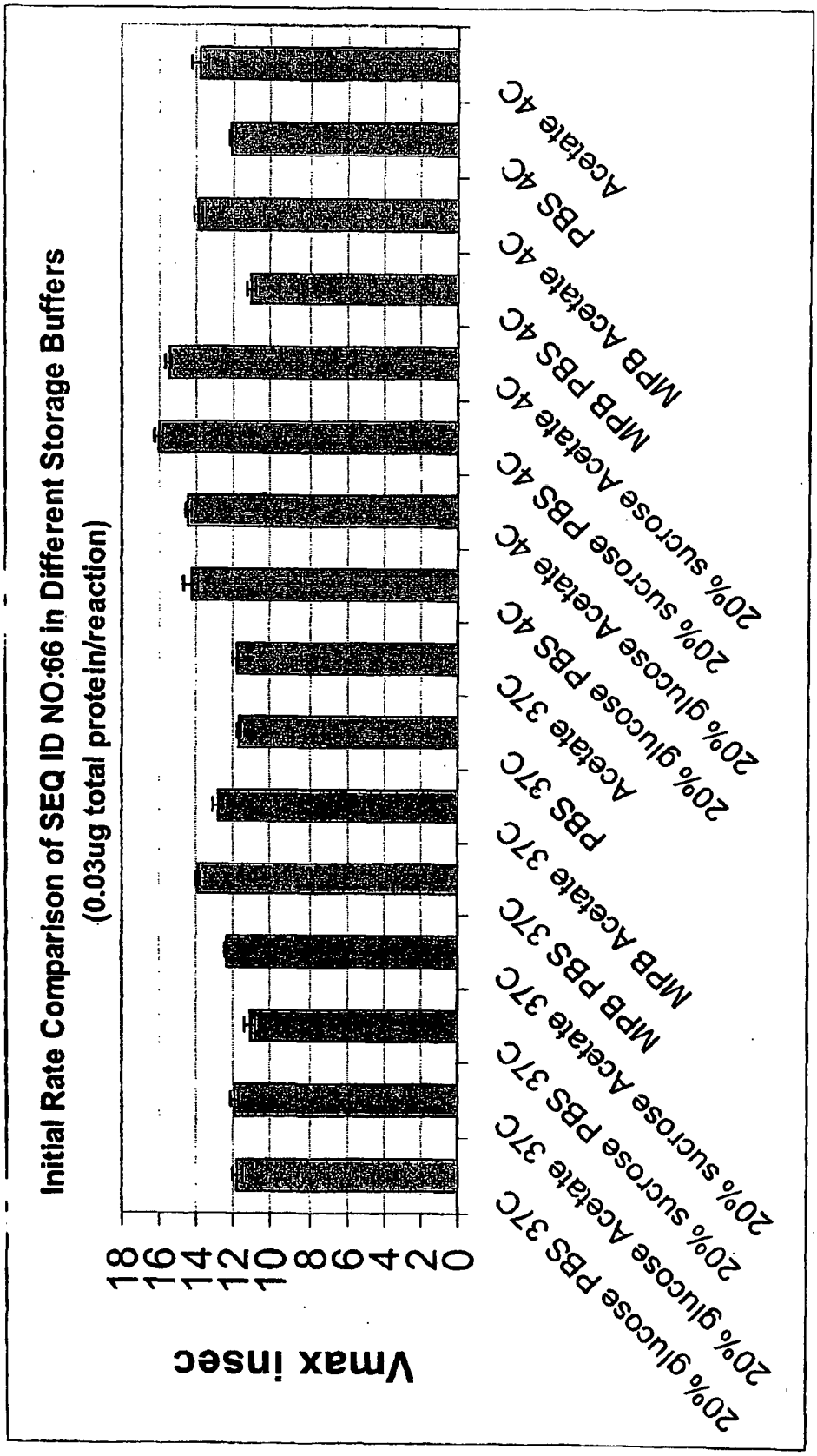

Initial Rate Comparison of SEQ ID NO:66 in Different Storage Buffers
(0.03ug total protein/reaction)
Vmax inrec
18
16
14
12
10
8
6
4
2
0
20% glucose PBS 37C
20% glucose Acetate 37C
20% sucrose PBS 37C
20% sucrose Acetate 37C
MPB PBS 37C
MPB Acetate 37C
PBS 37C
Acetate 37C
20% glucose PBS 4C
20% glucose Acetate 4C
20% sucrose PBS 4C
20% sucrose Acetate 4C
MPB PBS 4C
MPB Acetate 4C
PBS 4C
Acetate 4C

Figure 17

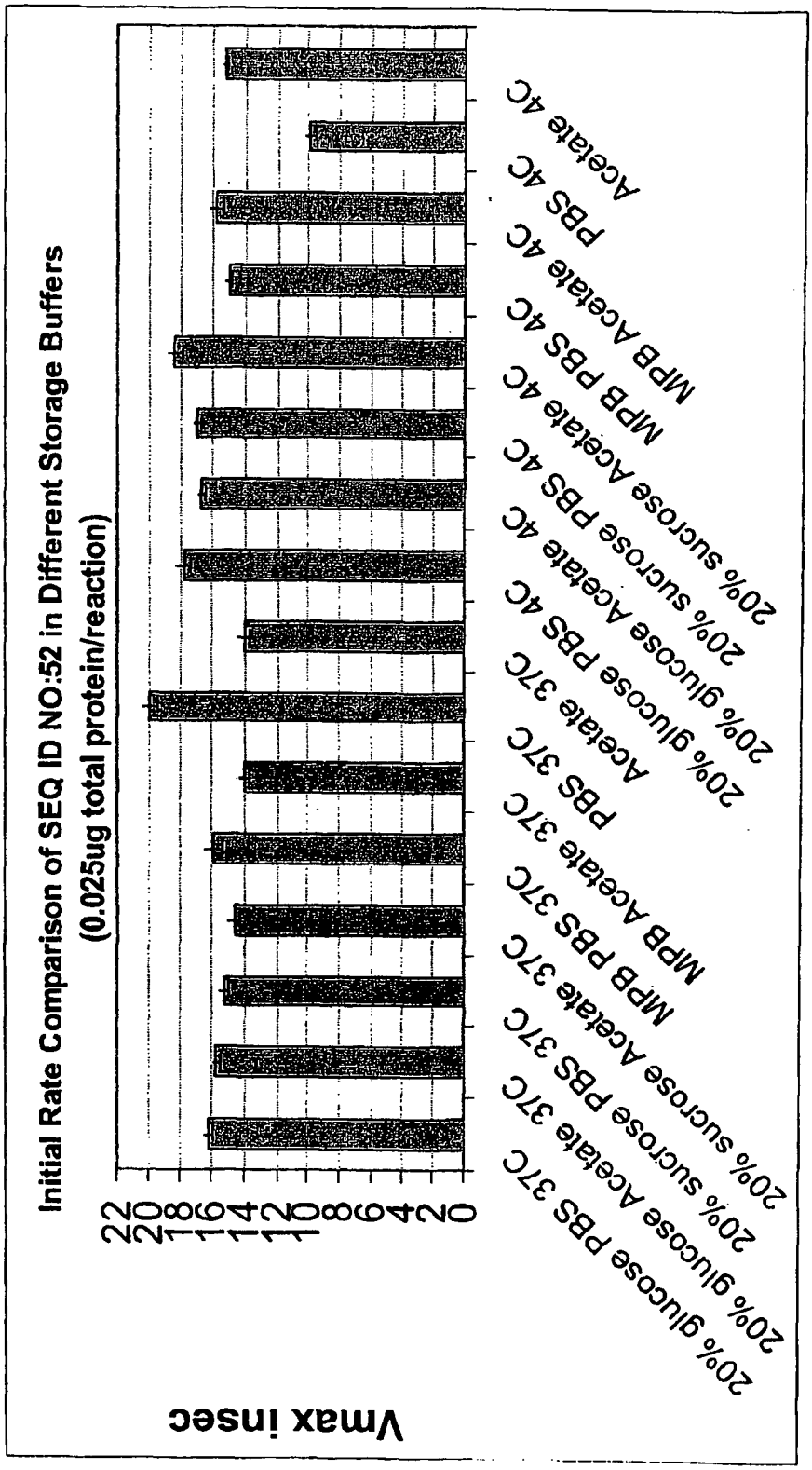

Initial Rate Comparison of SEQ ID NO:52 in Different Storage Buffers
(0.025ug total protein/reaction)
Vmax in sec
22
20
18
16
14
12
10
8
6
4
2
0
20% glucose PBS 37C
20% glucose Acetate 37C
20% sucrose PBS 37C
20% sucrose Acetate 37C
MPB PBS 37C
MPB Acetate 37C
PBS 37C
Acetate 37C
20% glucose PBS 4C
20% glucose Acetate 4C
20% sucrose PBS 4C
20% sucrose Acetate 4C
MPB PBS 4C
MPB Acetate 4C
PBS 4C
Acetate 4C

Figure 18

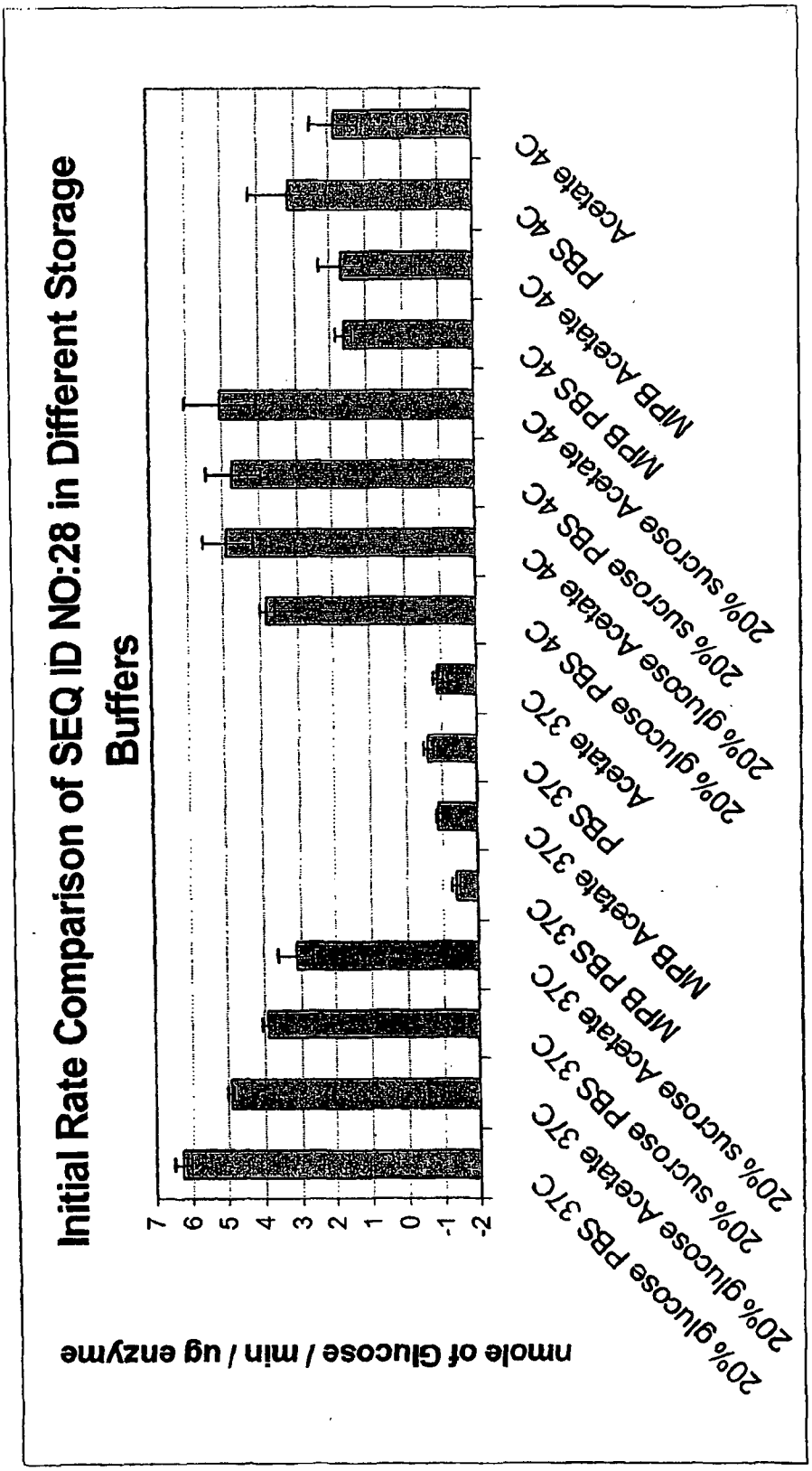

Initial Rate Comparison of SEQ ID NO:28 in Different Storage Buffers
nmole of Glucose / min / ug enzyme
7
6
5
4
3
2
1
0
-1
-2
20% glucose PBS 37C
20% glucose Acetate 37C
20% sucrose PBS 37C
20% sucrose Acetate 37C
MPB PBS 37C
MPB Acetate 37C
PBS 37C
Acetate 37C
20% glucose PBS 4C
20% glucose Acetate 4C
20% sucrose PBS 4C
20% sucrose Acetate 4C
MPB PBS 4C
MPB Acetate 4C
PBS 4C
Acetate 4C

Figure 19

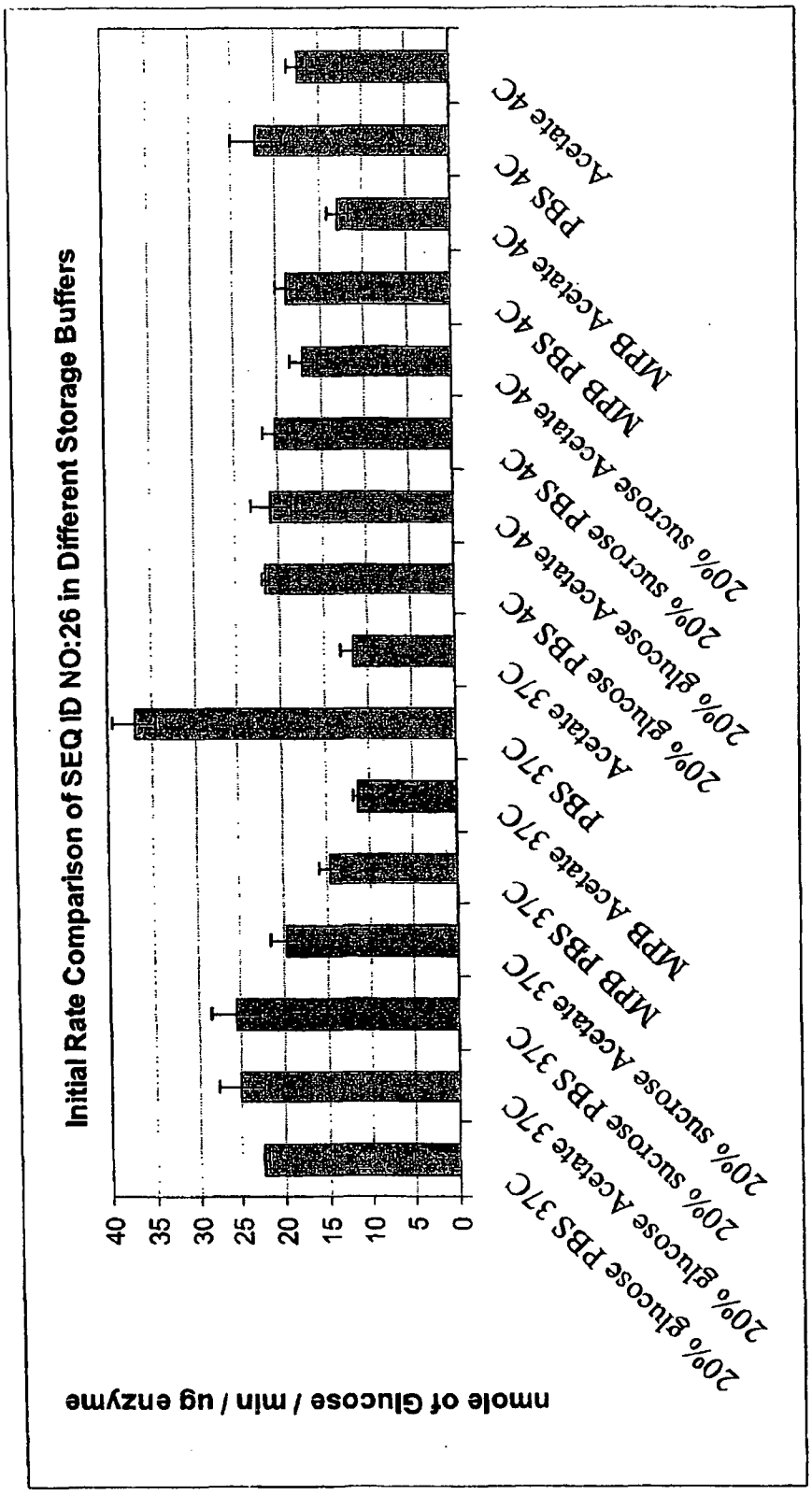

Initial Rate Comparison of SEQ ID NO:26 in Different Storage Buffers
nmole of Glucose / min / ug enzyme
0
5
10
15
20
25
30
35
40
20% glucose PBS 37C
20% glucose Acetate 37C
20% sucrose PBS 37C
20% sucrose Acetate 37C
MPB PBS 37C
MPB Acetate 37C
PBS 37C
Acetate 37C
20% glucose PBS 4C
20% glucose Acetate 4C
20% sucrose PBS 4C
20% sucrose Acetate 4C
MPB PBS 4C
MPB Acetate 4C
PBS 4C
Acetate 4C

Figure 20

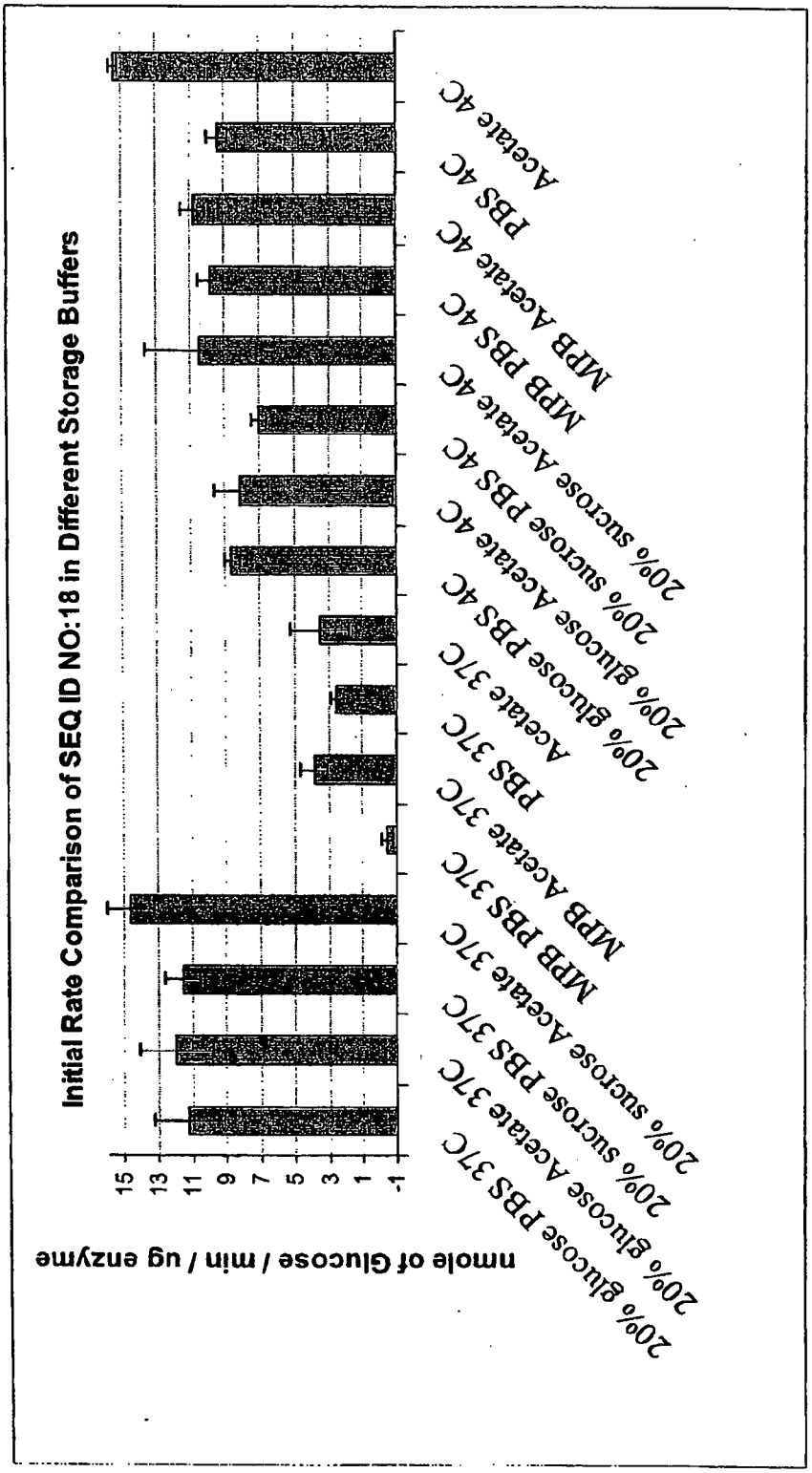

Initial Rate Comparison of SEQ ID NO:18 in Different Storage Buffers
nmole of Glucose / min / ug enzyme
15
13
11
9
7
5
3
1
-1
20% glucose PBS 37C
20% glucose Acetate 37C
20% sucrose PBS 37C
20% sucrose Acetate 37C
MPB PBS 37C
MPB Acetate 37C
PBS 37C
Acetate 37C
20% glucose PBS 4C
20% glucose Acetate 4C
20% sucrose PBS 4C
20% sucrose Acetate 4C
MPB PBS 4C
MPB Acetate 4C
PBS 4C
Acetate 4C

Figure 21

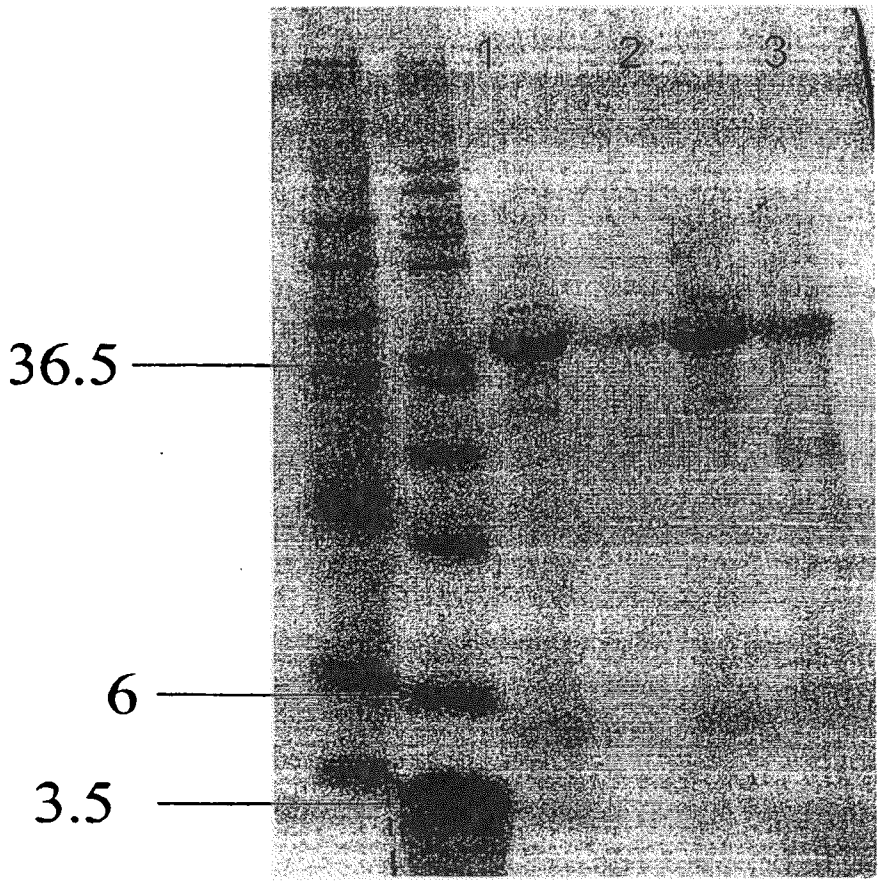


Figure 22

Trypsin digested LC MS\MS peptide identification

>SEQ ID NO:52 66kD pH1.3 pepsin cut
MKWTFSLLLLLSVFGQATHALTPAEWRSQSIYFLLTDRFGRTDNSTTAACDTTDRVYCGGSWQGIINHLDYIQGMGFTAI
WITPVTGQFYENTGDGTSYHGYWQQDIYDLNYNYGTAQDLKNLASALHERGMYLMVDVVANHMGYDGAGNTVDYSVF
NPFSSSSYFHPYCLISNYDNQTNVEDCWLGDTTVSLPDLDTTSTAVRDIWYDWVADLVANYSIDGLRVDTVKHVEKDFW\
PDYNSAAGVYCVGEVFSGDPAYTCPYQNYMDGVLNYPIYYQLLYAFESSSGSISDLYNMISSVASSCKDPTLLGNFIENH
DNPRFASYTSDYSQAKNVITFIFLSDGIPIVYAGQEQHYSGGSDPANREATWLSGYSTSATLYTWIASTNQIRSLAISKDAG
YVQAKNNPFYSDSNTIAMRKGTTAGAQVITVLSNKGASGSSYTLSLSGTGYSAGATLVETYTCTTVTVDSSGNLPVPMTS
GLPRVFVPSSWVNGSALCNTECTAATSLPVLEEELVTTTYGENIYLSGSISQLGSWNTASAVALSASQYTSSNPKWYVSV
TL***PVGTSFQYKFI*** **KKGSDGSVVWESDPNRSYTVPAGCEGATVTVADTWR**

5095 Da

N-linked Glycosylation site

3938.8977	571-607	0	IKKGSDGSVVWESDPNRSYT VPAGCEGATVTVADTWR
3212.4483	40-69	0	GRTDNSTTAACDTTDRVYCG GSWQGIINHL
3118.4500	341-369	0	SDGIPIVYAGQEQHYSGGSD PANREATWL
2525.0528	90-110	0	YENTGDGTSYHGYWQQDIYD L
2461.0798	135-157	0	MVDVVANHMGYDGAGNTVDY SVF
2397.2442	408-430	0	YSDSNTIAMRKGTTAGAQVI TVL

Figure 23A

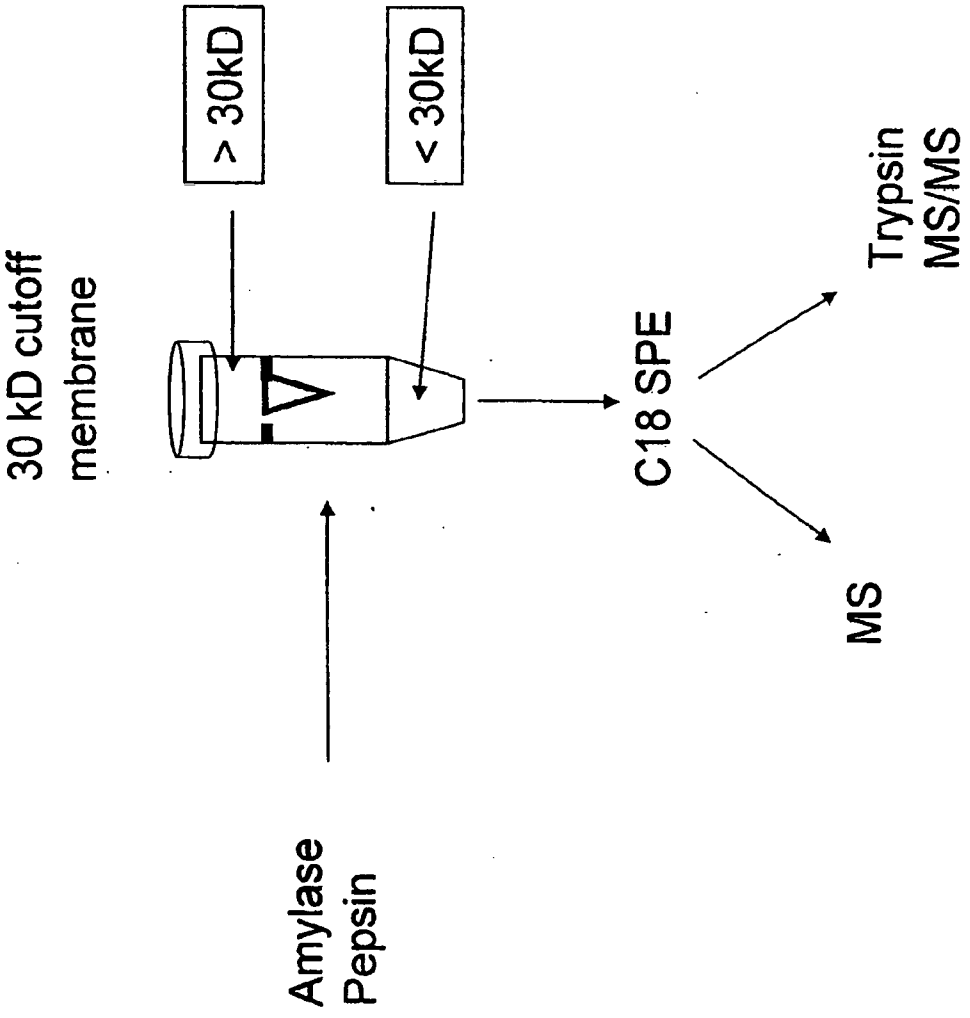
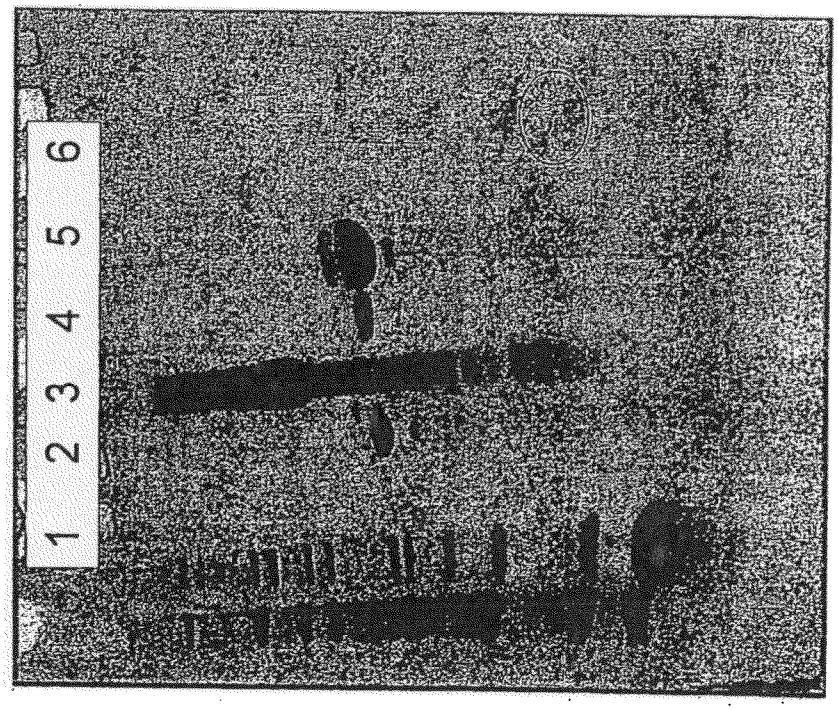

30 kD cutoff membrane
> 30kD
< 30kD
Amylase
Pepsin
C18 SPE
Trypsin
MS/MS
MS

Figure 23B



1) Pepsin
2) Amylase SEQ ID NO:52
3) SEQ ID NO:52 Pepsin
4) 30kd cut off top
5) 30kd flow through
6) C18 RP captured

FIGURE 23(C)

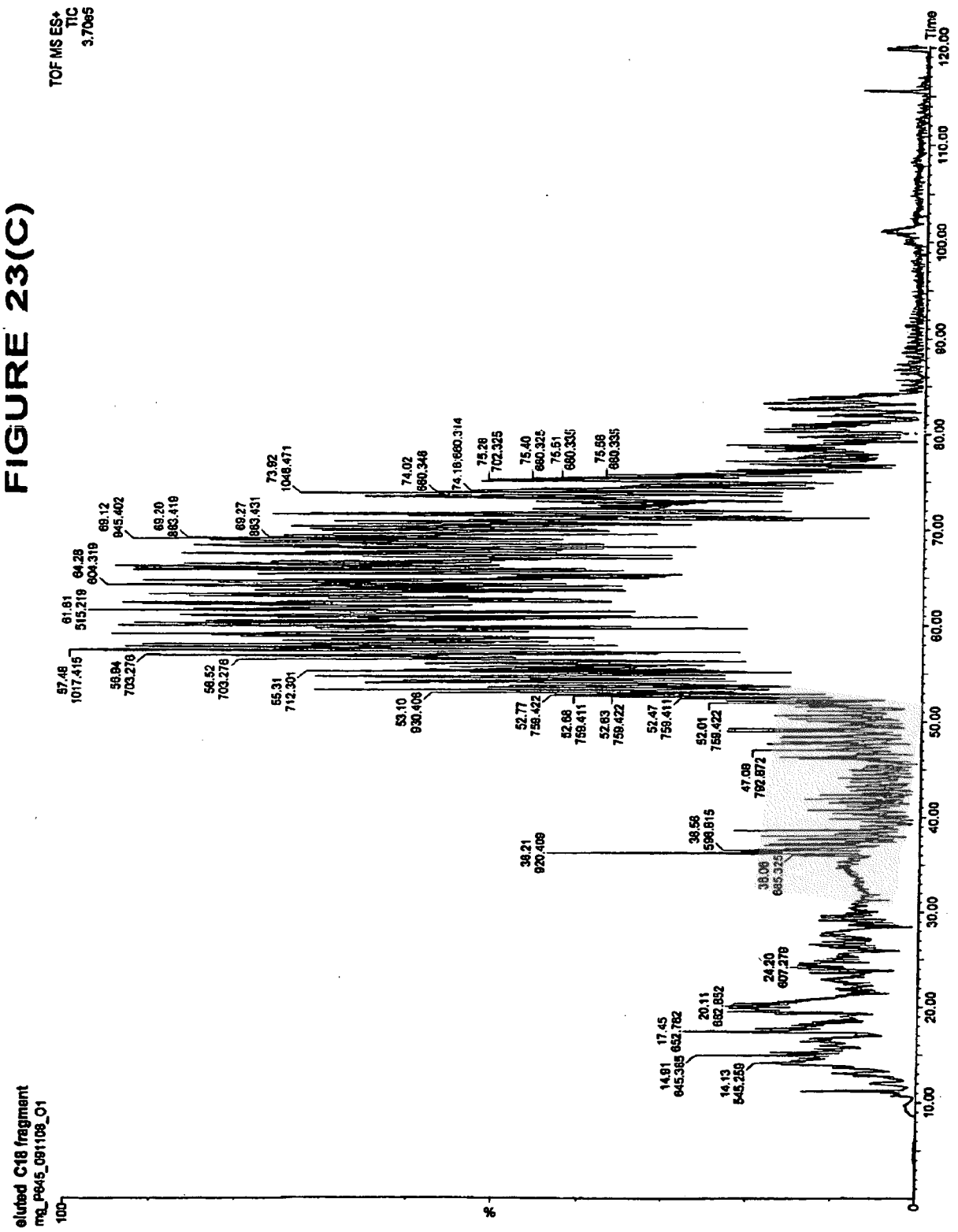

TOF MS ES+
TIC
3.70e5
eluted C18 fragment
mg_P645_091108_01
100
%
0
Time
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
110.00
120.00
57.48
1017.415
56.84
703.276
56.52
703.278
55.31
712.301
53.10
930.406
52.77
759.422
52.68
759.411
52.63
759.422
52.47
759.411
52.01
759.422
61.81
515.219
64.28
604.319
69.12
945.402
69.20
883.416
69.27
883.431
73.92
1048.471
74.02
660.346
74.18
660.314
75.28
702.325
75.40
660.325
75.51
660.335
75.68
660.335
47.09
792.872
38.56
598.815
38.21
920.409
38.08
685.325
24.20
607.279
20.11
682.852
17.45
632.782
14.91
645.365
14.13
645.259

Figure 23D

>SEQ ID NO:52 66kD pH1.3 pepsin cut
MKWTFSLLLLLSVFGQATHALTPAEWRSQSIYFLLTDRFGRTDNSTTAACDTTDRVYCGGSWQGIINHLDYIQGMGFTAI
WITPVTGQFYENTGDGTSYHGYWQQDIYDLNYNYGTAQDLKNLASALHERGMYLMVDVVANHMGYDGAGNTVDYSVF
NPFSSSSYFHPYCLISNYDNQTNVEDCWLGDTTVSLPDLDTTSTAVRDIWYDWVADLVANYSIDGLRVDTVKHVEKDFWA
PDYNSAAGVYCVGEVFSGDPAYTCPYQNYMDGVLNYPIYYQLLYAFESSSGSISDLYNMISSVASSCKDPTLLGNFIENH
DNPRFASYTSDYSQAKNVITFIFLSDGIPIVYAGQEQHYSGGSDPANREATWLSGYSTSATLYTWIASTNQIRSLAISKDAG
YVQAKNNPFYSDSNTIAMRKGTTAGAQVITVLSNKGASGSSYTLSLSGTGYSAGATLVETYTCTTVTVDSSGNLPVPMTS
GLPRVFVPSSWVNGSALCNTECTAATSLPVLFEELVTTTYGENIYLSGSISQLGSWNTASAVALSASQYTSSNPKWYVSV
TLPVGTSFQYKFIKKGSDGSVVWESDPNRSYTVPAGCEGATVTVADTWR

3938.8977	571-607	0		IKKGSDGSVVWESDPNRSYT VPAGCEGATVTVADTWR
3212.4483	40-69	0		GRTDNSTTAACDTTDRVYCG GSWQGIINHL
3118.4500	341-369	0		SDGIPIVY*AGQEQHYSGGSD PANR*EATWL
2525.0528	90-110	0		YENTGDGTSYHGYWQQDIYD L
2461.0798	135-157	0		MVDVVANHMGYDGAGNTVDY SVF
2397.2442	408-430	0		YSDSNTIAMRKGTTAGAQVI TVL

Figure 23E

Asn-Xaa-Ser/Thr sequons in the sequence output below are highlighted in blue.
Asparagines predicted to be N-glycosylated are highlighted in red.

putative glycosylated site

Output for 'Sequence'

SEQ ID NO:52

Name: Sequence Length: 607

MKWTFSLLLLLSVFGQATHALTPAEWRSQSIYFLLTDRFGRTDNSTTAACDTTDRVYCGGSWQGIINHLDYIQGMGFTAI
WITPVTGQFYENTGDGTSYHGYWQQDIYDLNYNYGTAQDLKNLASALHERGMYLMVDVVANHMGYDGAGNTVDYSVFNPF
SSSSYFHPYCLISNYDNQTNVEDCWLGDTTVSLPDLDTTSTAVRDIWYDWVADLVANYSIDGLRVDTVKHVEKDFWPDYN
SAAGVYCVGEVFSGDPAYTCPYQNYMDGVLNYPIYYQLLYAFESSSGSISDLYNMISSVASSCKDPTLLGNFIENHDNPR
FASYTSDYSQAKNVITFIFLSDGIPIVYAGQEQHYSGGSDPANREATWLSGYSTSATLYTWIASTNQIRSLAISKDAGYV
QAKNNPFYSDSNTIAMRKGTTAGAQVITVLSNKGASGSSYTLSLSGTGYSAGATLVETYTCTTVTVDSSGNLPVPMTSGL
PRVFVPSSWVNGSALCNTECTAATSLPVLFEELVTTTYGENIYLSGSISQLGSWNTASAVALSASQYTSSNPKWYVSVTL
PVGTSFQYKFIKKGSDGSVVWESDPNRSYTVPAGCEGATVTVADTWR

Figure 23F

SEQ ID NO:52

Name: Sequence Length: 607

MKWTFSLLLLLSVFGQATHALTPAEWRSQSIYFLLTDRFGRTDNSTTAACDTTDRVYCGGSWQGIINHLDYIQGMGFTAI
WITPVTGQFYENTGDGTSYHGYWQQDIYDLNYNYGTAQDLKNLASALHERGMYLMVDVVANHMGYDGAGNTVDYSVFHPF
SSSSYFHPYCLISNYDNQTNVEDCWLGDTTVSLPDLDTTSTAVRDIWYDWVADLVANYSIDGLRVDTVKHVEKDFWPDYN
SAAGVYCVGEVFSGDPAYTCPYQNYMDGVLNYPIYYQLLYAFESSSGSISDLYNMISSVASSCKDPTLLGNFIENHDNPR
FASYTSDYSQAKNVITFIFLSDGIPIVYAGQEQHYSGGSDPANREATWLSGYSTSATLYTWIASTNQIRSLAISKDAGYV
QAKNNPFYSDSNTIAMRKGTTAGAQVITVLSNKGASGSSYTLSLSGTGYSAGATLVETYTCTTVTVDSSGNLPVPMTSGL
PRVFVPSSWVNGSALCNTECTAATSLPVLFEELVTTTYGENIYLSGSISQLGSWNTASAVALSASQYTSSNPKWYVSVTL
PVGTSFQYKFIKKGSDGSVVWESDPNRSYTVPAGCEGATVTVADTWR

EP 2 479 267 B1

Figure 24

Enzyme	Initial rate* ± SD Granular starch	Initial rate* ± SD Soluble starch
SEQ ID NO:48	35.6 ± 3.8	60.7 ± 5.1
SEQ ID NO:26	28.8 ± 3.4	51.8 ± 7.4
SEQ ID NO:74	25.1 ± 2.5	84.3 ± 3.5
SEQ ID NO:18	24.3 ± 4.3	58.3 ± 3.0
SEQ ID NO:28	17.8 ± 4.3	33.8 ± 4.0
SEQ ID NO:14	6.6 ± 1.2	53.9 ± 4.1
SEQ ID NO:20	0	59.3 ± 8.5
A.niger	11.3 ± 2.7	43.3 ± 7.4

Figure 25

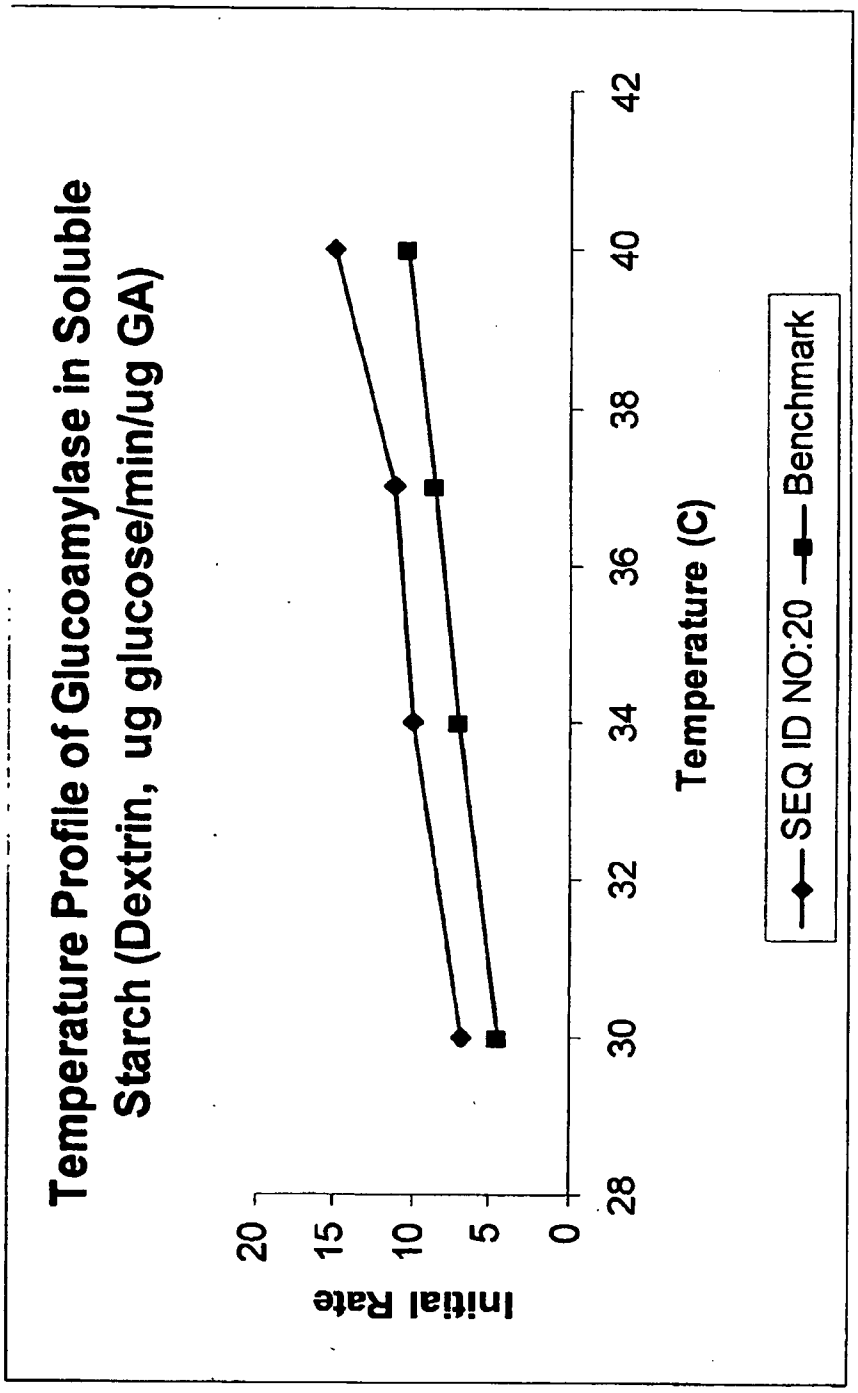

Temperature Profile of Glucoamylase in Soluble Starch (Dextrin, ug glucose/min/ug GA)
Initial Rate
0
5
10
15
20
28
30
32
34
36
38
40
42
Temperature (C)
SEQ ID NO:20
Benchmark

Figure 26

Summary of Purity				
	Before Purification		After Purification	
	A260/A280	Expression Ratio	A260/A280	Expression Ratio
SEQ ID NO:18	0.52	91%	0.59	92%
SEQ ID NO:26	0.56	85%	0.65	86%
SEQ ID NO:4	0.67	91%	0.73	85%

Summary of Activity				
	Raw Starch		Soluble	
	Purified	Unpurified	Purified	Unpurified
SEQ ID NO:18	10.7228 ±0.7925	6.4828 ± 0.5681	40.6725 ± 1.7016	24.7354 ± 3.821
SEQ ID NO:26	5.7837 ± 0.2271	4.0691 ± 0.2864	17.4833 ± 0.7831	14.1849 ± 0.3761
SEQ ID NO:4	6.47 ± 0.34	4.074 ± 0.35	127.37 ± 1.78	116.52 ± 1.27

FIGURE 27

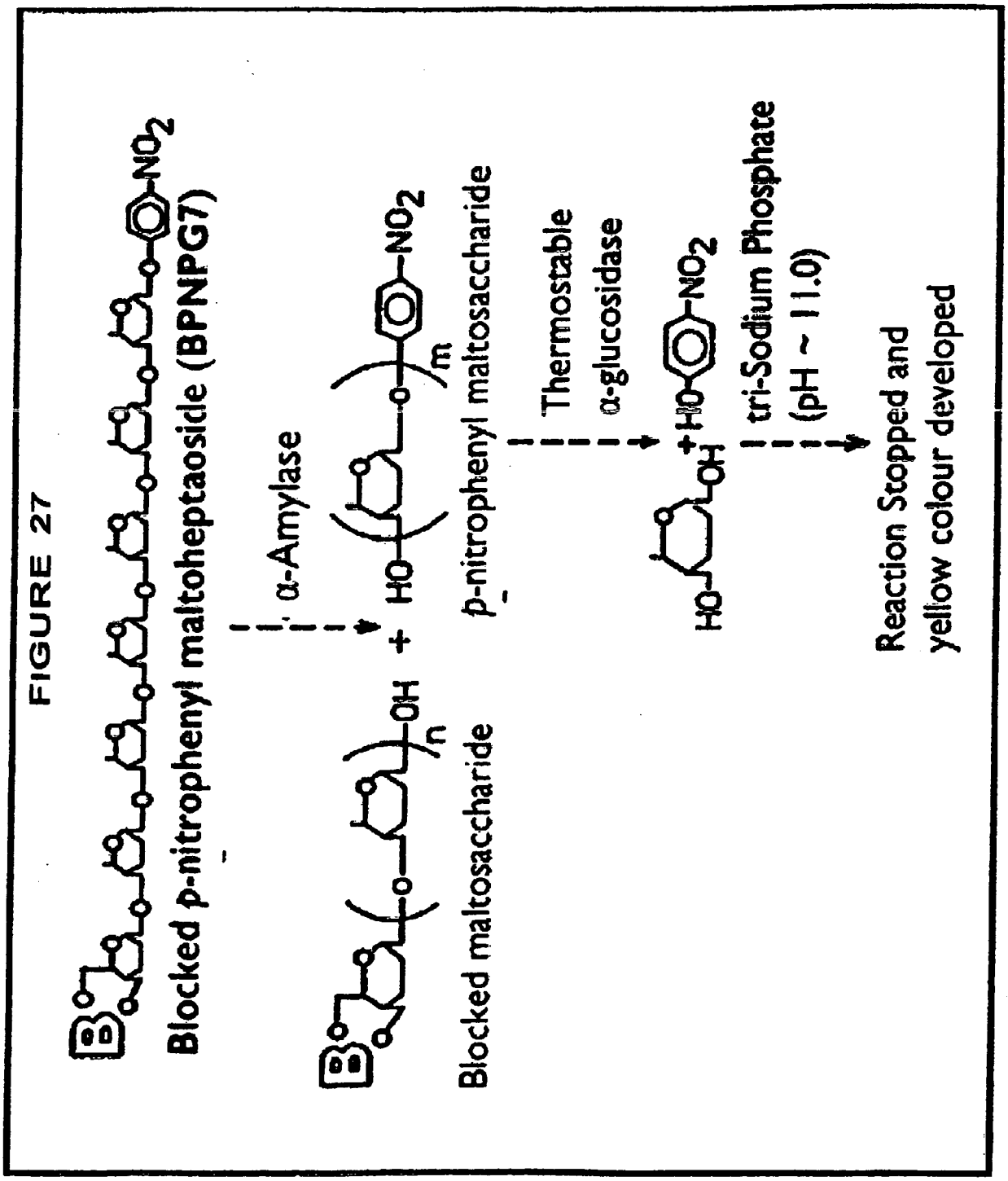

Blocked p-nitrophenyl maltoheptaoside (BPNPG7)
α-Amylase
Blocked maltosaccharide
p-nitrophenyl maltosaccharide
Thermostable
α-glucosidase
tri-Sodium Phosphate
(pH ~ 11.0)
Reaction Stopped and
yellow colour developed

Fig. 28A

Temperature Profile of Glucoamylases on Granular Starch – Initial Rate (ug glucose/min/ug glucoamylase)

Temperature (°C)	SEQ ID NO:74	SEQ ID NO:28	SEQ ID NO:26	Benchmark	SEQ ID NO:14	SEQ ID NO:18	SEQ ID NO:48	SEQ ID NO:20
30	3.04	1.64	3.12	1.4	0.96	3.92	4.6	inactive
34	3.92	2.24	4.36	1.56	1.08	5.2	5.64	inactive
37	4.36	2.76	5.16	1.84	1.36	5.24	6.84	inactive
40	5.12	3.32	5.92	1.84	1.44	5.72	7.84	inactive

Fig. 28B

Temperature Profile of Glucoamylases on Soluble Starch (Dextrin) – Initial Rate (ug glucose/min/ug glucoamylase)

Temperature (°C)	SEQ ID NO:20	Benchmark
30	6.840	4.380
34	9.920	6.980
37	11.080	8.600
40	15.040	10.380

Figure 29

Fig. 29A

pH Profile of Glucoamylases on Granular Starch – Percent Relative Activity

pH	SEQ ID NO:26	SEQ ID NO:74	SEQ ID NO:18	SEQ ID NO:28	SEQ ID NO:14	SEQ ID NO:48	Benchmark	SEQ ID NO:20
3.5	100.0000	78.6207	98.1308	88.1720	60.6061	99.3151	100.0000	inactive
4.0	88.1081	100.0000	100.0000	100.0000	66.6667	99.3151	100.0000	inactive
5.0	67.0270	91.0345	87.8505	91.3978	66.6667	100.0000	100.0000	inactive
6.0	43.2432	84.8276	79.4393	88.1720	100.0000	78.0822	40.0000	inactive
7.0	16.2162	37.2414	38.3178	61.2903	45.4545	34.2466	18.6667	inactive

Fig. 29B

pH Profile of Glucoamylases on Soluble Starch (Dextrin) – Percent Relative Activity

pH	SEQ ID NO:74	SEQ ID NO:14	SEQ ID NO:18	SEQ ID NO:28	SEQ ID NO:20	SEQ ID NO:26	SEQ ID NO:48	Benchmark
3.5	83.4997	72.1981	81.9000	79.4340	91.6548	98.2773	91.3527	91.3255
4.0	85.6287	76.7159	88.3000	91.1321	98.7874	100.0000	89.8116	100.0000
5.0	90.3526	83.0582	100.0000	100.0000	100.0000	85.4799	100.0000	80.8967
6.0	100.0000	100.0000	80.9000	98.6792	72.9672	46.4315	78.5103	67.9337
7.0	75.5156	81.2337	60.6922	68.8679	29.8859	11.8130	29.1952	18.3236

Figure 30

Temperature Profile of Amylases on Granular Starch – Percent Relative Activity

Temp. (°C)	SEQ ID NO:56	SEQ ID NO:70	SEQ ID NO:62	SEQ ID NO:66	SEQ ID NO:2	SEQ ID NO:52	SEQ ID NO:78	SEQ ID NO:76	Sigma-A6211	Megazyme E-ANAAM
30	6.44	0.82	2.24	0.26	6.05	8.99	0.14	1.26	0.42	0.167432
34	10.98	1.29	3.26	0.47	6.97	11.21	0.27	2.20	0.49	0.229423
37	14.61	1.88	3.25	0.75	7.46	11.53	0.36	2.85	0.55	0.307667
40	17.66	2.36	3.11	1.27	6.93	11.40	0.51	3.26	0.54	0.29692

Figure 31

Fig. 31A

pH Profile of Amylases on Granular Starch – Percent Relative Activity

pH	SEQ ID NO:56	SEQ ID NO:70	SEQ ID NO:62	SEQ ID NO:66	SEQ ID NO:2	SEQ ID NO:52	SEQ ID NO:78	SEQ ID NO:76	Sigma-A6211	Megazyme E-ANAAM
3.5	0.00	3.69	0.00	24.31	31.59	89.70	6.89	6.87	72.06	51.25
4.0	0.00	3.88	0.00	70.83	86.95	100.00	9.85	9.33	94.34	74.69
5.0	93.07	45.60	50.20	100.00	100.00	96.97	87.70	72.80	100.00	100.00
6.0	100.00	100.00	100.00	97.10	95.08	76.59	100.00	100.00	78.16	62.43
7.0	92.52	99.12	85.70	64.29	79.10	43.67	61.30	71.84	71.04	47.70

Fig. 31B

pH Profile of Amylases on Soluble Starch – Percent Relative Activity

pH	SEQ ID NO:86	SEQ ID NO:70	SEQ ID NO:62	SEQ ID NO:66	SEQ ID NO:2	SEQ ID NO:52	SEQ ID NO:78	SEQ ID NO:76	Sigma-A6211	Megazyme E-ANAAM
3.5	0.00	2.23	0.00	11.00	51.35	100.00	1.22	1.25	38.55	46.04
4.0	6.94	2.57	0.00	47.94	89.60	96.63	18.89	3.62	63.72	60.97
5.0	74.57	37.01	35.90	100.00	100.00	83.16	84.63	83.03	100.00	100.00
6.0	97.83	65.88	93.03	86.33	94.27	60.31	100.00	100.00	57.80	50.62
7.0	100.00	100.00	100.00	56.96	87.91	52.17	66.50	27.79	11.53	13.24

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO 03012071 A2 **[0008]**
- US 4458066 A **[0137]**
- US 5721118 A **[0139]**
- US 6025155 A **[0139]**
- US 4962028 A **[0147]**
- US 5633440 A **[0147]**
- US 5608148 A, John **[0150] [0339]**
- US 5602321 A **[0150]**
- US 5589583 A, Klee **[0150]**
- EP 0385962 A, Monsanto **[0165]**
- EP 0359472 A, Lubrizol **[0165]**
- WO 9307278 A, Ciba-Geigy **[0165]**
- EP 255378 A **[0173]**
- US 7102057 B **[0173] [0185]**
- US 5625136 A **[0173]**
- US 4943674 A **[0174]**
- US 4535060 A **[0174]**
- US 4769061 A **[0174]**
- US 4801590 A **[0174]**
- US 5107065 A **[0174]**
- US 20070006346 A **[0177]**
- US 5589379 A **[0178]**
- WO 9627673 A **[0180]**
- WO 9301294 A **[0180]**
- WO 9403619 A **[0180]**
- WO 02061102 A **[0180]**
- WO 0152620 A **[0180]**
- US 5641876 A **[0181]**
- US 5639949 A **[0181] [0185]**
- WO 2005096704 A **[0185] [0335]**
- US 5217879 A **[0190]**
- US 5750870 A **[0201]**
- US 4683195 A, Sambrook; Ausubel **[0212]**
- US 4683202 A **[0212]**
- WO 9703211 A **[0262]**
- WO 9639154 A **[0262]**
- US 4987071 A, Cech **[0266]**
- US 6506559 B **[0267]**
- US 6511824 B **[0267]**
- US 6515109 B **[0267]**
- US 6489127 B **[0267]**
- US 6361974 B **[0269] [0276] [0297] [0301]**
- US 5830696 A **[0269]**
- US 6291242 B **[0270]**
- US 6287862 B **[0270]**
- US 6287861 B **[0270]**
- US 5955358 A **[0270]**
- US 5830721 A **[0270] [0274]**
- US 5824514 A **[0270]**
- US 5811238 A **[0270] [0274]**
- US 5605793 A **[0270] [0274]**
- US 5834252 A, Stemmer **[0274]**
- US 5837458 A, Minshull **[0274]**
- WO 9522625 A, Stemmer and Crameri **[0274]**
- WO 9633207 A, Stemmer and Lipschutz **[0274]**
- WO 9720078 A, Stemmer and Crameri **[0274]**
- WO 9735966 A, Minshull and Stemmer **[0274]**
- WO 9941402 A, Punnonen **[0274]**
- WO 9941383 A, Punnonen **[0274]**
- WO 9941369 A, Punnonen **[0274]**
- WO 9941368 A, Punnonen **[0274]**
- EP 752008 A, Stemmer and Crameri **[0274]**
- EP 0932670 A **[0274]**
- WO 9923107 A, Stemmer **[0274]**
- WO 9921979 A, Apt **[0274]**
- WO 9831837 A, del Cardayre **[0274]**
- WO 9827230 A, Patten and Stemmer **[0274]**
- WO 0000632 A **[0274]**
- WO 0009679 A **[0274]**
- WO 9842832 A, Arnold **[0274]**
- WO 9929902 A, Arnold **[0274]**
- WO 9841653 A, Vind **[0274]**
- WO 9841622 A, Borchert **[0274]**
- WO 9842727 A, Pati and Zarling **[0274]**
- US 09407800 B **[0275]**
- US 6379964 B **[0275]**
- US 6319714 B **[0275]**
- US 6368861 B **[0275]**
- US 6376246 B **[0275]**
- US 6423542 B **[0275]**
- US 6426224 B **[0275]**
- US 0001203 W **[0275]**
- US 6436675 B **[0275]**
- US 0001202 W **[0275]**
- US 09618579 B **[0275]**
- US 0001138 W **[0275]**
- US 09656549 B **[0275]**
- US 6177263 B **[0275]**
- US 6153410 A **[0275]**
- US 6280926 B **[0276]**
- US 5939250 A **[0276] [0317]**
- US 6171820 B **[0277]**
- US 6579258 B **[0277]**
- US 6773900 B **[0284] [0297] [0301]**
- US 6740506 B **[0284] [0297] [0301]**
- US 6713282 B **[0284] [0297] [0301]**
- US 6635449 B **[0284] [0297] [0301]**
- US 6605449 B **[0284] [0297] [0301]**

- US 6537776 B **[0284] [0297] [0301]**
- US 5965408 A **[0311] [0317]**
- WO 9116427 A **[0313]**
- US 5795737 A **[0325]**
- US 6211428 B **[0327]**
- US 6187992 B **[0327]**
- US 6156952 A **[0327]**
- US 6118044 A **[0327]**
- US 6111166 A **[0327]**
- US 6107541 A **[0327]**
- US 5959171 A **[0327]**
- US 5922854 A **[0327]**
- US 5892070 A **[0327]**
- US 5880327 A **[0327]**
- US 5891698 A **[0327]**
- US 5639940 A **[0327]**
- US 5573933 A **[0327]**
- US 5387742 A **[0327]**
- US 5087571 A **[0327]**
- US 20060195940 A **[0329]**
- US 6309872 B **[0330]**
- WO 9811235 A **[0335]**
- WO 200318766 A **[0335]**
- US 4940935 A **[0336]**
- US 5188642 A **[0336]**
- US 5767378 A **[0337]**
- US 5994629 A **[0337]**
- US 5015580 A **[0339]**
- US 5681730 A, Ellis **[0339]**
- US 5712135 A **[0344]**
- US 9409174 W **[0388]**
- US 6277628 B **[0412]**
- US 6277489 B **[0412]**
- US 6261776 B **[0412]**
- US 6258606 B **[0412]**
- US 6054270 A **[0412]**
- US 6048695 A **[0412]**
- US 6045996 A **[0412]**
- US 6022963 A **[0412]**
- US 6013440 A **[0412]**
- US 5965452 A **[0412]**
- US 5959098 A **[0412]**
- US 5856174 A **[0412]**
- US 5830645 A **[0412]**
- US 5770456 A **[0412]**
- US 5632957 A **[0412]**
- US 5556752 A **[0412]**
- US 5143854 A **[0412]**
- US 5807522 A **[0412]**
- US 5800992 A **[0412]**
- US 5744305 A **[0412]**
- US 5700637 A **[0412]**
- US 5434049 A **[0412]**
- WO 9951773 A **[0412]**
- WO 9909217 A **[0412]**
- WO 9746313 A **[0412]**
- WO 9617958 A **[0412]**
- US 20010018642 A **[0412]**
- US 20010019827 A **[0412]**
- US 20010016322 A **[0412]**
- US 20010014449 A **[0412]**
- US 20010014448 A **[0412]**
- US 20010012537 A **[0412]**
- US 20010008765 A **[0412]**
- US 4946778 A **[0422]**
- US 6057103 A **[0432]**
- US 6413928 B **[0434]**
- US 6399561 B **[0434]**
- US 6365561 B **[0434]**
- US 6380147 B **[0434]**
- US 6333301 B **[0438]**
- US 6329333 B **[0438]**
- US 6326341 B **[0438]**
- US 6297038 B **[0438]**
- US 6309871 B **[0438] [0482]**
- US 6204232 B **[0438]**
- US 6197070 B **[0438]**
- US 5856164 A **[0438]**
- US 6077316 A **[0439]**
- US 6147206 A **[0471]**
- US 6197352 B **[0473]**
- US 2615810 A **[0473]**
- US 3026205 A **[0473]**
- US 6241849 B **[0479]**
- US 6066233 A **[0479]**
- US 5582681 A **[0479]**
- WO 9514807 A **[0481]**
- US 5709796 A **[0483]**
- US 6264925 B **[0485]**
- US 5762991 A **[0488]**
- US 5536650 A **[0488]**
- US 5405624 A **[0488]**
- US 5021246 A **[0488]**
- US 4788066 A **[0488]**
- US 6581687 B **[0493]**
- US 20070089910 A, Hewson **[0513]**
- US 20070084638 A, Bohnsack; C. **[0514]**
- US 20070081157 A, Csutak; S. **[0515]**
- US 20070075706 A, Chen, S. **[0516]**
- US 20070068675 A, Barry, M. **[0517]**
- US 20070066491 A, Bicerano; J. **[0518]**
- US 20070039735 A, Robertson; B. **[0519]**
- US 20070027036 A, Polizzotti; R. **[0520]**
- US 4099583 A **[0525]**
- US 6530437 B **[0526]**
- US 6588501 B **[0526]**
- US 6422326 B **[0526]**
- US 6156708 A **[0526]**
- US 5910467 A **[0526]**
- US 5881826 A **[0526]**
- US 6497289 B **[0527]**
- US 6372901 B **[0542]**
- US 5610225 A **[0542]**
- US 6660506 B **[0549] [0581]**
- US 6423145 B **[0549] [0581]**
- US 5536325 A **[0550] [0582]**

- US 6409841 B **[0551] [0583]**
- US 5705369 A **[0552] [0584]**
- US 6712866 B **[0553] [0585]**
- US 6333181 B **[0554] [0586]**
- US 6090595 A **[0555] [0589]**
- US 20060014260 A **[0587]**
- US 20050069998 A **[0588]**
- US 20020164730 A **[0588]**
- WO 0043496 A **[0591]**
- WO 8100857 A **[0591]**
- US 6776979 B **[0593]**
- US 6468964 B **[0593]**
- US 12152662 B **[0917]**
- US 60892823 B **[0917]**
- US 60877068 B **[0917]**

Non-patent literature cited in the description

- Molecular basis for allelic polymorphism of the maize Globulin-1 gene. **BELANGER.** Genetics. 1991, vol. 129, 863-872 **[0035]**
- **LOPES.** Identification of two opaque2 modifier loci in Quality Protein Maize. *Mol. Gen. Genet.,* 1995, vol. 247, 603-613 **[0035]**
- **TAKAIWA.** Analysis of the 5' flanking region responsible for the endosperm-specific expression of a rice glutelin chimeric gene in transgenic tobacco. *Plant Mol. Biol.,* 1991, vol. 16, 49-58 **[0035]**
- **MATA.** *Toxicol. Appl. Pharmacol.,* 1997, vol. 144, 189-197 **[0129]**
- **STRAUSS-SOUKUP.** *Biochemistry,* 1997, vol. 36, 8692-8698 **[0129]**
- **SAMSTAG.** *Antisense Nucleic Acid Drug Dev,* 1996, vol. 6, 153-156 **[0129]**
- **ADAMS.** *J. Am. Chem. Soc.,* 1983, vol. 105, 661 **[0137]**
- **BELOUSOV.** *Nucleic Acids Res.,* 1997, vol. 25, 3440-3444 **[0137]**
- **FRENKEL.** *Free Radic. Biol. Med.,* 1995, vol. 19, 373-380 **[0137]**
- **BLOMMERS.** *Biochemistry,* 1994, vol. 33, 7886-7896 **[0137]**
- **NARANG.** *Meth. Enzymol.,* 1979, vol. 68, 90 **[0137]**
- **BROWN.** *Meth. Enzymol.,* 1979, vol. 68, 109 **[0137]**
- **BEAUCAGE.** *Tetra. Lett.,* 1981, vol. 22, 1859 **[0137]**
- MOLECULAR CLONING: A LABORATORY MANUAL. Cold Spring Harbor Laboratory, 1989, vol. 1-3 **[0138]**
- CURRENT PROTOCOLS IN MOLECULAR BIOLOGY. John Wiley & Sons, Inc, 1997 **[0138]**
- LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES. Theory and Nucleic Acid Preparation. Elsevier, 1993 **[0138]**
- **ROSENFELD.** *Nat. Genet.,* 1997, vol. 15, 333-335 **[0139]**
- **WOON.** *Genomics,* 1998, vol. 50, 306-316 **[0139]**
- **KERN.** *Biotechniques,* 1997, vol. 23, 120-124 **[0139] [0412]**
- **WILLIAMS.** *Biochemistry,* 1995, vol. 34, 1787-1797 **[0141]**
- **DOBELI.** *Protein Expr. Purif.,* 1998, vol. 12, 404-414 **[0141]**
- **KROLL.** *DNA Cell. Biol.,* 1993, vol. 12, 441-53 **[0141]**
- **HUANG.** *Plant Mol. BioL,* 1996, vol. 33, 125-139 **[0147]**
- **ZHONG.** *Mol. Gen. Genet.,* 1996, vol. 251, 196-203 **[0147]**
- **SOLOCOMBE.** *Plant Physiol.,* 1994, vol. 104, 1167-1176 **[0147]**
- **MARTINEZ.** *J. Mol. Biol,* 1989, vol. 208, 551-565 **[0147]**
- **MANJUNATH.** *Plant Mol. Biol.,* 1997, vol. 33, 97-112 **[0147]**
- **KUMAGAI.** *Proc. Natl. Acad. Sci. USA,* 1995, vol. 92, 1679-1683 **[0148]**
- **VERDAGUER.** *Plant Mol. Biol.,* 1996, vol. 31, 1129-1139 **[0148]**
- **KIRCH.** *Plant Mol. Biol.,* 1997, vol. 33, 897-909 **[0149]**
- **BLAZQUEZ.** *Plant Cell,* 1998, vol. 10, 791-800 **[0150]**
- **CARDON.** *Plant J,* 1997, vol. 12, 367-77 **[0150]**
- **MANDEL.** *Plant Molecular Biology,* 1995, vol. 29, 995-1004 **[0150]**
- **JOHN.** *Proc. Natl. Acad. Sci. USA,* 1997, vol. 89, 5769-5773 **[0150]**
- **DELISLE.** *Int. Rev. Cytol.,* 1990, vol. 123, 39-60 **[0150]**
- **BUSK.** *Plant J.,* 1997, vol. 11, 1285-1295 **[0150]**
- **GUERRERO.** *Mol. Gen. Genet.,* 1990, vol. 224, 161-168 **[0150]**
- **BLUME.** *Plant J.,* 1997, vol. 12, 731-746 **[0150]**
- **FICKER.** *Plant Mol. Biol.,* 1997, vol. 35, 425-431 **[0150]**
- **REISER.** *Cell,* 1995, vol. 83, 735-742 **[0150]**
- **LIU.** *Plant Physiol.,* 1997, vol. 115, 397-407 **[0151] [0179]**
- **CHEN.** *Plant J.,* 1996, vol. 10, 955-966 **[0151] [0179]**
- **STREIT.** *Mol. Plant Microbe Interact.,* 1997, vol. 10, 933-937 **[0151]**
- **SHEEN.** *Science,* 1996, vol. 274, 1900-1902 **[0151] [0179]**
- **DE VEYLDER.** *Plant Cell Physiol.,* 1997, vol. 38, 568-577 **[0152] [0154] [0180]**
- **MASGRAU.** *Plant J.,* 1997, vol. 11, 465-473 **[0152] [0154] [0180]**
- **STANGE.** *Plant J.,* 1997, vol. 11, 1315-1324 **[0152] [0154] [0180]**
- **GAFFNEY.** *Science,* 1993, vol. 261, 754-756 **[0158]**

- **JOSHI.** *N.A.R.,* 1987, vol. 15, 6643-6653 **[0162]**
- **GUO.** *Plant J.,* 2003, vol. 34, 383-92 **[0167]**
- **CHEN.** *Plant J.,* 2003, vol. 36, 731-40 **[0167]**
- **JORDANO.** *Plant Cell,* 1989, vol. 1, 855-866 **[0172]**
- **BUSTOS.** *Plant Cell,* 1989, vol. 1, 839-854 **[0172]**
- **GREEN.** *EMBO J.,* 1988, vol. 7, 4035-4044 **[0172]**
- **MEIER.** *Plant Cell,* 1991, vol. 3, 309-316 **[0172]**
- **ZHANG.** *Plant Physiology,* 1996, vol. 110, 1069-1079 **[0172]**
- **KRIDL.** *Seed Science Research,* 1991, vol. 1, 209 **[0173]**
- **VODKIN.** *Prog. Clin. Biol. Res.,* 1983, 87 **[0173]**
- **LINDSTROM.** *Der. Genet.,* 1990, vol. 11, 160 **[0173]**
- **DENNIS.** *Nucleic Acids Res.,* 1984, vol. 12, 3983 **[0173]**
- **SIMPSON.** *Science,* 1986, vol. 233, 34 **[0173]**
- **BANSAL.** *Proc. Natl. Acad. Sci. USA,* 1992, vol. 89, 3654 **[0173]**
- **ODELL.** *Nature,* 1985, vol. 313, 810 **[0173] [0181]**
- **POULSEN.** *Mol. Gen. Genet.,* 1986, vol. 205, 193-200 **[0173]**
- **CASHMORE.** Gen. Eng. of Plants. Plenum Press, 1983, 29-38 **[0173]**
- **LANGRIDGE.** *Proc. Natl. Acad. Sci. USA,* 1989, vol. 86, 3219-3223 **[0173]**
- **VANTUNEN.** *EMBO J.,* 1988, vol. 7, 1257 **[0173]**
- **KELLER.** *Genes Dev.,* 1989, vol. 3, 1639 **[0173]**
- **WENZLER.** *Plant Mol. Biol.,* 1989, vol. 13, 347 **[0173]**
- **YAMAMOTO.** *Nucleic Acids Res.,* 1990, vol. 18, 7449 **[0173]**
- **REINA.** *Nucleic Acids Res.,* 1990, vol. 18, 6425 **[0173]**
- **LOPES.** *Mol. Gen. Genet.,* 1995, vol. 247, 603-613 **[0173]**
- **KRIZ.** *Mol. Gen. Genet.,* 1987, vol. 207, 90 **[0173]**
- **WANDELT.** *Nucleic Acids Res.,* 1989, vol. 17, 2354 **[0173]**
- **LANGRIDGE.** *Cell,* 1983, vol. 34, 1015 **[0173]**
- **REINA.** *Nucleic Acids Res.,* 1990, vol. 18, 7449 **[0173]**
- **BELANGER.** *Genetics,* 1991, vol. 129, 863 **[0173]**
- **SUNILKUMAR.** *Transgenic Res.,* 2002, vol. 11, 347-359 **[0173]**
- **SULLIVAN.** *Mol. Gen. Genet.,* 1989, vol. 215, 431 **[0173]**
- **HUDSPETH ; GRULA.** *Plant Molec. Biol.,* 1989, vol. 12, 579-589 **[0173]**
- **CHANDLER.** *Plant Cell,* 1989, vol. 1, 1175 **[0173]**
- **CZAKO.** *Mol. Gen. Genet.,* 1992, vol. 235, 33 **[0173]**
- **TAKAIWA.** *Plant Mol. Biol.,* 1991, vol. 16 (1), 49-58 **[0173]**
- **FRANKEN.** *EMBO J.,* 1991, vol. 10, 2605 **[0173]**
- **SIMS ; GOLDBURG.** *Nuc. Acid Res.,* 1989, vol. 17 (11), 4368 **[0173]**
- **JOHN ; CROW.** *PNAS,* 1992, vol. 89, 5769-5773 **[0175]**
- **YAMAMOTO.** *Plant Cell Physiol.,* 1994, vol. 35, 773-778 **[0177]**
- **FEJES.** *Plant Mol. Biol.,* 1990, vol. 15, 921-932 **[0177]**
- **LUBBERSTEDT.** *Plant Physiol.,* 1994, vol. 104, 997-1006 **[0177]**
- **LUAN.** *Plant Cell,* 1992, vol. 4, 971-981 **[0177]**
- **MATSUOKA.** *Proc. Natl. Acad. Sci USA,* 1993, vol. 90, 9586-9590 **[0177]**
- **CERDAN.** *Plant Mol. Biol.,* 1997, vol. 33, 245-255 **[0177]**
- **TRUERNIT.** *Planta,* 1995, vol. 196, 564-570 **[0177]**
- **STREIT.** *Mol. Plant Microbe Interact,* 1997, vol. 10, 933-937 **[0179]**
- **WANG.** *Mol. Cell. Biol.,* 1992, vol. 12, 3399 **[0181]**
- **MCELROY.** *Plant Cell,* 1990, vol. 2, 163-171 **[0181]**
- **MCELROY.** *Mol. Gen. Genet.,* 1991, vol. 231, 150-160 **[0181]**
- **LAWTON.** *Plant Mol. Biol.,* 1987, vol. 9, 315-324 **[0181]**
- **EBERT.** *PNAS USA,* 1987, vol. 84, 5745-5749 **[0181]**
- **WALKER.** *PNAS,* 1987, vol. 84, 6624-6628 **[0181]**
- **YANG ; RUSSELL.** *PNAS,* 1990, vol. 87, 4144-4148 **[0181]**
- **BINET.** *Plant Science,* 1991, vol. 79, 87-94 **[0181]**
- **CHRISTENSEN.** *Plant Molec. Biol.,* 1989, vol. 12, 619-632 **[0181]**
- **CALLIS.** *J. Biol. Chem.,* 1990, vol. 265, 12486-12493 **[0181]**
- **NORRIS.** *Plant Mol. BioL,* 1993, vol. 21, 895-906 **[0181]**
- **GALLIE.** *Nucl. Acids Res.,* 1987, vol. 15, 8693-8711 **[0184]**
- **SKUZESKI.** *Plant Molec. Biol.,* 1990, vol. 15, 65-79 **[0184]**
- **UNGER.** *Plant Molec. Biol.,* 1989, vol. 13, 411-418 **[0185]**
- **ROGERS.** *Proc. Natl. Acad. Sci. USA,* 1985, vol. 82, 6512-651 **[0185]**
- **COMAI.** *J. Biol. Chem.,* 1988, vol. 263, 15104-15109 **[0185]**
- **VAN DEN BROECK.** *Nature,* 1985, vol. 313, 358-363 **[0185]**
- **KOEHLER ; HO.** *Plant Cell,* 1990, vol. 2, 769-783 **[0185]**
- **SHINSHI.** *Plant Molec. Biol.,* 1990, vol. 14, 357-368 **[0185]**
- **COVEY.** *Proc. Natl. Acad. Sci. USA,* 1990, vol. 87, 1633-1637 **[0197]**
- **ANGELL.** *EMBO J.,* 1997, vol. 16, 3675-3684 **[0198]**
- **CASPER.** *Gene,* 1996, vol. 173, 69-73 **[0198]**
- **HILLMAN.** *Virology,* 1989, vol. 169, 42-50 **[0198]**
- **DOLJA.** *Virology,* 1997, vol. 234, 243-252 **[0198]**
- **MORINAGA.** *Microbiol Immunol.,* 1993, vol. 37, 471-476 **[0198]**
- **CECCHINI.** *Mol. Plant Microbe Interact.,* 1997, vol. 10, 1094-1101 **[0198]**
- **RUBIN.** *Mol. Cell. Biol.,* 1997, vol. 17, 6294-6302 **[0198]**

- **KUNZE.** *Curr. Top. Microbiol. Immunol.,* 1996, vol. 204, 161-194 **[0198]**
- **SCHLAPPI.** *Plant Mol. Biol.,* 1996, vol. 32, 717-725 **[0198]**
- **WEISING.** *Ann. Rev. Genet.,* 1988, vol. 22, 421-477 **[0201]**
- **DAVIS, L. ; DIBNER, M. ; BATTEY, I.** *Basic Methods in Molecular Biology,* 1986 **[0202]**
- PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS. Academic Press, 1990 **[0212]**
- PCR STRATEGIES. Academic Press, Inc, 1995 **[0212]**
- **WU.** *Genomics,* 1989, vol. 4, 560 **[0212]**
- **LANDEGREN.** *Science,* 1988, vol. 241, 1077 **[0212]**
- **BARRINGER.** *Gene,* 1990, vol. 89, 117 **[0212]**
- **KWOH.** *Proc. Natl. Acad. Sci. USA,* 1989, vol. 86, 1173 **[0212]**
- **GUATELLI.** *Proc. Natl. Acad. Sci. USA,* 1990, vol. 87, 1874 **[0212]**
- **SMITH.** *J. Clin. Microbiol.,* 1997, vol. 35, 1477-1491 **[0212]**
- **BURG.** *Mol. Cell. Probes,* 1996, vol. 10, 257-271 **[0212]**
- **BERGER.** *Methods Enzymol.,* 1987, vol. 152, 307-316 **[0212]**
- **SOOKNANAN.** *Biotechnology,* 1995, vol. 13, 563-564 **[0212]**
- **STRYER ; LUBERT.** Biochemistry. W. H Freeman & Co, **[0215]**
- **PEARSON ; LIPMAN.** *Proc. Natl. Acad. Sci. USA,* 1988, vol. 85 (8), 2444-2448 **[0216]**
- **ALTSCHUL et al.** *J. Mol. Biol.,* 1990, vol. 215 (3), 403-410 **[0216]**
- **THOMPSON et al.** *Nucleic Acids Res.,* 1994, vol. 22 (2), 4673-4680 **[0216]**
- **HIGGINS et al.** *Methods Enzymol.,* 1996, vol. 266, 383-402 **[0216]**
- **ALTSCHUL et al.** *Nature Genetics,* 1993, vol. 3, 266-272 **[0216]**
- **SMITH ; WATERMAN.** *Adv. Appl. Math.,* 1981, vol. 2, 482 **[0219]**
- **NEEDLEMAN ; WUNSCH.** *J. Mol. Biol.,* 1970, vol. 48, 443 **[0219]**
- **LIPMAN.** *Proc. Nat'l. Acad. Sci. USA,* 1988, vol. 85, 2444 **[0219]**
- **GIBBS.** *Human Genome Sequencing Project,* 1995 **[0219]**
- **FRASER et al.** *M. genitalium,* 1995 **[0219]**
- **BULT et al.** *M. jannaschii,* 1996 **[0219]**
- **FLEISCHMANN et al.** *H. influenzae,* 1995 **[0219]**
- **BLATTNER et al.** *E. coli,* 1997 **[0219]**
- **MEWES et al.** *S. cerevisiae,* 1997 **[0219]**
- **ADAMS et al.** *D. melanogaster,* 2000 **[0219]**
- **ALTSCHUL.** *Nuc. Acids Res.,* 1977, vol. 25, 3389-3402 **[0220]**
- **ALTSCHUL.** *J. Mol. Biol.,* 1990, vol. 215, 403-410 **[0220]**
- **HENIKOFF ; HENIKOFF.** *Proc. Natl. Acad. Sci. USA,* 1989, vol. 89, 10915 **[0220]**
- **KARLIN ; ALTSCHUL.** *Proc. Natl. Acad. Sci. USA,* 1993, vol. 90, 5873 **[0220]**
- **GONNET et al.** *Science,* 1992, vol. 256, 1443-1445 **[0220]**
- **HENIKOFF ; HENIKOFF.** *Proteins,* 1993, vol. 17, 49-61 **[0220]**
- Matrices for Detecting Distance Relationships: Atlas of Protein Sequence and Structure. National Biomedical Research Foundation, 1978 **[0220]**
- **ALTSCHUL et al.** *J. Mol. Biol.,* 1990, vol. 215, 403 **[0232]**
- **PEARSON ; LIPMAN.** *Proc. Natl. Acad. Sci. USA,* 1988, vol. 85, 2444 **[0232]**
- **BRUTLAG et al.** *Comp. App. Biosci.,* 1990, vol. 6, 237-245 **[0232]**
- **HO.** *Methods Enzymol.,* 2000, vol. 314, 168-183 **[0261]**
- **SMITH.** *Eur. J. Pharm. Sci.,* 2000, vol. 11, 191-198 **[0261]**
- **MATA.** *Toxicol Appl Pharmacol,* 1997, vol. 144, 189-197 **[0262]**
- Antisense Therapeutics. Humana Press, 1996 **[0262]**
- **GOLD.** *J. of Biol. Chem.,* 1995, vol. 270, 13581-13584 **[0263]**
- **ROSSI.** *Aids Research and Human Retroviruses,* 1992, vol. 8, 183 **[0266]**
- **HAMPEL.** *Biochemistry,* 1989, vol. 28, 4929 **[0266]**
- **HAMPEL.** *Nuc. Acids Res.,* 1990, vol. 18, 299 **[0266]**
- **PERROTTA.** *Biochemistry,* 1992, vol. 31, 16 **[0266]**
- **GUERRIER-TAKADA.** *Cell,* 1983, vol. 35, 849 **[0266]**
- **SHUEY.** *Drug Discov. Today,* 2002, vol. 7, 1040-1046 **[0267]**
- **RICE.** *Proc. Natl. Acad. Sci. USA,* 1992, vol. 89, 5467-5471 **[0270]**
- **CRAMERI.** *Biotechniques,* 1995, vol. 18, 194-196 **[0270]**
- **STEMMER.** Molecular breeding of viruses for targeting and other clinical properties. *Tumor Targeting,* 1999, vol. 4, 1-4 **[0271]**
- **NESS.** *Nature Biotechnology,* 1999, vol. 17, 893-896 **[0271]**
- **CHANG.** Evolution of a cytokine using DNA family shuffling. *Nature Biotechnology,* 1999, vol. 17, 793-797 **[0271]**
- **MINSHULL.** Protein evolution by molecular breeding. *Current Opinion in Chemical Biology,* 1999, vol. 3, 284-290 **[0271]**
- **CHRISTIANS.** Directed evolution of thymidine kinase for AZT phosphorylation using DNA family shuffling. *Nature Biotechnology,* 1999, vol. 17, 259-264 **[0271]**
- **CRAMERI.** DNA shuffling of a family of genes from diverse species accelerates directed evolution. *Nature,* 1998, vol. 391, 288-291 **[0271]**

- **CRAMERI.** Molecular evolution of an arsenate detoxification pathway by DNA shuffling. *Nature Biotechnology,* 1997, vol. 15, 436-438 **[0271]**
- **ZHANG.** Directed evolution of an effective fucosidase from a galactosidase by DNA shuffling and screening. *Proc. Natl. Acad. Sci. USA,* 1997, vol. 94, 4504-4509 **[0271]**
- **PATTEN et al.** Applications of DNA Shuffling to Pharmaceuticals and Vaccines. *Current Opinion in Biotechnology,* 1997, vol. 8, 724-733 **[0271]**
- **CRAMERI et al.** Construction and evolution of antibody-phage libraries by DNA shuffling. *Nature Medicine,* 1996, vol. 2, 100-103 **[0271]**
- **GATES et al.** Affinity selective isolation of ligands from peptide libraries through display on a lac repressor 'headpiece dimer. *Journal of Molecular Biology,* 1996, vol. 255, 373-386 **[0271]**
- Sexual PCR and Assembly PCR. **STEMMER.** In: The Encyclopedia of Molecular Biology. VCH Publishers, 1996, 447-457 **[0271]**
- **CRAMERI ; STEMMER.** Combinatorial multiple cassette mutagenesis creates all the permutations of mutant and wildtype cassettes. *BioTechniques,* 1995, vol. 18, 194-195 **[0271]**
- **STEMMER et al.** Single-step assembly of a gene and entire plasmid form large numbers of oligodeoxyribonucleotides. *Gene,* 1995, vol. 164, 49-53 **[0271]**
- **STEMMER.** The Evolution of Molecular Computation. *Science,* 1995, vol. 270, 1510 **[0271]**
- **STEMMER.** Searching Sequence Space. *Bio/Technology,* 1995, vol. 13, 549-553 **[0271]**
- **STEMMER.** Rapid evolution of a protein in vitro by DNA shuffling. *Nature,* 1994, vol. 370, 389-391 **[0271]**
- **STEMMER.** DNA shuffling by random fragmentation and reassembly: In vitro recombination for molecular evolution. *Proc. Natl. Acad. Sci. USA,* 1994, vol. 91, 10747-10751 **[0271]**
- **LING et al.** Approaches to DNA mutagenesis: an overview. *Anal Biochem.,* 1997, vol. 254 (2), 157-178 **[0272]**
- **DALE et al.** Oligonucleotide-directed random mutagenesis using the phosphorothioate method. *Methods Mol. Biol.,* 1996, vol. 57, 369-374 **[0272]**
- **SMITH.** In vitro mutagenesis. *Ann. Rev. Genet.,* 1985, vol. 19, 423-462 **[0272]**
- **BOTSTEIN ; SHORTLE.** Strategies and applications of in vitro mutagenesis. *Science,* 1985, vol. 229, 1193-1201 **[0272]**
- **CARTER.** Site-directed mutagenesis. *Biochem. J.,* 1986, vol. 237, 1-7 **[0272]**
- The efficiency of oligonucleotide directed mutagenesis. **KUNKEL.** Nucleic Acids & Molecular Biology. Springer Verlag, 1987 **[0272]**
- **KUNKEL.** Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA,* 1985, vol. 82, 488-492 **[0272]**
- **KUNKEL et al.** Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods in Enzymol.,* 1987, vol. 154, 367-382 **[0272]**
- **BASS et al.** Mutant Trp repressors with new DNA-binding specificities. *Science,* 1988, vol. 242, 240-245 **[0272]**
- *Methods in Enzymol.,* 1983, vol. 100, 468-500 **[0272]**
- *Methods in Enzymol.,* 1987, vol. 154, 329-350 **[0272]**
- **ZOLLER ; SMITH.** Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any DNA fragment. *Nucleic Acids Res.,* 1982, vol. 10, 6487-6500 **[0272]**
- **ZOLLER ; SMITH.** Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. *Methods in Enzymol.,* 1983, vol. 100, 468-500 **[0272]**
- **ZOLLER ; SMITH.** Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. *Methods in Enzymol.,* 1987, vol. 154, 329-350 **[0272]**
- **TAYLOR et al.** The use of phosphorothioate-modified DNA in restriction enzyme reactions to prepare nicked DNA. *Nucl. Acids Res.,* 1985, vol. 13, 8749-8764 **[0272]**
- **TAYLOR et al.** The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. *Nucl. Acids Res.,* 1985, vol. 13, 8765-8787 **[0272]**
- **NAKAMAYE.** Inhibition of restriction endonuclease Nci I cleavage by phosphorothioate groups and its application to oligonucleotide-directed mutagenesis. *Nucl. Acids Res.,* 1986, vol. 14, 9679-9698 **[0272]**
- **SAYERS et al.** Y-T Exonucleases in phosphorothioate-based oligonucleotide-directed mutagenesis. *Nucl. Acids Res.,* 1988, vol. 16, 791-802 **[0272]**
- **SAYERS et al.** Strand specific cleavage of phosphorothioate-containing DNA by reaction with restriction endonucleases in the presence of ethidium bromide. *Nucl. Acids Res.,* 1988, vol. 16, 803-814 **[0272]**
- **KRAMER et al.** The gapped duplex DNA approach to oligonucleotide-directed mutation construction. *Nucl. Acids Res.,* 1984, vol. 12, 9441-9456 **[0272]**
- **KRAMER ; FRITZ.** Oligonucleotide-directed construction of mutations via gapped duplex DNA. *Methods in Enzymol.,* 1987, vol. 154, 350-367 **[0272]**
- **KRAMER et al.** Improved enzymatic in vitro reactions in the gapped duplex DNA approach to oligonucleotide-directed construction of mutations. *Nucl. Acids Res.,* 1988, vol. 16, 7207 **[0272]**
- **FRITZ et al.** Oligonucleotide-directed construction of mutations: a gapped duplex DNA procedure without enzymatic reactions in vitro. *Nucl. Acids Res.,* 1988, vol. 16, 6987-6999 **[0272]**
- **KRAMER.** Point Mismatch Repair. *Cell,* 1984, vol. 38, 879-887 **[0273]**
- **CARTER et al.** Improved oligonucleotide site-directed mutagenesis using M13 vectors. *Nucl. Acids Res.,* 1985, vol. 13, 4431-4443 **[0273]**

- **CARTER.** Improved oligonucleotide-directed mutagenesis using M13 vectors. *Methods in Enzymol.,* 1987, vol. 154, 382-403 **[0273]**
- **EGHTEDARZADEH.** Use of oligonucleotides to generate large deletions. *Nucl. Acids Res.,* 1986, vol. 14, 5115 **[0273]**
- **WELLS et al.** Importance of hydrogen-bond formation in stabilizing the transition state of subtilisin. *Phil. Trans. R. Soc. Lond. A,* 1986, vol. 317, 415-423 **[0273]**
- **NAMBIAR et al.** Total synthesis and cloning of a gene coding for the ribonuclease S protein. *Science,* 1984, vol. 223, 1299-1301 **[0273]**
- **KHORANA.** Total synthesis and expression of a gene for the a-subunit of bovine rod outer segment guanine nucleotide-binding protein (transducin). *Nucl. Acids Res.,* 1988, vol. 14, 6361-6372 **[0273]**
- **WELLS et al.** Cassette mutagenesis: an efficient method for generation of multiple mutations at defined sites. *Gene,* 1985, vol. 34, 315-323 **[0273]**
- **GRUNDSTROM et al.** Oligonucleotide-directed mutagenesis by microscale 'shot-gun' gene synthesis. *Nucl. Acids Res.,* 1985, vol. 13, 3305-3316 **[0273]**
- **ARNOLD.** Protein engineering for unusual environments. *Current Opinion in Biotechnology,* 1993, vol. 4, 450-455 **[0273]**
- Oligonucleotide-directed double-strand break repair in plasmids of Escherichia coli: a method for site-specific mutagenesis. *Proc. Natl. Acad. Sci. USA,* vol. 83, 7177-7181 **[0273]**
- *Methods in Enzymology,* vol. 154 **[0273]**
- **LEUNG, D.W. et al.** *Technique,* 1989, vol. 1, 11-15 **[0309]**
- **CALDWELL, R. C. ; JOYCE G.F.** *PCR Methods Applic.,* 1992, vol. 2, 28-33 **[0309]**
- **REIDHAAR-OLSON.** *Science,* 1988, vol. 241, 53-57 **[0310]**
- **STEMMER.** *Proc. Natl. Acad. Sci. USA,* 1994, vol. 91, 10747-10751 **[0312]**
- **ARKIN.** *Proc. Natl. Acad. Sci. USA,* 1992, vol. 89, 7811-7815 **[0315]**
- **DELEGRAVE.** *Biotechnology Res.,* 1993, vol. 11, 1548-1552 **[0316]**
- **ARNOLD.** *Current Opinion in Biotechnology,* 1993, vol. 4, 450-455 **[0316]**
- **BACA.** *Int. J. Parasitol.,* 2000, vol. 30, 113-118 **[0325]**
- **HALE.** *Protein Expr. Purif.,* 1998, vol. 12, 185-188 **[0325]**
- **NARUM.** *Infect. Immun.,* 2001, vol. 69, 7250-7253 **[0325]**
- **OUTCHKOUROV.** *Protein Expr. Purif.,* 2002, vol. 24, 18-24 **[0325]**
- **FENG.** *Biochemistry,* 2000, vol. 39, 15399-15409 **[0325]**
- **HUMPHREYS.** *Protein Expr. Purif.,* 2000, vol. 20, 252-264 **[0325]**
- **POLLOCK.** *J. Immunol. Methods,* 1999, vol. 231, 147-157 **[0327]**
- **BAGUISI.** *Nat. Biotechnol.,* 1999, vol. 17, 456-461 **[0327]**
- **MESSING ; VIERRA.** *Gene,* 1982, vol. 19, 259-268 **[0336]**
- **BEVAN.** *Nature,* 1983, vol. 304, 184-187 **[0336]**
- **WHITE.** *Nucl. Acids Res,* 1990, vol. 18, 1062 **[0336]**
- **SPENCER.** *Theor. Appl. Genet,* 1990, vol. 79, 625-631 **[0336]**
- **BLOCHINGER ; DIGGELMANN.** *Mol Cell Biol,* vol. 4, 2929-2931 **[0336]**
- **BOUROUIS.** *EMBO J.,* 1983, vol. 2 (7), 1099-1104 **[0336]**
- **CHRISTOU.** *Plant Mol. Biol.,* 1997, vol. 35, 197-203 **[0339]**
- **PAWLOWSKI.** *Mol. Biotechnol.,* 1996, vol. 6, 17-30 **[0339]**
- **KLEIN.** *Nature,* 1987, vol. 327, 70-73 **[0339]**
- **TAKUMI.** *Genes Genet. Syst.,* 1997, vol. 72, 63-69 **[0339]**
- **ROUWENDAL.** *Plant Mol. Biol.,* 1997, vol. 33, 989-999 **[0341]**
- **PORTA.** Use of viral replicons for the expression of genes in plants. *Mol. Biotechnol.,* 1996, vol. 5, 209-221 **[0341]**
- **HORSCH.** *Science,* 1984, vol. 233, 496-498 **[0342]**
- **FRALEY.** *Proc. Natl. Acad. Sci. USA,* 1983, vol. 80, 4803 **[0342]**
- Gene Transfer to Plants. Springerlag, 1995 **[0342]**
- **HIEI.** *Plant Mol. Biol.,* 1997, vol. 35, 205-218 **[0344]**
- **HORSCH.** *Science,* 1984, vol. 233, 496 **[0344]**
- **FRALEY.** *Proc. Natl. Acad. Sci USA,* 1983, vol. 80, 4803 **[0344]**
- **PARK.** *Plant Mol. Biol.,* 1996, vol. 32, 1135-1148 **[0344]**
- **EVANS et al.** Protoplasts Isolation and Culture, Handbook of Plant Cell Culture. MacMillilan Publishing Company, 1983, 124-176 **[0345]**
- **BINDING.** Regeneration of Plants, Plant Protoplasts. CRC Press, 1985, 21-73 **[0345]**
- **KLEE.** *Ann. Rev. of Plant Phys.,* 1987, vol. 38, 467-486 **[0345]**
- **WELSH J. R.** Fundamentals of Plant Genetics and Breeding. John Wiley & Sons, 1981 **[0346]**
- Crop Breeding. American Society of Agronomy Madison, Wis, 1983 **[0346]**
- **MAYO O.** The Theory of Plant Breeding. Clarendon Press, 1987 **[0346]**
- **SINGH, D. P.** Breeding for Resistance to Diseases and Insect Pests. Springer-Verlag, 1986 **[0346]**
- **WRICKE ; WEBER.** Quantitative Genetics and Selection Plant Breeding. Walter de Gruyter and Co, 1986 **[0346]**
- **PALMGREN.** *Trends Genet.,* 1997, vol. 13, 348 **[0352]**
- **CHONG.** *Transgenic Res.,* 1997, vol. 6, 289-296 **[0352]**

- **CARUTHERS.** *Nucleic Acids Res. Symp. Ser.,* 1980, 215-223 **[0369]**
- **HORN.** *Nucleic Acids Res. Symp. Ser.,* 1980, 225-232 **[0369]**
- **BANGA, A.K.** Therapeutic Peptides and Proteins, Formulation, Processing and Delivery Systems. Technomic Publishing Co, 1995 **[0369]**
- **ROBERGE.** *Science,* 1995, vol. 269, 202 **[0369]**
- **MERRIFIELD.** *Methods Enzymol.,* 1997, vol. 289, 3-13 **[0369]**
- **SPATOLA.** Chemistry and Biochemistry of Amino Acids. *Peptides and Proteins,* 1983, vol. 7, 267-357 **[0374]**
- **CREIGHTON, T.E.** Proteins - Structure and Molecular Properties. W.H. Freeman and Company, 1993 **[0378]**
- Posttranslational Covalent Modification of Proteins. Academic Press, 1983, 1-12 **[0378]**
- **MERRIFIELD, R. B.** *J. Am. Chem. Soc.,* 1963, vol. 85, 2149-2154 **[0379]**
- **STEWART, J. M. ; YOUNG, J. D.** Solid Phase Peptide Synthesis. Pierce Chemical Co, 11-12 **[0379]**
- **H. M. GEYSEN et al.** *Proc. Natl. Acad. Sci.,* 1984, vol. 81, 3998 **[0379]**
- **NIELSEN.** Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering,* 1997, vol. 10 (1), 1-6 **[0392]**
- **NIELSEN et al.** Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering,* 1997, vol. 10 (1), 1-6 **[0396]**
- **VAN DE VEN.** *Crit. Rev. Oncog.,* 1993, vol. 4 (2), 115-136 **[0399]**
- **A. BATEMAN ; E. BIRNEY ; L. CERRUTI ; R. DURBIN ; L. ETWILLER ; S.R. EDDY ; S. GRIFFITHS-JONES ; K.L. HOWE ; M. MARSHALL ; E.L.L. SONNHAMMER.** *Nucleic Acids Research,* 2002, vol. 30 (1), 276-280 **[0400]**
- **JOHNSTON.** *Curr. Biol.,* 1998, vol. 8, R171-R174 **[0412]**
- **SCHUMMER.** *Biotechniques,* 1997, vol. 23, 1087-1092 **[0412]**
- **SOLINAS-TOLDO.** *Genes, Chromosomes & Cancer,* 1997, vol. 20, 399-407 **[0412]**
- **BOWTELL.** *Nature Genetics Supp.,* 1999, vol. 21, 25-32 **[0412]**
- Fundamental Immunology. Raven Press, 1993 **[0414]**
- **WILSON.** *J. Immunol. Methods,* 1994, vol. 175, 267-273 **[0414]**
- **YARMUSH.** *J. Biochem. Biophys. Methods,* 1992, vol. 25, 85-97 **[0414]**
- **WARD et al.** *Nature,* 1989, vol. 341, 544-546 **[0414]**
- **COLIGAN.** CURRENT PROTOCOLS IN IMMUNOLOGY. Wiley/Greene, 1991 **[0416]**
- BASIC AND CLINICAL IMMUNOLOGY. Lange Medical Publications **[0416]**
- **GODING.** MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE. Academic Press, 1986 **[0416]**
- **KOHLER.** *Nature,* 1975, vol. 256, 495 **[0416]**
- **HARLOW.** ANTIBODIES, A LABORATORY MANUAL. Cold Spring Harbor Publications, 1988 **[0416]**
- **HOOGENBOOM.** *Trends Biotechnol.,* 1997, vol. 15, 62-70 **[0416]**
- **KATZ.** *Annu. Rev. Biophys. Biomol. Struct.,* 1997, vol. 26, 27-45 **[0416]**
- **COLE.** Monoclonal Antibodies and Cancer Therapy. Alan R. Liss, Inc, 1985, 77-96 **[0421]**
- **KREUZER.** *Br. J. Haematol.,* 2001, vol. 114, 313-318 **[0429]**
- **XIA.** *Transplantation,* 2001, vol. 72, 907-914 **[0429]**
- **SILBERSTEIN.** *Baker's Digest,* 1964, vol. 38, 66-72 **[0473]**
- **DOMINIC W.S. WONG ; SARAH B. BATT ; GEORGE H. ROBERTSON.** *J. Agric. Food Chem.,* 2000, vol. 48, 4540-4543 **[0629]**
- **JEFFREY D. FOX ; JOHN F. ROBYT.** *Anal. Biochem.,* 1991, vol. 195, 93-96 **[0629]**
- **MARLIDA.** *World J. Microbiol. Biotechnol.,* 2000, vol. 16, 573-578 **[0698]**
- *J. Agric. Food Chem.,* vol. 48, 4540-4543 **[0705]**
- **FOX.** Miniaturization of three carbohydrate analyses using a micro sample plate reader. *Anal. Biochem.,* 1991, vol. 195, 93-96 **[0705]**
- In Determination with Glucose Oxidase and Peroxidase. **BERGMEYER.** Methods of. Enzymatic Analysis. 1974, 1205-1212 **[0707]**
- **WONG.** *J. Agric. Food Chem.,* 2000, vol. 48, 4540-4543 **[0725]**
- **FOX.** *Anal. Biochem.,* 1991, vol. 195, 93-96 **[0725]**
- **FRANDESEN.** *Biochemistry,* 1995, vol. 34, 10162-10169 **[0795]**
- **SIERKS ; SVENSSON.** *Biochemistry,* 1996, vol. 35, 1865-1871 **[0795]**
- **FAGERSTROM ; KALKKINEN.** *Biotechnol. Appl. Biochem.,* 1995, vol. 21, 223-231 **[0795]**
- *JBC,* 2002, vol. 277 (29), 26501-26507 **[0810] [0887]**
- **FRANDSEN TP ; CHRISTENSEN T ; STOFFER B ; LEHMBECK J ; DUPONT C ; HONZATKO RB ; SVENSSON B.** *Biochemistry,* 1995, vol. 34, 10162-9 **[0909]**
- **SIERKS MR ; SVENSSON B.** *Biochemistry,* 1996, vol. 35, 1865-71 **[0909]**
- **FAGERSTROM R ; KALKKINEN N.** *Biotechnol Appl Biochem.,* 1995, vol. 21, 223-31 **[0909]**

SBGK
Szabadalmi
Ügyvivői Iroda

SZTNH-100007129

716.284/SZE

Amilázok és glükoamilázok, az ezeket kódoló nukleinsavak, valamint eljárások ezek előállítására és alkalmazására

SZABADALMI IGÉNYPONTOK

1. Izolált, szintetikus vagy rekombináns nukleinsav, amely a következőket tartalmazza:

(a) egy nukleinsav szekvencia, amely legalább 98%-os, 99%-os, vagy 100%-os szekvencia azonosságot mutat a 47. számú szekvenciával, ahol a nukleinsav legalább egy, glükoamiláz aktivitással rendelkező polipeptidet kódol;

(b) egy nukleinsav szekvencia, amely a 48. számú szekvenciával rendelkező polipeptidet kódolja;

(c) nukleinsav szekvencia, amely teljesen komplementer az (a) vagy (b) pont szerinti nukleinsav szekvenciával; vagy

(d) a 47. számú szekvencia-vázlaton megadott nukleinsav szekvencia.

2. Az 1. igénypont szerinti izolált, szintetikus vagy rekombináns nukleinsav, amelyben a glükoamiláz aktivitás tartalmazza a glükozidos kötések hidrolizálását, és adott esetben az egy poliszacharidban, egy oligoszacharidban, vagy egy keményítőben levő glükozidos kötések hidrolizálását, vagy adott esetben egy α-1,4-glükozidos kötés és/vagy egy α-1,6-glükozidos kötés hidrolizálását, vagy ahol a glükoamiláz aktivitás tartalmazza egy maltóz- vagy egy D-glükóz-egység lehasítását egy poliszacharid, egy oligoszacharid vagy egy keményítő nem-redukáló végéről.

3. Egy vektor, egy expressziós kazetta vagy egy klónozó hordozó, amely tartalmaz egy nukleinsavat, amely tartalmazza az 1. igénypont szerinti nukleinsav szekvenciát;

ahol a nukleinsav adott esetben működés szempontjából egy promoterhez van kapcsolva;

ahol a promoter adott esetben egy növényi promoter vagy egy virális promoter;

ahol a promoter adott esetben egy indukálható promoter vagy egy szövet-specifikus promoter; vagy

ahol adott esetben a szövet-specifikus promoter adott esetben magspecifikus, egy endosperma-specifikus promoter, egy levélspecifikus promoter, egy gyökérspecifikus promoter, egy szárspecifikus-, vagy egy lemetszés által indukált promoter.

4. Egy transzformált sejt, amely tartalmaz egy nukleinsavat, amely tartalmazza az 1. igénypont szerinti nukleinsav szekvenciát, vagy egy, a 3. igénypont szerinti vektor, expressziós kazetta vagy klónozó hordozó, ahol a sejt adott esetben egy baktérium sejt, egy emlős sejt, egy gom-

basejt, egy élesztősejt, egy rovarsejt vagy egy növénysejt, ahol a növénysejt adott esetben egy burgonya- rizs-, kukorica-, búza- vagy árpasejt.

5. Egy glükoamiláz aktivitással rendelkező izolált, szintetikus vagy rekombináns polipeptid, amely a következőket tartalmazza:

(i) egy aminosav szekvencia, amely legalább 99%-os, vagy teljes szekvencia azonosságot mutat a 48. számú szekvenciával, a polipeptid teljes hosszában;

(ii) egy aminosav szekvencia, amelyet az 1. igénypont szerinti nukleinsav kódol;

(iii) az (i) – (ii) pontok bármelyike szerinti polipeptid, amely polipeptid a 48. számú szekvencia-vázlaton bemutatott szekvenciával rendelkezik, vagy ennek glükoamiláz aktivitással rendelkező enzimatikusan aktív fragmensei.

6. Az 5. igénypont szerinti izolált, szintetikus vagy rekombináns polipeptid, amelyben a glükoamiláz aktivitás tartalmazza a glükozidos kötések hidrolizálását, és adott esetben a glükozidos kötés egy α-1,4-glükozidos kötés vagy egy α-1,6-glükozidos kötés, és/vagy ahol adott esetben a glükoamiláz aktivitás tartalmazza glükozidos kötések hidrolizálását egy poliszacharidban, egy oligoszacharidban, vagy egy keményítőben, és/vagy ahol a glükoamiláz aktivitás tartalmazza egy maltóz- vagy egy D-glükóz-egység lehasítását egy poliszacharid, egy oligoszacharid vagy egy keményítő nem-redukáló végéről.

7. Eljárás egy rekombináns polipeptid előállítására, azzal jellemezve, hogy a következő lépéseket tartalmazza: (a) biztosítunk egy nukleinsavat, működés szempontjából egy promoterhez kapcsolva, ahol a nukleinsav tartalmazza az 1. igénypont szerinti nukleinsav szekvenciát; és (b) az (a) pont szerinti nukleinsavat olyan körülmények között expresszáljuk, amelyek lehetővé teszik a polipeptid expresszióját, ezáltal előállítva egy rekombináns polipeptidet, ezenfelül tartalmazza egy gazdasejt transzformálását az (a) pont szerinti nukleinsavval, majd az (a) pont szerinti nukleinsav expresszióját, ezáltal előállítva egy rekombináns polipeptidet egy transzformált sejtben.

8. Eljárás egy poliszacharid, egy oligoszacharid vagy egy keményítő hidrolizálására, azzal jellemezve, hogy a következő lépéseket tartalmazza:

(a) biztosítjuk az 5. igénypont szerinti polipeptidet;

(b) biztosítunk egy készítményt, amely tartalmaz egy poliszacharidot, egy oligoszacharidot vagy egy keményítőt; ahol a készítmény adott esetben tartalmaz egy α-1,4-glükozidos vagy egy α-1,6-glükozidos kötést, és

(c) az (a) lépés szerinti polipeptidet érintkezésbe hozzuk a (b) lépés szerinti készítménnyel, olyan körülmények között, amely körülmények között a polipeptid hidrolizálja a poliszacharidot, az oligoszacharidot vagy a keményítőt.

9. Eljárás egy poliszacharid, egy oligoszacharid vagy egy keményítő elfolyósítására vagy eltávolítására, azzal jellemezve, hogy a következő lépéseket tartalmazza:

(a) biztosítjuk az 5. igénypont szerinti polipeptidet;

(b) biztosítunk egy készítményt, amely tartalmaz egy poliszacharidot, egy oligoszacharidot vagy egy keményítőt; és

(c) az (a) lépés szerinti polipeptidet érintkezésbe hozzuk a (b) lépés szerinti készítménnyel, olyan körülmények között, amely körülmények között a polipeptid elfolyósítja vagy eltávolítja a poliszacharidot, az oligoszacharidot vagy a keményítőt.

10. Eljárás egy tárgy mosására, azzal jellemezve, hogy a következő lépéseket tartalmazza:

(a) biztosítunk egy készítményt, amely tartalmazza az 5. igénypont szerinti polipeptidet;

(b) biztosítunk egy tárgyat; és

(c) az (a) lépés szerinti polipeptidet érintkezésbe hozzuk a (b) lépés szerinti tárggyal, olyan körülmények között, amely körülmények között a készítmény képes lemosni a tárgyat.

11. Eljárás egy poliszacharid, oligoszacharid vagy egy keményítő hidrolízisére egy tápban vagy egy élelmiszerben mielőtt egy állat azt elfogyasztja, azzal jellemezve, hogy a következő lépéseket tartalmazza:

(a) beszerzünk egy keményítőt tartalmazó táp-alapanyagot, ahol a poliszacharidban, oligoszacharidban vagy keményítőben levő poliszacharid, oligoszacharid vagy keményítő hidrolizálható az 5. igénypont szerinti peptiddel; és

(b) az (a) lépés szerinti polipeptidet elegendő mennyiségben és elegendő ideig hozzáadjuk a táp vagy az élelmiszer anyagához, hogy a poliszacharid, oligoszacharid vagy keményítő hidrolízisét okozza, és kezelt élelmiszer vagy táp keletkezzen, ezáltal hidrolizálva a poliszacharidot, oligoszacharidot vagy keményítőt az élelmiszerben vagy a tápban, mielőtt az állat elfogyasztja, ahol az élelmiszer vagy a táp adott esetben rizs, kukorica, árpa, búza, zöldségek vagy burgonya.

12. Eljárás textília ırtelenítésére, azzal jellemezve, hogy a következő lépéseket tartalmazza:

(a) biztosítjuk az 5. igénypont szerinti polipeptidet;

(b) biztosítunk egy textíliát; és

(c) érintkezésbe hozzuk az (a) lépés szerinti polipeptidet és a (b) lépés szerinti szövetet, olyan körülmények között, amely körülmények között a glükoamiláz képes ırtelenıteni a szövetet.

13. Eljárás lignocellulóz rostok kezelésére, azzal jellemezve, hogy a következő lépéseket tartalmazza:

(a) biztosítjuk az 5. igénypont szerinti polipeptidet;

(b) biztosítunk egy lignocellulóz rostot; és

(c) érintkezésbe hozzuk az (a) lépés szerinti polipeptidet és a (b) lépés szerinti rostot, olyan körülmények között, amely körülmények között a polipeptid képes kezelni a rostot, ezzel javítva a rost tulajdonságait.

14. Eljárás magas maltóz-tartalmú vagy magas glükóz-tartalmú szirup előállítására azzal jellemezve, hogy a következő lépéseket tartalmazza:

(a) biztosítjuk az 5. igénypont szerinti polipeptidet;

(b) biztosítunk egy poliszacharidot, oligoszacharidot vagy keményítőt tartalmazó készítményt; a poliszacharid, oligoszacharid vagy keményítő adott esetben rizsből, kukoricából, árpából, búzából, zöldségekből vagy édesburgonyából származik, és

(c) érintkezésbe hozzuk az (a) lépés szerinti polipeptidet és a (b) lépés szerinti készítményt, olyan körülmények között, amely körülmények között az (a) pont szerinti polipeptid képes hidrolizálni a (b) pont szerinti készítményt, ezzel előállítva egy magas maltóz-tartalmú vagy magas glükóz-tartalmú szirupot.

15. Eljárás egy poliszacharidot, egy oligoszacharidot vagy keményítőt tartalmazó gyártási folyadékok folyási tulajdonságainak javítására, azzal jellemezve, hogy a következő lépéseket tartalmazza:

(a) biztosítjuk az 5. igénypont szerinti polipeptidet;

(b) biztosítunk egy poliszacharidot, oligoszacharidot vagy keményítőt tartalmazó gyártási folyadékot; adott esetben a gyártási folyadék egy föld alatti formációból származik; és

(c) érintkezésbe hozzuk az (a) lépés szerinti polipeptidet és a (b) lépés szerinti gyártási folyadékot, olyan körülmények között, amely körülmények között a polipeptid képes hidrolizálni a gyártási folyadékban levő poliszacharidot, oligoszacharidot vagy keményítőt, ezáltal sűrűségének csökkentésével áramlási tulajdonságait javítva.

16. Eljárás egy készítmény viszkozitásának megváltoztatására, azzal jellemezve, hogy a következő lépéseket tartalmazza:

(i) (a) biztosítunk egy készítményt és az 5. igénypont szerinti polipeptidet; és

(b) a készítményt az 5. igénypont szerinti készítménnyel kezeljük; vagy

(ii) az (i) pont szerinti eljárás, ahol a készítmény egy talaj vagy egy fúróiszap.

17. Eljárás glükoamiláz alkalmazására erjesztésben vagy alkohol előállításában, azzal jellemezve, hogy a következő lépéseket tartalmazza:

(a) biztosítjuk az 5. igénypont szerinti polipeptidet;

(b) biztosítunk egy erjesztésben vagy alkohol előállításában használt poliszacharidot, oligoszacharidot vagy keményítőt tartalmazó készítményt; ahol a poliszacharidot, oligoszacharidot vagy keményítőt tartalmazó készítmény adott esetben egy sör;

(c) kombináljuk az (a) lépés szerinti polipeptidet egy (b) lépés szerinti készítménnyel, olyan körülmények között, amely körülmények között a polipeptid képes hidrolizálni a poliszacharidot, oligoszacharidot vagy keményítőt az erjesztésben vagy alkohol előállításában használt készítményben.

18. Eljárás egy készítmény bio-fehérítésére, azzal jellemezve, hogy a következő lépéseket tartalmazza:

(i) biztosítunk egy készítményt, ahol a készítmény egy papír vagy egy cellulóz termék, valamint biztosítjuk az 5. igénypont szerinti polipeptidet;

(ii) a készítményt az 5. igénypont szerinti polipeptiddel kezeljük.

19. Eljárás papír és rostok nyomdafesték-mentesítésére, azzal jellemezve, hogy a következő lépéseket tartalmazza:

(a) biztosítjuk az 5. igénypont szerinti polipeptidet;

(b) biztosítunk egy papírt vagy rostokat tartalmazó készítményt;

(c) az (a) lépés szerinti polipeptidet és a (b) lépés szerinti készítményt érintkezésbe hozzuk olyan körülmények között, amely körülmények között a polipeptid képes nyomdafesték-mentesíteni a papírt vagy a rostot.

20. Eljárás üzemanyag előállítására, azzal jellemezve, hogy a következő lépéseket tartalmazza:

(i) (a) biztosítjuk az 5. igénypont szerinti polipeptidet; (b) biztosítunk egy poliszacharidot, oligoszacharidot vagy keményítőt tartalmazó készítményt; és (c) az (a) pont szerinti polipeptidet érintkezésbe hozzuk a (b) pont szerinti készítménnyel, olyan körülmények között, amely körülmények között a polipeptid hidrolizálja a poliszacharidot, oligoszacharidot vagy keményítőt; vagy

(ii) az (i) pont szerinti eljárás, ahol a polipeptid egy hőstabil enzim;

(iii) az (i) pont szerinti eljárás, ahol az üzemanyag etanol-alapú.

21. Eljárás egy lignocellulóz biomassza anyag feldolgozására, azzal jellemezve, hogy a következő lépéseket tartalmazza:

(i) (a) biztosítunk egy készítményt, amely tartalmazza az 5. igénypont szerinti polipeptidet, valamint biztosítunk egy biomassza anyagot; és (b) a készítményt érintkezésbe hozzuk a biomassza anyaggal;

(ii) az (i) pont szerinti eljárás, ahol a biomassza anyag egy mezőgazdasági termény, vagy abból származik, vagy egy élelmiszer- vagy táp-előállítás melléktermék, vagy egy lignocellulóz hulladék, vagy egy növényi maradék, vagy papírhulladék, vagy papírhulladék termék,

és adott esetben a biomassza burgonyát, szójababot, árpát, rozsot, zabot, búzát, cukorrépát vagy cukornádat tartalmaz;

(iii) az (i) pont szerinti eljárás, ahol a polipeptid aktivitása glükoamiláz aktivitás;

(iv) a (ii) pont szerint eljárás, ahol a növényi maradék szárakat, leveleket, héjakat, kukoricacsöveket, fát, faforgácsot, cellulózt és fűrészport tartalmaz; vagy a papírhulladék kidobott vagy használt fénymásolat papírt, számítógépes nyomtató papírt, jegyzetfüzet papírt, jegyzettömb papírt, írógép papírt, újságokat, magazinokat, kartonpapírt és papíralapú csomagoló anyagokat tartalmaz; vagy

(v) az (i) – (iv) pontok szerinti eljárás, ahol a biomassza anyag feldolgozása bioetanolt, biopropanolt, biobutanolt vagy biodízel olajat eredményez;

(vi) az (i) – (v) pontok szerinti eljárás, ahol a biomassza anyag egy lignocellulózt tartalmaz.

22. Eljárás bioetanol, biopropanol, biobutanol vagy biodízel olaj előállítására, azzal jellemezve, hogy a következő lépéseket tartalmazza:

(i) (a) biztosítunk egy 5. igénypont szerinti polipeptidet, és biztosítunk egy poliszacharidot vagy oligoszacharidot tartalmazó készítményt; és

(b) a poliszacharidot vagy oligoszacharidot tartalmazó készítményt érintkezésbe hozzuk az 5. igénypont szerinti polipeptiddel;

(ii) az (i) pont szerinti eljárás, ahol a poliszacharidot vagy oligoszacharidot tartalmazó készítmény egy növényt, növényi terméket vagy növény-származékot tartalmaz;

(iii) az (ii) pont szerinti eljárás, ahol a növény vagy növényi termék cukornád növényeket vagy növényi termékeket, répákat vagy cukorrépákat, búzát, kukoricát, szójababot, burgonyát, rizst vagy árpát tartalmaz;

(iv) az (i) – (iii) pontok bármelyike szerinti eljárás, ahol a polipeptid aktivitása glükoamiláz aktivitás; vagy

(v) az (i) – (iv) pontok bármelyike szerinti eljárás, ahol a poliszacharid vagy az oligoszacharid egy fermentálható cukrot tartalmaz.

23. Eljárás üzemanyag előállítására, azzal jellemezve, hogy a következő lépéseket tartalmazza

(i) (a) biztosítjuk az 5. igénypont szerinti polipeptidet, és biztosítunk egy készítményt, amely tartalmaz egy fermentálható cukrot, és

(b) a fermentálható cukrot tartalmazó készítményt érintkezésbe hozzuk az 5. igénypont szerinti polipeptiddel;

(ii) az (i) pont szerinti eljárás, ahol a fermentálható cukrot tartalmazó készítmény egy növényt, növényi terméket vagy növény-származékot tartalmaz;

(iii) az (ii) pont szerinti eljárás, ahol a növény vagy növényi termék cukornád növényeket vagy növényi termékeket, répákat vagy cukorrépákat, búzát, kukoricát, szójababot, burgonyát, rizst vagy árpát tartalmaz;

(iv) az (i) – (iii) pontok bármelyike szerinti eljárás, ahol a polipeptid aktivitása glükoamiláz aktivitás; vagy

(v) az (i) – (iv) pontok bármelyike szerinti eljárás, ahol az üzemanyag bioetanol, benzin-etanol keverék, vagy bioetanolt, biopropanolt, biobutanolt vagy egy biodízelt tartalmaz..

24. Eljárás cukor előállítására, azzal jellemezve, hogy a következő lépéseket tartalmazza:

(i) (a) biztosítjuk az 5. igénypont szerinti polipeptidet;

(b) biztosítunk egy poliszacharidot vagy oligoszacharidot tartalmazó készítményt; és

(c) a (b) lépés szerinti készítményt érintkezésbe hozzuk az (a) lépés szerinti polipep-tiddel, ezzel cukrokat állítunk elő;

(ii) az (i) pont szerinti eljárás, ahol a készítmény egy keményítőt tartalmazó poliszacharidot tartalmaz;

(iii) az (i) – (iii) pontok bármelyike szerinti eljárás, ahol a cukor egy fermentálható cukor.

25. Készítmény, amely az 5. igénypont szerinti polipeptidet tartalmazza,

ahol a készítményt a következők közül választhatjuk ki: egy detergens, egy élelmiszer vagy táp, egy textília vagy szövet, egy papír vagy papírtermék, vagy papíripari rostanyag, egy magas maltóz- vagy magas glükóz-tartalmú folyadék vagy szirup, egy szikkadásgátló készítmény, egy alkoholtartalmú üdítőital, egy sör, egy gyógyászati készítmény, egy szájhigiéniai termék, egy fogkrém, egy fogászati krém, egy gél vagy egy fogpor, foggal kapcsolatos termék, szájvíz, fogmosás előtti vagy utáni öblítő készítmény; rágógumi, cukorka, vagy egy édesség, olajfúró folyadék, bio-fehérítő oldat, fertőtlenítőszer, egy biodefense vagy bio-méregtelenítő szer, tejtermék, vagy biomassza anyag.

26. Egy késleltetett vagy szabályozott felszabadulású, az 5. igénypont szerinti polipeptidet tartalmazó készítmény, amely a következő komponenseket tartalmazza:

(a) egy kívánt adalékanyag, latex polimer bevonattal bevonva;

(b) az (a) pont szerinti késleltetett vagy szabályozott felszabadulású készítmény, ahol a kívánt adalékanyag egy enzim;

(c) az (a) vagy (b) pont szerinti késleltetett vagy szabályozott felszabadulású készítmény, ahol a kívánt adalékanyag egy kis molekula, egy gyógyszer, egy poliszacharid, egy lipid, egy nukleinsav, egy vitamin, egy antibiotikum vagy egy rovarirtó;

(d) az (a), (b) vagy (c) pont szerinti késleltetett vagy szabályozott felszabadulású készítmény;

ahol a kívánt adalékanyag egy pellet vagy egy mátrix;

(e) az (a) – (d) pontok bármelyike szerinti késleltetett vagy szabályozott felszabadulású készítmény, ahol a pellet vagy a mátrix ehető anyagot tartalmaz;

(f) az (a) – (e) pontok bármelyike szerinti késleltetett vagy szabályozott felszabadulású készítmény, ahol a latex polimer bevonó egy latex festéket tartalmaz; vagy

(g) az (a) – (f) pontok bármelyike szerinti késleltetett vagy szabályozott felszabadulású készítmény, ahol a latex polimer bevonó egy (met)akrilát, egy vinilacetát, egy sztirol, egy etilén, egy vinilklorid, egy butadién, egy vinilidén-klorid, egy vinil-verzatát, egy vinil-propionát, egy terc-butil-akrilát, egy akrilonitril, egy neoprén, egy maleát, egy fumarát, vagy ezek kombinációja vagy származéka.

