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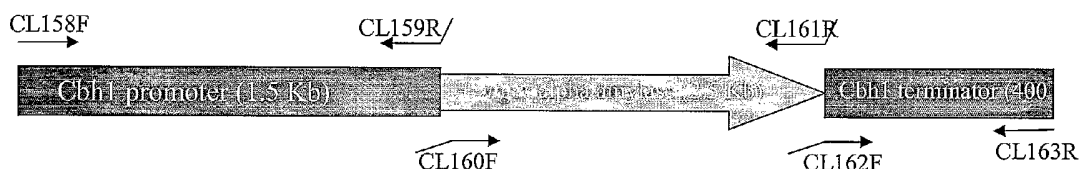
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(54) Title: POLYPEPTIDES HAVING ALPHA-AMYLASE AND GRANULAR STARCH HYDROLYZING ACTIVITY

A. niger alpha amylase expression cassette



(57) Abstract: The present invention relates to a polypeptide having alpha-amylase activity obtained from a strain of *Aspergillus niger*.

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POLYPEPTIDES HAVING ALPHA-AMYLASE AND GRANULAR STARCH HYDROLYZING ACTIVITY

The present application claims priority to United States Provisional Patent
5 Application Serial No. 60/654,105, filed February 18, 2005.

Field of the Invention

The present invention relates to polypeptides having alpha-amylase activity and
10 exhibiting granular starch hydrolyzing activity and biologically active fragments thereof,
the heterologous expression of the polypeptides in host microorganisms as well as
enzyme compositions comprising the polypeptides and uses of said polypeptides.

Background of the Invention

15 Alpha-amylases (α -1,4-glucan-4-glucanohydrolase, EC 3.2.1.1.) hydrolyze
internal α -1,4-glucosidic linkages in starch, largely at random to produce smaller
molecular weight dextrans. These polypeptides are of considerable commercial value and
are used in starch processing, in alcohol production, as cleaning agents, in the textile
industry for starch desizing, in the paper and pulp industry, and in baking.

20 Alpha-amylases are produced by a wide variety of microorganisms including
Bacillus and *Aspergillus*. Despite the advances made in the prior art concerning new and
modified alpha-amylases there is a need in the art for alpha-amylases having a
performance suitable for specific applications.

25 Summary of the Invention

In a first aspect, the invention relates to an isolated polypeptide having alpha-
amylase activity and granular starch hydrolyzing activity comprising an amino acid
sequence which is at least 85% identical, preferably at least 90% identical or at least 95%
30 identical or even at least 98% identical to SEQ ID NO: 8 and biologically active
fragments thereof. The alpha amylase includes a catalytic domain, a linker and a starch-
binding domain.

In some embodiments, the catalytic domain of the alpha-amylase has at least 95%
sequence identity to SEQ ID NO: 5, the starch-binding domain has at least 80% sequence

identity to SEQ ID NO: 7, and the linker has at least 90% sequence identity to SEQ ID NO: 6. In other embodiments, the alpha-amylase will comprise a signal sequence and preferably the signal sequence will have an amino acid sequence which is at least 90% identical to the sequence of SEQ ID NO: 4. In yet other embodiments, the alpha-amylase is obtained from an *Aspergillus niger* strain.

In a second aspect, the invention relates to a polynucleotide which encodes a polypeptide having alpha-amylase activity and granular starch hydrolyzing activity. In one embodiment, the polynucleotide encodes an alpha-amylase having at least 95% sequence identity with SEQ ID NO: 8. In other embodiments, the polynucleotide has the sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

In a third aspect, the invention relates to a polypeptide having alpha-amylase activity comprising an amino acid sequence having at least 95% sequence identity to the sequence of SEQ ID NO: 5.

In a fourth aspect, the invention relates to a method of recombinantly producing an alpha-amylase having granular starch hydrolyzing activity comprising expressing a polynucleotide encoding a polypeptide having alpha-amylase activity in a filamentous fungal host cell, wherein the polypeptide comprises an amino acid sequence which is at least 85% identical, preferably 90% identical or at least 95% identical or even 98% identical to SEQ ID NO: 8 and comprises both a catalytic domain and a starch-binding domain. In some embodiments, the filamentous host cell is of the genus *Aspergillus*, such as *A. awamori*, *A. niger* or *A. oryzae*. In other embodiments, the filamentous host cell is of the genus *Trichoderma*, such as *T. reesei*.

In a fifth aspect, the invention relates to a filamentous fungal host cell comprising a heterologous polynucleotide encoding a polypeptide having alpha-amylase activity and granular starch hydrolyzing activity, the polypeptide comprising an amino acid which is at least 85% identical, preferably at least 90% identical or at least 95% identical or even 98% identical to SEQ ID NO: 8.

In a sixth aspect, the invention relates to an enzyme composition comprising a polypeptide having alpha-amylase activity and optionally having granular starch hydrolyzing activity, which comprises an amino acid sequence which is at least 85% identical, preferably at least 90% identical or at least 95% identical or even at least 98% identical to SEQ ID NO: 8 and biologically active fragments thereof. In one embodiment, of this aspect the amino acid sequence includes a catalytic domain, a linker and a starch-

binding domain and in another embodiment of this aspect the amino acid sequence includes a catalytic domain.

In a seventh aspect, the invention relates to uses of the polypeptide of the invention having alpha-amylase activity and optionally granular starch hydrolyzing activity. Uses of the polypeptide in various processes include uses in starch conversion processes; processes for producing specialty syrups, processes for producing ethanol, and in fermentation processes for producing organic compounds, such as ascorbic acid, glutamic acid and glycerol.

Brief Description of the Drawings

Figures 1A-1B represent genomic DNA isolated from an *Aspergillus niger* (SEQ ID NO: 1), which encodes a polypeptide having alpha-amylase activity and granular starch hydrolyzing activity.

Figures 2A-2B represent the cDNA sequence (SEQ ID NO: 2) from the DNA sequence of figure 1, wherein 8 putative introns are removed.

Figure 3 illustrates the translated alpha-amylase protein (SEQ ID NO: 3). The mature protein sequence, which excludes the signal sequence is represented by SEQ ID NO: 8). In the figure, the signal sequence is underlined (SEQ ID NO: 4); the catalytic domain is in bold (SEQ ID NO: 5); the linker region is in italics (SEQ ID NO: 6) and the starch-binding domain is double underlined (SEQ ID NO: 7).

Figure 4 illustrates the *A. niger* alpha-amylase expression cassette used in *Trichoderma reesei* transformation.

Figure 5 illustrates NuPage gels of sample transformants, wherein transformants are represented in lanes 1 – 12 and the untransformed parent control is in lane 13. The Mark 12 molecular weight markers (Invitrogen) MW are observed on both ends of the gel. Standard bands are indicated in kDa on the left. The expressed *A. niger* alpha amylase is observed in lane 5 as indicated by the band marked with the arrow.

Figure 6 illustrates raw starch degradation by the alpha-amylase represented in Figure 3, (labeled putative alpha in the figure) compared to a starch blank and an alpha amylase control as measured by HPLC.

Detailed Description of the Invention

In some aspects, the present invention relies on routine techniques and methods used in the field of genetic engineering and molecular biology. The following resources include descriptions of general methodology useful in accordance with the invention: Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL (2nd Ed., 1989);
5 Kreigler, GENE TRANSFER AND EXPRESSION; A LABORATORY MANUAL (1990) and Ausubel *et al.*, Eds. CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (1994). These general references provide definitions and methods known to those in the art. However, it is not intended that the present invention be limited to any particular methods, protocols, and reagents described, as these may vary.

10 Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994) and Hale & Markham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY (1991)
15 provide one of skill with general dictionaries of many of the terms used in this invention.

Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

The invention will now be described in detail by way of reference only using the
20 following definitions and examples. All patents and publications, including all sequences disclosed within such patents and publications, referred to herein are expressly incorporated by reference.

Numeric ranges are inclusive of the numbers defining the range.

25 Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

The headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the specification as a whole.

30

Definitions -

The term "alpha-amylase (*e.g.*, E.C. class 3.2.1.1)" refers to polypeptides that catalyze the hydrolysis of alpha-1, 4-glucosidic linkages.

The term "acid-stable alpha amylase" refers to an alpha-amylase that is active in the pH range of pH 3.0 to 7.0 and preferably 3.5 to 6.0.

The term "starch binding domain (SBD)" refers to a region of a polypeptide, which binds to a starch substrate.

5 The term "catalytic domain" refers to a structural region of a polypeptide which is distinct from the SBD and which contains the active site for substrate hydrolysis.

The term "linker" refers to a short amino acid sequence, which generally has between 3 and 40 amino acid residues, which covalently bind an amino acid sequence comprising a SBD with an amino acid sequence comprising a catalytic domain.

10 The term "granular starch" refers to raw (uncooked) starch, *e.g.*, starch granules that have not been subject to temperatures that result in gelatinization.

A "biologically active fragment" means an amino acid fragment of an alpha-amylase encompassed by the invention, said fragment also having alpha-amylase activity.

15 The term "granular starch hydrolyzing (GSH) activity" refers to the ability of a polypeptide to hydrolyze starch in granular form.

As used herein the term "starch" refers to any material comprised of the complex polysaccharide carbohydrates of plants, comprised of amylose and amylopectin with the formula $(C_6H_{10}O_5)_x$, wherein X can be any number. In particular, the term refers to any plant-based material including but not limited to grains, grasses, tubers and roots and
20 more specifically wheat, barley, corn, rye, rice, sorghum, brans, cassava, millet, potato, sweet potato, and tapioca.

The term "glucoamylase" refers to the amyloglucosidase class of enzymes (*e.g.*, EC.3.2.1.3, glucoamylase, 1, 4-alpha-D-glucan glucohydrolase). These are exo-acting enzymes, which release glucosyl residues from the non-reducing ends of amylose and
25 amylopectin molecules. The enzyme also hydrolyzes alpha-1, 6 and alpha-1,3 linkages although at much slower rate than alpha-1, 4 linkages.

Glucoamylase activity may be measured using the well-known assay which is based on the ability of glucoamylase to catalyze the hydrolysis of p-nitrophenyl-alpha-D-glucopyranoside (PNPG) to glucose and p-nitrophenol. At an alkaline pH, the
30 nitrophenol; forms a yellow color that is proportional to glucoamylase activity and is monitored at 400nm and compared against an enzyme standard measured as a GAU. In an embodiment, a "Glucoamylase Activity Unit" (GAU) is defined as the amount of enzyme

that will produce 1 gm of reducing sugar, calculated as glucose per hour from a soluble starch substrate (4% ds) at pH 4.2 and 60°C.

The term "recombinant" when used in reference to a cell, nucleic acid, protein or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

The terms "protein" and "polypeptide" are used interchangeably herein. The conventional one-letter or three-letter code for amino acid residues is used herein.

A "signal sequence" means a sequence of amino acids bound to the N-terminal portion of a protein, which facilitates the secretion of the mature form of the protein outside the cell. The definition of a signal sequence is a functional one. The mature form of the extracellular protein lacks the signal sequence which is cleaved off during the secretion process.

A "gene" refers to a DNA segment that is involved in producing a polypeptide and includes regions preceding and following the coding regions as well as intervening sequences (introns) between individual coding segments (exons).

The term "nucleic acid" encompasses DNA, cDNA, RNA, single stranded or double stranded and chemical modifications thereof. The terms "nucleic acid" and "polynucleotide" may be used interchangeably herein. The term cDNA refers to a DNA molecule which lacks the intron sequences that are usually present in the corresponding genomic DNA. A "coding" sequence is a nucleic acid sequence, which directly specifies the amino acid sequence of its protein product.

The term "heterologous" with reference to a polynucleotide or protein refers to a polynucleotide or protein that does not naturally occur in a host cell and includes a polynucleotide or polypeptide that has been introduced into a host cell by recombinant DNA technology. It is intended that the term encompass proteins that are encoded by naturally occurring genes, mutated genes, and/or synthetic genes.

The term "endogenous" with reference to a polynucleotide or protein refers to a polynucleotide or protein that occurs naturally in the host cell.

A "vector" refers to a polynucleotide sequence designed to introduce nucleic acids into one or more cell types. Vectors include cloning vectors, expression vectors, shuttle vectors, plasmids, phage particles, cassettes and the like.

5 An "expression vector" as used herein means a DNA construct comprising a DNA sequence which is operably linked to a suitable control sequence capable of effecting expression of the DNA in a suitable host. Such control sequences may include a promoter to effect transcription, an optional operator sequence to control transcription, a sequence encoding suitable ribosome binding sites on the mRNA, enhancers and sequences which control termination of transcription and translation.

10 A "promoter" is a regulatory sequence that is involved in binding RNA polymerase to initiate transcription of a gene. The promoter may be an inducible promoter or a constitutive promoter.

"Under transcriptional control" is a term well understood in the art that indicates that transcription of a polynucleotide sequence, usually a DNA sequence, depends on its being operably linked to an element which contributes to the initiation of, or promotes transcription.

"Under translational control" is a term well understood in the art that indicates a regulatory process that occurs after mRNA has been formed.

20 The term "operably linked" refers to juxtaposition wherein the elements are in an arrangement allowing them to be functionally related. For example, a promoter is operably linked to a coding sequence if it controls the transcription of the sequence.

The term "selective marker" refers to a gene capable of expression in a host that allows for ease of selection of those hosts containing an introduced nucleic acid or vector.

25 A polynucleotide or a polypeptide having a certain percent (*e.g.* 80%, 85%, 90%, 95%, or 99%) of sequence identity with another sequence means that, when aligned, that percentage of bases or amino acid residues are the same in comparing the two sequences. This alignment and the percent homology or identity can be determined using any suitable software program known in the art, for example those described in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel *et al.* (eds) 1987, Supplement 30, section 7.7.18). Preferred programs include the GCG Pileup program, FASTA (Pearson *et al.* (1988) *Proc. Natl. Acad. Sci USA* 85:2444-2448), and BLAST (BLAST Manual, Altschul *et al.*, Natl. Cent. Biotechnol. Inf., Natl Lib. Med. (NCIB NLM NIH), Bethesda, MD, and Altschul *et al.*, (1997) *NAR* 25:3389-3402). Another preferred alignment

program is ALIGN Plus (Scientific and Educational Software, PA), preferably using default parameters. Another sequence software program that finds use is the TFASTA Data Searching Program available in the Sequence Software Package Version 6.0 (Genetics Computer Group, University of Wisconsin, Madison, WI).

5 One skilled in the art will recognize that sequences encompassed by the invention are also defined by the ability to hybridize under stringent hybridization conditions with an exemplified sequence (*e. g.*, SEQ ID NO: 2). A nucleic acid is hybridizable to another nucleic acid sequence when a single stranded form of the nucleic acid can anneal to the other nucleic acid under appropriate conditions of temperature and solution ionic strength.
10 Hybridization and washing conditions are well known in the art (See, *e.g.*, Sambrook (1989) *supra*, particularly chapters 9 and 11). In some embodiments, stringent conditions correspond to a T_m of 65°C and 0.1x SSC, 0.1% SDS.

“Host strain” or “host cell” means a host cell which is suitable for transformation with an expression vector or DNA construct. In one embodiment of the invention, “host
15 cell” means both the cells and protoplasts created from the cells of a filamentous fungal strain.

The term “culturing” refers to growing a population of microbial cells under suitable conditions in a liquid or solid medium. In one embodiment, culturing refers to fermentative bioconversion of a starch substrate containing granular starch to an end-
20 product (typically in a vessel or reactor). Fermentation is the enzymatic and anaerobic breakdown of organic substances by microorganisms to produce simpler organic compounds. While fermentation occurs under anaerobic conditions, it is not intended that the term be solely limited to strict anaerobic conditions, as fermentation also occurs in the presence of oxygen.

25 The phrase “simultaneous saccharification and fermentation (SSF)” refers to a process in the production of products in which a microbial organism, such as an ethanol producing microorganism and at least one enzyme such as a enzyme having alpha-amylase activity are in the same process step. In one embodiment of the present invention, SSF refers to the contemporaneous hydrolysis of granular starch substrates to
30 saccharides including glucose and the fermentation of the saccharides into alcohol in the same reactor vessel.

The term “contacting” refers to the placing of the respective enzyme(s) in sufficiently close proximity to the respective substrate to enable the enzyme(s) to convert

the substrate to the end-product. Those skilled in the art will recognize that mixing solutions of the enzyme with the respective substrates can effect contacting.

The term "enzymatic conversion" in general refers to the modification of a substrate by enzyme action. The term as used herein also refers to the modification of a granular starch substrate by the action of an enzyme.

As used herein the term "saccharification" refers to enzymatic conversion of starch to glucose.

The term "gelatinization" means solubilization of a starch molecule by cooking to form a viscous suspension.

The term "liquefaction" refers to the stage in starch conversion in which gelatinized starch is hydrolyzed to give low molecular weight soluble dextrins.

The terms "end-product" or "desired end-product" refer to any carbon-source derived molecule product which is enzymatically converted from a starch containing substrate.

As used herein "ethanologenic microorganism" refers to a microorganism with the ability to convert sugars or oligosaccharides to ethanol. The ethanologenic microorganisms are ethanologenic by virtue of their ability to express one or more enzymes that individually or together convert sugar to ethanol.

As used herein the term "ethanol producer" or "ethanol producing microorganism" refers to any organism or cell that is capable of producing ethanol from a hexose or pentose. Generally, ethanol-producing cells contain an alcohol dehydrogenase and a pyruvate decarboxylase. Examples of ethanol producing microorganisms include fungal microorganisms such as yeast. A preferred yeast includes strains of *Saccharomyces*, particularly, *S. cerevisiae*.

The terms "recovered", "isolated", and "separated" as used herein refer to a compound, protein, cell, nucleic acid or amino acid that is removed from at least one component with which it is naturally associated.

As used herein, the terms "transformed", "stably transformed" and "transgenic" used in reference to a cell means the cell has a non-native (*e.g.*, heterologous) nucleic acid sequence integrated into its genome or as an episomal plasmid that is maintained through multiple generations.

As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process includes both transcription and translation.

The term "introduced" in the context of inserting a nucleic acid sequence into a cell, means "transfection", or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid sequence into a eukaryotic or prokaryotic cell wherein the nucleic acid sequence may be incorporated into the genome of the cell (*e.g.*, chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (*e.g.*, transfected mRNA).

As used herein the term "specific activity" means an enzyme unit defined as the number of moles of substrate converted to product by an enzyme preparation per unit time under specific conditions. Specific activity is expressed as units (U)/mg of protein.

As used herein the term "enzyme unit" refers to the amount of enzyme that produces 1 micromole of product per minute under the specified conditions of the assay.

In one embodiment, a granular starch hydrolyzing enzyme unit (GSHE U) is defined as being the amount of GSHE required to produce 1 mg of glucose per minute from granular starch under assay conditions of, for example 25°C at pH 5.0. In an alternate embodiment, a GSHE U is defined as being the amount of a GSHE required to produce 1 mg glucose/min from a granular starch substrate at 50°C at pH 4.5.

"ATCC" refers to American Type Culture Collection located at Manassas, VA 20108 (ATCC; <www.atcc.org>).

"NRRL" refers to the Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research (and previously known as USDA Northern Regional Research Laboratory), Peoria, ILL.

"A", "an" and "the" include plural references unless the context clearly dictates otherwise.

As used herein the term "comprising" and its cognates are used in their inclusive sense; that is, equivalent to the term "including" and its corresponding cognates.

Polypeptides having alpha-amylase activity.

In one embodiment, the present invention relates to a polypeptide having alpha-amylase activity. The polypeptide may also have granular starch hydrolyzing (GSH) activity. Alpha-amylase enzymes, E.C. class 3.2.1.1, have also been described as those

effecting the exo- or endohydrolysis of 1,4- α -D-glucosidic linkages in polysaccharides containing 1,4- α -linked D-glucose units.

In some embodiments, the polypeptide having alpha-amylase activity comprises an amino acid sequence which is at least 85% identical, at least 90% identical, at least 5 95% identical, at least 96% identical, at least 97% identical, at least 98% identical and at least 99% identical to SEQ ID NO: 8. In some embodiments, the polypeptide will have the sequence of SEQ ID NO: 8. The polypeptide includes a catalytic domain, a linker and a starch-binding domain.

In some embodiments, the polypeptide having alpha-amylase activity and 10 optionally granular starch hydrolyzing activity will differ from SEQ ID NO: 8 by including conservative amino acid replacements that do not significantly affect the activity of the polypeptide. Conservative replacements include, for example within the following groups a) acidic amino acids (Asp and Glu); b) basic amino acids (Arg, Lys and His); c) polar amino acids (Asn and Gln); d) aromatic amino acids (Phe, Tyr, Trp and 15 His); and e) small amino acids (Ala, Ser and Gly). See, H. Neurath and R. L. Hill, 1979, THE PROTEINS, Academic Press, NY. Table 1 illustrates exemplary conservative amino acid substitutions that are recognized in the art. In addition substitution may be by one or more non-conservative amino acid substitutions, deletions, or insertions, which do not abolish the alpha amylase activity.

20

TABLE 1
CONSERVATIVE AMINO ACID REPLACEMENTS

For Amino Acid	Code	Replace with any of
Alanine	A	D-Ala, Gly, beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, b-Ala, Acp

Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, cis-3, 4, or 5-phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

In some embodiments, the catalytic domain of the alpha-amylase will have at least 95%, at least 96%, at least 97%, at least 98% and at least 99% sequence identity to SEQ ID NO: 5. In other embodiments, the starch-binding domain will have at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98% and at least 99% sequence identity to SEQ ID NO: 7. In further embodiments, the linker will have at least 90%, at least 95%, at least 96%, at least 97%, at least 98% and at least 99% sequence identity to SEQ ID NO: 6. In some embodiments, the linker will consist of a sequence having 1, 2 or 3 amino acid residues more or less than the linker of SEQ ID NO: 6. In preferred embodiments, the polypeptides of the invention having alpha-amylase activity and optionally granular starch hydrolyzing activity are secreted proteins. In other embodiments, the polypeptides are acid-stable alpha-amylases.

In some embodiments, the alpha-amylase will comprise a signal sequence and preferably the signal sequence will comprise an amino acid sequence which is at least 90%, at least 95%, at least 96%, at least 97%, at least 98% and at least 99% identical to the sequence of SEQ ID NO: 4.

In some embodiments, the invention relates to biologically active fragments of an alpha-amylase encompassed by the invention, said fragments also having alpha-amylase activity. In some embodiments, biologically active fragments include alpha-amylases

having at least 250 amino acid residues, at least 300 amino acid residues, at least 350 amino acid residues, at least 375 amino acid residues, and also at least 400 amino acid residues. In other embodiments, biologically active fragments include at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 97%, at least 98%, at least 99% of a polypeptide sequence having at least 95%, at least 97%, at least 98% and at least 99% sequence identity with the alpha-amylase having SEQ ID NO: 8.

Biologically active fragments of an alpha-amylase encompassed by the invention can be generated by methods known to those skilled in the art. The ability of a candidate fragment to exhibit a biological activity of an amylase can be assessed by methods known to those skilled in the art as described herein.

In some embodiments, the alpha-amylases of the invention, which include biologically active fragments, will comprise at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, and at least 100% of the alpha-amylase activity of the mature polypeptide of SEQ ID NO: 8. In some embodiments, a biologically active fragment possesses at least 40% or at least 90% of the amylase activity of the alpha-amylase having SEQ ID NO: 8 in any in vivo or in vitro amylase activity assay.

In other embodiments, a polypeptide of the invention has at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% at least 90%, at least 95% and preferably at least 100% of the granular starch hydrolyzing activity of the mature polypeptide of SEQ ID NO: 8.

In yet other embodiments, a polypeptide of the present invention is a wild-type alpha-amylase identified and isolated from a filamentous fungus. The polypeptide of the present invention is preferably isolated from an *Aspergillus* strain such as an *Aspergillus niger* strain. Further, the polypeptide of the invention may be prepared by techniques known in the art.

Polynucleotides encoding the alpha-amylase of the invention.

In another embodiment, the invention relates to isolated polynucleotides which encode a polypeptide of the invention having alpha-amylase activity and optionally granular starch hydrolyzing activity (GSH). In a preferred embodiment, the polynucleotide is set forth in SEQ ID NO: 1 or SEQ ID NO: 2. In other preferred

embodiments, the polynucleotide has a sequence which is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% and at least 99% identical to SEQ ID NO: 1 or SEQ ID NO: 2. In some preferred embodiments, the polynucleotide will encode a polypeptide having alpha-amylase activity and GSH activity comprising a polypeptide sequence having at least 90% sequence identity to SEQ ID NO: 8.

In other embodiments, the polynucleotide will encode a biologically active fragment having alpha-amylase activity. In some embodiments, the polynucleotide will encode a catalytic domain of an alpha-amylase encompassed by the invention. In some preferred embodiments, the polynucleotide will encode a biologically active fragment which has at least 95% sequence identity to the sequence having SEQ ID NO: 5.

One skilled in the art is well aware of the degeneracy of the genetic code and because of this degeneracy, more than one codon can be used to encode a particular polypeptide. The present invention encompasses polynucleotides which encode a particular amino acid sequence.

Techniques used to isolate or clone a polynucleotide encoding a polypeptide are known in the art and include isolation of genomic DNA, preparation from cDNA or a combination thereof. Additionally amplification techniques such as the polymerase chain reaction (PCR) using specific primers, ligase chain reaction (LCR), ligated activated transcription (LAT), nucleic acid sequence based amplification (NASBA) and antibody screening of expression libraries to detect cloned DNA may be used. (See, e.g. Innis et al., 1990, PCR: A GUIDE TO METHODS AND APPLICATIONS; Academic Press NY). Further, a polynucleotide sequence encoding a polypeptide of the invention may be prepared synthetically by established techniques.

Expression of an alpha-amylase of the invention.

According to the invention, a DNA sequence encoding a polypeptide having alpha-amylase activity and optionally granular starch hydrolyzing activity as described above can be expressed in enzyme form using a DNA construct (expression vector) which typically includes a promoter, signal sequence, and optionally other control sequences.

Nucleic acid constructs and expression vectors -

In a further embodiment, the invention relates to nucleic acid constructs comprising a polynucleotide encoding a polypeptide having alpha-amylase activity and

optionally granular starch hydrolyzing activity operably linked to a suitable promoter, which shows transcriptional activity in a host cell (e.g. a fungal host cell).

The promoter may be derived from genes encoding proteins either homologous or heterologous to the host cell. The promoter may be a truncated or hybrid promoter.

5 Preferably, the promoter is useful in a *Trichoderma* host or an *Aspergillus* host.

Suitable nonlimiting examples of promoters include *cbh1*, *cbh2*, *egl1*, *egl2*. In one embodiment, the promoter is one that is native to the host cell. For example, when *T. reesei* is the host, the promoter is a native *T. reesei* promoter. In one embodiment, the promoter is *T. reesei cbh1*, which is an inducible promoter and has been deposited in
10 GenBank under Accession No. D86235. An "inducible promoter" is a promoter that is active under environmental or developmental regulation. In another embodiment, the promoter is one that is heterologous to the fungal host cell. Other examples of useful promoters include promoters from the genes of *A. awamori* and *A. niger* glucoamylase genes (*glaA*) (Nunberg *et al.*, (1984) *Mol. Cell Biol.* 4:2306-2315 and Boel *et al.*, (1984)
15 *EMBO J.* 3:1581-1585); *Aspergillus oryzae* TAKA amylase; *Rhizomucor miehei* aspartic proteinase; *Aspergillus niger* neutral alpha-amylase; *Aspergillus niger* acid stable alpha-amylase; *Trichoderma reesei xln1* and the *cellobiohydrolase 1* gene promoter (EPA 137280A1) and mutant, truncated and hybrid promoters thereof.

In some embodiments, the DNA construct or expression vector includes a
20 termination sequence. The terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the polypeptide. Any terminator functional in the host cell may be used. In one embodiment, the termination sequence and the promoter sequence are derived from the same source. In another embodiment, the termination sequence is homologous to the host cell. A particularly suitable terminator sequence is
25 *cbh1* derived from a *Trichoderma* strain and particularly *T. reesei*. Other useful fungal terminators include the terminator from *A. niger* or *A. awamori* glucoamylase (Nunberg *et al.* (1984) *supra*, and Boel *et al.*, (1984) *supra*); *Aspergillus nidulans* anthranilate synthase; *Aspergillus niger* alpha-glucosidase and *Aspergillus oryzae* TAKA amylase.

In some embodiments, the polypeptide coding sequence is operably linked to a
30 signal sequence which directs the encoded polypeptide into the cell's secretory pathway. The 5' end of the coding sequence may naturally contain a signal sequence naturally linked in translation reading frame with the segment of the coding region which encodes the secreted polypeptide. The DNA encoding the signal sequence is preferably that which

is naturally associated with the polypeptide to be expressed. Preferably, the signal sequence is encoded by an *Aspergillus niger* alpha-amylase, *Aspergillus niger* neutral amylase or *Aspergillus niger* glucoamylase. In some embodiments, the signal sequence is the *Trichoderma cdh1* signal sequence which is operably linked to a *cdh1* promoter. More preferably, the signal sequence has at least 90%, at least 95%, at least 97%, at least 98% and at least 99% sequence identity to the signal sequence of SEQ ID NO: 4. In additional embodiments, a signal sequence and a promoter sequence comprising a DNA construct or vector to be introduced into a fungal host cell are derived from the same source.

In some embodiments, the DNA construct or expression vector may include polyadenylation sequences for filamentous fungal host cells which may be obtained for example from *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans*, *Aspergillus niger* alpha glucosidase and *Aspergillus niger* alpha amylase.

In some embodiments, a DNA construct or an expression vector includes a selectable marker. Examples of preferred selectable markers include ones which confer antimicrobial resistance (*e.g.*, hygromycin, chloramphenicol and phleomycin). Nutritional and metabolic selective markers also find use in the present invention including those markers known in the art as *amdS*, *argB* and *pyr4*.

Markers useful in vector systems for transformation of for example *Trichoderma* are known in the art (See, *e.g.*, Finkelstein, chapter 6 in BIOTECHNOLOGY OF FILAMENTOUS FUNGI, Finkelstein et al. Eds. Butterworth-Heinemann, Boston, MA (1992), Chap. 6.; and Kinghorn *et al.* (1992) APPLIED MOLECULAR GENETICS OF FILAMENTOUS FUNGI, Blackie Academic and Professional, Chapman and Hall, London). In one embodiment, the selective marker is the *amdS* gene, which encodes the enzyme acetamidase, allowing transformed cells to grow on acetamide as a nitrogen source. The use of *A. nidulans amdS* gene as a selective marker is described in Kelley *et al.*, (1985) *EMBO J.* 4:475 - 479 and Penttila *et al.*, (1987) *Gene* 61:155-164. Also useful are *pyrG* genes of *A. nidulans* and *A. oryzae*.

An expression vector comprising a DNA construct with a polynucleotide encoding a polypeptide having alpha-amylase activity and optionally having GSH activity according to the invention may be any vector which is capable of replicating autonomously in a given fungal host organism or of integrating into the DNA of the host. Reference is made to the Fungal Genetics Stock Center Catalogue of Strains (FGSC, <

www.fgsc.net>) for a list of vectors. Additional examples of suitable expression and/or integration vectors are provided in Sambrook *et al.*, (1989) *supra*, Ausubel (1987) *supra*, van den Hondel *et al.* (1991) in Bennett and Lasure (Eds.) MORE GENE MANIPULATIONS IN FUNGI, Academic Press pp. 396-428 and U.S. Patent No. 5,874,276. Particularly useful
5 vectors include pFB6, pBR322, PUC18, pUC100 and pENTR/D.

In some embodiments, the expression vector is a plasmid. In some embodiments, two types of expression vectors for obtaining expression of genes are contemplated.

The first expression vector comprises DNA sequences in which the promoter, polypeptide coding region, and terminator all originate from the gene to be expressed. In
10 some embodiments, gene truncation is obtained by deleting undesired DNA sequences (*e.g.*, DNA encoding unwanted domains) to leave the domain to be expressed under control of its own transcriptional and translational regulatory sequences.

The second type of expression vector is preassembled and contains sequences required for high-level transcription and a selectable marker. In some embodiments, the
15 coding region for an alpha-amylase or part thereof is inserted into this general-purpose expression vector such that it is under the transcriptional control of the expression construct promoter and terminator sequences. In some embodiments, genes or part thereof are inserted downstream of a strong promoter such as the *cbh1* promoter.

Methods used to ligate the DNA construct comprising a polynucleotide encoding
20 an alpha-amylase, a promoter, a terminator and other sequences and to insert them into a suitable vector are well known in the art. Linking is generally accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide linkers are used in accordance with conventional practice. (*See*, Sambrook (1989) *supra*, and Bennett and Lasure, MORE GENE MANIPULATIONS IN FUNGI, Academic Press, San Diego
25 (1991) pp 70 – 76.). Additionally, vectors can be constructed using known recombination techniques (*e.g.*, Invitrogen Life Technologies, Gateway Technology).

Host cells -

Host cells include those cells in which expression of an alpha-amylase according
30 to the present invention can be achieved. Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. In some embodiments, the host cell is a strain of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, various fungi and yeast. Some preferred host cells include fungal cells, particularly filamentous fungal cells.

The term "filamentous fungi" refers to all filamentous forms of the subdivision Eumycotina (See, Alexopoulos, C. J. (1962), INTRODUCTORY MYCOLOGY, Wiley, New York). These fungi are characterized by a vegetative mycelium with a cell wall composed of chitin, cellulose, and other complex polysaccharides. The filamentous fungi of the present invention are morphologically, physiologically, and genetically distinct from yeasts. Vegetative growth by filamentous fungi is by hyphal elongation and carbon catabolism is obligatory aerobic.

The filamentous fungal cell may be a cell of a species of, but not limited to, *Trichoderma* sp. (e.g., *Trichoderma reesei* (previously classified as *T. longibrachiatum* and currently also known as *Hypocrea jecorina*), *Trichoderma viride*, *Trichoderma koningii*, and *Trichoderma harzianum*); *Penicillium* sp., *Humicola* sp. (e.g., *Humicola insolens* and *Humicola grisea*); *Chrysosporium* sp. (e.g., *C. lucknowense*); *Gliocladium* sp., *Aspergillus* sp. (e.g., *A. oryzae*, *A. niger*, *A. awamori*, *A. nidulans*, *A. aculeatus* and reference is made to in Ward et al. (1993) Appl. Microbiol. Biotechnol. 39:738-743 and Goedegebuur et al., (2002) Curr Gene 41:89 – 98.), *Fusarium* sp. (e.g. *F. bactridiodes*, *F. cerealis*, *F. graminearum*, *F. oxysporum*, and *F. roseum*), *Mucor* sp. (e.g. *M. miehei*), *Neurospora* sp. (e.g. *N. crassa*), *Sclerotium (Athelia)* sp. (e.g., *A. rolfii*), *Hypocrea* sp., and *Emericella* sp. (See also, Innis et al., (1985) Sci. 228:21 -26).

As used herein, the term "*Trichoderma*" or "*Trichoderma* sp." refer to any fungal genus previously or currently classified as *Trichoderma*. In some embodiments, particular strains of *Trichoderma reesei* include ATCC No. 13631, ATCC No. 26921, ATCC No. 56764, ATCC No. 56765, ATCC No. 56767 and NRRL 15709. In some preferred embodiments, the host strain is a derivative of RL-P37. RL-P37 is disclosed in Sheir-Neiss et al. (1984) Appl. Microbiol. Biotechnology 20:46 – 53.

In some embodiments, the host cell is a member of the genus *Bacillus*, while in some embodiments, the *Bacillus* strain of interest is an industrial *Bacillus* strain. Examples of industrial *Bacillus* strains include, but are not limited to *B. licheniformis*, *B. subtilis*, *B. lentus*, *B. amyloliquefaciens*. In additional embodiments, the *Bacillus* host strain is selected from the group consisting of *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. coagulans*, *B. cirulans*, *B. pumilus*, *B. thuringiensis*, *B. clausii*, and *B. megaterium*, as well as other organisms within the genus *Bacillus*. In some preferred embodiments, *B. subtilis* or *B. licheniformis* is used. For example, U.S. Patents 5,264,366

and 4,760,025 (RE34,606), and US2002/0182734 (International Publication No. WO 02/14490) describe various *Bacillus* host strains that find use in the present invention.

In some embodiments, native genes will be deleted or inactivated in the host cell, for example the strain may be an alpha-amylase negative *Bacillus* strain (genes deleted);
5 an alpha-amylase and protease deleted *Bacillus* strain ($\Delta amyE$, Δapr , Δnpr) or a *Trichoderma* strain with one or more genes deleted (e.g., $\Delta cbh1$, $\Delta cbh2$, $\Delta gl1$, and $\Delta gl2$). See, for example, USP 5,847,276 and WO 05/001036. Methods for making gene deletions are known in the art and reference is made to USP 5,246,853, USP 5,475,101 and WO 92/06209.

10

Methods of transformation -

Introduction of a DNA construct or vector into a host cell includes techniques such as transformation; electroporation; nuclear microinjection; transduction; transfection, (e.g., lipofection mediated and DEAE-Dextrin mediated transfection);
15 incubation with calcium phosphate DNA precipitate; high velocity bombardment with DNA-coated microprojectiles; and protoplast fusion. General transformation techniques are known in the art (See, e.g., Ausubel et al., (1987), *supra*, chapter 9; and Sambrook (1989) *supra*, and Campbell et al., (1989) *Curr. Genet.* 16:53-56). The expression of heterologous protein in *Trichoderma* is described in USP 6,022,725; U.S. Pat. No.
20 6,268,328; Harkki et al. (1991); *Enzyme Microb. Technol.* 13:227-233; Harkki et al., (1989) *Bio Technol.* 7:596-603; EP 244,234; EP 215,594; and Nevalainen et al., "The Molecular Biology of *Trichoderma* and its Application to the Expression of Both Homologous and Heterologous Genes", in MOLECULAR INDUSTRIAL MYCOLOGY, Eds. Leong and Berka, Marcel Dekker Inc., NY (1992) pp. 129 - 148). Methods of
25 transformation of *Aspergillus* are described in Yelton, Hamer and Timberlake, (1984) *Proc. Natl. Acad. Sci. USA* 81: 1470-1474 and Cao et al., (2000) *Sci.* 9:991 - 1001; for *Fusarium* in Bajar, Podila and Kolattukudy, (1991) *Proc. Natl. Acad. Sci. USA* 88: 8202-8212, and for *Streptomyces* include Hopwood et al., 1985, Genetic Manipulation of *Streptomyces*: Laboratory Manual, The John Innes Foundation, Norwich, UK and
30 Fernandez-Abalos et al., *Microbiol* 149:1623 - 1632 (2003) and for *Bacillus* include Brigidi, DeRossi, Bertarini, Riccardi and Matteuzzi, (1990) *FEMS Microbiol. Lett.* 55: 135-138.

Preferably, genetically stable transformants are constructed with vector systems whereby the nucleic acid encoding an alpha-amylase is stably integrated into a host strain chromosome. Transformants are then purified by known techniques.

In one nonlimiting example, stable transformants including an *amdS* marker are distinguished from unstable transformants by their faster growth rate and the formation of circular colonies with a smooth, rather than ragged outline on solid culture medium containing acetamide. Additionally, in some cases a further test of stability is conducted by growing the transformants on solid non-selective medium (*i.e.*, medium that lacks acetamide), harvesting spores from this culture medium and determining the percentage of these spores which subsequently germinate and grow on selective medium containing acetamide. Alternatively, other methods known in the art may be used to select transformants.

In one specific embodiment, the preparation of *Trichoderma sp.* for transformation involves the preparation of protoplasts from fungal mycelia. (See, Campbell *et al.*, (1989) *Curr. Genet.* 16:53-56). In some embodiments, the mycelia are obtained from germinated vegetative spores. The mycelia are treated with an enzyme that digests the cell wall resulting in protoplasts. The protoplasts are then protected by the presence of an osmotic stabilizer in the suspending medium. These stabilizers include sorbitol, mannitol, potassium chloride, magnesium sulfate and the like.

Uptake of DNA into the host *Trichoderma sp.* strain is dependent upon the calcium ion concentration. Generally, between about 10 mM CaCl₂ and 50 mM CaCl₂ is used in an uptake solution. Besides the need for the calcium ion in the uptake solution, other compounds generally included are a buffering system such as TE buffer (10 Mm Tris, pH 7.4; 1 mM EDTA) or 10 mM MOPS, pH 6.0 buffer (morpholinepropanesulfonic acid) and polyethylene glycol (PEG).

Usually a suspension containing the *Trichoderma sp.* protoplasts or cells that have been subjected to a permeability treatment at a density of 10⁵ to 10⁷/mL are used in transformation. A volume of 100 µL of these protoplasts or cells in an appropriate solution (*e.g.*, 1.2 M sorbitol; 50 mM CaCl₂) are mixed with the desired DNA. Generally, a high concentration of PEG is added to the uptake solution. From 0.1 to 1 volume of 25% PEG 4000 can be added to the protoplast suspension. However, it is preferable to add about 0.25 volumes to the protoplast suspension. Additives such as dimethyl sulfoxide, heparin, spermidine, potassium chloride and the like may also be added to the

uptake solution and aid in transformation. Similar procedures are available for other fungal host cells. (See, *e.g.*, U.S. Patent Nos. 6,022,725 and 6,268,328, both of which are incorporated by reference).

Generally, the mixture is then incubated at approximately 0°C for a period of
5 between 10 to 30 minutes. Additional PEG is then added to the mixture to further enhance the uptake of the desired gene or DNA sequence. The 25% PEG 4000 is generally added in volumes of 5 to 15 times the volume of the transformation mixture; however, greater and lesser volumes may be suitable. The 25% PEG 4000 is preferably about 10 times the volume of the transformation mixture. After the PEG is added, the
10 transformation mixture is then incubated either at room temperature or on ice before the addition of a sorbitol and CaCl₂ solution. The protoplast suspension is then further added to molten aliquots of a growth medium. This growth medium permits the growth of transformants only.

15 Cell culture -

Generally, cells are cultured in a standard medium containing physiological salts and nutrients (See, *e.g.*, Pourquie, J. *et al.*, BIOCHEMISTRY AND GENETICS OF CELLULOSE DEGRADATION, eds. Aubert, J. P. *et al.*, Academic Press, pp. 71-86, 1988 and Ilmen, M. *et al.*, (1997) *Appl. Environ. Microbiol.* 63:1298-1306). Common commercially prepared
20 media (*e.g.*, Yeast Malt Extract (YM) broth, Luria Bertani (LB) broth and Sabouraud Dextrose (SD) broth) also find use in the present invention.

Culture conditions are also standard, (*e.g.*, cultures are incubated at approximately 28 °C in appropriate medium in shake cultures or fermenters until desired levels of alpha-amylase expression are achieved). Preferred culture conditions for a given
25 host, such as a filamentous fungus, are known in the art and may be found in the scientific literature and/or from sources such as the American Type Culture Collection and Fungal Genetics Stock Center.

After host cell growth (*e.g.* fungal growth) has been established, the cells are exposed to conditions effective to cause or permit the expression of an alpha-amylase as
30 defined herein. In cases where the coding sequence of an alpha-amylase is under the control of an inducible promoter, the inducing agent (*e.g.*, a sugar, metal salt or antimicrobial), is added to the medium at a concentration effective to induce alpha-amylase expression.

In some embodiments of the present invention, host cells (*e.g.*, fungal cells) expressing an alpha-amylase of the invention are grown under batch or continuous fermentation conditions. A classical batch fermentation is a closed system, wherein the composition of the medium is set at the beginning of the fermentation and is not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the medium is inoculated with the desired organism(s). In this method, fermentation is permitted to occur without the addition of any components to the system. Typically, a batch fermentation qualifies as a "batch" with respect to the addition of the carbon source and attempts are often made at controlling factors such as pH and oxygen concentration.

The metabolite and biomass compositions of the batch system change constantly up to the time the fermentation is stopped. Within batch cultures, cells progress through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase eventually die. In general, cells in log phase are responsible for the bulk of production of end product.

A variation on the standard batch system is the "fed-batch fermentation" system, which also finds use with the present invention. In this variation of a typical batch system, the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium.

Measurement of the actual substrate concentration in fed-batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO₂. Batch and fed-batch fermentations are common and well known in the art.

Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor and an equal amount of conditioned medium is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth and/or end product concentration. For example, in one embodiment, a limiting nutrient such as the carbon source or nitrogen source is maintained at a fixed rate and all other parameters are allowed to moderate. In other systems, a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive

to maintain steady state growth conditions. Thus, cell loss due to medium being drawn off must be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology.

Alpha-amylase production.

The invention relates to methods of producing a polypeptide of the invention comprising culturing a host cell which is capable of producing a polypeptide having alpha-amylase activity encompassed by the invention and recovering the polypeptide, Preferably the host cell is a *Bacillus* cell; an *Aspergillus* cell (e.g. an *Aspergillus niger* cell) or a *Trichoderma* cell (e.g., a *Trichoderma reesei* cell).

The invention further relates to methods of recombinantly producing a polypeptide having alpha-amylase activity and optionally granular starch hydrolyzing activity in a host cell comprising culturing a host cell under suitable culture conditions for the production of the alpha-amylase and recovering the alpha-amylase. In some preferred embodiments, the host cell is a filamentous fungal host cell is an *Aspergillus* cell and in other preferred embodiments, the filamentous fungal host cell is a *Trichoderma* cell.

Identification of alpha-amylase activity and GSH activity.

To evaluate the expression of an alpha-amylase by a cell line, assays can be carried out at the protein level, the RNA level or by use of functional bioassays particular to alpha amylase activity and/or production. In general assays employed include, Northern blotting, dot blotting (DNA or RNA analysis), RT-PCR (reverse transcriptase polymerase chain reaction), or *in situ* hybridization, using an appropriately labeled probe (based on the nucleic acid coding sequence) and conventional Southern blotting and autoradiography.

In addition, the production and/or expression of an alpha-amylase may be measured in a sample directly, for example, by assays directly measuring reducing sugars such as glucose in the culture media and by assays for measuring glucoamylase activity, expression and/or production.

Further, gene expression may be evaluated by immunological methods, such as immunohistochemical staining of cells, tissue sections or immunoassay of tissue culture medium, e.g., by Western blot or ELISA. Such immunoassays can be used to

qualitatively and quantitatively evaluate expression of an alpha-amylase. The details of such methods are known to those of skill in the art and many reagents for practicing such methods are commercially available.

Alpha-amylase activity may be measured for example by using the DNS method
5 as described in Miller, G. L. (1959) *Anal. Chem.* 31:426 - 428. Alternative methods using kits may also be used.

Substrates useful for assaying GSH activity include granular starch substrates such as corn starch, wheat starch, rice starch, and milo starch. For example, glucose concentration may be determined by any convenient method such as by using glucose
10 reagent kit No 15-UV (Sigma Chemical Co.) or an instrument such as Technicon Autoanalyzer. Also reference is made to glucose oxidase kits and glucose hexose kits commercially available from Instrumentation Lab. (Lexington, MA).

To determine the affinity of the starch binding domain to granular starch substrates the method described in Belshaw & Williams, 1990 *FEBS Lett.* 269:350 -353
15 may be used wherein various amount of the starch binding domain are incubated with the granular cornstarch in a buffer. The reaction is terminated by centrifugation and the protein concentration of the supernatant is determined and subtracted from the total protein to give the amount of starch bound protein.

In some embodiments of the invention, the polypeptides having alpha-amylase
20 activity and optionally granular starch hydrolyzing activity expressed in a *Trichoderma* or *Aspergillus* host will be greater than 1 gram protein per liter (g/L), greater than 2 g/L, greater than 5 g/L, greater than 10 g/L, greater than 20 g/L, greater than 25 g/L, greater than 30 g/L, greater than 50 g/L and also greater than 100 g/L of culture media.

25 Methods for recovering alpha-amylase.

In general, the alpha-amylase produced in cell culture is secreted into the medium and may be purified or isolated, *e.g.*, by removing unwanted components from the cell culture medium. In some cases, an alpha-amylase may be produced in a cellular form necessitating recovery from a cell lysate. In such cases the enzyme is purified from the
30 cells in which it was produced using techniques routinely employed by those of skill in the art. Examples include, but are not limited to, affinity chromatography (Tilbeurgh *et al.*, (1984) *FEBS Lett.* 16:215); ion-exchange chromatographic methods (Goyal *et al.*, (1991) *Biores. Technol.* 36:37; Fliess *et al.*, (1983) *Eur. J. Appl. Microbiol. Biotechnol.*

17:314; Bhikhabhai *et al.*, (1984) *J. Appl. Biochem.* 6:336; and Ellouz *et al.*, (1987) *Chromatography* 396:307), including ion-exchange using materials with high resolution power (Medve *et al.*, (1998) *J. Chromatography A* 808:153; hydrophobic interaction chromatography (Tomaz and Queiroz, (1999) *J. Chromatography A* 865:123; two-phase partitioning (Brumbauer, *et al.*, (1999) *Bioseparation* 7:287); ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; and gel filtration using, *e.g.*, Sephadex G-75.

10 Enzyme compositions.

In some aspects, the invention relates to enzyme compositions, which comprise the polypeptides of the invention having alpha-amylase activity and optionally granular starch hydrolyzing activity.

15 In some embodiments, the alpha-amylase is available as a cell free filtrate (for example wherein the alpha-amylase is isolated from a culture medium), and in other embodiments, the alpha-amylase is available in a culture medium containing the fungal host cells which express and secrete the alpha-amylase.

Enzyme compositions include compositions for starch conversion, compositions for granular starch hydrolysis, cleaning compositions, such as detergent, hard surface cleaning and dishwashing compositions, compositions for paper and pulp production, textile treatment compositions, brewing compositions, baking compositions, animal feed compositions, alcohol fermentation compositions and compositions for the production of sweeteners.

25 As understood by those in the art, the quantity of alpha-amylase used in the compositions and methods of the present invention will depend on the enzymatic activity of the alpha-amylase and the use of the composition. In some embodiments, the range of alpha-amylase present in an enzyme composition is from 0.001 to 15.0 SSU per gram of dry solids content of a slurry comprising a substrate contain granular starch.

30 Depending on the specific composition, one or more additional enzymes may be included. Non-limiting examples of these enzymes include a second amylase, such as a beta-amylase or maltogenic alpha-amylase; glucoamylases, proteases, cellulases, lipases, cutinases, esterases, hemicellulases, laccases, mannanases, cyclodextrin

glucanotransferases, pullulanases, oxidoreductases and glycosyltransferases. These enzymes may be obtained from fungal, bacterium or plant sources.

Industrial Applications and Uses.

5

Alpha-amylases are of considerable commercial value and the alpha-amylases of the invention may be used in starch processing, in alcohol production, as cleaning agents, in the sweetener industry, in the textile industry for starch desizing, in the paper and pulp industry and in baking applications.

10

In one preferred embodiment, an alpha-amylase of the invention is used for starch processing particularly for alcohol fermentation, such as for fuel or portable ethanol. The alpha-amylases encompassed by the invention may be used in various process steps including pretreatment, liquefaction and saccharification steps.

15

In some embodiments, the alpha-amylase of the invention may be used in a process for hydrolyzing granular starch from a granular starch substrate, such as cornstarch, at a temperature below the gelatinization temperature of the granular starch in the substrate. The granular starch to be used in the process may be highly refined starch or it may be a more crude starch containing milled whole grain. In some embodiments, the temperature of the starch hydrolysis process will be conducted in the range of 25°C to 70°C. In other embodiments, the temperature will be in greater than 30°C but less than 70°C, less than 68°C, less than 65°C, less than 60°C, less than 55°C, less than 50°C, and less than 45°C. In some embodiments, the alpha-amylase according to the invention may be used in a method for liquefying starch.

20

25

In some embodiments, the fermentation may be carried out sequentially to the hydrolysis of the starch containing substrates and in other embodiments the fermentation may be carried out simultaneously with the hydrolysis in a process known as simultaneous saccharification and fermentation (SSF). During SSF a starch containing substrate, generally in slurry form, is contacted simultaneously with an alpha-amylase of the invention and ethanol producing microorganisms to produce alcohol or other useful compounds.

30

Experimental

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be

construed as limiting the scope thereof. Indeed, it is contemplated that these teachings will find use in further optimizing the process systems described herein.

In the disclosure and experimental section which follows, the following abbreviations apply:

- 5 wt% (weight percent); °C (degrees Centigrade); rpm (revolutions per minute); H₂O (water); dH₂O (deionized water); dIH₂O (deionized water, Milli-Q filtration); aa (amino acid); bp (base pair); kb (kilobase pair); kD (kilodaltons); g or gm (grams); µg (micrograms); mg (milligrams); µL (microliters); ml and mL (milliliters); mm (millimeters); µm (micrometer); M (molar); mM (millimolar); µM (micromolar); U
10 (units); V (volts); MW (molecular weight); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); PAGE (polyacrylamide gel electrophoresis); DO (dissolved oxygen); phthalate buffer (sodium phthalate in water, 20 mM, pH 5.0); PBS (phosphate buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2]); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); w/v (weight to volume); w/w (weight
15 to weight); v/v (volume to volume); Genencor (Genencor International, Inc., Palo Alto, CA).

The following assays and methods are contemplated for use in the examples provided below:

- 20 Alpha-amylase activity: The measurement of alpha-amylase activity is based on the degree of hydrolysis of soluble potato starch substrate (4% ds) by an aliquot of the enzyme sample at pH 4.5, 50°C. The reducing sugar content is measured using the DNS method as described in Miller, G. L. (1959) *Anal. Chem.* 31:426 - 428. One unit of the enzyme activity (SSU, soluble starch unit) is equivalent to the reducing power of 1 mg of
25 glucose released per minute at the specific incubation conditions.

- Determination of total starch content: The enzyme-enzyme starch liquefaction and saccharification process is used to determine the total starch content. In a typical analysis, 2 g of the dry sample is taken in a 100 ml Kohlrausch flask and 45 ml of MOPS buffer,
30 pH 7.0 is added. The slurry is well stirred for 30 min. An alpha-amylase e.g. SPEZYME FRED (1:50 diluted in water), 1.0 ml is added and heated to boiling for 3 - 5 min. The flask is placed in an autoclave maintained at 121°C for 15 min. After autoclaving the flask is placed in a water bath at 95°C and 1 ml of the 1:50 diluted SPEZYME FRED is

added and incubated for 45 min. The pH is adjusted to pH 4.2 and the temperature is reduced to 60°C. This is followed by addition of 20 ml acetate buffer, pH 4.2.

Saccharification is carried out by adding 1.0 ml of 1:100 diluted OPTIDEX L-400 (Glucoamylase from Genencor International Inc.) and the incubation is continued for 18
5 hr at 60°C. The enzyme reaction is terminated by heating at 95°C for 10 min. The total sugar composition is determined by HPLC analysis using glucose as a standard. The soluble starch hydrolysate from water extraction of a sample at room temperature without enzymatic treatment is subtracted from the total sugar.

- 10 Residual starch iodine test: A sample of the fermentation broth is centrifuged in 2 ml plastic centrifuge tubes. The supernatant is decanted and the tube containing the pellet is placed in an ice bath. Several drops of 0.025N iodine solution (0.1N iodine from VWR Cat. No. VW3207-1 diluted 4X) is added to the pellet and mixed. A positive (+) starch shows a range of color from blue to purple and the intensity of color is directly
15 proportional to the concentration of starch. A negative result (-) remains yellowish.

Total protein analysis: The total nitrogen (N) in the sample preparations is determined using the Kjeldhal method (American Assoc. Cereal Chemists (AACC), (1983), Methods 22B60 8th Ed. St Paul, MN). Protein content is calculated by 6.25 X total N.

20

Ethanol and carbohydrate determinations - Ethanol and carbohydrate composition of the samples are determined using the HPLC method as described herein:

- a) a 1.5 mL Eppendorf centrifuge tube is filled with fermentor broth and cooled on ice for 10 min;
25 b) the sample tube is centrifuged for 1 min in Eppendorf table top centrifuge;
c) a 0.5 mL sample of the supernatant is transferred to a test tube containing 0.05 mL of Kill solution (1.1N H₂SO₄) and allowed to stand for 5 min;
d) 5.0 mL of water is added to the test tube sample and then filtered into a HPLC vial through 0.45 µm Nylon Syringe Filter; and
30 e) run on HPLC.

HPLC conditions:

a) Ethanol System: Column: Phenomenex Rezex Organic Acid Column (RHM-Monosaccharide) #00H-132 KO (Equivalent to Bio-Rad 87H); Column Temperature: 60°C; Mobile Phase: 0.01 N H₂SO₄; Flow Rate: 0.6 mL/min
Detector: RI; Injection Volume: 20 µL

5 b) Carbohydrate System: Column: Phenomenex Rezex Carbohydrate (RCM-Monosaccharide) #00H-0130-KO (Equivalent to Bio-Rad 87H); Column Temperature: 70°C; Mobile Phase: Nanopure DI H₂O; Flow Rate: 0.8 mL/min
Detector: RI; Injection Volume: 10 µL (3% DS material);

The column separates based on the molecular weight of the saccharides, which are
10 designated as DP1 (monosaccharides); DP2 (disaccharides); DP3 (trisaccharides) and DP+4 (oligosaccharide sugars having a degree of polymerization greater than 3).

EXAMPLE 1

Cloning the *Aspergillus niger* alpha-amylase gene.

15

Genomic DNA was extracted from frozen *A. niger* (NRRL 3, ATCC 9029) mycelia. The frozen mycelia were ground with dry ice in a coffee grinder and the DNA was extracted by the EasyDNA protocol (Invitrogen). An extra chloroform/phenol/isoamyl alcohol extraction was added to the standard protocol. PCR primers were
20 designed, based on homology of alpha-amylase sequences to contigs in an *A. niger* strain NRRL 3 shotgun sequencing genomic DNA database (purchased from Integrated Genomics). The forward primer contained a motif for directional cloning into a Gateway entry vector (Invitrogen).

Forward primer, Anaa5: CAC CAT GAG ACT ATC GAC TTC AAG (SEQ ID
25 NO: 9) and reverse primer, Anaa4b: TTA CCT CCA AGT GTC AAC CAC CGT CTC (SEQ ID NO: 10).

The PCR product (using *Pfu* Turbo DNA polymerase, Stratagene) included multiple bands. The largest band, 2.4 kb, was purified by gel extraction (QIAquick Gel Extraction Kit, Qiagen). The PCR product was cloned into pCR_BluntII_TOPO (Zero
30 Blunt TOPO PCR Cloning Kit, Invitrogen) for sequencing. The vector, pCR_BluntII_9029alpha, was transformed into chemically competent Top10 *E. coli* (Invitrogen) with kanamycin selection. Plasmid DNA from several clones was digested with EcoRI to confirm the correct size insert. The alpha-amylase insert was sequenced

(Sequetech, Mountain View, CA) (Figures 1 – 3). The new alpha-amylase (2365 bp) showed sequence homology with known *Aspergillus* alpha-amylases, having more identity in the exons and less identity in the 8 introns.

5

EXAMPLE 2

Construction of an expression cassette

The following procedures were used in constructing an expression cassette, which includes a polynucleotide encoding the alpha-amylase of the invention. The alpha
10 amylase DNA sequence in vector pCR_BluntII_9029alpha was transferred into an expression cassette (Figure 4) by fusion PCR. In the first step, three fragments of DNA were individually PCR amplified using the following primers:

For the *T. reesei cbh1* promoter region (1.49 kb fragment size)

15 CL158F 5' TAGAGTTGTGAAGTCGTAATCCCGC 3' (SEQ ID NO: 11) and
CL159R 5' CGATAGTCTCATTGACTATTGGGTTTCTGTGCCTC 3' (SEQ ID
NO: 12);

for the *A. niger* alpha-amylase region (2.37 kb fragment size)

CL160F 5' ACCCAATAGTCAATGAGACTATCGACTTCAAG 3' (SEQ ID NO:
20 13) and
CL161R 5' TCGCACGGAGCTTTACCTCCAAGTGTCACCACCGTCTC 3' (SEQ
ID NO: 14);

for the *T. reesei cbh1* terminator region (0.35 kb fragment size)

CL162F 5' TTGGAGGTAAAGCTCCGTGCGAAAGCCTGAC 3' (SEQ ID NO: 15)
25 and
CL163R 5' TACTGGGATACACGAAGAGCGG 3' (SEQ ID NO: 16)

Cbh1 promoter and terminator regions were amplified from *T. reesei* genomic DNA (extracted from strain *T. reesei* QM6a, ATCC number 13631). *A. niger* alpha-
30 amylase was amplified using the vector- pCR_BluntII_9029alpha as template. Pfu Ultra DNA polymerase (Stratagene) was used for amplification. After performing PCR, the individual reactions were run through agarose gels and the appropriately sized fragments were excised and purified (QIAquick Gel Extraction Kit, Qiagen).

Next a fusion PCR reaction was performed to join the three fragments into a single expression cassette fragment of the form: cbh1 promoter linked to the *A. niger* alpha-amylase linked to the cbh1 terminator (Figure 4). Approximately equimolar amounts of each of the three fragments was included in the PCR fusion reaction along with the primers:

596F 5' GTGAAGTCGGTAATCCCGCTGTATAG 3' (SEQ ID NO 17)

597R 5' GGATACACGAAGAGCGGCGATTC 3' (SEQ ID NO: 18)

Herculase DNA polymerase (Stratagene) was used according to the manufacture's instructions. The following PCR cycle program was used on a PTC-200 Thermocycler (MJ Research): Step 1 - 95°C for 2'; step 2 - 95°C for 30''; step 3 - 55°C for 30''; step 4 - 72°C for 5'; step 5 - go to step 2 for 9 cycles; step 6 - 95°C for 30''; step 7 - 55°C for 30''; step 8 - 72°C for 5' add 10'' per cycle; step 9 - go to step 6 for 19 cycles; step 10 - 72°C for 10'; step 11 - 4°C hold at this temperature; and step 12 end.

A 4.17 kb DNA fragment was amplified and purified from agarose gel (QIAquick Gel Extraction Kit, Qiagen). Additionally other non-specific reaction products were formed (two prominent fragments were sized 1.7 and 2.0 kb). These non-specific products were discarded.

The fungal selectable marker, *Aspergillus nidulans amdS* gene, including its promoter and terminator, was isolated by restriction digest and purified from agarose from a vector equivalent to p3SR2 (Hynes et al., (1983) *Mol. Cell. Biol.* 3:1430 – 1439). The *A. nidulans amdS* gene is further referenced in NCBI Genebank record M16371).

EXAMPLE 3

25 Co-Transformation and fermentation of *Trichoderma reesei*

Biolistic transformation of *T. reesei* with the *A.niger* alpha-amylase expression construction and the *A.nidulans amdS* marker was performed using the protocol outlined below.

30 A suspension of spores (approximately 3.5×10^8 spores/ml) from a quad deleted strain of *T. reesei* (WO 05/001036) was prepared. 100ul – 200ul of spore suspension was spread onto the center of plates of MM acetamide medium. MM acetamide medium had the following composition: 0.6 g/L acetamide; 1.68 g/L CsCl; 20 g/L glucose; 20 g/L

KH₂PO₄; 0.6 g/L CaCl₂·2H₂O; 1 ml/L 1000X trace elements solution; 20 g/L Noble agar; pH 5.5. 1000X trace elements solution contained 5.0 g/l FeSO₄·7H₂O, 1.6 g/l MnSO₄·H₂O, 1.4 g/l ZnSO₄·7H₂O and 1.0 g/l CoCl₂·6H₂O. The spore suspension was allowed to dry on the surface of the MM acetamide medium.

- 5 Transformation of *T. reesei* was performed using a Biolistic® PDS-1000/He Particle Delivery System from Bio-Rad (Hercules, CA) following the manufacturer's instructions. Briefly, 60 mg of M10 tungsten particles were placed in a microcentrifuge tube. 1mL of ethanol was added, the mixture was briefly vortexed and allowed to stand for 15 minutes. The particles were centrifuged at 15,000 rpm for 15 minutes. The
- 10 ethanol was removed and the particles were washed three times with sterile dH₂O before 1 mL of 50% (v/v) sterile glycerol was added. After ten seconds of vortexing, 25 ul of tungsten/glycerol particle suspension was removed and placed into a microcentrifuge tube. While continuously vortexing the 25 ul tungsten/glycerol particle suspension, the following were added; in order, allowing 5' incubations between additions; 2 ul (50-100
- 15 ng/ul) of *A.nidulans amdS* marker DNA, 2-3 ul *A.niger* alpha-amylase construct (50-100 ng/ul) DNA, 25 ul of 2.5M CaCl₂ and 10 ul of 0.1M spermidine. After another 5' incubation after spermidine addition, the particles were centrifuged for 3 seconds. The supernatant was removed; the particles were washed with 200ul of 70% (v/v) ethanol and then centrifuged for 3 seconds. The supernatant was removed; the particles were washed
- 20 with 200 ul of 100% ethanol and centrifuged for 3 seconds. The supernatant was removed and 24 ul 100% ethanol was added and mixed by pipetting. The tube was placed in an ultrasonic cleaning bath for approximately 15 seconds to further resuspend the particles in the ethanol. While the tube was in the ultrasonic bath, 8 ul aliquots of suspended particles were removed and placed onto the center of macrocarrier disks that
- 25 were held in a desiccator. Once the tungsten/DNA solution had dried onto the macrocarrier, it was placed in the bombardment chamber along with the plate of MM acetamide with spores and the bombardment process was performed using 1100 psi rupture discs according to the manufacturers instructions. After the bombardment of the plated spores with the tungsten/DNA particles, the plates were placed incubated at 28 C.
- 30 Large transformed colonies were picked to fresh plates of MM acetamide after 5 days (Penttila et al., (1987) Gene 61:155 – 164).

The large colonies were compared to each other. The one dozen large colonies showing the most colony opacity were chosen for shake flask evaluation.

EXAMPLE 4

Fermentation of *Trichoderma reesei*

5 The dozen transformants chose for evaluation and the untransformed parent strain were transferred to individual PDA plates and grown to sporulation (7 to 10 days). Next they were grown in two stages in shake flasks. In the first stage the transformants and the parent were grown in 250 ml four baffled bottom shake flasks (Bellco Biotechnology) containing 50 ml of *T. reesei* Proflo Media. Inoculation was performed by transferring
10 approximately 5 cm² of the sporulated mycelia and agar from the PDA plates. The flasks were grown for two days at 30°C with a shaking speed of 250 rpm (1" diameter orbit) (Innova 4900 shaker incubator, New Brunswick Scientific).

T. reesei Proflo Media, per liter: alpha-lactose, 30 g; (NH₄)₂SO₄, 6.5g; KH₂PO₄, 2 g; MgSO₄ · 7H₂O, 0.3 g; CaCl₂ · 2H₂O, 0.26 g; 10% Tween 80, 2 ml; FeSO₄ · 7H₂O, 5 mg; MnSO₄ · H₂O, 1.6 mg; ZnSO₄ · 7H₂O, 1.4 mg; Proflo, 22.5g and CaCO₃, 0.72 g.
15

 Next, 5 ml from the first stage shake flask was inoculated into a second stage shake flask consisting of a 250 ml four baffled bottom shake flask, containing 50 ml of Lactose defined Media. These flasks were grown for 5 days at 28°C with a shaking speed of 175 rpm (5 cm diameter orbit) (Multitron shaker incubator, Infors AG, Bottmingen, Switzerland). Lactose defined media, per liter: (NH₄)₂SO₄, 5g; PIPPS Buffers, 33g; Bacto Casamino Acids, 9 g; KH₂PO₄, 4.5g; CaCl₂ · 2H₂O, 1.32g; MgSO₄ · 7H₂O, 1g; Mazu DF204, 5 ml; 400X Salts Solution 2.5 ml; pH to 5.5; Sterile addition after autoclave and 40% lactose, 40ml. 400 X Salt Solution: citric acid (anhydrous), 175g; FeSO₄ · 7H₂O, 200g; MnSO₄ · H₂O, 1.4 mg; ZnSO₄ · 7H₂O, 16g; CuSO₄ · 5H₂O, 3.2g and
25 H₃BO₃, 0.8g.

 Five day samples were taken from the shake flasks. The supernate was separated from the mycelia by filtration through 0.45 um pore filters. Supernate samples were run on 4-12% NuPage Gels (Invitrogen), 1 mm thick using MOPS buffer and stained with SimplyBlue Safe Stain (Invitrogen) following the manufacturer's directions (Figure 5).
30 Transformants are observed in lanes 1 – 12 of the gel.

EXAMPLE 5

Raw Starch Hydrolyzing Activity of the *A. niger* alpha-amylase

Samples, which were obtained as described in the examples above, were analyzed for raw starch degradation by HPAEC-PAD, using a Dionex PA1, with a sodium hydroxide/sodium acetate gradient. Two shake flask samples (control strain and strain
5 containing the *A. niger* alpha-amylase of the invention (designated putative alpha-amylase)) were first concentrated about 10 fold using a spin column. Fifty microliters of the concentrate were then added to 2 ml of 5% corn starch in a 100 mM sodium acetate, pH 4.5. the tubes were incubated overnight at 34°C, 250 rpm. Reaction tubes were spun the next morning, filtered and diluted. Ten microliters were injected into the HPLC and
10 the results are illustrated in Figure 6.

IT IS CLAIMED:

1. An isolated DNA encoding an alpha-amylase having at least 95% sequence
5 identity to SEQ ID NO: 3.
2. The isolated DNA of claim 1, wherein the DNA encodes an alpha-amylase having
at least 98% sequence identity to SEQ ID NO: 3.
- 10 3. The isolated DNA of claim 1, wherein the DNA has the sequence of SEQ ID NO:
1.
4. The isolated DNA of claim 1, wherein the DNA has the sequence of SEQ ID NO:
2.
- 15 5. An isolated DNA encoding an alpha-amylase having at least 95% sequence
identity to SEQ ID NO: 5 and biologically active fragments thereof.
6. An expression vector comprising the DNA of claim 1.
- 20 7. An expression vector comprising the DNA of claim 5.
8. A host cell comprising the expression vector of claim 6 or claim 7.
- 25 9. The host cell of claim 8, wherein the host cell is a bacterial or fungal cell.
10. The host cell of claim 9, wherein the fungal cell is an *Aspergillus*, a *Trichoderma*
or a *Fusarium* cell.
- 30 11. An isolated protein having alpha-amylase activity and at least 95% sequence
identity to SEQ ID NO: 8 and biologically active fragments thereof.

12. The isolated protein of claim 11, wherein the protein has at least 95% sequence identity to SEQ ID NO: 8.

13. An isolated protein having alpha-amylase activity and at least 95% sequence
5 identity to SEQ ID NO: 5.

14. A cell culture comprising cells which express a heterologous alpha-amylase, said alpha-amylase having at least 90% sequence identity with SEQ ID NO: 8.

10 15. The cell culture of claim 14, wherein the cells are Trichoderma or Aspergillus cells.

16. An enzyme composition comprising the alpha-amylase of claim 11.

15 17. The enzyme composition of claim 16, wherein the alpha-amylase has at least 95% sequence identity to SEQ ID NO: 8.

18. The enzyme composition of claim 16, wherein the alpha-amylase at least 95% sequence identity to SEQ ID NO: 5.

20 19. The enzyme composition of claim 16 which is a starch hydrolyzing enzyme composition.

20. The enzyme composition of claim 16 which is a detergent composition.

25 21. The enzyme composition of claim 16 which is a cleaning composition.

22. The enzyme composition of claim 16 further comprising one or more additional enzymes selected from the group of glucoamylases, other amylases, proteases, lipases,
30 pullulanases, xylanases, cellulases and combinations thereof.

FIGURE 1A

Aspergillus niger alpha-amylase DNA sequence
(SEQ ID NO: 1)

ATGAGACTATCGACTTCAAGTCTCTTACTTTCCGTGTCTCTGCTGGGGAAGCTGGCC
CTCGGGCTGTTCGGCTGCAGAATGGCGCACTCAGTCGATTTACTTCCTATTGACGGAT
CGGTTTCGGTAGGACGGACAATTTCGACGACAGCTACATGCAATACGGGTGACCAAGTA
CGTTGGTATTGCAGGACTTTTATCATTTCATCTACTGACTTGAATAGATCTATTGTGG
TGGTAGTTGGCAAGGGATCATCAACCATGTTTGTAACCGCTCCATACTATCTGCTGT
GCGCGTGTCTGACTTTATTTGCTGCAGCTGGATTATATCCAGGGCATGGGATTACG
GCCATCTGGATCTCGCCTATCACTGAACAGCTGCCCCAGGATACTGCTGATGGTGAA
GCCTACCATGGATATTGGCAGCAGAAGATGTATGCGTTCCTCCTTTCCATATCGTAG
GCTTACTCTCAGACGGCGACTGACTTGACAGATACGACGTGAACTCCAACCTTCGGCA
CTGCAGATGACCTCAAGTCCCTCTCAGATGCGCTTCATGCCCCGCGGAATGTACCTCA
TGGTGGACGTCGTCCCTAACCACATGGTAAGTGCTGCTTCAGCATCCTAATCAGTGA
ATTCCAAGTGCCAACGCTAACTGTACCAGGGCTACGCCGGCAACGGCAACGATGTAG
ACTACAGCGTCTTCGACCCCTTCGATTCCCTCCTCTACTTCCACCCATACTGCCTGA
TCACAGATTGGGACAACCTTGACCATGGTCCAAGATTGTTGGGAGGGTGACACCATCG
TATCTCTGCCAGACCTAAACACCACCGAACTGCCGTGAGAACAATCTGGTATGACT
GGGTAGCCGACCTGGTATCCAATTATTCAGGTGCGAATTGCAATCCAATCTAAAATA
ATCATATACTAAGTGAAATCATCAGTCGACGGACTCCGCATCGACAGTGTCTCGAA
GTCGAACCAGACTTCTTCCCAGGGCTACCAGGAAGCAGCGGGTGTCTACTGCGTCGGC
GAAGTCGACAACGGCAACCCTGCCCTCGACTGCCCATAACCAGGAGTACCTGGACGGC
GTCCTCAACTATCCGATGTACATTCCCCTACACATTATTCAGATCTTCGCTAACTCC
AAACCAGCTACTGGCAACTCCTCTACGCCTTCGAATCCTCCAGCGGCAGCATCAGCG
ACCTCTACAACATGATCAAATCCGTCGCAAGCGACTGCTCCGATCCGACACTACTCG
GCAACTTCATCGAAAACCACGACAATCCCCGTTTTTGCCCTCGTATGTCCCACCCCTC
CCATCCCCCTCCCCACAATCACACTACTAATGCATCAAACAGCTACACATCCGACTA
CTCGCAAGCCAAAAACGTCCTCAGCTACATCTTCTCTCCGACGGCATCCCCATCGT
CTACGCCGGCGAAGAACAGCACTACTCCGGCGGCAAGGTGCCCTACAACCGCGAAGC
GACCTGGCTCTCAGGCTACGACACCTCCGCAGAGCTCTACACCTGGATAGCCACCAC
GAACGCGATCCGCAAACTAGCCATCTCAGCTGACTCGGCCTACATTACCTACGCGGT

FIGURE 1B

TCGTCCTTCCCTCCCCCCCACCCTACAAACACCCCCACATACTAACAACATCCCAAT
AATGAAATAGAATGACGCATTCTACACCGACAGCAACACCATCGCAATGCGCAAAGG
CACCTCAGGGAGCCAAGTCATCACCGTCTCTCCAACAAAGGCTCCTCAGGAAGCAG
CTACACCCTGACCCTCAGCGGAAGCGGCTATACATCCGGCACGAAGCTGATCGAAGC
GTATACATGCACGTCCGTGACCGTGGACTCGAGCGGCGATATCCCCGTGCCGATGGC
GTCGGGATTACCGAGAGTTCTTCTCCCCGCGTCCGTTCGTCGATAGCTCTTCGCTCTG
TGGCGGGAGCGGAAGTAATTCTCAACTACAACCACAACAACAGCTACCTCATCTTC
CACTGCGACATCCAAATCCGCATCAACCTCGTCTACGTCGACGGCATGCACAGCTAC
CTCTACCTCCCTCGCGGTCACGTTCGAAGAGCTCGTCACGACTACCTACGGGGAGGA
AATCTACCTGAGCGGATCGATCTCCAGCTTGGGGACTGGGATACGAGTGATGCGGT
GAAGATGTCCGCGGATGATTATACGTCGAGTAATCCGGAGTGGTTCGGTTACTGTGAC
GTTGCCGGTGGGGACAACCTTTGAGTATAAGTTTATTAAGGTGGAGTCGGATGGGAC
TGTTACTTGGGAGAGTGATCCGAATCGGGAGTATACGGTGCCCCGAGTGTGGGAGTGG
GGAGACGGTGGTTGACACTTGGAGGTAA

FIGURE 2A

cDNA sequence (SEQ ID NO: 2)

ATGAGACTATCGACTTCAAGTCTCTTACTTTCCGTGTCTCTGCTGGGGAAGCTGGCC
CTCGGGCTGTCTGGCTGCAGAATGGCGCACTCAGTCGATTTACTTCCTATTGACGGAT
CGGTTCGGTAGGACGGACAATTCGACGACAGCTACATGCAATACGGGTGACCAAATC
TATTGTGGTGGTAGTTGGCAAGGGATCATCAACCATCTGGATTATATCCAGGGCATG
GGATTCACGGCCATCTGGATCTCGCCTATCACTGAACAGCTGCCCCAGGATACTGCT
GATGGTGAAGCCTACCATGGATATTGGCAGCAGAAGATATACGACGTGAACTCCAAC
TTCGGCACTGCAGATGACCTCAAGTCCCTCTCAGATGCGCTTCATGCCCCGCGGAATG
TACCTCATGGTGGACGTCGTCCCTAACCACATGGGCTACGCCGGCAACGGCAACGAT
GTAGACTACAGCGTCTTCGACCCCTTCGATTCCCTCCTCTACTTCCACCCATACTGC
CTGATCACAGATTGGGACAACCTTGACCATGGTCCAAGATTGTTGGGAGGGTGACACC
ATCGTATCTCTGCCAGACCTAAACACCACCGAAACTGCCGTGAGAACAATCTGGTAT
GACTGGGTAGCCGACCTGGTATCCAATTATTTCAGTCGACGGACTCCGCATCGACAGT
GTCCTCGAAGTCGAACCAGACTTCTTCCCGGGCTACCAGGAAGCAGCGGGTGTCTAC
TGCGTCGGCGAAGTCGACAACGGCAACCCTGCCCTCGACTGCCCATACCAGGAGTAC
CTGGACGGCGTCCTCAACTATCCGATCTACTGGCAACTCCTCTACGCCTTCGAATCC
TCCAGCGGCAGCATCAGCGACCTCTACAACATGATCAAATCCGTCGCAAGCGACTGC
TCCGATCCGACACTACTCGGCAACTTCATCGAAAACCACGACAATCCCCGTTTTGCC
TCCTACACATCCGACTACTCGCAAGCCAAAACGTCCCTCAGCTACATCTTCTCTCC
GACGGCATCCCCATCGTCTACGCCGGCGAAGAACAGCACTACTCCGGCGGCAAGGTG
CCCTACAACCGCGAAGCGACCTGGCTCTCAGGCTACGACACCTCCGCAGAGCTCTAC
ACCTGGATAGCCACCACGAACGCGATCCGCAAACCTAGCCATCTCAGCTGACTCGGCC
TACATTACCTACGCGAATGACGCATTCTACACCGACAGCAACACCATCGCAATGCGC
AAAGGCACCTCAGGGAGCCAAGTCATCACCGTCCTCTCCAACAAAGGCTCCTCAGGA
AGCAGCTACACCCTGACCCTCAGCGGAAGCGGCTATACATCCGGCACGAAGCTGATC
GAAGCGTATACATGCACGTCCGTGACCGTGGACTCGAGCGGCGATATCCCCGTGCCG
ATGGCGTCGGGATTACCGAGAGTTCTTCTCCCCGCGTCCGTCTCGTATAGCTCTTCG
CTCTGTGGCGGGAGCGGAAGTAATTCCTCAACTACAACCACAACAACAGCTACCTCA
TCTTCCACTGCGACATCCAAATCCGCATCAACCTCGTCTACGTCGACGGCATGCACA
GCTACCTCTACCTCCCTCGCGGTACGTTTGAAGAGCTCGTCACGACTACCTACGGG
GAGGAAATCTACCTGAGCGGATCGATCTCCAGCTTGGGGACTGGGATACGAGTGAT

FIGURE 2B

GCGGTGAAGATGTCCGCGGATGATTATACGTCGAGTAATCCGGAGTGGTCGGTTACT
GTGACGTTGCCGGTGGGGACAACCTTTGAGTATAAGTTTATTAAGGTGGAGTCGGAT
GGGACTGTTACTTGGGAGAGTGATCCGAATCGGGAGTATACGGTGCCCGAGTGTGGG
AGTGGGGAGACGGTGGTTGACACTTGAGAGGTAA

FIGURE 3

Translated protein sequence (SEQ ID NO.: 3)

MRLSTSSLLLSVSLLGKLALGLSAAEWRTQSIYFLLTDRFGRTDNSTTATCNTGDQI
YCGGSWQGIINHLDYIQGMGFTAIWISPITEQLPQDTADGEAYHGYWQQKIYDVNSN
FGTADDLKSLSDALHARGMYLMVDVVPNHMGYAGNGNDVDYSVFDPPFDSSSYFHPYC
LITDWDNLTMVQDCWEGDTIVSLPDLNTTETAVRTIWYDWVADLVSNYSVDGLRIDS
VLEVEPDFFPQYQEAAGVYCVGEVDNGNPALDCPYQEYLDGVLNYPYIWQLLYAFES
SSGSISDLYNMIKSVASDCSDPTLLGNFIENHDNPRFASYTSDYSQAKNVLSYIFLS
DGIPIVYAGEEQHYSGGKVPYNREATWLSGYDTS AELYTWIATTN AIRKL AISADSA
YITYANDAFYTDSENTIAMRKGTSGSQVITVLSNKGSSGSSYTLTSLSGSGYTSGTKLI
EAYTCTSVTVDS S G DIPVPMASGLPRVLLPASVVDSSSLCGGSGSNSSTTTTTTATS
SSTATSKSASTSSTSTACTATSTSLAVTFEELVTTTTYGEEIYLSGSISQLGDWDTS
AVKMSADDYTSSNPEWSVTVTLPVGTTFEYKFIKVESDGTVTWESDPNREYTVPECG
SGETVVD TWR

FIGURE 4
A. niger alpha amylase expression cassette

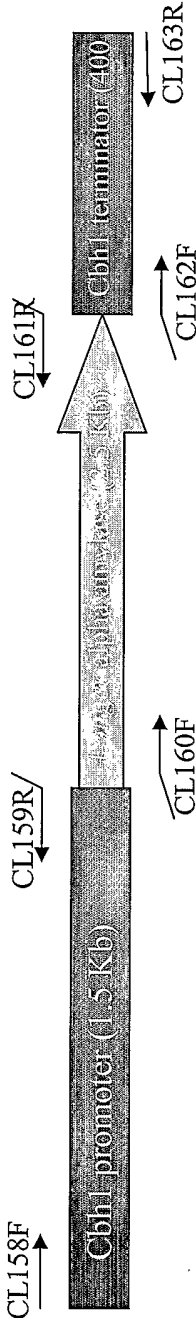


FIGURE 5

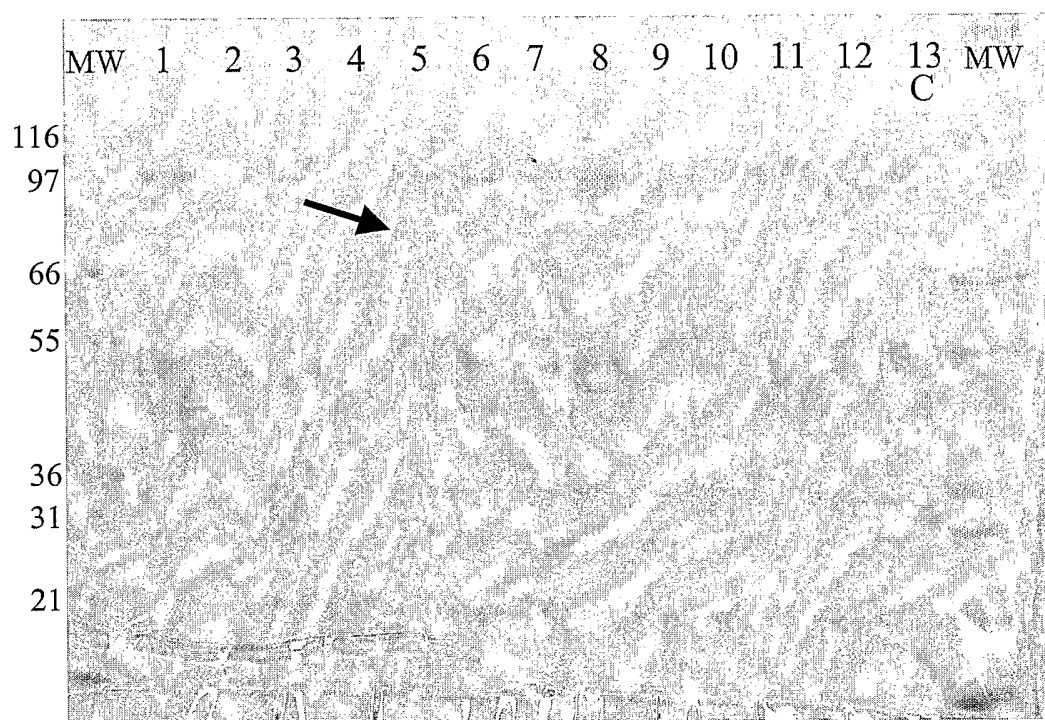
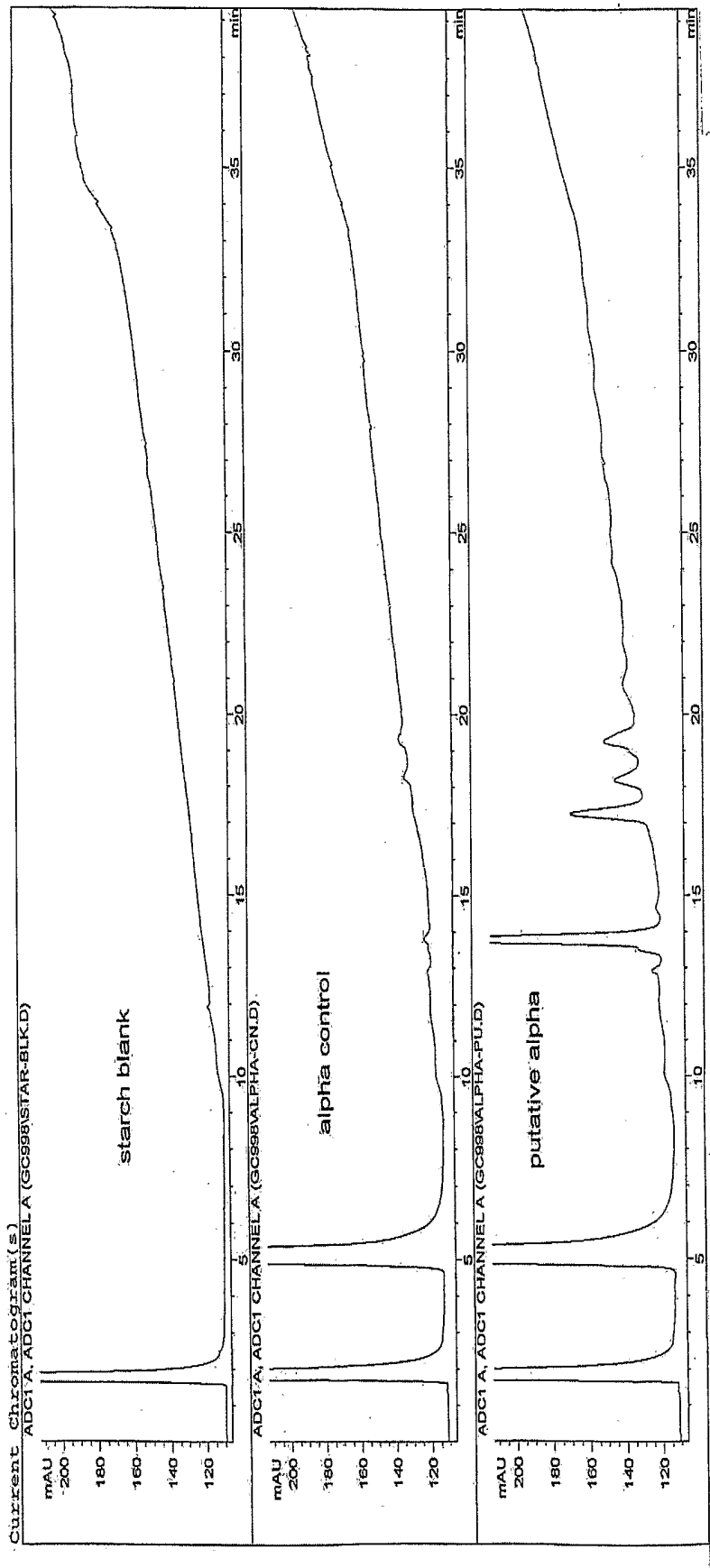


FIGURE 6



INTERNATIONAL SEARCH REPORT

International application No

PCT/US2006/005622

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/38

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, MEDLINE, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005/003311 A (NOVOZYMES A/S; NOVOZYMES NORTH AMERICA, INC; TAIRA, RIKAKO; TKAGI, SHI) 13 January 2005 (2005-01-13) page 36; example 6; sequence 42	5,7-10, 13
X	KANEKO AKIHIRO ET AL: "Molecular cloning and determination of the nucleotide sequence of a gene encoding an acids-stable alpha-amylase from Aspergillus kawachii" JOURNAL OF FERMENTATION AND BIOENGINEERING, SOCIETY OF FERMENTATION TECHNOLOGY, JP, vol. 81, no. 4, 1996, pages 292-298, XP002256177 ISSN: 0922-338X page 293, right-hand column, paragraph 2 - page 297, right-hand column; figure 4	14



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

G document member of the same patent family

Date of the actual completion of the international search

11 July 2006

Date of mailing of the international search report

18/07/2006

Name and mailing address of the ISA/

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Authorized officer

Stoyanov, B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2006/005622

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

a. type of material

☒

a sequence listing

☐

table(s) related to the sequence listing

b. format of material

☒

on paper

☒

in electronic form

c. time of filing/furnishing

☐

contained in the international application as filed

☐

filed together with the international application in electronic form

☒

furnished subsequently to this Authority for the purpose of search

2. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/005622

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/113551 A (NOVOZYMES A/S; VIKSOE-NIELSEN, ANDERS; ANDERSEN, CARSTEN; PEDERSEN, SV) 29 December 2004 (2004-12-29) sequence 22 -----	14

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: -

Present claims 5 and 11 relate to fully undefined "biologically active fragments" which renders said claims completely unclear. Consequently, the subject matter of claims related to said claims, namely claims 7-12 is also unclear. Said claims have been searcht with respect to the SEQ ID NOs. given in the specification.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2006/005622

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-4, 6 (completely); claims 8-10 (only partially)

Provision of alpha-amylase of SEQ ID NO.3.

2. claims: 5, 7, 13 (completely); claims 8-10, 18 (only partially)

Provision of alpha-amylase of SEQ ID NO.5.

3. claims: 11-12, 14.17, 19, 20-22 (completely); claim 18 (only partially)

Provision of alpha-amylase of SEQ ID NO.8.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2006/005622

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2005003311 A	13-01-2005	EP 1648996 A2	26-04-2006
WO 2004113551 A	29-12-2004	EP 1641932 A1	05-04-2006