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(54) Title: NEUTRAL PH SACCHARIFICATION AND FERMENTATION

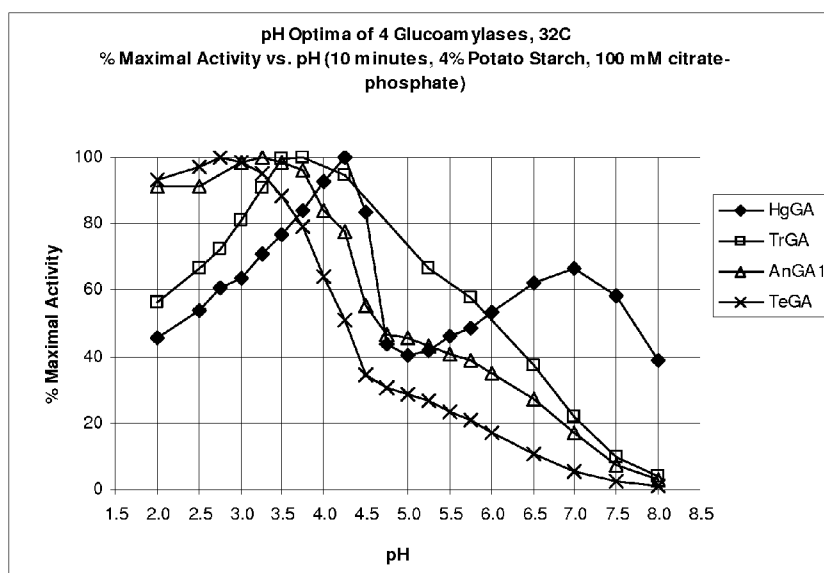


FIG. 1

(57) Abstract: Embodiments of the present disclosure relate to a process for producing downstream products, such as fermentable sugars and end products, from a starch substrate by saccharification and/or fermentation. The saccharification is effectively catalyzed by a glucoamylase at a pH in the range of 5.0 to 8.0. At a pH of 6.0 or above, the glucoamylase possesses at least 50% activity relative to its maximum activity. The saccharification and fermentation may be performed as a simultaneous saccharification and fermentation (SSF) process.

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NEUTRAL pH SACCHARIFICATION AND FERMENTATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. Provisional Appl. 61/371,639, filed August 6, 2010, the contents of which are hereby incorporated by reference in its entirety.

SEQUENCE LISTING

[0002] A Sequence Listing, comprising SEQ ID NOs: 1-9, is attached and incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0003] Glucoamylases capable of effectively hydrolyzing a starch substrate at a pH in the range of 5.0 to 8.0 are useful in simultaneous saccharification and fermentation (SSF) to produce an end product.

BACKGROUND

[0004] Industrial fermentations predominately use glucose as a feedstock for the production of a multitude of proteins, enzymes, alcohols, and other chemical end products. Typically, glucose is the product of starch processing, which is conventionally a two-step, enzymatic process that catalyzes the breakdown of starch, involving liquefaction and saccharification. During liquefaction, insoluble granular starch is slurried in water, gelatinized with heat, and hydrolyzed by a thermostable alpha-amylase. During saccharification, the soluble dextrans produced in liquefaction are further hydrolyzed by glucoamylases producing a high glucose syrup containing greater than 95 % glucose.

[0005] Glucoamylases are exo-acting carbohydrases, capable of hydrolyzing both the linear and branched glucosidic linkages of starch (*e.g.*, amylose and amylopectin). Commercially, glucoamylases are typically used in the acidic pH ranges (pH less than 5.0) to produce fermentable sugars from the enzyme liquefied starch substrate. The fermentable sugars, *e.g.*, low molecular weight sugars, such as glucose, may then be converted to fructose by other enzymes (*e.g.*, glucose isomerases); crystallized; or used in fermentations to produce

numerous end products (*e.g.*, alcohols, monosodium glutamate, succinic acid, vitamins, amino acids, 1,3-propanediol, and lactic acid).

[0006] A system that combines (1) saccharification and (2) fermentation is known as simultaneous saccharification and fermentation (SSF). SSF replaces the classical double-step fermentation, *i.e.*, production of fermentable sugars first and then conducting the fermentation process for producing the end product. In SSF, an inoculum can be added along with the starch hydrolyzing enzymes to concurrently saccharify a starch substrate and convert the saccharification products (*i.e.*, fermentable sugars) to the desired end product. The inoculum is typically a microorganism capable of producing the end product. The benefits of SSF include, but are not limited to, the following:

- 1) promote sustained microbial growth by providing a continuous feeding of glucose;
- 2) improving the carbon conversion efficiency by reducing the osmotic stress;
- 3) boosting fermentation capacity by accommodating higher dry solids; and
- 4) improving end product yield and facilitating downstream processing due to reduced production of reversion reaction products and non-fermentable sugars.

SSF is particularly promising where a high concentration substrate is present in a low reactor volume. *See e.g.*, John et al., *Biotechnol. Adv.* 27: 145-152 (2009).

[0007] There is still need to optimize SSF by choosing conditions, *e.g.*, temperature, pH, enzymes, etc., that are most suitable for both saccharification and fermentation. For example, the pH of the yeast fermentation matches the saccharifying glucoamylase enzyme activity during the production of fuel alcohol using grain as a feedstock. The need is acute particularly for fermentations that are optimally performed above pH 6.0. Most commercial saccharification enzymes, *e.g.*, *Aspergillus niger* glucoamylase (AnGA), only display significant saccharifying enzyme activity in the pH range of 4.2 to 5.5. The glucoamylases display significantly lowered activity at the fermentation pH above 6.0. Additional steps, such as pre-treatment or pre-saccharification for producing fermentation feed stocks, have been used when the optimal conditions for the fermentation and saccharification are not congruent. As disclosed in WO 2003/066816, for example, the slurry is subject to pasteurization at 65°C for 14 hours before the SSF is performed to produce 1,3-propanediol at

34°C. Similarly, the lactic acid-producing microorganism may be subject to forty to fifty serial transfers at an acidic pH before being applied in the SSF to produce lactic acid. *See* WO 2003/095659.

SUMMARY OF THE INVENTION

[0008] Glucoamylases such as *Humicola grisea* glucoamylase (HgGA), *Trichoderma reesei* glucoamylase (TrGA), and *Rhizopus oryzae/niveus*. glucoamylase (RhGA) display different pH profiles from other known glucoamylases, such as glucoamylases (GAs) from *Aspergillus niger* (AnGA) and *Talaromyces emersonii* (TeGA). At a pH of 6.0 or above, both HgGA and TrGA retain at least 50% of the activity relative to the maximum activity at pH 4.25 or pH 3.75, respectively. Both HgGA and TrGA are capable of saccharifying a starch substrate effectively at a pH in the range of 5.0 to 8.0. This property enables HgGA and TrGA to be used in simultaneous saccharification and fermentation (SSF) to produce end products from a starch substrate.

[0009] The embodiment contemplated herein provides a method of processing starch to produce fermentable sugars at pH 5.0 to 8.0. The fermentable sugars are produced by saccharifying a starch substrate in the presence of a glucoamylase, which possesses at least 50% activity at pH 6.0 or above relative to its maximum activity. Saccharifying may be carried out at a pH in a range of 6.0 to 7.5, or optionally 7.0 to 7.5. Saccharifying is performed at a temperature in a range of about 30°C to about 60°C, or optionally about 30°C to about 40°C.

[0010] In one aspect, the starch substrate is from corn, wheat, rye, barley, sorghum, cassava, tapioca, potato and any combination thereof. In another aspect, the starch substrate is granular starch or liquefied starch. In a further aspect, the starch substrate is about 15% to 50%, about 15% to 30%, or about 15% to 25% dry solid (DS).

[0011] In one embodiment, the method further comprises fermenting the fermentable sugars to an end product at the same pH saccharifying is performed. The end product may be selected from the group consisting of methanol, ethanol, butanol, monosodium glutamate, succinic acid, 1,3-propanediol, vitamins, amino acids, and lactic acid. Optionally, the end product is ethanol, 1,3-propanediol, or succinic acid. In another embodiment, saccharifying

and fermenting are carried out as a simultaneous saccharification and fermentation (SSF) process, which is typically carried out at pH 6.5 to 7.5.

[0012] In one aspect, the glucoamylase is selected from the group consisting of *Humicola grisea* glucoamylase (HgGA) comprising SEQ ID NO: 3, *Trichoderma reesei* glucoamylase (TrGA) comprising SEQ ID NO: 6, *Rhizopus p.* glucoamylase (RhGA) comprising SEQ ID NO: 9, and a variant thereof. The variant has at least 99% sequence identity to the parent glucoamylase. Optionally, the variant has one amino acid modifications compared to the parent glucoamylase. In another aspect, the HgGA is SEQ ID NO: 3, and is optionally produced from a *Trichoderma reesei* host cell. In a further aspect, the TrGA is SEQ ID No: 6. In yet another aspect, the RhGA is SEQ ID NO: 9.

[0013] In one embodiment, the glucoamylase is added at a range of about 0.1 to about 2.0, about 0.2 to about 1.0, or 0.5 to 1.0 GAU per gram of dry substance starch. In another embodiment, saccharifying further comprises adding an alpha-amylase. The alpha-amylase is from a *Bacillus* species, or a variant thereof. The alpha-amylase is a *Bacillus subtilis* alpha-amylase (AmyE), a *Bacillus amyloliquefaciens* alpha-amylase, a *Bacillus licheniformis* alpha-amylase, a *Bacillus stearothermophilus* alpha-amylase, or a variant thereof.

[0014] In another embodiment, the invention provides for methods of processing starch comprising saccharifying a starch substrate to fermentable sugars at pH 5.0 to 8.0 in the presence of glucoamylase and at least one other enzyme, wherein the glucoamylase possesses at least 50% activity at pH 6.0 or above relative to its maximum activity, wherein the glucoamylase is selected from the group consisting of *Humicola grisea* glucoamylase (HgGA) comprising SEQ ID NO: 3, *Trichoderma reesei* glucoamylase (TrGA) comprising SEQ ID NO: 6, *Rhizopus sp.* glucoamylase (RhGA) comprising SEQ ID NO: 9, and a variant thereof, and wherein the variant has at least 99% sequence identity to a parent glucoamylase, and wherein the other enzyme is selected from the group consisting of proteases, pullulanases, isoamylases, cellulases, hemicellulases, xylanases, cyclodextrin glycotransferases, lipases, phytases, laccases, oxidases, esterases, cutinases, xylanases, and alpha-glucosidases.

[0015] In another embodiment, the invention provides for methods of processing starch comprising saccharifying a starch substrate to fermentable sugars at pH 5.0 to 8.0 in the presence of glucoamylase and at least one other non-starch polysaccharide hydrolyzing

enzymes, wherein the glucoamylase possesses at least 50% activity at pH 6.0 or above relative to its maximum activity, wherein the glucoamylase is selected from the group consisting of *Humicola grisea* glucoamylase (HgGA) comprising SEQ ID NO: 3, *Trichoderma reesei* glucoamylase (TrGA) comprising SEQ ID NO: 6, *Rhizopus* sp. glucoamylase (RhGA) comprising SEQ ID NO: 9, and a variant thereof, and wherein the variant has at least 99% sequence identity to a parent glucoamylase, and wherein the non-starch polysaccharide hydrolyzing enzymes is selected from the group consisting of cellulases, hemicellulases and pectinases.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] The accompanying drawings are incorporated into the specification and provide non-limiting illustrations of various embodiments. In the drawings:

[0017] FIG. 1 depicts the pH profiles of HgGA, TrGA, AnGA, and TeGA, at 32°C. The pH profiles are presented as the percentage of the maximum activity under the saccharification conditions described in Example 1.

[0018] FIG. 2 depicts the presence of higher sugars after 48-hour saccharification reactions catalyzed by HgGA, TrGA, and AnGA. The saccharification reactions are described in Example 4.

[0019] FIG. 3 depicts scanning electron micrographs of corn, wheat, and cassava starch treated with HgGA and an alpha-amylase at pH 6.4. Starch samples are hydrolyzed by HgGA and an alpha-amylase under the conditions as described in Example 7.

DETAILED DESCRIPTION

[0020] The present disclosure relates to a glucoamylase capable of effectively saccharifying a starch substrate at a neutral pH, for example, between pH 5.0 and 8.0. At a pH of 6.0 or above, the glucoamylase retains at least about 50% activity relative to the maximum activity. The glucoamylases having the unusually properties may include, for example, HgGA, TrGA, and RhGA. Also disclosed includes a method of using the glucoamylase to perform simultaneous saccharification and fermentation (SSF), at a neutral pH, to produce an end product, for example, 1,3-propanediol, succinic acid, lysine, monosodium glutamate, and lactic acid.

[0021] In some aspects, the embodiments of the present disclosure rely 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); Kreigler, *GENE TRANSFER AND EXPRESSION; A LABORATORY MANUAL* (1990) and Ausubel et al., Eds. *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY* (1994). 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, N.Y. (1991) provide one of skill with a general dictionary 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 representative methods and materials are described. Numeric ranges are inclusive of the numbers defining the range. The headings provided herein are not limitations of the various aspects or embodiments, which can be had by reference to the specification as a whole.

1. Definitions and Abbreviations

[0022] In accordance with this detailed description, the following abbreviations and definitions apply. It should be noted that as used herein, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “an enzyme” includes a plurality of such enzymes.

1.1. Definitions

[0023] As used herein, “amino acid sequence” is synonymous with the term “polypeptide” and/or the term “protein.” In some instances, the term “amino acid sequence” is synonymous with the term “peptide”; in some instances, the term “amino acid sequence” is synonymous with the term “enzyme.”

[0024] As used herein, “nucleotide sequence” or “nucleic acid sequence” refers to a sequence of genomic, synthetic, or recombinant origin and may be double-stranded or single-stranded, whether representing the sense or anti-sense strand. As used herein, the term

“nucleic acid” may refer to genomic DNA, cDNA, synthetic DNA, or RNA. The residues of a nucleic acid may contain any of the chemically modifications commonly known and used in the art.

[0025] “Isolated” means that the material is at least substantially free from at least one other component that the material is naturally associated and found in nature.

[0026] “Purified” means that the material is in a relatively pure state, *e.g.*, at least about 90% pure, at least about 95% pure, at least about 98% pure, or at least about 99% pure.

[0027] “Oligosaccharide” means a carbohydrate molecule composed of 3-20 monosaccharides.

[0028] As used herein, “transformed cell” includes cells that have been transformed by use of recombinant DNA techniques. Transformation typically occurs by insertion of one or more nucleotide sequences into a cell. The inserted nucleotide sequence may be a heterologous nucleotide sequence, *i.e.*, is a sequence that may not be natural to the cell that is to be transformed, such as a fusion protein.

[0029] As used herein, “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 more specifically wheat, barley, corn, rye, rice, sorghum, brans, cassava, millet, potato, sweet potato, and tapioca.

[0030] As used herein, “granular starch” refers to uncooked (raw) starch, which has not been subject to gelatinization.

[0031] As used herein, “starch gelatinization” means solubilization of a starch molecule to form a viscous suspension.

[0032] As used herein, “gelatinization temperature” refers to the lowest temperature at which gelatinization of a starch substrate occurs. The exact temperature depends upon the specific starch substrate and further may depend on the particular variety and the growth conditions of plant species from which the starch is obtained.

[0033] “DE” or “dextrose equivalent” is an industry standard for measuring the concentration of total reducing sugars, calculated as the percentage of the total solids that

have been converted to reducing sugars. The granular starch that has not been hydrolyzed has a DE that is about zero (0), and D-glucose has a DE of about 100.

[0034] As used herein, “starch substrate” refers to granular starch or liquefied starch using refined starch, whole ground grains, or fractionated grains.

[0035] As used herein, “liquefied starch” refers to starch that has gone through solubilization process, for example, the conventional starch liquefaction process.

[0036] As used herein, “glucose syrup” refers to an aqueous composition containing glucose solids. Glucose syrup will have a DE of at least about 20. In some embodiments, glucose syrup may contain no more than about 21% water while at least about 25% reducing sugar calculated as dextrose. In one embodiment, glucose syrup may include at least about 90% D-glucose, and in another embodiment, glucose syrup may include at least about 95% D-glucose. In some embodiments, the terms glucose and glucose syrup are used interchangeably.

[0037] “Degree of polymerization (DP)” refers to the number (n) of anhydroglucopyranose units in a given saccharide. Examples of DP1 are the monosaccharides, such as glucose and fructose. Examples of DP2 are the disaccharides, such as maltose and sucrose. A DP4+ (>DP4) denotes polymers with a degree of polymerization of greater than four.

[0038] As used herein, “fermentable sugars” refer to saccharides that are capable of being metabolized under fermentation conditions. These sugars typically refer to glucose, maltose, and maltotriose (DP1, DP2 and DP3).

[0039] As used herein, “total sugar content” refers to the total sugar content present in a starch composition.

[0040] As used herein, “ds” refers to dissolved solids in a solution. The term “dry solids content (DS)” refers to the total solids of a slurry in % on a dry weight basis. The term “slurry” refers to an aqueous mixture containing insoluble solids.

[0041] As used herein, “starch-liquefying enzyme” refers to an enzyme that catalyzes the hydrolysis or breakdown of granular starch. Exemplary starch liquefying enzymes include alpha-amylases (EC 3.2.1.1).

[0042] “Amylase” means an enzyme that is, among other things, capable of catalyzing the degradation of starch. For example, β -Amylases, α -glucosidases (EC 3.2.1.20; α -D-glucoside glucohydrolase), glucoamylase (EC 3.2.1.3; α -D-(1 \rightarrow 4)-glucan glucohydrolase),

and product-specific amylases can produce malto-oligosaccharides of a specific length from starch.

[0043] “Alpha-amylases (EC 3.2.1.1)” refer to endo-acting enzymes that cleave α -D-(1 \rightarrow 4) O-glycosidic linkages within the starch molecule in a random fashion. In contrast, the exo-acting amylolytic enzymes, such as beta-amylases (EC 3.2.1.2; α -D-(1 \rightarrow 4)-glucan maltohydrolase) and some product-specific amylases like maltogenic alpha-amylase (EC 3.2.1.133) cleave the starch molecule from the non-reducing end of the substrate. These enzymes 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. Another term used to describe these enzymes is glycogenase. Exemplary enzymes include alpha-1, 4-glucan 4-glucanohydrolase.

[0044] As used herein, “glucoamylases” refer to the amyloglucosidase class of enzymes (EC 3.2.1.3, glucoamylase, α -1, 4-D-glucan glucohydrolase). These are exo-acting enzymes that release glucosyl residues from the non-reducing ends of amylose and/or amylopectin molecules. The enzymes are also capable of hydrolyzing α -1, 6 and α -1,3 linkages, however, at much slower rates than the hydrolysis of α -1, 4 linkages.

[0045] As used herein, the term “non-starch polysaccharide hydrolyzing enzymes” are enzymes capable of hydrolyzing complex carbohydrate polymers such as cellulose, hemicellulose, and pectin. For example, cellulases (endo and exo-glucanases, beta glucosidase) hemicellulases (xylanases) and pectinases are non-starch polysaccharide hydrolyzing enzymes.

[0046] As used herein, “maximum activity” refers to the enzyme activity measured under the most favorable conditions, for example, at an optimum pH. As used herein, “optimum pH” refers to a pH value, under which the enzyme displays the highest activity with other conditions being equal.

[0047] The phrase “mature form” of a protein or polypeptide refers to the final functional form of the protein or polypeptide. A mature form of a glucoamylase may lack a signal peptide and/or initiator methionine, for example. A mature form of a glucoamylase may be produced from its native host, for example, by endogenous expression. Alternatively, a mature form of a glucoamylase may be produced from a non-native host, for example, by exogenous expression. An exogenously expressed glucoamylase, while maintaining the

glucoamylase activity, may have a varied glycosylation pattern compared to the endogenous expressed counterpart.

[0048] The term “parent” or “parent sequence” refers to a sequence that is native or naturally occurring in a host cell.

[0049] As used herein, the terms “variant” is used in reference to glucoamylases that have some degree of amino acid sequence identity to a parent glucoamylase sequence. A variant is similar to a parent sequence, but has at least one substitution, deletion or insertion in their amino acid sequence that makes them different in sequence from a parent glucoamylase. In some cases, variants have been manipulated and/or engineered to include at least one substitution, deletion, or insertion in their amino acid sequence that makes them different in sequence from a parent. Additionally, a glucoamylase variant may retain the functional characteristics of the parent glucoamylase, *e.g.*, maintaining a glucoamylase activity that is at least about 50%, about 60%, about 70%, about 80%, or about 90% of that of the parent glucoamylase.

[0050] As used herein, “hydrolysis of starch” refers to the cleavage of glucosidic bonds with the addition of water molecules.

[0051] As used herein, “no-cook” refers to a process of converting a granular starch substrate, for example, raw starch, to fermentable sugars without the conventional high-temperature starch liquefaction process.

[0052] As used herein, “end product” or “desired end product” refers to a molecule or compound to which a starch substrate is converted into, by an enzyme and/or a microorganism.

[0053] As used herein, “contacting” or “admixing” 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 affect contacting or admixing.

1.2. Abbreviations

[0054] The following abbreviations apply unless indicated otherwise:

AkAA	<i>Aspergillus kawachii</i> alpha-amylase
AmyE	<i>Bacillus subtilis</i> alpha-amylase
AmyL	<i>Bacillus licheniformis</i> alpha-amylase

AmyR	SPEZYME® XTRA amylase
AmyS	<i>Geobacillus stearothermophilus</i> alpha-amylase
AnGA	<i>Aspergillus niger</i> glucoamylase
BAA	bacterial alpha-amylase
cDNA	complementary DNA
DE	Dextrose Equivalent
DI	distilled, deionized
DNA	deoxyribonucleic acid
DP3	degree of polymerization with three subunits
DPn	degree of polymerization with n subunits
DS or ds	dry solid
dss	dry solid starch
EC	enzyme commission for enzyme classification
g	gram
gpm	gallon per minute
GAU	glucoamylase units
HGA	<i>Humicola grisea</i> glucoamylase
HgGA	<i>Humicola grisea</i> glucoamylase
HPLC	high pressure liquid chromatography
kg	kilogram
MOPS	3-(N-morpholino)propanesulfonic acid
MT	metric ton
MW	molecular weight
NCBI	National Center for Biotechnology Information
nm	nanometer
OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
pI	isoelectric point
ppm	parts per million
RhGA	<i>Rhizopus</i> sp. glucoamylase

RNA	ribonucleic acid
RO	reverse osmosis
rpm	revolutions per minute
slpm	standard liters per minute
SSF	simultaneous saccharification and fermentation
TeGA	<i>Talaromyces emersonii</i> glucoamylase
TrGA	<i>Trichoderma reesei</i> glucoamylase
w/v	weight/volume
w/w	weight/weight
wt	wild-type
μL	microliter

2. Enzymes in Starch Processing

2.1. Glucoamylase

2.1.1. Structure and Function

[0055] Glucoamylases are produced by numerous strains of bacteria, fungi, yeast and plants. Many fungal glucoamylases are fungal enzymes that are extracellularly produced, for example from strains of *Aspergillus* (Svensson et al., *Carlsberg Res. Commun.* 48: 529-544 (1983); Boel et al., *EMBO J.* 3: 1097-1102 (1984); Hayashida et al., *Agric. Biol. Chem.* 53: 923-929 (1989); U.S. Patent No. 5,024,941; U.S. Patent No. 4,794,175 and WO 88/09795); *Talaromyces* (U.S. Patent No. 4,247,637; U.S. Patent No. 6,255,084; and U.S. Patent No. 6,620,924); *Rhizopus* (Ashikari et al., *Agric. Biol. Chem.* 50: 957-964 (1986); Ashikari et al., *App. Microbio. Biotech.* 32: 129-133 (1989) and U.S. Patent No. 4,863,864); *Humicola* (WO 05/052148 and U.S. Patent No. 4,618,579); and *Mucor* (Houghton-Larsen et al., *Appl. Microbiol. Biotechnol.* 62: 210-217 (2003)). Many of the genes that code for these enzymes have been cloned and expressed in yeast, fungal and/or bacterial cells.

[0056] Commercially, glucoamylases are very important enzymes and have been used in a wide variety of applications that require the hydrolysis of starch (*e.g.*, for producing glucose and other monosaccharides from starch). Glucoamylases are used to produce high fructose corn sweeteners, which comprise over 50% of the sweetener market in the United States. In general, glucoamylases may be, and commonly are, used with alpha-amylases in starch

hydrolyzing processes to hydrolyze starch to dextrins and then glucose. The glucose may then be converted to fructose by other enzymes (*e.g.*, glucose isomerases); crystallized; or used in fermentations to produce numerous end products (*e.g.*, ethanol, citric acid, succinic acid, ascorbic acid intermediates, glutamic acid, glycerol, 1,3-propanediol and lactic acid).

[0057] Glucoamylases consist of as many as three distinct structural domains, a catalytic domain of approximately 450 residues that is structurally conserved in all glucoamylases, generally followed by a linker region consisting of between 30 and 80 residues that are connected to a starch binding domain of approximately 100 residues. The structure of the *Trichoderma reesei* glucoamylase (TrGA) with all three regions intact was determined to 1.8 Angstrom resolution. *See* WO 2009/048488 and WO 2009/048487. Using the determined coordinates, the structure was aligned with the coordinates of the catalytic domain of the glucoamylase from *Aspergillus awamori* strain X100 that was determined previously (Aleshin, A.E., Hoffman, C., Firsov, L.M., and Honzatko, R.B. Refined crystal structures of glucoamylase from *Aspergillus awamori* var. X100. *J. Mol. Biol.* 238: 575-591 (1994)). *See id.* The structure of the catalytic domains of these two glucoamylases overlap very closely, and it is possible to identify equivalent residues based on this structural superposition. *See id.* It is further believed that all glucoamylases share the basic structure. *See id.*

[0058] Given the well-known structure and function relationship of glucoamylases, glucoamylase variants having altered properties have been successfully created and characterized. The variants may display improved properties as compared to the parent glucoamylases. The improved properties may include and are not limited to increased thermostability and increased specific activity. For example, methods for making and characterizing TrGA variants with altered properties have been described in WO 2009/067218.

2.1.2. Glucoamylases having the desired pH profile

[0059] The embodiments of the present disclosure utilize a glucoamylase capable of effectively saccharifying a starch substrate at a neutral pH, for example, between pH 5.0 and 8.0, 5.5 and 7.5, 6.0 and 7.5, 6.5 and 7.5, or 7.0 and 7.5. At a pH of 6.0 or above, the glucoamylase retains at least about 50%, about 51%, about 52%, about 53%, about 54%, or about 55% of the activity relative to the maximum activity. The glucoamylases having the

desired pH profile include, but are not limited to, *Humicola grisea* glucoamylase (HgGA), *Trichoderma reesei* glucoamylase (TrGA), and *Rhizopus* sp. glucoamylase (RhGA).

[0060] HgGA may be the glucoamylase comprising the amino acid sequence of SEQ ID NO: 3, which is described in detail in U.S. Patent Nos. 4,618,579 and 7,262,041. This HgGA is also described as a granular starch hydrolyzing enzyme (GSHE), because it is capable of hydrolyzing starch in granular form. The genomic sequence coding the HgGA from *Humicola grisea* var. *thermoidea* is presented as SEQ ID NO: 1, which contains three putative introns (positions 233-307, 752-817, and 950-1006). The native HgGA from *Humicola grisea* var. *thermoidea* has the amino acid sequence of SEQ ID NO: 2, which includes a signal peptide containing 30 amino acid residues (positions 1 to 30 of SEQ ID NO: 2). Cleavage of the signal peptide results in the mature HgGA having the amino acid sequence of SEQ ID NO: 3. The embodiments of the present disclosure also include a HgGA produced from a *Trichoderma* host cell, e.g., a *Trichoderma reesei* cell. See U.S. Patent No 7,262,041.

[0061] TrGA may be the glucoamylase from *Trichoderma reesei* QM6a (ATCC, Accession No. 13631). This TrGA comprising the amino acid sequence of SEQ ID NO: 6, which is described in U.S. Patent No. 7,413,879, for example. The cDNA sequence coding the TrGA from *Trichoderma reesei* QM6a is presented as SEQ ID NO: 4. The native TrGA has the amino acid sequence of SEQ ID NO: 5, which includes a signal peptide containing 33 amino acid residues (positions 1 to 33 of SEQ ID NO: 4). See *id.* Cleavage of the signal peptide results in the mature TrGA having the amino acid sequence of SEQ ID NO: 6. See *id.* The catalytic domain of TrGA is presented as SEQ ID NO: 7. See *id.* The embodiments of the present disclosure also include an endogenously expressed TrGA. See *id.*

[0062] RhGA may be the glucoamylase from *Rhizopus niveus* or *Rhizopus oryzae*. See U.S. Patent Nos. 4,514,496 and 4,092,434. The native RhGA from *R. oryzae* has the amino acid sequence of SEQ ID NO: 8, which includes a signal peptide containing 25 amino acid residues (positions 1 to 25 of SEQ ID NO:8). Cleavage of the signal peptide results in the mature RhGA having the amino acid sequence of SEQ ID NO: 9. A typical RhGA may be the glucoamylase having trade names CU.CONC (Shin Nihon Chemicals, Japan) or M1 (Biocon India, Bangalore, India).

[0063] The glucoamylase of the embodiment of the present disclosure may also be a variant of HgGA, TrGA, or RhGA. The variant has at least 99% sequence identity to the parent glucoamylase. In some embodiments, the variant has at least 98%, at least 97%, at least 96%, at least 95%, at least 94%, at least 93%, at least 92%, at least 91%, or at least 90% sequence identity to the parent glucoamylase. Optionally, the variant has one, two, three, four, five, or six amino acids modification compared to the mature form of the parent glucoamylase. Optionally, the variant has more than six amino acids (e.g., 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60) modification compared to the mature form of the parent glucoamylase. The variant possesses the desired pH profile and capability of saccharifying a starch substrate at a pH in the range of 5.0 to 8.0. In some embodiments, the variants may possess other improved properties, such as improved thermostability and improved specificity.

2.1.3. Production of Glucoamylase

[0064] Glucoamylases suitable for the embodiments of the present disclosure may be produce with recombinant DNA technology in various host cells.

[0065] In some embodiments, the host cells are selected from bacterial, fungal, plant and yeast cells. The term host cell includes both the cells, progeny of the cells and protoplasts created from the cells that are used to produce a variant glucoamylase according to the disclosure. In some embodiments, the host cells are fungal cells and typically filamentous fungal host 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 disclosure are morphologically, physiologically, and genetically distinct from yeasts. Vegetative growth by filamentous fungi is by hyphal elongation and carbon catabolism is obligatory aerobic. In the embodiments of the present disclosure, the filamentous fungal parent cell may be a cell of a species of, but not limited to, *Trichoderma*, (e.g., *Trichoderma reesei*, the asexual morph of *Hypocrea jecorina*, previously classified as *T. longibrachiatum*, *Trichoderma viride*, *Trichoderma koningii*, *Trichoderma harzianum*) (Sheir-Neirs et al., (1984) *Appl. Microbiol. Biotechnol* 20:46-53; ATCC No. 56765 and ATCC No. 26921); *Penicillium* sp., *Humicola* sp. (e.g., *H. insolens*, *H. lanuginosa* and *H.*

grisea); *Chrysosporium* sp. (e.g., *C. lucknowense*), *Gliocladium* sp., *Aspergillus* sp. (e.g., *A. oryzae*, *A. niger*, *A. sojae*, *A. japonicus*, *A. nidulans*, and *A. awamori*) (Ward et al., (1993) Appl. Microbiol. Biotechnol. 39:738-743 and Goedegebuur et al., (2002) Genet 41:89-98), *Fusarium* sp., (e.g., *F. roseum*, *F. gramineum*, *F. cerealis*, *F. oxysporum* and *F. venenatum*), *Neurospora* sp., (*N. crassa*), *Hypocrea* sp., *Mucor* sp., (*M. miehei*), *Rhizopus* sp. and *Emericella* sp. (see also, Innis et al., (1985) Sci. 228:21-26). The term “*Trichoderma*” or “*Trichoderma* sp.” or “*Trichoderma* spp.” refers to any fungal genus previously or currently classified as *Trichoderma*. In other embodiments, the host cell will be a genetically engineered host cell wherein native genes have been inactivated, for example by deletion in fungal cells. Where it is desired to obtain a fungal host cell having one or more inactivated genes known methods may be used (e.g. methods disclosed in U.S. Patent Nos. 5,246,853 and 5,475,101, and WO 92/06209). Gene inactivation may be accomplished by complete or partial deletion, by insertional inactivation or by any other means that renders a gene nonfunctional for its intended purpose (such that the gene is prevented from expression of a functional protein). In some embodiments, when the host cell is a *Trichoderma* cell and particularly a *T. reesei* host cell, the *cbh1*, *cbh2*, *egl1* and *egl2* genes will be inactivated and/or typically deleted. Typically, *Trichoderma reesei* host cells having quad-deleted proteins are set forth and described in U.S. Patent No. 5,847,276 and WO 05/001036. In other embodiments, the host cell is a protease deficient or protease minus strain.

[0066] To produce the glucoamylase of the embodiments of the present disclosure with the recombinant DNA technology, a DNA construct comprising nucleic acid encoding the amino acid sequence of the designated glucoamylase can be constructed and transferred into, for example, a *Trichoderma reesei* host cell. The vector may be any vector which when introduced into a *Trichoderma reesei* host cell can be integrated into the host cell genome and can be replicated. 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, and Ausubel (1987) supra, and 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. The nucleic acid encoding the glucoamylase can be operably linked to a suitable promoter, which shows transcriptional activity in *Trichoderma reesei* host cell. The promoter may be

derived from genes encoding proteins either homologous or heterologous to the host cell. Suitable non-limiting examples of promoters include *cbh1*, *cbh2*, *egl1*, *egl2*. In one embodiment, the promoter may be a native *T. reesei* promoter. Typically, the promoter can be *T. reesei cbh1*, which is an inducible promoter and has been deposited in GenBank under Accession No. D86235. An “inducible promoter” may refer to a promoter that is active under environmental or developmental regulation. In another embodiment, the promoter can be one that is heterologous to *T. reesei* host cell. Other examples of useful promoters include promoters from *A. awamori* and *A. niger* glucoamylase genes (*see, e.g.*, Nunberg et al., (1984) *Mol. Cell Biol.* 4:2306-2315 and Boel et al., (1984) *EMBO J.* 3:1581-1585). Also, the promoters of the *T. reesei xln1* gene and the cellobiohydrolase 1 gene may be useful (EPA 13f280A1).

[0067] In some embodiments, the glucoamylase coding sequence can be operably linked to a signal sequence. The signal sequence may be the native signal peptide of the glucoamylase (residues 1-20 of SEQ ID NO: 2 for HgGA, or residues 1-33 of SEQ ID NO: 5 for TrGA, for example). Alternatively, the signal sequence may have at least 90% or at least 95% sequence identity to the native signal sequence. In additional embodiments, a signal sequence and a promoter sequence comprising a DNA construct or vector to be introduced into the *T. reesei* host cell are derived from the same source. For example, in some embodiments, the signal sequence can be the *cdhl* signal sequence that is operably linked to a *cdhl* promoter.

[0068] In some embodiments, the expression vector may also include a termination sequence. In one embodiment, the termination sequence and the promoter sequence can be derived from the same source. In another embodiment, the termination sequence can be homologous to the host cell. A particularly suitable terminator sequence can be *cbh1* derived from *T. reesei*. Other exemplary fungal terminators include the terminator from *A. niger* or *A. awamori* glucoamylase gene.

[0069] In some embodiments, an expression vector may include a selectable marker. Examples of representative selectable markers include ones that confer antimicrobial resistance (e.g., hygromycin and phleomycin). Nutritional 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 *Trichoderma* are known in the art (*see, e.g.*, Finkelstein, chapter 6 in BIOTECHNOLOGY OF FILAMENTOUS FUNGI, Finkelstein et al.

Eds. Butterworth-Heinemann, Boston, Mass. (1992), Chap. 6.; and Kinghorn et al. (1992) APPLIED MOLECULAR GENETICS OF FILAMENTOUS FUNGI, Blackie Academic and Professional, Chapman and Hall, London). In a representative embodiment, the selective marker may be 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 for example in Kelley et al., (1985) *EMBO J.* 4:475-479 and Penttila et al., (1987) *Gene* 61:155-164.

[0070] An expression vector comprising a DNA construct with a polynucleotide encoding the glucoamylase 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. In some embodiments, the expression vector can be a plasmid. In typical embodiments, two types of expression vectors for obtaining expression of genes are contemplated.

[0071] The first expression vector may comprise DNA sequences in which the promoter, glucoamylase-coding region, and terminator all originate from the gene to be expressed. In some embodiments, gene truncation can be 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.

[0072] The second type of expression vector may be preassembled and contains sequences needed for high-level transcription and a selectable marker. In some embodiments, the coding region for the glucoamylase gene or part thereof can be 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 may be inserted downstream of a strong promoter, such as the strong *cbhI* promoter.

[0073] Methods used to ligate the DNA construct comprising a polynucleotide encoding the glucoamylase, a promoter, a terminator and other sequences and to insert them into a suitable vector are well known in the art. Linking can be 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 (1991) pp 70-76.). Additionally, vectors can be constructed using known recombination techniques (e.g., Invitrogen Life Technologies, Gateway Technology).

[0074] 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); 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 U.S. Pat. Nos. 6,022,725; 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).

[0075] In some embodiments, genetically stable transformants can be constructed with vector systems whereby the nucleic acid encoding glucoamylase is stably integrated into a host strain chromosome. Transformants are then purified by known techniques.

[0076] In one non-limiting 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 can be 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.

[0077] 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 may be 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). It is believed that the polyethylene glycol acts to fuse the cell membranes, thus permitting the contents of the medium to be delivered into the cytoplasm of

the *Trichoderma* sp. strain and the plasmid DNA is transferred to the nucleus. This fusion frequently leaves multiple copies of the plasmid DNA integrated into the host chromosome.

[0078] Usually a suspension containing the *Trichoderma* sp. protoplasts or cells that have been subjected to a permeability treatment at a density of 10^5 to 10^7 /mL, typically, 2×10^6 /mL are used in transformation. A volume of 100 μ L of these protoplasts or cells in an appropriate solution (e.g., 1.2 M sorbitol; 50 mM CaCl_2) are mixed with the desired DNA. Generally, a high concentration of PEG may be added to the uptake solution. From 0.1 to 1 volume of 25% PEG 4000 can be added to the protoplast suspension. It is also typical 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. Pat. Nos. 6,022,725 and 6,268,328.

[0079] Generally, the mixture can be then incubated at approximately 0°C for a period of between 10 to 30 minutes. Additional PEG may then be added to the mixture to further enhance the uptake of the desired gene or DNA sequence. The 25% PEG 4000 can be 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 may be typically about 10 times the volume of the transformation mixture. After the PEG is added, the transformation mixture can then be incubated either at room temperature or on ice before the addition of a sorbitol and CaCl_2 solution. The protoplast suspension can then be further added to molten aliquots of a growth medium. This growth medium permits the growth of transformants only.

[0080] 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 media (e.g., Yeast Malt Extract (YM) broth, Luria Bertani (LB) broth and Sabouraud Dextrose (SD) broth also find use in the present embodiments.

[0081] Culture-conditions are also standard, (e.g., cultures are incubated at approximately 28°C in appropriate medium in shake cultures or fermentors until desired levels of glucoamylase expression are achieved). After fungal growth has been established, the cells

are exposed to conditions effective to cause or permit the expression of the glucoamylase. In cases where the glucoamylase coding sequence is under the control of an inducible promoter, the inducing agent (e.g., a sugar, metal salt or antimicrobial), can be added to the medium at a concentration effective to induce glucoamylase expression.

[0082] In general, the glucoamylase produced in cell culture may be secreted into the medium and may be purified or isolated, e.g., by removing unwanted components from the cell culture medium. In some cases, the glucoamylase can be produced in a cellular form, necessitating recovery from a cell lysate. In such cases, the enzyme may be purified from the cells in which it was produced using techniques routinely employed by those of skill in the art. Examples of these techniques 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 (*see*, Tomaz and Queiroz, (1999) *J. Chromatography A* 865: 123; two-phase partitioning (*see*, 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 (e.g., Sephadex G-75).

2.2. Alpha-amylases

[0083] Alpha-amylases constitute a group of enzymes present in microorganisms and tissues from animals and plants. They are capable of hydrolyzing alpha-1,4-glycosidic bonds of glycogen, starch, related polysaccharides, and some oligosaccharides. Although all alpha-amylases possess the same catalytic function, their amino acid sequences vary greatly. The sequence identity between different amylases can be virtually non-existent, e.g., falling below 25%. Despite considerable amino acid sequence variation, alpha-amylases share a common overall topological scheme that has been identified after the three-dimensional structures of alpha-amylases from different species have been determined. The common three-dimensional structure reveals three domains: (1) a "TIM" barrel known as domain A, (2) a long loop region known as domain B that is inserted within domain A, and (3) a region

close to the C-terminus known as domain C that contains a characteristic beta-structure with a Greek-key motif.

[0084] “Termamyl-like” alpha-amylases refer to a group of alpha-amylases widely used in the starch-processing industry. The *Bacillus licheniformis* alpha-amylase having an amino acid sequence of SEQ ID NO: 2 of U.S. Patent No. 6,440,716 is commercially available as Termamyl®. Termamyl-like alpha-amylases commonly refer to a group of highly homologous alpha-amylases produced by *Bacillus* spp. Other members of the group include the alpha-amylases from *Geobacillus stearothermophilus* (previously known as *Bacillus stearothermophilus*; both names are used interchangeably in the present disclosure) and *Bacillus amyloliquefaciens*, and those derived from *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513, and DSM 9375, all of which are described in detail in U.S. Patent No. 6,440,716 and WO 95/26397.

[0085] Although alpha-amylases universally contain the three domains discussed above, the three-dimensional structures of some alpha-amylases, such as AmyE from *Bacillus subtilis*, differ from Termamyl-like alpha-amylases. These enzymes are collectively referred as non-Termamyl-like alpha-amylases. “AmyE” for the purpose of this disclosure means a naturally occurring alpha-amylase (EC 3.2.1.1; 1, 4- α -D-glucan glucanohydrolase) from *Bacillus subtilis*. Representative AmyE enzymes and the variants thereof are disclosed in U.S. Patent Application 12/478,266 and 12/478,368, both filed June 4, 2009, and 12/479,427, filed June 5, 2009.

[0086] Other commercially available amylases can be used, e.g., TERMAMYL® 120-L, LC and SC SAN SUPER®, SUPRA®, and LIQUEZYME® SC available from Novo Nordisk A/S, FUELZYME® FL from Diversa, and CLARASE® L, SPEZYME® FRED, SPEZYME® ETHYL, GC626, and GZYME® G997 available from Danisco, US, Inc., Genencor Division.

2.3. Other enzymes and enzyme combinations

[0087] In embodiments of the present disclosure, other enzyme(s) may also be supplemented in starch processing, during saccharification and/or fermentation. These supplementary enzymes may include proteases, pullulanases, isoamylases, cellulases, hemicellulases, xylanases, cyclodextrin glycotransferases, lipases, phytases, laccases, oxidases, esterases, cutinases, xylanases, and/or alpha-glucosidases. *See e.g.*, WO

2009/099783. Skilled artisans in the art are well aware of the methods using the above-listed enzymes.

[0088] The glucoamylases disclosed herein can be used in combination with any other enzyme. For example, glucoamylase may be used in combination with amylases (e.g., alpha-amylases). In one embodiment, saccharification and/or fermentation or the simultaneous saccharification and fermentation (SSF) process use glucoamylase and one or more non-starch polysaccharide hydrolyzing enzymes. These enzymes are capable of hydrolyzing complex carbohydrate polymers such as cellulose, hemicellulose, and pectin. Non-limiting examples include cellulases (e.g., endo and exo-glucanases, beta glucosidase) hemicellulases (e.g., xylanases) and pectinases. In another embodiment, saccharification and/or fermentation or the SSF process use glucoamylase, alpha-amylase and one or more non-starch polysaccharide hydrolyzing enzymes. In another embodiment, saccharification and/or fermentation or the SSF process use glucoamylase with phytases, proteases, isoamylases and pullulanases.

[0089] In some embodiments, the saccharification and/or fermentation or the SSF process can use at least two non-starch polysaccharide hydrolyzing enzymes. In some embodiments, the saccharification and/or fermentation or the SSF process can use at least three non-starch polysaccharide hydrolyzing enzymes.

[0090] Cellulases are enzyme compositions that hydrolyze cellulose (β -1,4-D-glucan linkages) and/or derivatives thereof, such as phosphoric acid swollen cellulose. Cellulases include the classification of exo-cellobiohydrolases (CBH), endoglucanases (EG) and β -glucosidases (BG) (EC3.2.1.91, EC3.2.1.4 and EC3.2.1.21). Examples of cellulases include cellulases from *Penicillium*, *Trichoderma*, *Humicola*, *Fusarium*, *Thermomonospora*, *Cellulomonas*, *Hypocrea*, *Clostridium*, *Thermomonospora*, *Bacillus*, *Cellulomonas* and *Aspergillus*. Non-limiting examples of commercially available cellulases sold for feed applications are beta-glucanases such as ROVABIO[®] (Adisseo), NATUGRAIN[®] (BASF), MULTIFECT[®] BGL (Danisco Genencor) and ECONASE[®] (AB Enzymes). Some commercial cellulases includes ACCELERASE[®]. The cellulases and endoglucanases described in US20060193897A1 also may be used.

[0091] Beta-glucosidases (cellobiase) hydrolyzes cellobiose into individual monosaccharides. Various beta glucanases find use in the invention in combination with

phytases. Beta glucanases (endo-cellulase-enzyme classification EC 3.2.1.4) also called endoglucanase I, II, and III, are enzymes that will attack the cellulose fiber to liberate smaller fragments of cellulose which is further attacked by exo-cellulase to liberate glucose. Commercial beta-glucanases useful in the methods of the invention include OPTIMASH[®] BG and OPTIMASH[®] TBG (Danisco, US, Inc. Genencor Division).

[0092] Hemicellulases are enzymes that break down hemicellulose. Hemicellulose categorizes a wide variety of polysaccharides that are more complex than sugars and less complex than cellulose, that are found in plant walls. In some embodiments, a xylanase find use as a secondary enzyme in the methods of the invention. Any suitable xylanase can be used in the invention. Xylanases (e.g. endo- β -xylanases (E.C. 3.2.1.8), which hydrolyze the xylan backbone chain, can be from bacterial sources (e.g., *Bacillus*, *Streptomyces*, *Clostridium*, *Acidothermus*, *Microtetraspora* or *Thermonospora*) or from fungal sources (*Aspergillus*, *Trichoderma*, *Neurospora*, *Humicola*, *Penicillium* or *Fusarium* (See, e.g., EP473 545; U.S. Pat. No. 5,612,055; WO 92/06209; and WO 97/20920)). Xylanases useful in the invention include commercial preparations (e.g., MULTIFECT[®] and FEEDTREAT[®] Y5 (Danisco Genencor), RONOZYME[®] WX (Novozymes A/S) and NATUGRAIN WHEAT[®] (BASF)). In some embodiments the xylanase is from *Trichoderma reesei* or a variant xylanase from *Trichoderma reesei*, or the inherently thermostable xylanase described in EP1222256B1, as well as other xylanases from *Aspergillus niger*, *Aspergillus kawachii*, *Aspergillus tubigensis*, *Bacillus circulans*, *Bacillus pumilus*, *Bacillus subtilis*, *Neocallimastix patriciarum*, *Penicillium species*, *Streptomyces lividans*, *Streptomyces thermoviolaceus*, *Thermomonospora fusca*, *Trichoderma harzianum*, *Trichoderma reesei*, and *Trichoderma viridae*.

[0093] Phytases that can be used include those enzymes capable of liberating at least one inorganic phosphate from inositol hexaphosphate. Phytases are grouped according to their preference for a specific position of the phosphate ester group on the phytate molecule at which hydrolysis is initiated, (e.g., as 3-phytases (EC 3.1.3.8) or as 6-phytases (EC 3.1.3.26)). A typical example of phytase is myo-inositol-hexakiphosphate-3-phosphohydrolase. Phytases can be obtained from microorganisms such as fungal and bacterial organisms (e.g. *Aspergillus* (e.g., *A. niger*, *A. terreus*, and *A. fumigatus*), *Myceliophthora* (*M. thermophila*), *Talaromyces* (*T. thermophilus*) *Trichoderma* spp (*T. reesei*). And *Thermomyces* (See e.g.,

WO 99/49740)). Also phytases are available from *Penicillium* species, (e.g., *P. hordei* (See e.g., ATCC No. 22053), *P. piceum* (See e.g., ATCC No. 10519), or *P. brevis-compactum* (See e.g., ATCC No. 48944) (See, e.g. U.S. Pat. No. 6,475,762). Additional phytases that find use in the invention are available from *Peniophora*, *E. coli*, *Citrobacter*, *Enterbacter* and *Buttiauxella* (see e.g., WO2006/043178, filed Oct. 17, 2005). Additional phytases useful in the invention can be obtained commercially (e.g. NATUPHOS[®] (BASF), RONOZYME[®] P (Novozymes A/S), PHZYME[®] (Danisco A/S, Diversa) and FINASE[®] (AB Enzymes).

[0094] Various acid fungal proteases (AFP) can be used as part of the combination as well. Acid fungal proteases include for example, those obtained from *Aspergillus*, *Trichoderma*, *Mucor* and *Rhizopus*, such as *A. niger*, *A. awamori*, *A. oryzae* and *M. miehei*. AFP can be derived from heterologous or endogenous protein expression of bacteria, plants and fungi sources. IAFP secreted from strains of *Trichoderma* can be used. Suitable AFP includes naturally occurring wild-type AFP as well as variant and genetically engineered mutant AFP. Some commercial AFP enzymes useful in the invention include FERMGEN[®] (Danisco US, Inc, Genencor Division), and FORMASE[®] 200.

[0095] Proteases can also be used with glucoamylase and any other enzyme combination. Any suitable protease can be used. Proteases can be derived from bacterial or fungal sources. Sources of bacterial proteases include proteases from *Bacillus* (e.g., *B. amyloliquefaciens*, *B. lentus*, *B. licheniformis*, and *B. subtilis*). Exemplary proteases include, but are not limited to, subtilisin such as a subtilisin obtainable from *B. amyloliquefaciens* and mutants thereof (U.S. Pat. No. 4,760,025). Suitable commercial protease includes MULTIFECT[®] P 3000 (Danisco Genencor) and SUMIZYME[®] FP (Shin Nihon). Sources of suitable fungal proteases include, but are not limited to, *Trichoderma*, *Aspergillus*, *Humicola* and *Penicillium*, for example.

[0096] Debranching enzymes, such as an isoamylase (EC 3.2.1.68) or pullulanase (EC 3.2.1.41), can also be used in combination with the glucoamylases in the saccharification and/or fermentation or SSF processes of the invention. A non-limiting example of a pullulanase that can be used is Promozyme[®].

3. Starch Processing

3.1. Starch Substrates and Raw Materials

[0097] Those of skill in the art are well aware of available methods that may be used to prepare starch substrates for use in the processes disclosed herein. For example, a useful starch substrate may be obtained from tubers, roots, stems, legumes, cereals, or whole grain. More specifically, the granular starch comes from plants that produce high amounts of starch. For example, granular starch may be obtained from corn, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, bean, banana, or potatoes. Corn contains about 60-68% starch; barley contains about 55-65% starch; millet contains about 75-80% starch; wheat contains about 60-65% starch; and polished rice contains about 70-72% starch. Specifically contemplated starch substrates are cornstarch, wheat starch, and barley starch. The starch from a grain may be ground or whole and includes corn solids, such as kernels, bran and/or cobs. The starch may be highly refined raw starch or feedstock from starch refinery processes. Various starches also are commercially available. For example, cornstarch may be available from Cerestar, Sigma, and Katayama Chemical Industry Co. (Japan); wheat starch may be available from Sigma; sweet potato starch may be available from Wako Pure Chemical Industry Co. (Japan); and potato starch may be available from Nakaari Chemical Pharmaceutical Co. (Japan).

3.2. Milling

[0098] The starch substrate can be a crude starch from milled whole grain, which contains non-starch fractions, *e.g.*, germ residues and fibers. Milling may comprise either wet milling or dry milling. In wet milling, whole grain can be soaked in water or dilute acid to separate the grain into its component parts, *e.g.*, starch, protein, germ, oil, kernel fibers. Wet milling efficiently separates the germ and meal (*i.e.*, starch granules and protein) and can be especially suitable for production of syrups. In dry milling, whole kernels are ground into a fine powder and processed without fractionating the grain into its component parts. Dry milled grain thus will comprise significant amounts of non-starch carbohydrate compounds, in addition to starch. Most ethanol comes from dry milling. Alternatively, the starch to be processed may be a highly refined starch quality, for example, at least about 90%, at least about 95%, at least about 97%, or at least about 99.5% pure.

3.3. Gelatinization and Liquefaction

[0099] In some embodiments of the invention, gelatinization and/or liquefaction may be used. As used herein, the term “liquefaction” or “liquefy” means a process by which starch is converted to less viscous and soluble shorter chain dextrans. In some embodiments, this process involves gelatinization of starch simultaneously with or followed by the addition of alpha-amylases. Additional liquefaction-inducing enzymes, *e.g.*, a phytase, optionally may be added. In some embodiments, gelatinization is not used. In other embodiments, a separate liquefaction step is not used. Starches can be converted to shorter chains at the same time that saccharification and/or fermentation is performed. In some embodiments, the starch is being converted directly to glucose. In other embodiments, a separate liquefaction step is used prior to saccharification.

[0100] In some embodiments, the starch substrate prepared as described above may be slurried with water. The starch slurry may contain starch as a weight percent of dry solids of about 10-55%, about 20-45%, about 30-45%, about 30-40%, or about 30-35%. In some embodiments, the starch slurry is at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, or at least about 55%.

[0101] To optimize alpha-amylase stability and activity, the pH of the slurry may be adjusted to the optimal pH for the alpha-amylases. Alpha-amylases remaining in the slurry following liquefaction may be deactivated by lowering pH in a subsequent reaction step or by removing calcium from the slurry. The pH of the slurry should be adjusted to a neutral pH (*e.g.*, pH 5.0 to 8.0 and any pH in between this range) when the glucoamylases of the invention are used.

[0102] The slurry of starch plus the alpha-amylases may be pumped continuously through a jet cooker, which may be steam heated from about 85°C to up to about 105°C. Gelatinization occurs very rapidly under these conditions, and the enzymatic activity, combined with the significant shear forces, begins the hydrolysis of the starch substrate. The residence time in the jet cooker can be very brief. The partly gelatinized starch may be passed into a series of holding tubes maintained at about 85-105°C and held for about 5 min. to complete the gelatinization process. These tanks may contain baffles to discourage back mixing. As used herein, the term “secondary liquefaction” refers the liquefaction step subsequent to primary

liquefaction, when the slurry is allowed to cool to room temperature. This cooling step can be about 30 minutes to about 180 minutes, *e.g.*, about 90 minutes to 120 minutes. Milled and liquefied grain is also known as mash.

3.4. Saccharification

[0103] Following liquefaction, the mash can be further hydrolyzed through saccharification to produce fermentable sugars that can be readily used in the downstream applications. The saccharification of the present embodiments can be carried out at a pH in the range of 5.0 to 8.0, 5.5 to 7.5, 6.0 to 7.5, 6.5 to 7.5, or 7.0 to 7.5, by using a glucoamylase as described above. In other embodiments, the pH used can be 5.0, 5.25, 5.50, 5.75, 6.0, 6.50, 7.0, 7.50 or 8.0.

[0104] In one embodiment, at pH 6.0 or higher, the glucoamylase possesses at least about 50%, about 51%, about 52%, about 53%, about 54%, or about 55% activity relative to its maximum activity at the optimum pH. In another embodiment, for a pH range of 6.0 to 7.5, HgGA can have at least 53% activity relative to its maximum activity. In another embodiment, at pH 6.0, TrGA can have at least 50% activity relative to its maximum activity. In one embodiment, a glucoamylase (*e.g.* HgGA) has 67% maximal activity at pH 7.0. In another embodiment, a glucoamylase (*e.g.*, TrGA) has 66% maximal activity at pH 5.25.

[0105] In one embodiment, the glucoamylase may be dosed at the range of about 0.2 to 2.0 GAU /g dss, about 0.5 to 1.5 GAU /g dss, or 1.0 to 1.5 GAU /g dss. In one embodiment, glucoamylase (*e.g.*, TrGA) can be used at a dose of about 1 to 5 GAU/ gds starch. In another embodiment, glucoamylase (*e.g.*, TrGA) can be used at a dose of about 1 GAU/ gds starch, 2 GAU/ gds starch, 3 GAU/ gds starch, 4 GAU/ gds starch, or 5 GAU/ gds starch. In one embodiment, glucoamylase (*e.g.*, HgGA) can be used at a dose of about 0.25 to 1 GAU/ gds starch. In another embodiment, glucoamylase (*e.g.*, HgGA) can be used at a dose of about 0.25 GAU/ gds starch, 0.5 GAU/ gds starch, 0.75 GAU/ gds starch, or 1 GAU/ gds starch. The saccharification may be performed at about 30 to about 60°C, or about 40 to about 60°C. In some embodiments, the saccharification occurs at pH 7.0 at 32°C. In other embodiments, the saccharification occurs at pH 6.5 at 58°C.

[0106] A full saccharification step may typically range 24 to 96 hours, 24 to 72 hours, or 24 to 48 hours. In some embodiments, saccharification occurs after about 2, 4, 6, 7.7, 8, 110, 14, 16, 18, 20, 22, 23.5, 24, 26, 28, 30, 31.5, 34, 36, 38, 40, 42, 44, 46, or 48 hours. In some

embodiments, the saccharification step and fermentation step are combined and the process is referred to as simultaneous saccharification and fermentation (SSF).

[0107] It is understood that generally, as time elapses, the enzymes (glucoamylase with or without other enzymes, such as alpha-amylases or non-starch polysaccharide hydrolyzing enzyme) reduces the higher sugars to lower DP sugars (such as DP1). The sugar profile can be varied by using different parameters, such as, but not limited to, starting starch substrate, temperature, amount of glucoamylase, type of glucoamylase, and pH. For example, in one embodiment, at 32 degrees Celsius and pH 7.0, the sugar or oligosaccharide distribution during the saccharification process can be between about 0.36% to about 96.50% DP1, about 3.59% to about 11.80% DP2, about 0.12% to about 7.75%, and/or about 2.26% to about 88.30% for higher sugars for HgGA. In another embodiment, at 32 degrees Celsius and pH 7.0, the sugar distribution during the saccharification process can be between about 0.36% to about 79.19% DP1, between about 3.59% to about 9.92% DP2, about 0.17% to about 9.10% DP3 and/or about 17.15% to about 88.30% for higher sugars for TrGA. Thus, in one embodiment, using HgGA, the DP1 content can reach more than 90% after 24 hours. After 45 hours, the DP1 content can reach more than 96%, while the content of higher sugars can decrease to less than 3%. Using TrGA, more than 70% DP1 can be obtained after 24 hours. After 45 hours, the DP1 content can reach about 80%, while the content of higher sugars can drop to less than 20%.

[0108] In another embodiment, at 58 degrees Celsius and pH 6.5, the sugar distribution during the saccharification process can be between about 60.66% to about 93.67% DP1, between about 1.49% to about 8.87% DP2, about 0.33% to about 1.93% DP3 and/or about 4.51% to about 28.17% for higher sugars for HgGA. In other embodiments, at 58 degrees Celsius and pH 6.5, the sugar or oligosaccharide distribution during the saccharification process can be between about 37.08% to about 75.25% DP1, about 5.48% to about 10.19% DP2, about 0.46% to about 5.06%, and/or about 18.37% to about 47.47% for higher sugars for TrGA. Thus, in one embodiment, using HgGA, the DP1 content can reach more than 90% after 24 hours. After 48 hours, the DP1 content can reach more than 93%, while the content of higher sugars can decrease to less than 5%. Using TrGA, more than 70% DP1 can be obtained after 24 hours. After 45 hours, the DP1 content can reach about 75%, while the content of higher sugars can drop to about 18%.

[0109] In yet another embodiment, at 58 degrees Celsius and pH 6.5, glucoamylases disclosed herein can be used to saccharify a starch substrate where high sugars (e.g., DP4+) is reduced. In some embodiments, the sugar or oligosaccharide distribution during the saccharification process can be between about 81.10% to about 90.36% DP1, about 1.99% to about 3.96% DP2, about 0.49% to about 0.61% DP3, about 4.48% to about 16.13% DP4+ for TrGA. In other embodiments, the sugar or oligosaccharide distribution during the saccharification process can be between about 93.15% to about 95.33% DP1, about 2.10% to about 3.94% DP2, about 0.53% to about 1.00% DP3, about 0.94% to about 3.76% DP4+ for HgGA.

[0110] In yet another embodiment, at 58 degrees Celsius and pH 6.4, the sugar or oligosaccharide distribution during the saccharification process can be between about 93.79% to about 96.9% DP1, about 1.55% to about 3.02% DP2, about 0.2% to about 0.49% DP3 and about 0% to about 3.98% DP4+ for HgGA. In some cases, about 93% solubility and about 96.9% glucose yield can be achieved within 24 hours. Continuous saccharification can result in 99% solubility and about 96.8% glucose after about 48 hours.

[0111] In another embodiment, at 58 degrees Celsius and pH 6.4, the sugar or oligosaccharide distribution during the saccharification process can be between about 75.08% to about 96.5% DP1, 1.57% to about 9.16% DP2, 0.67% to about 15.76% DP3+. In some cases, HgGA can maintain a significant amount of glucoamylase activity for about 52 hours at pH6.4 to yield continued production of DP1 products, DP2 products, and increase of percentage of soluble solids. Increased amounts of HgGA can result in increased rates of percentage solubilization and DP1 production.

3.5. Fermentation

[0112] In some embodiments of the present disclosure, the fermentable sugars may be subject to 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 may be inoculated with the desired organism(s). In this method, fermentation can be 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 the end product.

[0113] A variation on the standard batch system is the “fed-batch fermentation” system, which may be used in some embodiments of the present disclosure. In this variation of a typical batch system, the substrate can be added in increments as the fermentation progresses. Fed-batch systems are particularly 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 may be 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. Both batch and fed-batch fermentations are common and well known in the art.

[0114] On the other hand, continuous fermentation is an open system where a defined fermentation medium can be added continuously to a bioreactor and an equal amount of conditioned medium can be 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 can be maintained at a fixed rate while 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, may be 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.

[0115] In further embodiments, by use of appropriate fermenting microorganisms as known in the art, the fermentation end product may include without limitation alcohol, 1,3-propanediol, succinic acid, lactic acid, amino acids, proteins, functional oligosaccharides, and derivatives thereof. *See e.g.*, WO 2008/086811 (methanol, ethanol, propanol, and butanol fermentation); WO 2003/066816, U.S. Patent Nos. 5,254,467 and 6,303,352 (1,3-propanediol fermentation); U.S. Patent Nos. RE 37,393, 6,265,190, and 6,596,521 (succinic acid fermentation); U.S. Patent No. 5,464,760, WO 2003/095659, Mercier et al., *J. Chem. Tech. Biotechnol.* 55: 111-121, Zhang and Cheryan, *Biotechnol. Lett.* 13: 733-738 (1991), Linko and Javanainen, *Enzyme Microb. Technol.* 19: 118-123 (1996), and Tsai and Moon, *Appl. Biochem. Biotechnol.* 70-72: 417-428 (1998) (lactic acid fermentation); U.S. Patent Nos. 7,320,882, 7,332,309, 7,666,634, and Zhang et al., *Appl. Microbiol. Biotechnol.* 77: 355-366 (2007) (fermentation of various amino acids). The above enumerated list are only examples and one skilled in the art will be aware of a number of fermenting microorganisms that may be appropriately used to obtain a desired end product.

3.6. Simultaneous saccharification and fermentation (SSF)

[0116] During SSF, the hydrolyzing enzymes are added along with the end product producer, commonly a microorganism. Enzymes release fermentable lower molecular weight sugars, *i.e.*, fermentable sugars DP1-3, from the starch substrate, while the microorganism simultaneously uses the fermentable sugars for growth and production of the end product. Typically, fermentation conditions are selected that provide an optimal pH and temperature for promoting the best growth kinetics of the producer host cell strain and catalytic conditions for the enzymes produced by the culture. *See e.g.*, Doran et al., *Biotechnol. Progress* 9: 533-538 (1993). Table 1 presents exemplary fermentation microorganism and their optimal pH for fermentation. Because the glucoamylases disclosed herein possess significant activity at a neutral pH and an elevated temperature, they would be useful in the SSF for those microorganisms having an optimal fermenting pH in the range of 5.5 to 7.5.

Table 1. Exemplary fermentation organisms and their optimal pH.

End products	Fermentation Organisms	Optimal pH of the fermentation
Lysine and salts thereof	<i>Corynebacterium glutamicum</i>	6.8-7.0
	<i>Bacillus lacterosprous</i>	7.0-7.2
	<i>Methylophilotrophus</i>	7
Lactic Acid	<i>Lactobacillus amylophilus</i>	6.0-6.5
	<i>Bacillus coagulans</i>	6.4-6.6
	<i>Bacillus thermoamylovorans</i>	5.0-6.5
	<i>Bacillus smithii</i>	5.0-6.5
	<i>Geobacillus stearothermophilus</i>	5.0-6.5
Monosodium Glutamate (MSG)	<i>Corynebacterium pekinense</i>	7
	<i>Corynebacterium crenatum</i>	7
	<i>Brevibacterium tianjinense</i>	7
	<i>Corynebacterium glutamicum</i> HU7251	7.0-7.2
	<i>Arthrobacter sp</i>	7
Succinic acid	<i>Escherichia coli</i>	6.0-7.5
1,3-Propanediol	<i>Escherichia coli</i>	6.5-7.5
2-Keto-gulonic acid	<i>Escherichia coli</i>	5.0-6.0

[0117] In further embodiments, by use of appropriate fermenting microorganisms as known in the art to produce the desired end product, those of skill in the art are well capable of adjusting the SSF conditions, *e.g.*, temperature, nutrient composition, light conditions, oxygen availability, etc.

4. Methods used in the Examples

[0118] The following materials, assays, and methods are used in the examples provided below:

HPLC method to measure saccharide composition

[0119] The composition of the reaction products of oligosaccharides was measured by a HPLC system (Beckman System Gold 32 Karat Fullerton, CA). The system, maintained at 50°C, was equipped with a Rezex 8 u8% H Monosaccharides column and a refractive index (RI) detector (ERC-7515A, Anspec Company, Inc.). Diluted sulfuric acid (0.01 N) was applied as the mobile phase at a flow rate of 0.6 ml/min. 20 µl of 4.0% solution of the reaction mixture was injected onto the column. The column separates saccharides based on their molecular weights. The distribution of saccharides and the amount of each saccharide were determined from previously run standards.

Determination of glucoamylase activity units (GAU)

[0120] Glucoamylase activity units (GAU) were determined based on the activity of a glucoamylase enzyme to catalyze the hydrolysis of *p*-nitrophenyl- α -D-glucopyranoside (PNPG) to glucose and *p*-nitrophenol. At an alkaline pH, *p*-nitrophenol forms a yellow color that is measured spectrophotometrically at 405 nm. The amount of *p*-nitrophenol released correlates with the glucoamylase activity.

Protein concentration determination

[0121] The protein concentration in a sample was determined using the Bradford QuickStart™ Dye Reagent (Bio-Rad, California, USA). For example, a 10 µL sample of the enzyme was combined with 200 µL Bradford QuickStart™ Dye Reagent. After thorough mixing, the reaction mixture was incubated for at least 10 minutes at room temperature. Air bubbles were removed and the optical density (OD) was measured at 595 nm. The protein concentration was then calculated using a standard curve generated from known amounts of bovine serum albumin.

Purification of HgGA for characterization studies

[0122] The material concentrated by ultrafiltration (UFC) was desalted / buffer-exchanged using a BioRad DP-10 desalting column and 25 mM Tris pH 8.0. 100 mg of total protein was applied to a Pharmacia Hi Prep 16/10 S Sepharose FF column, which was equilibrated with the above buffer at 5 ml/min. Glucoamylase was eluted with a 4-column volume (CV)

gradient buffer containing 0-200 mM NaCl. Multiple runs were performed and the purest fractions, as determined via SDS-PAGE/coomassie blue staining analysis, were pooled and concentrated using VivaSpin 10K MWCO 25 ml spin tubes. The final material was passed over a Novagen HisBind 900 chromatography cartridge that had been washed with 250 mM EDTA and rinsed with above buffer. 2 ml of final material was obtained, having a protein concentration of 103.6 mg/ml, and a glucoamylase activity of 166.1 GAU/ml (determined by a PNPG based assay). Specific activities were determined using a standardized method using *p*-nitrophenyl-alpha-D-glucopyranoside (PNPG) as a substrate and reported in GAU units.

Determination of glucose concentration

[0123] Glucose concentration in a saccharification reaction mixture was determined with the ABTS assay. Samples or glucose standards in 5 μ L were placed in wells of a 96-well microtiter plate (MTP). Reactions were initiated with the addition of 95 μ L of the reactant containing 2.74 mg/ml 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma P1888), 0.1 U/ml horseradish peroxidase type VI (Sigma P8375), and 1 U/ml glucose oxidase (Sigma G7141). OD_{405 nm} was immediately monitored at a 9-second interval for 300 seconds using a Spectramax plate reader. Because the rate of OD_{405nm} increase is proportional to the glucose concentration, the sample's glucose concentration was determined by comparing with the glucose standard, and was reported as mg/ml.

Lactic acid fermentation conditions

[0124] Strains of *Lactobacillus rhamnosus* and *Bacillus coagulans* were obtained from China General Microbiological Culture Collection Center.

[0125] Seed media (MRS 006): Casein 10.0 g, Beef extract 10.0 g, Yeast extract 5.0 g, Glucose 5.0 g, Sodium acetate 5.0 g, diammonium citrate 2.0 g, Tween® 80 1.0 g, K₂HPO₄ 2.0 g, MgSO₄·7H₂O 0.2 g, MnSO₄·H₂O 0.05 g, Distilled water 1.0 L, pH 6.8; additional 20 g agar were added for inoculum media.

[0126] Fermentation medium: corn steep liquor 40 g, casein 10.0 g, Beef extract 10.0 g, Yeast extract 10.0 g, cassava starch 150 g, Tween® 80 1.5 g, MnSO₄·H₂O 0.3 g, Calcium

carbonate 20 g, Distilled water 1.0 L, pH 6.5. Starch (cassava starch or cornstarch) or glucose was added based on different test conditions.

[0127] Starch (cassava starch, cornstarch, or wheat starch), glucose, agar, corn steep liquor, casein, Beef extract, Yeast extract, Glucose, Sodium acetate, diammonium hydrogen citrate, Tween® 80, K₂HPO₄, MgSO₄·7H₂O, MnSO₄·H₂O, CaCO₃, and Agar powder were all of analytic grade and procured locally.

[0128] The inoculum of *Lactobacillus rhamnosus* was transferred to each 100 mL seed culture and cultivated at 37°C, 200 rpm, for 12 to 24 hrs, until OD₆₀₀ reached about 0.5. 10-20 mL seed culture was added to each fermentor with 1 L fermentation medium.

EXAMPLES

Example 1: Comparison of the pH and activity profiles of various glucoamylases at 32°C

[0129] The pH and activity profiles of glucoamylases (GAs) from *Humicola grisea* (HgGA), *Trichoderma reesei* (TrGA), *Aspergillus niger* (AnGA) and *Talaromyces emersonii* (TeGA) were determined at 32°C. As the substrate, 8% potato starch (Sigma Cat. No. S2630) was solubilized by heating. A series of citrate/phosphate buffers at 0.25 or 0.5 pH increments, ranging from pH 2.0 to 8.0, were prepared. Purified enzymes were diluted to 0.1 or 0.02 GAU/ml in water (TeGA was dosed at 0.2 GAU/ml). HgGA, TrGA, AnGA, and TeGA were dosed at 0.0125, 0.0076, 0.0109, and 0.0055 mg/ml, respectively. 10 µL buffer of various pH was placed in 0.2 ml PCR tube strips (AB Gene, Cat. No. AB-0451, 800-445-2812) with 15 µL of diluted enzyme. The reactions were initiated by the addition of 25 µL soluble potato starch. The reactions were incubated on a PCR type thermocycler heating block for exactly ten minutes, then terminated by the addition of 10 µL 0.5 M NaOH. The glucose released in the reaction was determined using the ABTS assay, and the glucoamylase activities were determined. The pH and activity profiles are presented in Table and FIG. 1 as the percentage of the maximum activity for each glucoamylase.

Table 2. pH profiles of HgGA, TrGA, AnGA, and TeGA at 32°C. The values represent % of the maximum activity for each enzyme.

pH	HgGA	TrGA	AnGA	TeGA
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pH	HgGA	TrGA	AnGA	TeGA
2.00	45	56	91	93
2.50	54	67	91	97
2.75	60	72		100
3.00	63	81	98	98
3.25	71	91	100	95
3.50	77	99	99	88
3.75	84	100	96	79
4.00	93		84	64
4.25	100	95	78	51
4.50	84		55	34
4.75	44		46	30
5.00	40		45	29
5.25	42	66	43	27
5.50	46		41	23
5.75	48	58	39	21
6.00	53	51	35	17
6.50	62	38	27	11
7.00	67	22	17	5
7.50	58	10	7	2
8.00	39	4	3	1

[0130] As shown in Table 2 and FIG. 1, both TeGA and AnGA exhibited significantly reduced activity in the pH range of 6.0 to 8.0. At a pH 5.0 or above, TeGA retained no more than 29% activity relative to its maximum activity. At a pH 6.0 or above, TeGA retained no more than 17% activity relative to its maximum activity. Similarly, at a pH 6.0 or above, AnGA displayed no more than 35% activity relative to its maximum activity. In the pH range of 6.0 to 7.5, HgGA retained at least 53% activity relative to its maximum activity. At pH 6.0, TrGA also displayed at least 50% activity relative to its maximum activity. The above observation suggests that both HgGA and TrGA may be suitable for producing fermentable sugars at a neutral pH range (as described herein for neutral pH glucoamylases) under fermentation conditions.

Example 2: Comparison of hydrolysis of solubilized starch at 32°C, pH 7.0

[0131] The ability of various glucoamylases to hydrolyze solubilized starch substrate (liquefact) at a neutral pH was compared. Corn starch was liquefied by following a conventional high-temperature jet cooking process using CLEARFLOW™ AA to a liquefact of DE 12-15. Saccharification of the liquefact (25% DS) was carried out using TrGA, HgGA, and AnGA at 1.0 GAU/g ds at 32°C, pH 7.0. Samples were withdrawn at different time intervals during the saccharification and subject to HPLC analysis. The composition of the oligosaccharides is presented in Table 3.

Table 3. Composition of oligosaccharides in saccharification.

GA	Time (hr)	% Sugars, pH 7.0, 32 °C			
		DP1	DP2	DP3	Higher Sugars
HgGA	0	0.36	3.59	7.75	88.30
	2	51.10	10.20	6.87	31.85
	5.25	64.90	11.80	0.13	23.13
	21.25	89.30	1.10	0.30	9.34
	25.25	91.20	0.98	0.23	7.61
	29.25	92.60	0.90	0.31	6.12
	45.25	96.50	1.15	0.12	2.26
TrGA	0	0.36	3.59	7.75	88.30
	2	38.06	7.49	9.10	45.35
	5.25	47.17	9.92	6.13	36.78
	21.25	69.43	8.33	0.17	22.07
	25.25	71.69	7.14	0.17	21.01
	29.25	73.57	6.16	0.18	20.09
	45.25	79.19	3.45	0.20	17.15
AnGA	0	0.36	3.59	7.75	88.30
	2	14.12	4.57	8.88	72.43
	5.25	28.38	8.01	10.30	53.31
	21.25	58.97	11.49	0.28	29.26
	25.25	60.94	10.53	0.28	28.25
	29.25	62.82	9.54	0.23	27.41
	45.25	74.14	4.08	0.24	21.54

[0132] Using HgGA, the DP1 content reached more than 90% after 24 hrs. After 45 hours, the DP1 content reached more than 96%, while the content of higher sugars decreased to less than 3%. Using TrGA, more than 70% DP1 was obtained after 24 hours. After 45 hours, the

DP1 content reaches about 80%, while the content of higher sugars dropped to less than 20%. For AnGA, less than 75% of DP1 was obtained after 45 hours, while higher sugars remained more than 20%. The data in Table 3 indicate that both HgGA and TrGA are more effective than AnGA to hydrolyze solubilized starch to glucose, at a neutral pH.

Example 3: Comparison of hydrolysis of liquefied starch at 58°C, pH 6.5

[0133] Corn starch liquefact (~9.1DE) obtained by SPEZYME[®] FRED (Danisco US Inc., Genencor Division) treatment was adjusted to pH 6.5 with NaOH and equilibrated at a 58°C water bath. AnGA (OPTIDEX[™] L-400, Danisco US Inc., Genencor Division), TrGA, and HgGA were added at 0.5 GAU/g ds to each flask containing corn starch liquefact. Saccharification was carried out up to 48 hours with periodical sampling for HPLC analysis. 0.5 mL enzyme-deactivated sample was diluted with 4.5 ml of RO water. The diluted sample was then filtered through 0.45 µm Whatman filters and subject to HPLC analysis. The HPLC analysis was conducted as described in Methods used in the Examples. The composition of the oligosaccharides is presented in Table 4.

Table 4. Composition of oligosaccharides in saccharification.

	Hour	Percent Sugar Composition			
		%DP1	%DP2	%DP3	%HS
Liquefact	0	0.49	3.02	5.52	90.98
HgGA	2	60.66	8.87	1.93	28.17
	4	69.92	7.43	0.69	21.75
	6	75.96	5.80	0.38	17.85
	7.7	77.56	5.15	0.47	16.35
	14	84.31	2.96	0.42	11.57
	23.5	88.70	2.20	0.43	8.67
	31.5	90.01	1.87	0.40	6.90
	48	93.67	1.49	0.33	4.51
TrGA	2	37.08	10.19	5.06	47.47
	4	49.25	12.12	2.12	36.42
	6	55.30	12.16	1.09	31.10
	7.7	58.06	11.74	0.76	29.12
	14	63.83	9.96	0.46	25.28
	23.5	68.52	8.18	0.53	22.77
	31.5	70.35	7.24	0.54	21.32
	48	75.25	5.48	0.50	18.37
AnGA	2	41.33	11.83	4.40	42.20
	4	50.08	12.95	1.60	35.04
	6	53.32	12.70	0.83	33.16
	7.7	54.80	12.41	0.62	31.91
	14	58.85	11.20	0.40	29.15
	23.5	61.70	10.44	0.46	27.41
	31.5	62.34	10.11	0.50	26.58
	48	64.23	9.83	0.59	25.01

[0134] Using HgGA, the DP1 content reached more than 90% after 24 hrs. After 48 hours, the DP1 content reached more than 93%, while the content of higher sugars decreased to less than 5%. Using TrGA, more than 70% DP1 was obtained after 24 hours. After 45 hours, the DP1 content reaches about 75%, while the content of higher sugars dropped to about 18%.

For AnGA, less than 65% of DP1 was obtained after 45 hours, while higher sugars remained more than 25%. The data in Table 4 indicate that both HgGA and TrGA are more effective than AnGA, at a neutral pH and 58°C, to hydrolyze solubilized starch to glucose. This observation is consistent with data presented in Table 3, where saccharification was performed at 32°C.

Example 4: Comparison of high sugars (DP4+) reduction at 58°C, pH 6.5

[0135] Various concentrations of AnGA, TrGA, and HgGA were used to saccharify a starch substrate at 58°C, pH 6.5, and the reduction of high sugars (DP4+) was compared. The starch substrate was a 25% cornstarch liquefact, which was liquefied by SPEZYME® FRED (Danisco US Inc., Genencor Division). Glucoamylases were added as shown in Table 5, from 0.25 GAU/gds to 10.0 GAU/gds. The saccharification reaction was conducted at 58°C, pH 6.5. Samples were withdrawn at various time points and the sugar composition was determined by HPLC analysis. The composition of the oligosaccharides is presented in Table 5 and FIG. 2.

Table 5. Composition of oligosaccharides in saccharification.

Glucoamylase	GAU/gds starch	Percent Sugar Composition at 48 hr			
		DP1	DP2	DP3	DP4+
AnGA	1	64.25	5.10	0.00	30.65
	2.5	73.36	1.74	0.41	24.49
	5	81.26	1.05	0.46	17.22
	7.5	85.53	1.48	0.44	12.13
	10	89.32	2.03	0.42	8.22
TrGA	1	81.10	2.28	0.49	16.13
	2	86.65	1.99	0.49	10.87
	3	90.36	2.86	0.49	8.30
	4	90.48	3.17	0.52	5.83
	5	90.95	3.96	0.61	4.48
HgGA	0.25	93.15	2.10	1.00	3.76
	0.5	95.33	2.58	0.64	1.45

Glucoamylase	GAU/gds starch	Percent Sugar Composition at 48 hr			
		DP1	DP2	DP3	DP4+
	0.75	95.08	3.36	0.53	1.02
	1	94.57	3.94	0.56	0.94

[0136] The results presented in Table 5 and FIG. 2 indicated that AnGA resulted in more than 8% of higher sugars (DP4+), at 58°C, pH 6.5, even at a high dosage of glucoamylase, 10.0 GAU/gds. In contrast, lower than 5% of higher sugars (DP4+) was observed for 5 GAU/gds TrGA. HgGA resulted in the lowest levels of higher sugars (DP4+). For example, at 0.5 GAU/gds HgGA, the saccharification mixture contained less than 1.5% of higher sugars (DP4+), which is comparable to the resulted obtained under the current industrial high glucose processing conditions (pH 4.5, 60°C) using AnGA.

Example 5: Continuous production of glucose from granular Cassava starch by HgGA at a neutral pH

[0137] The capability of HgGA to convert granular unmodified cassava starch to glucose and short chain glucose polymers at a neutral pH was further characterized. A 27 % dry substance aqueous slurry of cassava starch was first adjusted to pH 6.4 with sodium carbonate. SPEZYME™ Alpha (Danisco US Inc., Genencor Division) was added at 2 AAU/g ds, and HgGA was added at 1 GAU/g ds. The reaction was carried out for 48 hours at 58 °C with continuous stirring. At selected time intervals, samples of the slurry were removed. The removed sample was added to a 2.5 ml micro-centrifuge tube and centrifuged for 4 minutes at 13,000 rpm. Refractive index (RI) of the supernatant was determined at 30°C. The remaining supernatant was filtered through a 13 mm syringe filter with a 0.45 µm GHP membrane into a 2.5 ml micro-centrifuge tube and boiled for 10 minutes to terminate the amylase activity. 0.5 mL enzyme-deactivated sample was diluted with 4.5 ml of RO water. The diluted sample was then filtered through 0.45 µm Whatman filters and subject to HPLC analysis. The HPLC analysis was conducted as described in Methods used in the Examples.

[0138] The total dry substance was determined by taking about 1 ml of the starch slurry into a 2.5 ml spin tube, adding 1 drop of SPEZYME® FRED (Danisco US Inc., Genencor

Division) from a micro dispo-pipette, and boiling 10 minutes. Refractive index at 30°C was determined. The dry substance of the supernatant and the whole sample (total) was determined using appropriate DE tables. The CRA 95 DE Table was used for the supernatant and corrected for consumption of water of hydrolysis. % soluble was calculated as: $100 \times$ (the dry substance of the supernatant) / (the total dry substance). The composition of the oligosaccharides is presented in Table 6.

Table 6 Saccharide distribution for HgGA-mediated saccharification of cassava granular starch.

hrs	Saccharide Distribution				Soluble %
	DP1	DP2	DP3	DP4+	
2.50	93.799	1.726	0.499	3.976	56.20
7.50	96.166	1.551	0.480	1.802	78.80
12.00	96.731	1.639	0.411	1.220	85.10
23.50	96.928	2.204	0.326	0.541	92.80
48.00	96.772	3.023	0.205	0.000	99.00

[0139] As shown in Table 6, the reaction achieved about 93% solubility and yielded about 96.9% glucose within 24 hours. Continuation of saccharification resulted in 99% solubility and about 96.8% glucose after 48 hours.

Example 6: Continuous production of glucose from granular cornstarch by HgGA at a neutral pH

[0140] Corn granular starch was used to characterize HgGA. The experiments were carried out using 32% ds corn granular starch. Water (64.44 g) and starch (35.56 g; at 90% ds) were mixed and the pH of the slurry was increased to 6.4. The starch slurry was placed in a water bath maintained at 58°C and enzymes were added. The enzymes included SPEZYME™ Alpha (Danisco US Inc., Genencor Division) and HgGA. The starch slurry was maintained at 58°C for 48 hrs and samples were drawn at 3, 6, 10, 24, 32, and 52 hrs to analyze the % soluble and saccharide profile. The results are presented in Table 7.

Table 7. Saccharide distribution for HgGA-mediated saccharification of corn granular starch

HgGA (GAU/g ds)	Alpha-amylase (AAU/g ds)	hour	% Soluble	DP1	DP2	DP3+
1	2	3	56.82	94.74	1.57	3.69
		6	69.45	95.52	1.76	2.61
		10	75.96	96.50	1.79	1.43
		24	91.50	95.72	2.79	0.93
		32	92.71	95.50	3.08	0.86
		52	99.66	93.94	4.42	0.67
0.75	2	3	53.35	92.74	2.00	5.25
		6	65.87	94.69	1.77	3.43
		10	73.11	95.80	1.73	2.12
		24	89.09	95.70	2.53	1.59
		32	91.01	95.75	2.64	1.01
		52	98.65	95.44	3.44	1.12
0.5	2	3	49.06	88.36	3.36	8.29
		6	61.98	92.48	2.18	5.35
		10	68.18	94.08	1.90	3.67
		24	84.14	95.56	2.03	2.23
		32	87.90	95.49	2.25	2.11
		52	95.17	95.30	2.81	1.12
0.25	2	3	44.01	75.08	9.16	15.76
		6	53.92	84.31	5.25	10.45
		10	60.97	88.25	3.72	7.81
		24	76.63	93.11	2.25	4.48
		32	80.00	93.66	2.17	4.05
		52	88.37	94.55	2.31	2.89

[0141] As shown in Table 7, HgGA maintains a significant amount of glucoamylase activity for 52 hrs at pH 6.4, evidenced by the continued production of DP1 and DP2, as well as the continued increase of % soluble solids. The data also suggest that the rates of DP1 production and % solubilization of granular starch depend on the amount of HgGA. An increased amount of HgGA resulted in increased rates of % solubilization and DP1 production.

Example 7: Characterization of granular starch hydrolysis by HgGA and SPEZYME™ Alpha at a neutral pH by scanning electron microscopy

[0142] Granular starch from corn, wheat, and cassava was treated with HgGA and SPEZYME™ Alpha. A 28 % dry substance aqueous slurry of granular starch was first adjusted to pH 6.4 with sodium carbonate. SPEZYME™ Alpha (Danisco US Inc., Genencor Division) was added at 2 AAU/g ds, and HgGA was added at 1 GAU/g ds. Treatment was carried out at 58 °C with continuous stirring. Samples of the slurry were removed at various time points and subject to scanning electron microscopy (SEM). Slurry samples were laid on SEM sample stubs using double-sided carbon tape. Excess sample was removed by gently dusting the mounted sample with compressed air. Mounted samples were sputter coated with gold (15 nm) for 2 min at 25 mV, using an Emitech K550 Sputter Coater (Squorum Technologies). The scanning electron micrographs are presented in FIG. 3. Before treatment, starch surface was smooth and homogenous. Upon HgGA and SPEZYME™ Alpha treatment, the surface morphology of the granules changed over time. The enzyme blend first created small dimples (0.2-0.5 µm in diameter) on the surface of the starch granules. Quantity and size of the dimples increased over time. At a late stage of the treatment, for example, 48 hours for cassava granular starch, empty shells were spotted. Micrographs of empty shells indicated a complete digestion of the interior of the granule. The mechanism of enzymatic action appears to be starch granule surface peeling. Once the surface has been weakened by external peeling, the amylases penetrate and hydrolyze the interior of the granule (*i.e.*, amylolysis) leaving hollowed out shells.

Example 8: Lactic acid fermentation using various glucoamylases with the SSF process

[0143] Glucoamylases from various sources were tested for their use in lactic acid fermentation under a neutral pH. Lactic acid fermentation was carried out using SSF process. The substrate was 15% ds of cassava starch. Liquefaction was carried out at 85°C for 90 min with the alpha-amylase GC 358 (Danisco US Inc., Genencor Division) dosed at 0.25 kg/ton. For saccharification and fermentation, 0.3 GAU/g HgGA, 0.3 GAU/g OPTIDEX™ L-400 *Aspergillus niger* glucoamylase (AnGA) (Danisco US Inc., Genencor Division), and 0.3 GAU/g of TrGA was used. The SSF were carried out at pH of 6.5 at 40°C, using

inoculum of *Lactobacillus rhamnosus*. Samples were withdrawn at various time points for HPLC analysis, and the results are presented in Table 8.

Table 8. Lactic acid production during SSF

	time(h)	DP >3 w/v%	DP-3 w/v%	DP-2 w/v%	Glucose w/v%	Fructose %w/v	succinic acid %w/v	lactic acid mg/mL	glycerol w/v%	acetic acid w/v%	ethanol w/v%	lactic acid g
AnGA	4	3.80	0.67	0.00	6.40	0.05	0.11	9.71	0.00	0.08	0.08	10.68
	21	3.36	0.00	0.00	1.23	0.00	0.08	78.62	0.00	0.00	0.16	95.13
	45	2.83	0.00	0.17	0.19	0.00	0.09	106.41	0.00	0.00	0.18	130.89
TrGA	4	0.31	0.00	0.29	0.99	0.00	0.00	9.10	0.00	0.03	0.00	10.01
	21	0.15	0.03	0.04	0.32	0.00	0.00	88.73	0.00	0.04	0.04	107.36
	45	0.06	0.02	0.01	0.07	0.01	0.00	116.97	0.00	0.03	0.06	143.88
HgGA	4	3.75	0.65	0.00	6.94	0.00	0.11	9.64	0.00	0.08	0.06	10.60
	21	2.75	0.00	0.00	1.19	0.00	0.09	79.92	0.00	0.00	0.18	96.70
	45	1.51	0.43	0.56	0.16	0.00	0.09	108.53	0.00	0.00	0.23	133.49

[0144] Data presented in Table 8 indicates that when same amount of glucoamylases were used, both HgGA and TrGA resulted in more lactic acid production than AnGA at a neutral pH.

Example 9: Effect of alpha-amylase on lactic acid fermentation in no-cook process

[0145] Various alpha-amylases were combined with TrGA to produce lactic acid from cornstarch through a no-cook process. The lactic acid fermentation was performed with 2 GAU/g or 1 GAU/g TrGA glucoamylase, combined with various alpha-amylases, fungal alpha-amylase GC 626, thermostable bacterial alpha amylase SPEZYME® XTRA, and bacterial amylase AmyE (all from Danisco US Inc., Genencor Division). Each alpha-amylase was dosed at 1 kg/MT. Raw cornstarch was used as the substrate at 15% DS. Fermentation was carried out at 40°C for 45 hrs using inoculum of *Lactobacillus rhamnosus*. Samples were withdrawn at various time points for HPLC analysis, and the results are presented in Table 9.

Table 9. Lactic acid production during SSF

	time(h)	DP >3 w/v%	DP-3 w/v%	DP-2 w/v%	Glucose w/v%	Fructose %w/v	succinic acid %w/v	lactic acid mg/mL	glycerol w/v%	acetic acid w/v%	ethanol w/v%	lactic acid g
1 kg/MT GC626	3.5	0.41	0.00	0.00	2.01	0.00	0.01	6.95	0.00	0.00	0.00	7.65
	21	0.30	0.00	0.04	1.19	0.01	0.04	33.43	0.00	0.21	2.11	38.44
	28	0.38	0.00	0.00	0.00	0.06	0.09	39.00	0.00	0.25	2.48	45.24
	45.5	0.42	0.00	0.00	0.00	0.08	0.18	43.67	0.00	0.31	2.59	51.53
1 kg/MT SPEZYME® XTRA	3.5	9.31	0.00	1.25	7.64	0.35	0.21	9.24	0.00	0.38	0.29	10.17
	21	8.50	0.00	0.00	0.04	0.25	0.30	39.61	0.07	3.00	1.77	45.94
	28	11.91	0.00	0.06	0.05	0.15	0.30	47.91	0.10	3.92	5.40	56.54
	45.5	12.33	0.00	0.05	0.03	0.13	0.26	49.34	0.00	3.61	6.76	59.71
1 kb/MT AmyE	3.5	0.30	0.00	0.17	0.55	0.07	0.03	9.13	0.00	0.02	0.00	
	21	0.29	0.07	0.03	0.01	0.03	0.04	44.05	0.00	0.15	0.10	10.04
	28	0.29	0.12	0.02	0.00	0.02	0.03	55.46	0.00	0.15	0.10	51.54
	45.5	0.37	0.13	0.02	0.03	0.01	0.04	72.91	0.00	0.28	0.21	66.00

[0146] Data presented in Table 9 indicate that AmyE combined with TrGA resulted in the highest lactic acid yield, while fungal alpha-amylase GC 626 resulted in the lowest lactic acid yield.

[0147] Furthermore, the lactic acid fermentation was further performed using 1 kg/t AmyE with 1 GAU/g of HgGA, AnGA, or TrGA with the no-cook process. The fermentation was performed at pH 6.5, 40°C, with 15% DS of cornstarch. Samples were withdrawn at various time points for HPLC analysis, and the results are presented in Table 10.

Table 10. Lactic acid production during SSF

	time(h)	DP >3 w/v%	DP-3 w/v%	DP-2 w/v%	Glucose w/v%	Fructose %w/v	succinic acid %w/v	lactic acid mg/mL	glycerol w/v%	acetic acid w/v%	ethanol w/v%
HgGA	5	0.32	0.00	0.04	1.06	0.11	0.05	17.96	0.00	0.04	0.02
	22	0.43	0.10	0.02	0.02	0.02	0.04	60.90	0.00	0.15	0.14
	30	0.37	0.12	0.03	0.05	0.06	0.05	74.03	0.00	0.17	0.19
	51	0.45	0.17	0.03	0.02	0.02	0.06	104.74	0.00	0.28	0.35
TrGA	5	0.03	0.00	0.02	0.06	0.01	0.00	9.13	0.00	0.04	0.00
	21	0.03	0.01	0.00	0.00	0.00	0.00	44.05	0.00	0.15	0.01
	29	0.04	0.01	0.00	0.00	0.00	0.00	55.46	0.00	0.17	0.01
	52	0.04	0.01	0.00	0.00	0.00	0.00	76.40	0.00	0.28	0.03
AnGA	5	0.03	0.00	0.01	0.04	0.01	0.01	9.82	0.00	0.04	0.00
	21	0.03	0.01	0.00	0.00	0.00	0.00	34.26	0.00	0.15	0.02
	29	0.03	0.01	0.00	0.00	0.00	0.00	34.96	0.00	0.17	0.03
	52	0.03	0.01	0.00	0.00	0.00	0.00	24.52	0.00	0.28	0.32

[0148] Data presented in Table 10 indicate HgGA combined with AmyE resulted in the highest lactic acid yield, while AnGA combined with AmyE resulted in the lowest lactic acid yield.

Example 10: Comparison of various fermentation processes – glucose as substrate vs. conventional full saccharification process from starch

[0149] Glucose or the glucose syrup resulting from conventional liquefaction and full saccharification of cassava starch was used as the substrate for lactic acid fermentation. When glucose was used as the substrate, the inoculum of *Lactobacillus rhamnosus* was transferred to each 100 mL seed culture and cultivated at 37°C, 200rpm. Then 10 mL seed culture was added to each fermentor with 1 L fermentation medium. The fermentation temperature was controlled at 40°C. For the glucose syrup from conventional full saccharification of cassava starch, liquefaction was carried at 85°C for 90 min with 15% DS cassava starch, using alpha-amylase GC358 (Danisco US Inc., Genencor Division) at a dose of 0.25 kg/ton. When the mash was cool down to 60°C, saccharification was performed for about 18 h with 1 kg/ton AnGA. Fermentation was performed as using 15% glucose. Samples were withdrawn at various time points for HPLC analysis, and the results are presented in Table 11.

Table 11. Comparison of lactic acid fermentation using (1) glucose and (2) the conventional full saccharification product of cassava starch

	time(h)	DP >3 w/v%	DP-3 w/v%	DP-2 w/v%	Glucose w/v%	Fructose %w/v	succinic acid %w/v	lactic acid mg/mL	glycerol w/v%	acetic acid w/v%	ethanol w/v%
glucose	4	0.21	0.00	0.05	11.72	0.00	0.00	6.26	0.00	0.00	0.00
	21	0.28	0.11	0.07	1.85	0.00	0.04	74.73	0.00	0.07	0.19
	24	0.28	0.13	0.08	0.43	0.05	0.02	83.61	0.00	0.07	0.20
	28	0.28	0.12	0.07	0.01	0.05	0.03	81.92	0.00	0.08	0.25
	46	0.26	0.12	0.07	0.01	0.05	0.04	85.54	0.00	0.18	0.23
fully saccharified cassava starch	4	0.81	0.15	0.33	15.45	0.00	0.02	8.65	0.00	0.03	0.00
	21	0.92	0.26	0.31	5.14	0.00	0.03	82.61	0.00	0.03	0.05
	31	0.96	0.27	0.31	3.82	0.01	0.04	95.12	0.00	0.04	0.06
	45	0.91	0.26	0.29	1.10	0.07	0.04	111.70	0.00	0.05	0.07

[0150] Data presented in Table 8 indicate that there were still fermentable sugars remaining (1.10%) after 46 hours in the fermentation using conventional full saccharification product, while the glucose level dropped to 0.01% in the fermentation directly using glucose. Furthermore, compared with the fermentation directly using glucose, the fermentation using conventional full saccharification product resulted in a greater lactic acid yield and less amount of impurities (*i.e.*, glycerol, acetic acid, and ethanol).

Example 11. Comparison of various fermentation processes – SSF vs. glucose syrup from direct granular starch conversion

[0151] Three different processes using 15% DS cassava starch as the substrate for lactic acid fermentation were investigated. For SSF process, pH was adjusted to 6.5, liquefaction was carried at 85°C for 90 minutes using 15% DS cassava starch, and alpha-amylase GC358 (Danisco US Inc., Genencor Division) was dosed at 0.25 kg/t. 0.3 GAU/g TrGA was applied when the mash was cool down to 40°C for SSF. For the no-cook full saccharification process, pH was adjusted to 6.5, and 15% DS cassava starch was supplemented with 1.0 GAU/g HgGA and 0.5 kg/MT SPEZYME® XTRA (Danisco US Inc., Genencor Division). The saccharification was performed at 60°C for 18 hrs before fermentation. For the no-cook process, the slurry was adjusted to pH 6.5, supplemented with 2.0 GAU/g HgGA and 1.0 kg/MT AmyE, and then subject to fermentation directly. The inoculum of *Lactobacillus rhamnosus* was transferred to each 100 mL seed culture and cultivated at 37°C, 200 rpm. Then 10 mL seed culture was added to each fermentor with 1 L fermentation medium. The fermentation temperature was controlled at 40°C. Samples were withdrawn at various time points for HPLC analysis, and the results are presented in Table 12.

Table 12. Comparison of lactic acid fermentation using (1) SSF, (2) no-cook full saccharification fermentation, and (2) no-cook direct fermentation

	time(h)	DP >3 w/v%	DP-3 w/v%	DP-2 w/v%	Glucose w/v%	Fructose %w/v	succinic acid % w/v	lactic acid mg/mL	glycerol w/v%	acetic acid w/v%	ethanol w/v%
SSF	4	0.31	0.00	0.29	0.99	0.00	0.00	9.10	0.00	0.03	0.00
	21	0.15	0.03	0.04	0.32	0.00	0.00	88.73	0.00	0.04	0.04
	31	0.12	0.03	0.02	0.25	0.00	0.00	98.54	0.00	0.04	0.05
	45	0.06	0.02	0.01	0.07	0.01	0.00	116.97	0.00	0.03	0.06
No-cook full saccharification fermentation	0	1.41	0.00	1.16	11.21	0.00	0.00	0.00	0.00	0.02	0.00
	5	1.71	0.00	1.07	10.65	0.00	0.01	6.98	0.00	0.03	0.00
	21	1.20	0.38	0.52	0.09	0.09	0.05	89.66	0.00	0.00	0.04
	28	0.74	0.32	0.30	0.11	0.01	0.01	92.91	0.00	0.00	0.00
	52	0.96	0.38	0.13	0.01	0.00	0.03	96.28	0.00	0.00	0.05
No-cook direct fermentation	5	0.32	0.00	0.01	3.11	0.11	0.04	10.48	0.00	0.03	0.00
	22	0.44	0.11	0.03	0.03	0.05	0.06	74.87	0.00	0.08	0.13
	30	0.43	0.11	0.04	0.05	0.05	0.07	91.35	0.00	0.08	0.15
	51	0.47	0.12	0.02	0.00	0.02	0.05	116.37	0.00	0.11	0.30

[0152] Results presented in Table 9 indicate that the lactic acid yield was similar among all three processes. Traditionally liquefied cassava starch resulted in 116.97 mg/ml of lactic acid at 45 hours. For the cassava starch subject to the no-cook process, direct fermentation without saccharification resulted in 116.37 mg/ml of lactic acid at 51 hours, while fermentation of the fully saccharified substrate resulted in 96.28 mg/ml of lactic acid at 52 hours. The results further suggest that the no-cook process starting with raw starch may save at least 18 hours that was spent for saccharification.

Example 12. Fermentation using starch substrates with a high dry solid (DS) value

[0153] The granular cornstarch slurry having a DS of 18%, 20%, or 25% was adjusted to pH 6.5, supplemented with 2.0 GAU/g HgGA and 1.0 kg/MT AmyE, and then subject to fermentation directly. For the fermentation, the inoculum of *Lactobacillus rhamnosus* was transferred to each 100 mL seed culture and cultivated at 37°C, 200 rpm. Then 10 mL seed culture was added to each fermentor with 1 L fermentation medium. The fermentation temperature was controlled at 40°C. Samples were withdrawn at various time points for HPLC analysis, and the results are presented in Table 13.

Table 13. Lactic acid production using 18%, 20% and 25% DS cornstarch.

DS	Time(h)	Glucose %w/v	lactic acid mg/mL	Productivity (g/L·h)
18%	5	4.74	8.75	1.75
	20	0.20	83.41	4.28
	28	0.13	103.62	3.70
	45	0.02	118.25	2.63
	53	0.02	122.89	2.34
	69	0.01	128.01	1.86
20%	4	4.64	7.31	1.83
	22	0.13	89.27	4.15
	29	0.20	109.15	3.76
	45	0.05	125.88	2.80
	53	0.24	137.60	2.60
	117	0.05	144.36	1.23

25%	4	3.58	7.71	1.93
	22	0.24	102.40	4.76
	29	0.48	118.89	4.10
	45	1.16	137.15	3.05
	53	4.01	151.25	2.85
	117	0.53	164.94	1.41

[0154] The data presented in Table 13 indicate that HgGA combined with AmyE could be used to ferment lactic acid directly from granular starch having a high DS. This observation further suggests an increase of plant capacity without additional capital cost. As shown in Table 13, even at a high DS (25% ds), the rate of lactic acid production reached 4.76 g/L per hour, at 22 hrs.

Example 13. Fermentation under various pH conditions

[0155] The no-cook direct fermentation process was further performed at pH 6.5 and pH 7.0. The slurry was adjusted to pH 6.5 or pH 7.0, supplemented with 1.5 GAU/g TrGA and 1.0 kg/MT SPEZYME® XTRA, and then subject to fermentation directly. The inoculum of *Lactobacillus rhamnosus* was transferred to each 100 mL seed culture and cultivated at 37°C, 200 rpm. Then 10 mL seed culture was added to each fermentor with 1 L fermentation medium. The fermentation temperature was controlled at 40°C. Samples were withdrawn at various time points for HPLC analysis, and the results are presented in Table 14.

Table 14. Lactic acid production at pH 6.5 and pH 7.0.

	time(h)	DP >3 w/v%	DP-3 w/v%	DP-2 w/v%	Glucose w/v%	Fructose %w/v	succinic acid % w/v	lactic acid mg/mL	acetic acid w/v%	ethanol w/v%
pH 6.5	4	0.73	0.00	0.17	4.20	0.03	0.03	7.98	0.02	0.00
	22	1.01	0.32	0.27	0.52	0.08	0.07	87.71	0.03	0.00
	29	0.74	0.31	0.13	0.83	0.08	0.08	97.57	0.03	0.00
	45	0.60	0.27	0.11	0.36	0.00	0.08	112.13	0.03	0.03
	53	0.52	0.25	0.10	0.28	0.00	0.09	117.45	0.03	0.03
	70	0.43	0.23	0.08	0.24	0.00	0.08	120.23	0.03	0.00
pH 7.0	5	0.77	0.00	0.49	3.11	0.01	0.02	8.42	0.02	0.00
	22	0.81	0.29	0.06	0.34	0.03	0.06	91.60	0.04	0.04
	28	0.73	0.28	0.04	0.17	0.01	0.03	103.02	0.03	0.04
	45	0.50	0.24	0.04	0.24	0.02	0.06	118.11	0.02	0.04
	52	0.38	0.18	0.03	0.39	0.02	0.06	120.56	0.02	0.04
	69	0.36	0.19	0.03	0.24	0.02	0.06	124.86	0.04	0.04

[0156] As shown in Table 14, the lactic acid production results over time were similar at pH 6.5 and pH 7.0. The data suggest that the operation pH for the no-cook direct fermentation can be flexible, for example, in the range of 6.5 to 7.0.

Example 14. No-cook process adapted to glucose-based strains

[0157] Two lactic acid-producing microorganism, *Bacillus coagulans* and *Lactobacillus rhamnosus*, were used ferment lactic acid with the no-cook process. The cornstarch slurry was adjusted to pH 6.5, supplemented with 1 GAU/g HgGA and 1.0 kg/MT AmyE, and then subject to fermentation directly. The inoculum of *Bacillus coagulans* or *Lactobacillus rhamnosus* was transferred to each 100 mL seed culture and cultivated at 37°C, 200 rpm. Then 10 mL seed culture was added to each fermentor with 1 L fermentation medium. The fermentation temperature was controlled at 40°C. Fermentation was also performed as using 15% glucose.

Samples were withdrawn at various time points for HPLC analysis, and the results are presented in Table 15.

Table 15. Lactic acid production by different microorganism using different feed stocks

Microorganism	Substrate Process	Time(h)	Glucose %w/v	lactic acid mg/mL	Productivity (g/L·h)
<i>Bacillus coagulans</i>	15% glucose convention process	5	13.89	7.38	1.48
		22	11.22	19.19	0.87
		29	6.72	48.47	1.67
	Cornstarch No-cook	5	3.33	8.29	1.66
		22	1.90	38.80	1.76
		29	1.08	53.93	1.86
<i>Lactobacillus rhamnosus</i>	15% glucose convention process	4	11.72	6.16	1.76
		21	1.85	74.63	3.55
		28	0.01	81.81	2.92
	Cornstarch	3	1.69	8.67	3.47

	No-cook	19	0.40	78.44	4.13
		27	0.20	98.49	3.65

[0158] The data presented in Table 15 indicate that no-cook process resulted in (1) a higher lactic acid yield, and (2) a lower or at least equivalent level of residual glucose, for both *Bacillus coagulans* and *Lactobacillus rhamnosus*. This observation suggests that the no-cook process could adapt to glucose-based strains.

Example 15: Succinic acid fermentation using HgGA

[0159] The following is a prophetic example. This experiment will be carried out in 1L bioreactor to monitor succinic acid formation from granular starch using enzymes with glucoamylase activity mentioned in Example 5, at desired fermentation conditions of pH 6.7 and temperature 34°C. For this experiment, raw granular starch in slurry form (maximum final concentration 80 g/L glucose) in 0.5 × TM2 fermentation medium, will be pasteurized (*i.e.* the mixture held at 34°C for 30 min for germination of any contaminant present in the starch slurry, and then pasteurized at 65°C for 14 hr). The pasteurized starch will be added to the pre-sterilized 1L bioreactor. The pH of the starch slurry plus medium will be adjusted to 6.7 and controlled at 6.65 with NH₄OH. Then, the desired enzymes mentioned in Example 6 will be added as 0.2 micron filtered solution (20ml) in DI water. An inoculum of succinic acid-producing strain 36 1.6ppc *E. coli*, taken from frozen vial, will be prepared in TM2 + 10g/L glucose medium. After the inoculum grows to OD 3-4, measured at 550 nm, 70 ml will be added to the bioreactor. At 3.7 hours into the run, the air being sparged at 0.6 slpm will be switched to nitrogen gas at 0.6 slpm. During the fermentation, samples will be taken from the vessel, centrifuged and the supernatants will be refrigerated to terminate the enzyme action. The supernatants will be subjected to HPLC analysis to estimate the bioconversion of granular starch by measuring glucose formation and its conversion to succinate at 34°C and pH 6.7.

Example 16: 1,3-propanediol fermentation using HgGA

[0160] The following is a prophetic example. This experiment will be carried out in 1L fermentor to monitor 1,3-propanediol formation from granular starch using enzymes with

glucoamylase activity at the desired fermentation pH 6.7 and temperature 34°C. For this experiment, granular starch in slurry form (for maximum final concentration 100 g/L glucose) in 0.5 × TM2 fermentation medium will be pasteurized (the slurry mixture held at 34°C for 30 min for germination of any contaminant present in the starch slurry, and then inactivated at 65°C for 14 hr). Then, pasteurized starch will be added to the pre-sterilized 1L fermentor. The pH of the slurry plus fermentation medium will be adjusted to 6.7 and controlled at 6.65 with NH₄OH. Then, the desired enzyme activity and requirements specific for 1,3-propanediol production (30 mg spectinomycin and 2 mg vitamin B12) will be added in DI water. An inoculum of 1,3-propanediol-producing *E. coli* strain TTaldABml/p109F1 taken from a frozen vial, will be prepared in soytone-yeast extract-glucose medium. After the inoculum grows to OD 3-4 (measured at 550 nm), 70 ml will be transferred to the 1L fermentor. During the fermentation, samples will be taken from the fermentor, centrifuged, and supernatants will be subjected to HPLC analysis. This will determine the fermentative bioconversion of granular starch by measuring glucose formation and its conversion to glycerol (1,3-propanediol pathway intermediate) and then to 1,3-propanediol.

SEQUENCE LISTING

A Sequence Listing, comprising SEQ ID NOs: 1-9, is provided herein and is incorporated herein by reference in its entirety.

SEQ ID NO: 1: genomic sequence coding the full-length *Humicola grisea* glucoamylase; putative introns are in bold; corresponds to SEQ ID NO. 1 of U.S. Patent 7,262,041

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SEQ ID NO: 2: amino acid sequence of full-length *Humicola grisea* glucoamylase; the native signal sequence is in bold; corresponds to SEQ ID NO. 2 of U.S. Patent 7,262,041

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SEQ ID NO: 3: amino acid sequence of mature *Humicola grisea* glucoamylase; the native signal sequence is cleaved; corresponds to SEQ ID NO. 3 of U.S. Patent 7,262,041

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cagtttgccc agacgggcaa ggtggcgggc aacgccgcgg ccctgggcaa ctggagcacg     1680
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ggctccgtga cctgggagag tgatcccaac cacacttaca cggttcctgc ggtggttgt     1860
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SEQ ID NO: 5: *Trichoderma reesei* glucoamylase, full length; with signal peptide

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          35          40          45
Asn Leu Leu Cys Asn Val Gly Pro Asp Gly Cys Arg Ala Phe Gly Thr
          50          55          60
Ser Ala Gly Ala Val Ile Ala Ser Pro Ser Thr Ile Asp Pro Asp Tyr
65          70          75          80
Tyr Tyr Met Trp Thr Arg Asp Ser Ala Leu Val Phe Lys Asn Leu Ile
          85          90          95
Asp Arg Phe Thr Glu Thr Tyr Asp Ala Gly Leu Gln Arg Arg Ile Glu
          100          105          110
Gln Tyr Ile Thr Ala Gln Val Thr Leu Gln Gly Leu Ser Asn Pro Ser
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Gly Ser Leu Ala Asp Gly Ser Gly Leu Gly Glu Pro Lys Phe Glu Leu
130          135          140
Thr Leu Lys Pro Phe Thr Gly Asn Trp Gly Arg Pro Gln Arg Asp Gly
145          150          155          160
Pro Ala Leu Arg Ala Ile Ala Leu Ile Gly Tyr Ser Lys Trp Leu Ile
          165          170          175
    
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 210 215 220
 Gln His Arg Ala Leu Val Glu Gly Ala Thr Leu Ala Ala Thr Leu Gly
 225 230 235 240
 Gln Ser Gly Ser Ala Tyr Ser Ser Val Ala Pro Gln Val Leu Cys Phe
 245 250 255
 Leu Gln Arg Phe Trp Val Ser Ser Gly Gly Tyr Val Asp Ser Asn Ile
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 Gln Pro Cys Ser Asp Lys Ala Leu Ser Asn Leu Lys Val Val Val Asp
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 Ala Val Ala Ile Gly Arg Tyr Ala Glu Asp Val Tyr Tyr Asn Gly Asn
 340 345 350
 Pro Trp Tyr Leu Ala Thr Phe Ala Ala Ala Glu Gln Leu Tyr Asp Ala
 355 360 365
 Ile Tyr Val Trp Lys Lys Thr Gly Ser Ile Thr Val Thr Ala Thr Ser
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 Ser Val Ala Val Thr Phe His Glu Leu Val Ser Thr Gln Phe Gly Gln
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 Thr Val Lys Val Ala Gly Asn Ala Ala Ala Leu Gly Asn Trp Ser Thr
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 580 585 590
 Lys Tyr Ile Asn Val Gly Gln Asp Gly Ser Val Thr Trp Glu Ser Asp
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 Pro Asn His Thr Tyr Thr Val Pro Ala Val Ala Cys Val Thr Gln Val
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SEQ ID NO: 6: *Trichoderma reesei* glucoamylase, mature protein; without signal peptide

<210> 2
 <211> 599
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 35 40 45
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Val Lys Val Ala Gly Asn Ala Ala Ala Leu Gly Asn Trp Ser Thr Ser
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 Trp Ile Gly Thr Val Asn Leu Glu Ala Gly Asp Val Val Glu Tyr Lys
 545 550 555 560
 Tyr Ile Asn Val Gly Gln Asp Gly Ser Val Thr Trp Glu Ser Asp Pro
 565 570 575
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 Lys Glu Asp Thr Trp Gln Ser
 595

SEQ ID NO: 7: *Trichoderma reesei* glucoamylase catalytic domain, 1-453 of mature TrGA, catalytic domain

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 <212> PRT
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 65 70 75 80
 Tyr Ile Thr Ala Gln Val Thr Leu Gln Gly Leu Ser Asn Pro Ser Gly
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 Leu Lys Pro Phe Thr Gly Asn Trp Gly Arg Pro Gln Arg Asp Gly Pro
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 130 135 140
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 His Arg Ala Leu Val Glu Gly Ala Thr Leu Ala Ala Thr Leu Gly Gln
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 Ser Gly Ser Ala Tyr Ser Ser Val Ala Pro Gln Val Leu Cys Phe Leu
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 Gln Arg Phe Trp Val Ser Ser Gly Gly Tyr Val Asp Ser Asn Ile Asn
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 Pro Cys Ser Asp Lys Ala Leu Ser Asn Leu Lys Val Val Val Asp Ser
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 420 425 430
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 435 440 445
 Ile Pro Ser Thr Cys
 450

SEQ ID NO: 8: native RhGA (P07683.2 GI:1168453)

1 mqlfnlplkv sfflvlsyfs llvsaasips sasvqldsyn ydgstfsgki yvknaiaykk
 61 vtviyadgsd nwnnngntia asysapisgs nyeywtfsas ingikefyik yevsgktyyd
 121 nnsanyqvs tskpttttat attttapsts tttppsrsep atfptgnsti sswikkqegi
 181 srfamlrnin ppgsatgfa aslstagpdy yyawtrdaal tsnvivyeyn ttlsknktil
 241 nvlkdyvtfs vktqststvc nclgepkfnp dasgytgawg rpqndgpaer attfilfads
 301 yltqtkdasy vtgtlkpaif kdldyvvnvw sngcfdlwee vngvhfytlm vmrkglllga
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 421 nlgsyddgff tpgsekilat avavedsfas lypinknlps ylgnsigryp edtyngngns
 481 qgnswflavt gyaelyyrai kewignggvt vssislppfk kfdssatsgk kytvgtstfn
 541 nlaqniaalaa drflstvqlh ahnngslaee fdrttglstg ardltwshas litasyakag
 601 apaa

SEQ ID NO: 9: mature RhGA (P07683.2 GI:1168453)

1 asipssasvq ldsynydgst fsgkiyvknai ayskkvtviy adgsdnwnnn gntiaasysa
 61 pisgsnyeyw tfsasingik efyikyevsg ktyydnnsa nyqvstskpt tttatatttt
 121 apststtpp srsepatfpt gnstisswik kqegisrfam lrninppgsa tgfiiaaslst
 181 agpdyyyawt rdaaltsnvi vyeynttlsg nktilnvlkd yvtfsvktqs tstvcnclge
 241 pkfnpdasy tgawgrpqnd gpaerattfi lfadsylytqt kdasyvtgtl kpaifkdldy
 301 vvnvwsngcf dlweevngvh fytlmvmrkg lllgadafkr ngdstrasty sstastiank
 361 issfwvssnn wiqvsqsvtg gvskkgldvs tllaanlgsv ddgfftppgse kilataavave
 421 dsfaslypin knlpsylgns igrypedtyn gngnsqgnsw flavtgyael yyraikewig
 481 nggvtvssis lpffkkkfdss atsgkkytvq tsdfnnlaqn ialaadrfls tvqlhahnn
 541 slaefdrtt glstgardlt wshaslitas yakagapaa

WHAT IS CLAIMED IS:

1. A method of processing starch comprising saccharifying a starch substrate to fermentable sugars at pH 5.0 to 8.0 in the presence of a glucoamylase, wherein the glucoamylase possesses at least 50% activity at pH 6.0 or above relative to its maximum activity, wherein the glucoamylase is selected from the group consisting of *Humicola grisea* glucoamylase (HgGA) comprising SEQ ID NO: 3, *Trichoderma reesei* glucoamylase (TrGA) comprising SEQ ID NO: 6, *Rhizopus* sp. glucoamylase (RhGA) comprising SEQ ID NO: 9, and a variant thereof, and wherein the variant has at least 99% sequence identity to a parent glucoamylase.
2. The method of claim 1, wherein the variant has one amino acid modification compared to the parent glucoamylase.
3. The method of claim 1, wherein the HgGA is SEQ ID NO: 3.
4. The method of claim 3, wherein the HgGA is produced from a *Trichoderma reesei* host cell.
5. The method of claim 1, wherein the TrGA is SEQ ID No: 6.
6. The method of claim 1, wherein the RhGA is SEQ ID NO: 9.
7. The method of claim 1, wherein saccharifying is carried out at a pH in a range of 6.0 to 7.5.
8. The method of claim 1, wherein saccharifying is carried out at a pH in a range of 7.0 to 7.5.
9. The method of claim 1 further comprising fermenting the fermentable sugars to an end product, and wherein saccharifying and fermenting are performed at the same pH.

10. The method of claim 9, wherein saccharifying and fermenting are carried out as a simultaneous saccharification and fermentation (SSF) process.
11. The method of claim 10, wherein the SSF process is carried out at a pH between 7.0 to 7.5.
12. The method of claim 1, wherein saccharifying is performed at a temperature in a range of about 30°C to about 60°C.
13. The method of claim 12, wherein saccharifying is performed at a temperature in a range of about 40°C to about 60°C.
14. The method of claim 1, wherein the starch substrate is about 15% to 50% dry solid (DS).
15. The method of claim 1, wherein the starch substrate is about 15% to 30% dry solid (DS).
16. The method of claim 1, wherein the starch substrate is about 15% to 25% dry solid (DS).
17. The method of claim 9, wherein the end product is selected from the group consisting of methanol, ethanol, butanol, monosodium glutamate, succinic acid, 1,3-propanediol, vitamins, amino acids, and lactic acid.
18. The method of claim 17, wherein the end product is ethanol.
19. The method of claim 17, wherein the end product is 1,3-propanediol.
20. The method of claim 17, wherein the end product is succinic acid.

21. The method of claim 1, wherein the starch substrate is granular starch or liquefied starch.
22. The method of claim 1, wherein the glucoamylase is dosed at a range of about 0.1 to about 2.0 GAU per gram of dry substance starch.
23. The method of claim 22, wherein the glucoamylase is dosed at a range of about 0.2 to about 1.0 GAU per gram of dry substance starch.
24. The method of claim 22, wherein the glucoamylase is dosed at a range of about 0.5 to 1.0 GAU per gram of dry substance starch.
25. The method of claim 1 further comprising adding an alpha-amylase.
26. The method of claim 25, wherein the alpha-amylase is from a *Bacillus* species, or a variant thereof.
27. The method of claim 26, wherein the alpha-amylase is a *Bacillus subtilis* alpha-amylase (AmyE), a *Bacillus amyloliquefaciens* alpha-amylase, a *Bacillus licheniformis* alpha-amylase, a *Bacillus stearothermophilus* alpha-amylase, or a variant thereof.
28. The method of claim 1, wherein the starch substrate is from corn, wheat, rye, barley, sorghum, cassava, tapioca, potato and any combination thereof.
29. A method of processing starch comprising saccharifying a starch substrate to fermentable sugars at pH 5.0 to 8.0 in the presence of glucoamylase and at least one other enzyme,
wherein the glucoamylase possesses at least 50% activity at pH 6.0 or above relative to its maximum activity, wherein the glucoamylase is selected from the group consisting of *Humicola grisea* glucoamylase (HgGA) comprising SEQ ID NO: 3, *Trichoderma reesei* glucoamylase (TrGA) comprising SEQ ID NO: 6, *Rhizopus* sp. glucoamylase (RhGA)

comprising SEQ ID NO: 9, and a variant thereof, and wherein the variant has at least 99% sequence identity to a parent glucoamylase, and

wherein the other enzyme is selected from the group consisting of proteases, pullulanases, isoamylases, cellulases, hemicellulases, xylanases, cyclodextrin glycotransferases, lipases, phytases, laccases, oxidases, esterases, cutinases, xylanases, and alpha-glucosidases.

30. A method of processing starch comprising saccharifying a starch substrate to fermentable sugars at pH 5.0 to 8.0 in the presence of glucoamylase and at least one other non-starch polysaccharide hydrolyzing enzymes,

wherein the glucoamylase possesses at least 50% activity at pH 6.0 or above relative to its maximum activity, wherein the glucoamylase is selected from the group consisting of *Humicola grisea* glucoamylase (HgGA) comprising SEQ ID NO: 3, *Trichoderma reesei* glucoamylase (TrGA) comprising SEQ ID NO: 6, *Rhizopus* sp. glucoamylase (RhGA) comprising SEQ ID NO: 9, and a variant thereof, and wherein the variant has at least 99% sequence identity to a parent glucoamylase, and

wherein the non-starch polysaccharide hydrolyzing enzymes is selected from the group consisting of cellulases, hemicellulases and pectinases.

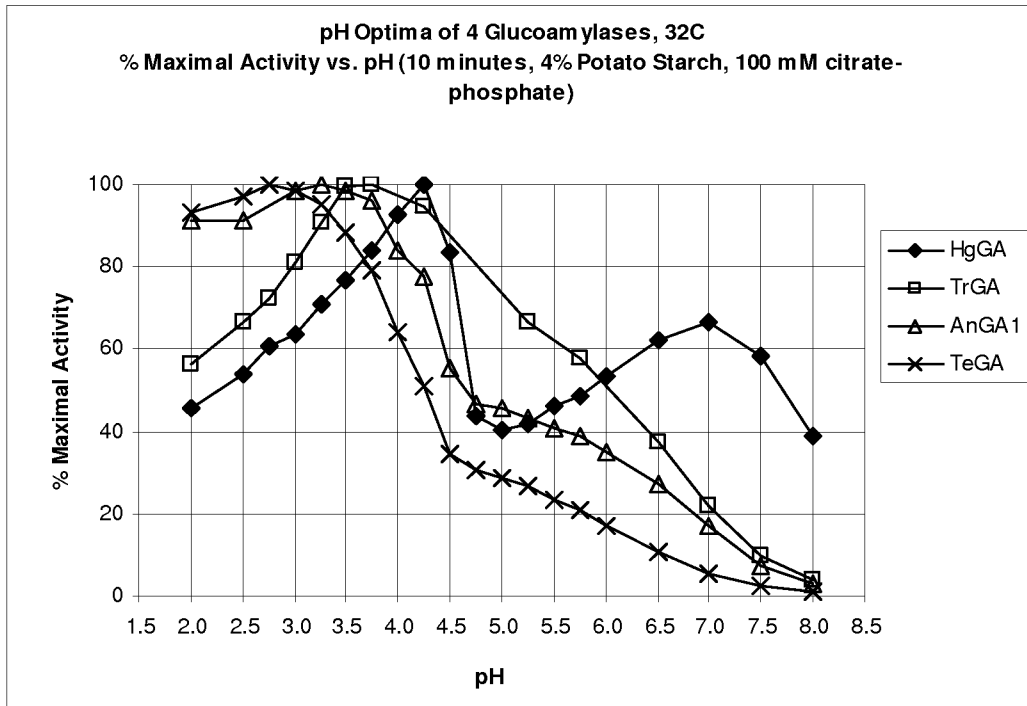


FIG. 1

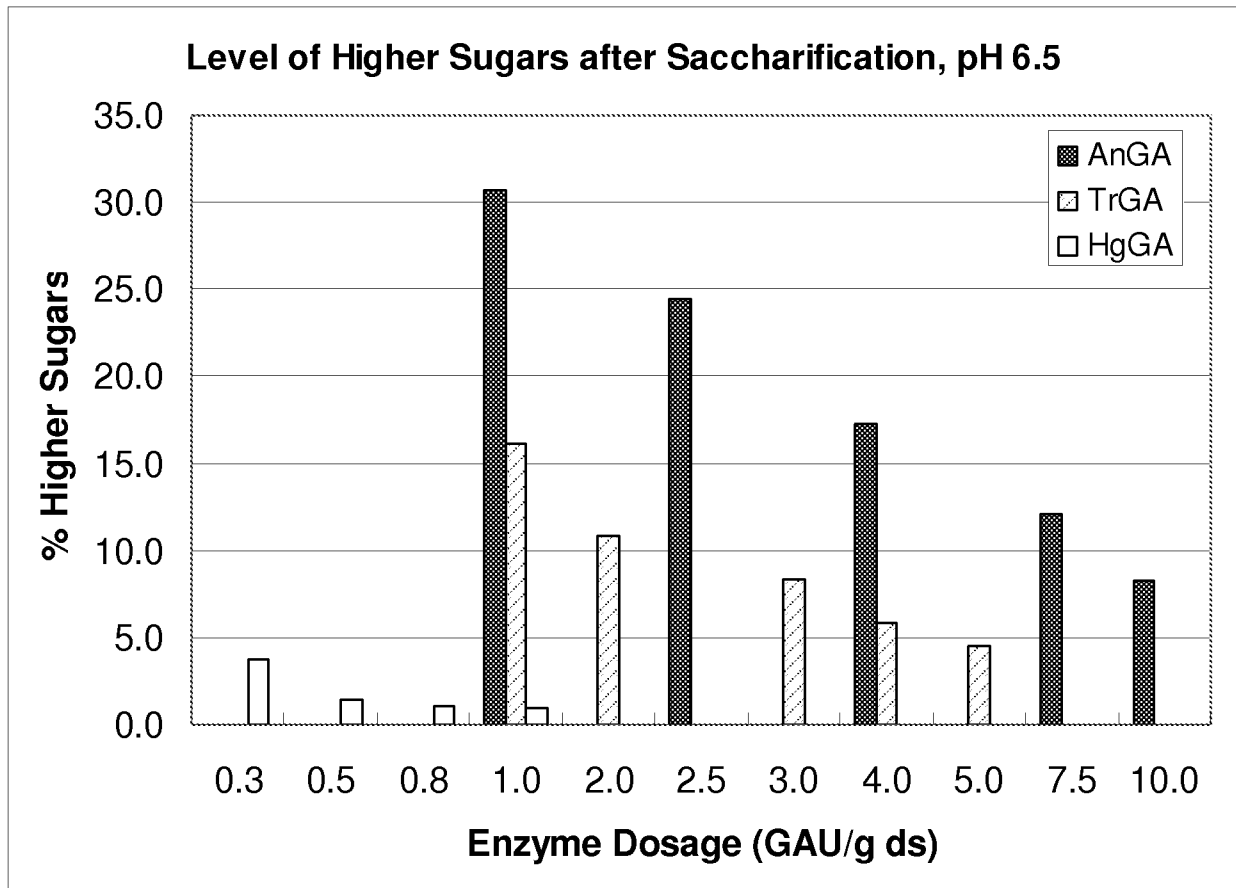


FIG. 2

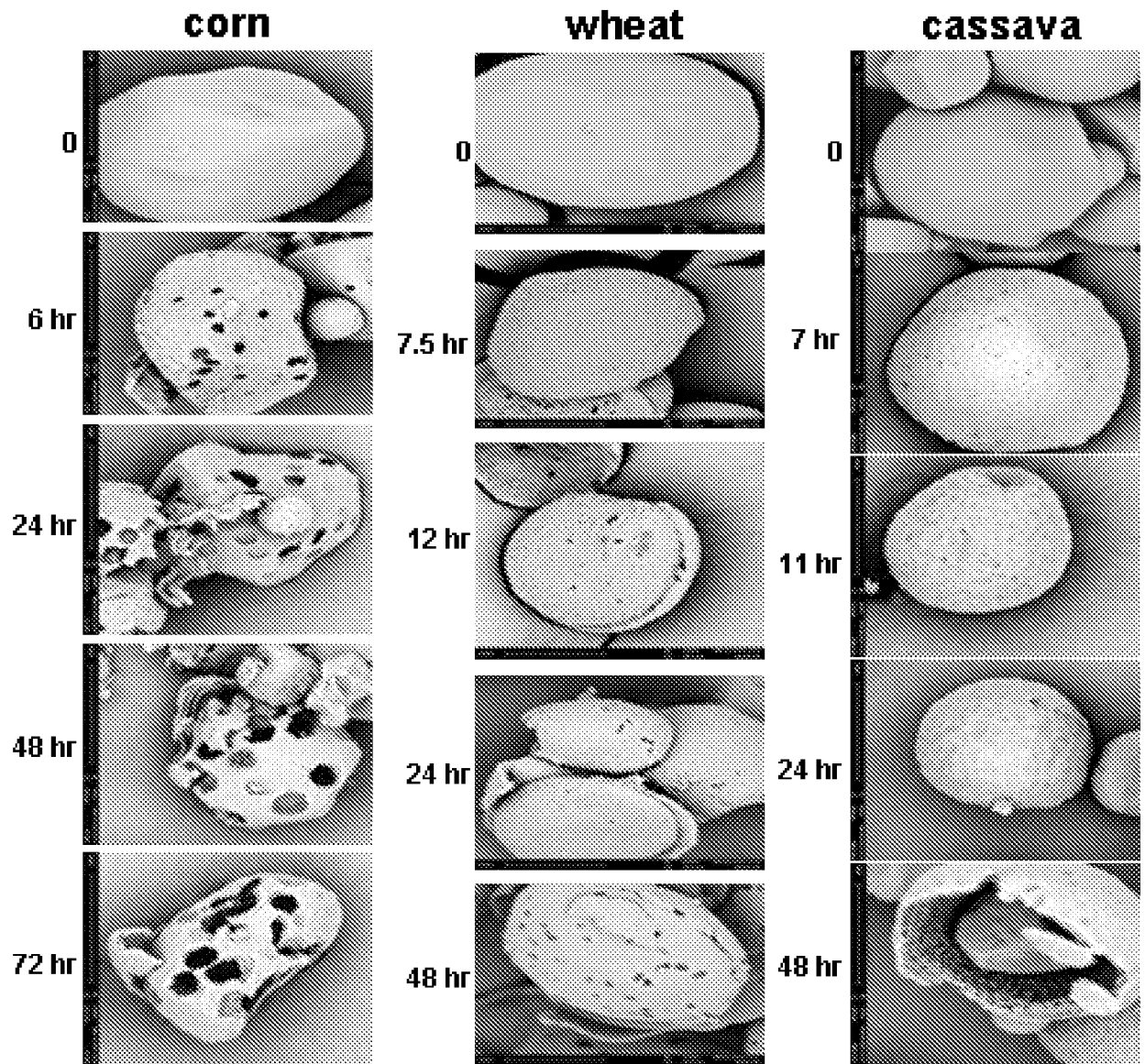


FIG. 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/046849

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/06
ADD.
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)
C12P
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, FSTA, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2005/208623 A1 (BALDWIN ET AL) 22 September 2005 (2005-09-22) * See page 1 (0006), pages 17-19 (SEQ ID NO 3 = SEQ 3 of the Application) and Figures 2B, 8A-8B *	1,3,4,7, 12,17
A	----- WO 2006/060062 A2 (GENENCOR INTERNATIONAL, INC.) 8 June 2006 (2006-06-08) * See Figures 3B (SEQ ID NO 4 = SEQ 6 of the Application) and 18 (pH) *	1-30
A	----- WO 2009/052101 A1 (DANISCO US, INC.) 23 April 2009 (2009-04-23) * See page 11 (SEQ ID NO 1 = SEQ 6 of the Application) and pages 22-23 (pH) *	1-30
	----- -/--	

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	"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
"E" earlier document but published on or after the international filing date	"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
"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)	"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.
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 29 November 2011	Date of mailing of the international search report 09/12/2011
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Korsner, Sven-Erik
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INTERNATIONAL SEARCH REPORT

International application No

PCT/US2011/046849

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 186 066 A1 (SUNTORY LIMITED) 2 July 1986 (1986-07-02) * See Figures 1a-c (= SEQ 8 of the Application; one mismatch at position 272 Ala/Gly) * -----	1-30
A	KIM ET AL: "Properties of a novel thermostable glucoamylase from the hyperthermophilic archaeon Sulfolobus solfataricus in relation to starch processing", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 70, 2004, pages 3933-3940, XP002664546, * See page 3933 (Abstract and Introduction) * -----	1-30
A	HUANG ET AL: "Simultaneous saccharification and fermentation of potato starch wastewater to lactic acid by Rhizopus oryzae and Rhizopus arrhizus", BIOCHEMICAL ENGINEERING JOURNAL, vol. 23, 2005, pages 265-276, XP027849102, * See pages 268-269 (section 3.1/pH) * -----	1-30

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2011/046849

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2005208623 A1	22-09-2005	US 2005208623 A1 US 2007099272 A1	22-09-2005 03-05-2007

WO 2006060062 A2	08-06-2006	AU 2005310284 A1 WO 2006060062 A2	08-06-2006 08-06-2006

WO 2009052101 A1	23-04-2009	AR 068924 A1 CA 2702949 A1 CN 101827935 A EP 2201108 A1 JP 2011500058 A US 2009203101 A1 WO 2009052101 A1	16-12-2009 14-04-2010 08-09-2010 30-06-2010 06-01-2011 13-08-2009 23-04-2009

EP 0186066 A1	02-07-1986	AT 86663 T BR 8506273 A CA 1267372 A1 DE 3587168 D1 DE 3587168 T2 EP 0186066 A1 JP 1904059 C JP 6030586 B JP 61141888 A MX 165920 B US 4863864 A	15-03-1993 26-08-1986 03-04-1990 15-04-1993 09-09-1993 02-07-1986 08-02-1995 27-04-1994 28-06-1986 10-12-1992 05-09-1989
