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(54) Title: PROTEASES IN CORN PROCESSING

FIG. 1

(57) Abstract: The present invention relates to reducing the viscosity of a corn-like grain slurry during a liquefaction and/or fermentation. The invention also relates to increasing the rate and/or yield of fermentation products, including, but not limited to ethanol, lactic acid and citric acid from corn-like grains.
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SL, SK), with international search report (Art. 21(3))
PROTEASES IN CORN PROCESSING

CROSS REFERENCE TO RELATED APPLICATIONS
[001] This application claims benefit of priorities from the U. S. Provisional application Serial No. 61/899755 filed 04 November 2013 and International application Serial No. PCT/CN2014/089754, filed 29 October, 2014 and both of which are incorporated by reference herewith in their entirety.

BACKGROUND OF THE INVENTION
[002] More and more biochemicals (for example, alcohols - ethanol, butanol, etc., acids - lactic acid, citric acid, etc.), are being produced from fermentation processes using renewable feedstocks to replace the traditional processes using limited fossil fuel. One major cost of the bioproduction is the feedstock. The fermentation feedstock is usually high quality glucose or liquefact from refined starch. Some producers are using cruder materials like whole ground corn, cassava chips, etc., in the fermentations, for examples, citric acid, other organic acids, and butanol fermentation. The key issue of this kind of process is that there is one filtration process to remove the fiber, fat, protein, unsolubolized starch and other impurities before pumping the syrup to fermentor. Otherwise, these impurities will affect the functions of bacteria, yeast or other fermentation organism. The disadvantages of the current process is that the filtration is needed to be done at high temperature, with high viscosity of the syrup, which leads to high residual starch in the filtered cake and loss of sugars.

[003] The commercial viability of producing ethanol as a fuel source from agricultural crops has generated renewed worldwide interest due to a variety of reasons that include continued and increased dependence on limited oil supplies and the fact that ethanol production is a renewable energy source.


[005] Wet milling process involves a series of soaking (steeping) steps to soften the cereal grain wherein soluble starch is removed followed by recovery of the germ, fiber (bran) and gluten (protein). The remaining starch is further processed by drying, chemical and/or enzyme treatments. The starch may then be used for alcohol production, high fructose corn syrup or commercial pure grade starch or other fermentation products.
Dry grain milling involves a number of basic steps, which include: grinding, cooking, liquefaction, saccharification, fermentation and separation of liquid and solids to produce alcohol and other co-products. Generally, whole cereal, such as corn cereal, is ground to a fine particle size and then mixed with liquid in a slurry tank. The slurry is subjected to high temperatures in a jet cooker along with liquefying enzymes (e.g. alpha-amylases) to solubilize and hydrolyze the starch in the cereal to dextrins. The mixture is cooled down and further treated with saccharifying enzymes (e.g. glucoamylases) to produce fermentable glucose. The mash containing glucose is then fermented for approximately 24 to 120 hours in the presence of ethanol producing microorganisms. The solids in the mash are separated from the liquid phase and ethanol and useful co-products such as distillers' grains are obtained.

Improvements to the above fermentation processes have been accomplished by combining the saccharification step and fermentation step in a process referred to as simultaneous saccharification and fermentation or simultaneous saccharification, yeast propagation and fermentation. These improved fermentation processes have advantages over the previously described dry or wet milling fermentation processes because significant sugar concentrations do not develop in the fermenter thereby avoiding sugar inhibition of yeast growth. In addition, bacterial growth is reduced due to lack of easily available glucose. Increased ethanol production may result by use of the simultaneous saccharification and fermentation processes.

The corn kernel has two kinds of endosperm portion: hard and soft endosperm. The soft endosperm contains spherical starch granule in protein matrix. The hard endosperm contains polygonal starch granules packed in a highly complex protein matrix. During drying on the cob and in the storage bins the protein matrix in hard endosperm are stretched and tightly aligned with starch granules which themselves change shapes from nice spherical shapes to polygonal shapes due to stretching during drying. Adding a protease to break the protein structure may result in release of more starch to be available for enzymes and yeast.

Viscosity of corn slurry increase rapidly when milled corn, corn flour, corn starch, or other starch granular substrates are heated above 60°C at various dry solids level in water. Typically alpha amylase is added to minimize the initial rise in the slurry viscosity (aka peak viscosity) as well as the viscosity of the final hot slurry produced (aka final viscosity). Controlling both of these viscosity values is important in the corn cook process (gelatinization and pasting) for pumpability in the process pipes, slurry mixer as well as the steam pressured jet cooker. It is known in the literature that addition of protease aids the liquefaction process by producing high FAN (free amino nitrogen) resulting in higher ethanol yield. However, addition
of proteases to reduce slurry viscosity has not been observed in the past. Thus, there is a need in
the field to develop processes that reduce viscosity during liquefaction of corn starch.

[0010] The present invention develops a new process, using proteases and/or other enzymes
before or during the liquefaction process, solubilizing more starch and reducing the viscosity of
the syrup, thus reducing the residual starch in the filtered cake. In addition, the protease
produces more Free amino nitrogen (FAN), which is helpful to the fermentation organism, thus
improving the fermentation process.

SUMMARY OF THE INVENTION

[0011] In one aspect, the invention comprises a method for producing a fermentation product
from corn or similar grain, comprising: liquifying the corn-like grain slurry at a pH of about 4.0
to about 7.0 at a temperature in the range from about 25°C to about 65°C using: an alpha
amylase, a protease; and optionally a beta-glucanase; saccharifying and fermenting the slurry
using enzymes, including a glucoamylase and a fermenting organism to produce the
fermentation product.

[0012] In one aspect, the invention comprises a method for increasing the yield and/or rate of
ethanol production from corn or similar grain, comprising: liquifying the corn-like grain slurry
at a pH of about 4.0 to about 7.0 at a temperature in the range from about 25°C to about 65°C
using: an alpha amylase, a protease; and optionally a beta-glucanase; saccharifying and
fermenting the slurry using enzymes, including a glucoamylase and a fermenting organism to
produce ethanol, wherein the yield and/or rate of ethanol production is greater than the rate
observed without the addition of protease.

[0013] In another aspect, the invention comprises a method for reducing the viscosity of the
corn-like grain slurry during a fermentation, comprising: liquifying the corn-like grain slurry at a
pH of about 4.0 to about 7.0 at a temperature in the range from about 25°C to about 65°C using:
an alpha amylase, a protease; and optionally a beta-glucanase; saccharifying and fermenting the
slurry using enzymes, including a glucoamylase and a fermenting organism to produce the
fermentation product, wherein the viscosity of the slurry is reduced by at least 20% compared to
a slurry not treated with a protease. In another aspect, the viscosity is reduced by 30%, 50% or
60%.

[0014] In another aspect, the invention comprises a method for reducing the viscosity of the
corn-like grain slurry during a liquefaction process, comprising: liquifying the corn-like gain
slurry at a pH of about 4.0 to about 7.0 at a temperature in the range from about 25°C to about
65°C using an alpha amylase, a protease; and optionally a beta-glucanase;
wherein the viscosity of the slurry is reduced by at least 20% compared to a slurry not treated with a protease. In another aspect, the viscosity is reduced by 30%, 50% or 60%.

[0015] In another aspect, the invention comprises a method for reducing the viscosity of the corn-like grain slurry during a liquefaction process, comprising: liquifying the corn-like gain slurry at a pH of about 4.0 to about 7.0 at a temperature in the range from about 25°C to about 95°C using an alpha amylase, a protease; and optionally a beta-glucanase; wherein the viscosity of the slurry is reduced by at least 20% compared to a slurry not treated with a protease. In another aspect, the viscosity is reduced by 30%, 40%, 50% or 60%.

[0016] In another aspect of the invention, the invention comprises a method for reducing foam in a corn-like grain slurry during a liquefaction process, comprising: liquifying the corn-like gain slurry at a pH of about 4.0 to about 7.0 at a temperature in the range from about 25°C to about 90°C using an alpha amylase, a protease; and optionally a beta-glucanase; and wherein the foam in the slurry is reduced by at least 20%, 30%, 50% or 60% compared to a slurry not treated with a protease.

[0017] In another aspect of the invention, the invention comprises a method for reducing amount of residual starch after a liquefaction process of a corn-like grain slurry, comprising: liquifying the corn-like gain slurry at a pH of about 4.0 to about 7.0 at a temperature in the range from about 25°C to about 90°C using: an alpha amylase, a protease; and optionally a beta-glucanase; and wherein the amount of residual starch of the slurry is reduced by at least 20%, 30%, 50% or 60% compared to a slurry not treated with a protease.

[0018] In another aspect of the invention, the protease is a metalloprotease. In another aspect, the metalloprotease is a thermolysin or Proteinase T. In another aspect, the protease is a variant thermolysin with one or more substitutions. In another aspect, the protease is NprE. In another aspect, the protease is an NprE varaint with one or more substitutions. In another aspect, the protease is Fermgen, an acid fungal protease. These substitutions are disclosed in various patent applications, for example, WO 2002007/044993, WO 2009/058679, WO 2009/058661, WO 2009/058518, WO 2009/058303, and US provisional application 61/722,660 filed on 05 November 2012, all of which are herein incorporated by reference in their entirety.

[0019] In another aspect of the invention, the fermentation product is an alcohol, an organic acid, an amino acid, a sugar, an antibiotic, an enzyme, a vitamin or a hormone.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] Figure 1: Titration of Proteinase T with 15 µgrams of Cytophaga spp alpha amylase.
[0021] Figure 2: Addition of protease (1mg purified NprE) to 15 µgrams of Cytophaga spp
alpha amylase.

[0022] Figure 3: Dose dependent reduction of peak and final viscosity values.

[0023] Figure 4: The final ethanol yield at 48 hr for different liquefaction and SSF treatments.

[0024] Figure 5: The final ethanol yield at 48 hr for different liquefaction and SSF treatments.

[0025] Figure 6: The final ethanol yield at 48 hr for both Proteinase T and FAN used in SSF with Fermgen.

**DETAILED DESCRIPTION**

[0026] Described are compositions and methods relating to protease enzymes. Exemplary applications for the protease enzymes are for starch liquefaction, to reduce viscosity and increase the yield and/or rate of ethanol production. These and other aspects of the compositions and methods are described in detail, below.

[0027] Prior to describing the various aspects and embodiments of the present compositions and methods, the following definitions and abbreviations are described.

1. **Definitions and Abbreviations**

[0028] In accordance with this detailed description, the following abbreviations and definitions apply. Note that 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, and reference to "the dosage" includes reference to one or more dosages and equivalents thereof known to those skilled in the art, and so forth.

[0029] The present document is organized into a number of sections for ease of reading; however, the reader will appreciate that statements made in one section may apply to other sections. In this manner, the headings used for different sections of the disclosure should not be construed as limiting.

[0030] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. The following terms are provided below.

1.1. **Abbreviations and Acronyms**

[0031] The following abbreviations/acronyms have the following meanings unless otherwise specified: ABTS: 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; AE or AEO: alcohol ethoxylate; AES or AEOS: alcohol ethoxysulfate; AkAA: *Aspergillus kawachii* a-amylase; AnGA: *Aspergillus niger* glucoamylase; AOS: a-olefinsulfonate; AS: alkyl sulfate; cDNA: complementary DNA; CMC: carboxymethylcellulose; DE: dextrose equivalent; DNA:
deoxyribonucleic acid; DPn: degree of saccharide polymerization having n subunits; ds or DS: dry solids; DTMPA: diethyleneetriaminepentaacetic acid; EC: Enzyme Commission; EDTA: ethylenediaminetetraacetic acid; EO: ethylene oxide (polymer fragment); EOF: End of Fermentation; GA: glucoamylase; GAU/g ds: glucoamylase activity unit/gram dry solids; HFCS: high fructose corn syrup; HgGA: *Humicola grisea* glucoamylase; IPTG: isopropyl β-D-thiogalactoside; IRS: insoluble residual starch; kDa: kilodalton; LAS: linear alkylbenzenesulfonate; LAT, BLA: *B. licheniformis* amylase; MW: molecular weight; MWU: modified Wohlgemuth unit; 1.6x10^-7 mg/MWU = unit of activity; NCBI: National Center for Biotechnology Information; NOBS: nonanoyloxybenzenesulfonate; NTA: nitroiloacetic acid; OxAm: Purastar HPAM 5000L (Danisco US Inc.); PAHBAH: p-hydroxybenzoic acid hydrazide; PEG: polyethylene glycol; PI: isoelectric point; PI: performance index; ppm: parts per million, *e.g.*, µg protein per gram dry solid; PVA: poly(vinyl alcohol); PVP: poly(vinylpyrrolidone); RCF: relative centrifugal/centripetal force (*i.e.*, x gravity); RNA: ribonucleic acid; SAS: alkanesulfonate; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; SSF: simultaneous saccharification and fermentation; SSU/g solid: soluble starch unit/gram dry solids; sp.: species; TAED: tetraacetylethylenediamine; Tm: melting temperature; TrGA: *Trichoderma reesei* glucoamylase; w/v: weight/volume; w/w: weight/weight; v/v: volume/volume; wt%: weight percent; °C: degrees Centigrade; H₂O: water; dH₂O or DI: deionized water; dH₂O: deionized water, Milli-Q filtration; g or gm: grams; µg: micrograms; mg: milligrams; kg: kilograms; µL and µl: microliters; mL and ml: milliliters; mm: millimeters; µm: micrometer; M: molar; mM: millimolar; µM: micromolar; U: units; sec: seconds; min(s): minute/minutes; hr(s): hour/hours; DO: dissolved oxygen; Ncm: Newton centimeter; ETOH: ethanol; eq.: equivalents; N: normal; MWCO: molecular weight cut-off; SSRL: Stanford Synchrotron Radiation Lightsource; PDB: Protein Database; CAZy: Carbohydrate-Active Enzymes database; Tris-HCl: tris(hydroxymethyl)aminomethane hydrochloride; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mS/cm: milli-Siemens/cm; CV: column volumes.

1.2. Definitions

[0032] As used herein, the terms "protease" and "proteinase" refer to an enzyme protein that has the ability to break down proteins or peptides. A protease has the ability to conduct "proteolysis," which begins protein catabolism by hydrolysis of peptide bonds that link amino acids together in a peptide or polypeptide chain forming the protein. This activity of a protease as a protein-digesting enzyme is referred to as "proteolytic activity." Many procedures exist for measuring proteolytic activity (*See e.g.*, Kalisz, "Microbial Proteinases," *In*: Fiechter (ed.), *Advances in Biochemical Engineering/Biotechnology*).
(1988)). For example, proteolytic activity may be ascertained by comparative assays which analyze the respective protease’s ability to hydrolyze a commercially available substrate. Exemplary substrates useful in the analysis of protease or proteolytic activity, include, but are not limited to, di-methyl casein (Sigma C-9801), bovine collagen (Sigma C-9879), bovine elastin (Sigma E-1625), and bovine keratin (ICN Biomedical 9021 11). Colorimetric assays utilizing these substrates are well known in the art (See e.g., WO 99/3401 1 and U.S. Pat. No. 6,376,450, both of which are incorporated herein by reference). The pHNA assay (See e.g., Del Mar et al, Anal. Biochem. 99:316-320 [1979]) also finds use in determining the active enzyme concentration for fractions collected during gradient elution. This assay measures the rate at which p-nitroaniline is released as the enzyme hydrolyzes the soluble synthetic substrate, succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (suc-AAPF-pNA). The rate of production of yellow color from the hydrolysis reaction is measured at 410 nm on a spectrophotometer and is proportional to the active enzyme concentration. In addition, absorbance measurements at 280 nanometers (nm) can be used to determine the total protein concentration. The active enzyme/total protein ratio gives the enzyme purity.

As used herein, the term "thermolysin" refers any member of the M4 protease family as described in MEROPS - The Peptidase Data base (See, Rawlings et al., MEROPS: the peptidase database, Nucl Acids Res, 34 Database issue, D270-272 [2006]), of which thermolysin (TLN; EC 3.4.24.27) is the prototype. The amino acid sequence of thermolysin, (EC 3.4.24.27) the neutral metallo endo-peptidase secreted from Bacillus thermoproteolyticus was first reported by Titani et al (Titani et al. (1972), Amino-acid sequence of thermolysin. Nature New Biol, 238:35-37). Subsequently, the gene for this enzyme was cloned by O'Donohue et al (0'Donohue,MJ) (1994) Cloning and expression in Bacillus subtilis of the npr gene from Bacillus thermoproteolyticus Rokko coding for the thermostable metalloprotease thermolysin. Biochem. J, 300:599-603) and the sequence set forth as UniProtKB/Swiss-Prot Accession No. P00800. The only differences between the protein sequences reported by Titani et al and O'Donohue et al are the confirmation of Asn at position 37 (instead of Asp) and Gin at position 119 (instead of Glu). As such the terms "thermolysin," "stearolysin," "bacillolysin," "proteinase-T", "PrT", "Thermolysin-like protease", and "TLPs", are used interchangeably herein to refer to the neutral metalloprotease enzyme of Bacillus thermoproteolyticus.

The terms "amylase" or "amylolytic enzyme" refer to an enzyme that is, among other things, capable of catalyzing the degradation of starch. a-amyloses are hydrolases that cleave the a-D-(1 → 4) O-glycosidic linkages in starch. Generally, a-amyloses (EC 3.2.1.1; a-D-(1 → 4) glucan glucanohydrolase) are defined as endo-acting enzymes cleaving a-D-(1 → 4) O-glycosidic linkages within the starch molecule in a random fashion yielding polysaccharides containing three or more (1-4)-a-linked D-glucose units. In contrast, the exo-acting amylolytic enzymes, such as β-amylases (EC 3.2.1.2; a-D-(1 → 4) glucan maltohydrolase) and some product- specific amylases like maltogenic a-amylose (EC 3.2.1.133) cleave the polysaccharide molecule from the non-reducing end of the substrate, β-amylases, a-glucosidases (EC 3.2.1.20; a-D-glucoside
glucohydrolase), glucoamylase (EC 3.2.1.3; α-D-(1 → 4)-glucan glucohydrolase), and product-specific amylases like the maltotetraosidases (EC 3.2.1.60) and the maltohexaosidases (EC 3.2.1.98) can produce malto-oligosaccharides of a specific length or enriched syrups of specific maltooligosaccharides.

[0035] "Enzyme units" herein refer to the amount of product formed per time under the specified conditions of the assay. For example, a "glucoamylase activity unit" (GAU) is defined as the amount of enzyme that produces 1 g of glucose per hour from soluble starch substrate (4% DS) at 60°C, pH 4.2. A "soluble starch unit" (SSU) is the amount of enzyme that produces 1 mg of glucose per minute from soluble starch substrate (4% DS) at pH 4.5, 50°C. DS refers to "dry solids."

[0036] The term "starch" refers to any material comprised of the complex polysaccharide carbohydrates of plants, comprised of amylase and amylopectin with the formula \((C_6H_{10}O_5)_n\), wherein X can be any number. The term includes plant-based materials such as grains, cereal, grasses, tubers and roots, and more specifically materials obtained from wheat, barley, corn, rye, rice, sorghum, brans, cassava, millet, milo, potato, sweet potato, and tapioca. The term "starch" includes granular starch. The term "granular starch" refers to raw, i.e., uncooked starch, e.g., starch that has not been subject to gelatinization.

[0037] The terms, "wild-type," "parental," or "reference," with respect to a polypeptide, refer to a naturally-occurring polypeptide that does not include a man-made substitution, insertion, or deletion at one or more amino acid positions. Similarly, the terms "wild-type," "parental," or "reference," with respect to a polynucleotide, refer to a naturally-occurring polynucleotide that does not include a man-made nucleoside change. However, note that a polynucleotide encoding a wild-type, parental, or reference polypeptide is not limited to a naturally-occurring polynucleotide, and encompasses any polynucleotide encoding the wild-type, parental, or reference polypeptide.

[0038] Reference to the wild-type polypeptide is understood to include the mature form of the polypeptide. A "mature" polypeptide or variant, thereof, is one in which a signal sequence is absent, for example, cleaved from an immature form of the polypeptide during or following expression of the polypeptide.

[0039] The term "variant," with respect to a polypeptide, refers to a polypeptide that differs from a specified wild-type, parental, or reference polypeptide in that it includes one or more naturally-occurring or man-made substitutions, insertions, or deletions of an amino acid. Similarly, the term "variant," with respect to a polynucleotide, refers to a polynucleotide that differs in nucleotide sequence from a specified wild-type, parental, or reference polynucleotide.
The identity of the wild-type, parental, or reference polypeptide or polynucleotide will be apparent from context.

[0040] In the case of the present a-amylases, "activity" refers to a-amylase activity, which can be measured as described, herein.

[0041] The term "recombinant," when used in reference to a subject cell, nucleic acid, protein or vector, indicates that the subject has been modified from its native state. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell, or express native genes at different levels or under different conditions than found in nature. Recombinant nucleic acids differ from a native sequence by one or more nucleotides and/or are operably linked to heterologous sequences, e.g., a heterologous promoter in an expression vector. Recombinant proteins may differ from a native sequence by one or more amino acids and/or are fused with heterologous sequences. A vector comprising a nucleic acid encoding an amylase is a recombinant vector.

[0042] The terms "recovered," "isolated," and "separated," refer to a compound, protein (polypeptides), cell, nucleic acid, amino acid, or other specified material or component that is removed from at least one other material or component with which it is naturally associated as found in nature. An "isolated" polypeptides, thereof, includes, but is not limited to, a culture broth containing secreted polypeptide expressed in a heterologous host cell.

[0043] The term "purified" refers to material (e.g., an isolated polypeptide or polynucleotide) that is in a relatively pure state, e.g., at least about 90% pure, at least about 95% pure, at least about 98% pure, or even at least about 99% pure.

[0044] The term "enriched" refers to material (e.g., an isolated polypeptide or polynucleotide) that is in about 50% pure, at least about 60% pure, at least about 70% pure, or even at least about 70% pure.

[0045] The terms "thermostable" and "thermostability," with reference to an enzyme, refer to the ability of the enzyme to retain activity after exposure to an elevated temperature. The thermostability of an enzyme, such as an amylase enzyme, is measured by its half-life (t^*) given in minutes, hours, or days, during which half the enzyme activity is lost under defined conditions. The half-life may be calculated by measuring residual a-amylase activity following exposure to (i.e., challenge by) an elevated temperature.

[0046] A "pH range," with reference to an enzyme, refers to the range of pH values under which the enzyme exhibits catalytic activity.
The terms "pH stable" and "pH stability," with reference to an enzyme, relate to the ability of the enzyme to retain activity over a wide range of pH values for a predetermined period of time (e.g., 15 min., 30 min., 1 hour).

The term "amino acid sequence" is synonymous with the terms "polypeptide," "protein," and "peptide," and are used interchangeably. Where such amino acid sequences exhibit activity, they may be referred to as an "enzyme." The conventional one-letter or three-letter codes for amino acid residues are used, with amino acid sequences being presented in the standard amino-to-carboxy terminal orientation (i.e., N→C).

The term "nucleic acid" encompasses DNA, RNA, heteroduplexes, and synthetic molecules capable of encoding a polypeptide. Nucleic acids may be single stranded or double stranded, and may be chemical modifications. The terms "nucleic acid" and "polynucleotide" are used interchangeably. Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and the present compositions and methods encompass nucleotide sequences that encode a particular amino acid sequence. Unless otherwise indicated, nucleic acid sequences are presented in 5'-to-3' orientation.

"Hybridization" refers to the process by which one strand of nucleic acid forms a duplex with, i.e., base pairs with, a complementary strand, as occurs during blot hybridization techniques and PCR techniques. Stringent hybridization conditions are exemplified by hybridization under the following conditions: 65°C and 0.1X SSC (where 1X SSC = 0.15 M NaCl, 0.015 M N\textsubscript{a}\textsubscript{2} citrate, pH 7.0). Hybridized, duplex nucleic acids are characterized by a melting temperature (T\textsubscript{m}), where one half of the hybridized nucleic acids are unpaired with the complementary strand. Mismatched nucleotides within the duplex lower the T\textsubscript{m}.

A "synthetic" molecule is produced by in vitro chemical or enzymatic synthesis rather than by an organism.

The terms "transformed," "stably transformed," and "transgenic," used with reference to a cell means that the cell contains a non-native (e.g., heterologous) nucleic acid sequence integrated into its genome or carried as an episome that is maintained through multiple generations. The non-native sequence may be endogenous sequence.

The term "introduced" in the context of inserting a nucleic acid sequence into a cell, means "transfection", "transformation" or "transduction," as known in the art.

A "host strain" or "host cell" is an organism into which an expression vector, phage, virus, or other DNA construct, including a polynucleotide encoding a polypeptide of interest (e.g., an amylase) has been introduced. Exemplary host strains are microorganism cells (e.g.,
bacteria, filamentous fungi, and yeast) capable of expressing the polypeptide of interest and/or fermenting saccharides. The term "host cell" includes protoplasts created from cells. [0055] The term "heterologous" with reference to a polynucleotide or protein refers to a polynucleotide or protein that does not naturally occur in a host cell.

[0056] The term "endogenous" with reference to a polynucleotide or protein refers to a polynucleotide or protein that occurs naturally in the host cell. [0057] The term "expression" refers to the process by which a polypeptide is produced based on a nucleic acid sequence. The process includes both transcription and translation. [0058] A "selective marker" or "selectable marker" refers to a gene capable of being expressed in a host to facilitate selection of host cells carrying the gene. Examples of selectable markers include but are not limited to antimicrobials (e.g., hygromycin, bleomycin, or chloramphenicol) and/or genes that confer a metabolic advantage, such as a nutritional advantage on the host cell. [0059] A "vector" refers to a polynucleotide sequence designed to introduce nucleic acids into one or more cell types. Vectors include cloning vectors, expression vectors, shuttle vectors, plasmids, phage particles, cassettes and the like. [0060] An "expression vector" refers to a DNA construct comprising a DNA sequence encoding a polypeptide of interest, which coding sequence is operably linked to a suitable control sequence capable of effecting expression of the DNA in a suitable host. Such control sequences may include a promoter to effect transcription, an optional operator sequence to control transcription, a sequence encoding suitable ribosome binding sites on the mRNA, enhancers and sequences which control termination of transcription and translation. [0061] The term "operably linked" means that specified components are in a relationship (including but not limited to juxtaposition) permitting them to function in an intended manner. For example, a regulatory sequence is operably linked to a coding sequence such that expression of the coding sequence is under control of the regulatory sequences. [0062] A "signal sequence" is a sequence of amino acids attached to the N-terminal portion of a protein, which facilitates the secretion of the protein outside the cell. The mature form of an extracellular protein lacks the signal sequence, which is cleaved off during the secretion process. [0063] "Biologically active" refer to a sequence having a specified biological activity, such an enzymatic activity. [0064] The term "specific activity" refers to the number of moles of substrate that can be converted to product by an enzyme or enzyme preparation per unit time under specific conditions. Specific activity is generally expressed as units (U)/mg of protein.
As used herein, "water hardness" is a measure of the minerals (e.g., calcium and magnesium) present in water.

As used herein, an "effective amount of amylase," or similar expressions, refers to an amount of amylase sufficient to produce a visible, or otherwise measurable amount of starch hydrolysis in an particular application. Starch hydrolysis may result in, e.g., a visible cleaning of fabrics or dishware, reduced viscosity of a starch slurry or mash, and the like.

A "swatch" is a piece of material such as a fabric that has a stain applied thereto. The material can be, for example, fabrics made of cotton, polyester or mixtures of natural and synthetic fibers. The swatch can further be paper, such as filter paper or nitrocellulose, or a piece of a hard material such as ceramic, metal, or glass. For amylases, the stain is starch based, but can include blood, milk, ink, grass, tea, wine, spinach, gravy, chocolate, egg, cheese, clay, pigment, oil, or mixtures of these compounds.

A "smaller swatch" is a section of the swatch that has been cut with a single hole punch device, or has been cut with a custom manufactured 96-hole punch device, where the pattern of the multi-hole punch is matched to standard 96-well microtiter plates, or the section has been otherwise removed from the swatch. The swatch can be of textile, paper, metal, or other suitable material. The smaller swatch can have the stain affixed either before or after it is placed into the well of a 24-, 48- or 96-well microtiter plate. The smaller swatch can also be made by applying a stain to a small piece of material. For example, the smaller swatch can be a stained piece of fabric 5/8" or 0.25" in diameter. The custom manufactured punch is designed in such a manner that it delivers 96 swatches simultaneously to all wells of a 96-well plate. The device allows delivery of more than one swatch per well by simply loading the same 96-well plate multiple times. Multi-hole punch devices can be conceived of to deliver simultaneously swatches to any format plate, including but not limited to 24-well, 48-well, and 96-well plates. In another conceivable method, the soiled test platform can be a bead made of metal, plastic, glass, ceramic, or another suitable material that is coated with the soil substrate. The one or more coated beads are then placed into wells of 96-, 48-, or 24-well plates or larger formats, containing suitable buffer and enzyme.

"A cultured cell material comprising an amylase" or similar language, refers to a cell lysate or supernatant (including media) that includes an amylase as a component. The cell material may be from a heterologous host that is grown in culture for the purpose of producing the amylase.

"Percent sequence identity" means that a particular sequence has at least a certain percentage of amino acid residues identical to those in a specified reference sequence, when
aligned using the CLUSTAL W algorithm with default parameters. See Thompson et al. (1994) Nucleic Acids Res. 22:4673-4680. Default parameters for the CLUSTAL W algorithm are: Gap opening penalty: 10.0; Gap extension penalty: 0.05; Protein weight matrix: BLOSUM series; DNA weight matrix: IUB; Delay divergent sequences %:40; Gap separation distance: 8; DNA transitions weight: 0.50; List hydrophilic residues: G, P, S, N, D, Q, E, K, R; Use negative matrix: OFF; Toggle Residue specific penalties: ON; Toggle hydrophilic penalties: ON; Toggle end gap separation penalty OFF.

Deletions are counted as non-identical residues, compared to a reference sequence. Deletions occurring at either terminus are included. For example, a variant 500-amino acid residue polypeptide with a deletion of five amino acid residues from the C-terminus would have a percent sequence identity of 99% (495/500 identical residues x 100) relative to the parent polypeptide. Such a variant would be encompassed by the language, "a variant having at least 99% sequence identity to the parent."

"Fused" polypeptide sequences are connected, i.e., operably linked, via a peptide bond between two subject polypeptide sequences.

The term "filamentous fungi" refers to all filamentous forms of the subdivision Eumycotina, particular species within Pezizomycotina subdivision.

The term "degree of polymerization" (DP) refers to the number (n) of anhydro-glucopyranose units in a given saccharide. Examples of DPI are the monosaccharides glucose and fructose. Examples of DP2 are the disaccharides maltose and sucrose. The term "DE," or "dextrose equivalent," is defined as the percentage of reducing sugar, i.e., D-glucose, as a fraction of total carbohydrate in a syrup.

The term "dry solids content" (ds) refers to the total solids of a slurry in a dry weight percent basis. The term "slurry" refers to an aqueous mixture containing insoluble solids.

The phrase "simultaneous saccharification and fermentation (SSF)" refers to a process in the production of biochemicals in which a microbial organism, such as an ethanologenic microorganism, and at least one enzyme, such as an amylase, are present during the same process step. SSF includes the contemporaneous hydrolysis of starch substrates (granular, liquefied, or solubilized) to saccharides, including glucose, and the fermentation of the saccharides into alcohol or other biochemical or biomaterial in the same reactor vessel.

An "ethanologenic or fermenting microorganism" refers to a microorganism with the ability to convert a sugar or oligosaccharide to ethanol.

The term "fermented beverage" refers to any beverage produced by a method comprising a fermentation process, such as a microbial fermentation, e.g., a bacterial and/or fungal
fermentation. "Beer" is an example of such a fermented beverage, and the term "beer" is meant to comprise any fermented wort produced by fermentation/brewing of a starch-containing plant material. Often, beer is produced exclusively from malt or adjunct, or any combination of malt and adjunct.

[0079] The term "malt" refers to any malted cereal grain, such as malted barley or wheat.

[0080] The term "adjunct" refers to any starch and/or sugar containing plant material that is not malt, such as barley or wheat malt. Examples of adjuncts include common corn grits, refined corn grits, brewer's milled yeast, rice, sorghum, refined corn starch, barley, barley starch, dehusked barley, wheat, wheat starch, torrifled cereal, cereal flakes, rye, oats, potato, tapioca, cassava and syrups, such as corn syrup, sugar cane syrup, inverted sugar syrup, barley and/or wheat syrups, and the like.

[0081] The term "mash" refers to aqueous slurry of any starch and/or sugar containing plant material, such as grist, e.g., comprising crushed barley malt, crushed barley, and/or other adjunct or a combination thereof, mixed with water later to be separated into wort and spent grains.

[0082] The term "wort" refers to the unfermented liquor run-off following extracting the grist during mashing.

[0083] "Iodine-positive starch" or "IPS" refers to (1) amylose that is not hydrolyzed after liquefaction and saccharification, or (2) a retrograded starch polymer. When saccharified starch or saccharide liquor is tested with iodine, the high DPn amylose or the retrograded starch polymer binds iodine and produces a characteristic blue color. The saccharide liquor is thus termed "iodine-positive saccharide," "blue saccharide," or "blue sac."

[0084] The terms "retrograded starch" or "starch retrogradation" refer to changes that occur spontaneously in a starch paste or gel on ageing.

[0085] The term "about" refers to ± 15% to the referenced value.

2. Enzymes of the Invention

Proteases

[0086] Proteases are currently classified into six broad groups: Serine proteases, Threonine proteases, Cysteine proteases, Aspartate proteases, Glutamic proteases, Metalloproteases. The mechanism used to cleave a peptide bond involves making an amino acid residue that has the cysteine and threonine (proteases) or a water molecule (aspartic acid, metallo- and glutamic acid proteases) nucleophilic so that it can attack the peptide carboxyl group. One way to make a nucleophile is by a catalytic triad, where a histidine residue is used to activate serine, cysteine, or threonine as a nucleophile.

[0087] Proteases of the invention may be wild type proteases or a variant thereof. Any suitable
protease may be adapted to perform in the methods of the invention.

[0088] In another aspect of the invention, the protease is a metalloprotease. In another aspect, the metalloprotease is a thermolysin or Proteinase T. In another aspect, the protease is a variant thermolysin with one or more substitutions. In another aspect, the protease is NprE. In another aspect, the protease is an NprE varaint with one or more substitutions. These substitutions of thermolysin and/or NprE are disclosed in various patent applications, for example, WO 2007/044993, WO 2009/058679, WO 2009/058661, WO 2009/058518, WO 2009/058303, and US provisional application 61/722,660 filed on 05 November 2012, all of which are herein incorporated by reference in their entirety.

[0089] In some embodiments, the present enzymes have a defined degree of amino acid sequence identity to other enzymes, for example, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99%, amino acid sequence identity. In some embodiments, the present enzymes are derived from a parental enzyme having a defined degree of amino acid sequence identity, for example, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99%, amino acid sequence identity.

[0090] In some embodiments, the present enzymes comprise conservative substitution of one or several amino acid residues relative to the amino acid sequence of a parent enzyme. Exemplary conservative amino acid substitutions are listed in the Table 1. Some conservative mutations can be produced by genetic manipulation, while others are produced by introducing synthetic amino acids into a polypeptide other means.

Table 1. Conservative amino acid substitutions

<table>
<thead>
<tr>
<th>For Amino Acid</th>
<th>Code</th>
<th>Replace with any of</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>A</td>
<td>D-Ala, Gly, beta-Ala, L-Cys, D-Cys</td>
</tr>
<tr>
<td>Arginine</td>
<td>R</td>
<td>D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn</td>
</tr>
<tr>
<td>Asparagine</td>
<td>N</td>
<td>D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>D</td>
<td>D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln</td>
</tr>
<tr>
<td>Cysteine</td>
<td>C</td>
<td>D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Q</td>
<td>D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>E</td>
<td>D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln</td>
</tr>
<tr>
<td>Glycine</td>
<td>G</td>
<td>Ala, D-Ala, Pro, D-Pro, b-Ala, Acp</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>I</td>
<td>D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met</td>
</tr>
<tr>
<td>Leucine</td>
<td>L</td>
<td>D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met</td>
</tr>
<tr>
<td>Lysine</td>
<td>K</td>
<td>D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn</td>
</tr>
<tr>
<td>Methionine</td>
<td>M</td>
<td>D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>F</td>
<td>D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, cis-3,4, or 5-phenylproline</td>
</tr>
</tbody>
</table>
In some embodiments, the present enzymes comprises a deletion, substitution, insertion, or addition of one or a few amino acid residues. In some embodiments, the present enzymes are derived from the amino acid sequence of the parent enzyme by conservative substitution of one or several amino acid residues. In some embodiments, the present enzymes are derived from the amino acid sequence of the parent enzyme by deletion, substitution, insertion, or addition of one or a few amino acid residues. In all cases, the expression "one or a few amino acid residues" refers to 10 or less, i.e., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, amino acid residues.

In some embodiments, the present enzymes are encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid sequence that is complementary to a nucleic acid that encodes the parent enzyme.

The present enzymes may be "precursor," "immature," or "full-length," in which case they include a signal sequence, or "mature," in which case they lack a signal sequence. Mature forms of the polypeptides are generally the most useful. Unless otherwise noted, the amino acid residue numbering used herein refers to the mature forms of the respective amylase polypeptides. The present amylase polypeptides may also be truncated to remove the N or C-termini, so long as the resulting polypeptides retain enzyme activity.

The present enzyme may be a "chimeric" or "hybrid" polypeptide, in that it includes at least a portion of a first enzyme polypeptide, and at least a portion of a second enzyme polypeptide (such chimeric enzymes have recently been "rediscovered" as domain-swap enzymes). The present enzymes may further include heterologous signal sequence, an epitope to allow tracking or purification, or the like. Exemplary heterologous signal sequences are from *B. licheniformis* amylase (LAT), *B. subtilis* (AmyE or AprE), and *Streptomyces CelA*.

In another aspect, nucleic acids encoding an enzyme polypeptide is provided. The nucleic acid may encode the enzyme having the amino acid sequence having a specified degree of amino acid sequence identity. In some embodiments, the nucleic acid encodes an enzyme having at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least

<table>
<thead>
<tr>
<th>For Amino Acid</th>
<th>Code</th>
<th>Replace with any of</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proline</td>
<td>P</td>
<td>D-Pro, L-I-thioazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid</td>
</tr>
<tr>
<td>Serine</td>
<td>S</td>
<td>D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys</td>
</tr>
<tr>
<td>Threonine</td>
<td>T</td>
<td>D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Y</td>
<td>D-Tyr, Phe, D-Phe, L-Dopa, His, D-His</td>
</tr>
<tr>
<td>Valine</td>
<td>V</td>
<td>D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met</td>
</tr>
</tbody>
</table>
93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99%, amino acid sequence identity to a parental enzyme. In some embodiments, the nucleic acid has at least 80%, at least 85%, at least 90%, at least 95%, or even at least 98% nucleotide sequence identity to parental enzyme.

[0096] In some embodiments, the present compositions and methods include nucleic acids that encode an enzyme having deletions, insertions, or substitutions, such as those mentioned, above. It will be appreciated that due to the degeneracy of the genetic code, a plurality of nucleic acids may encode the same polypeptide.

[0097] In another example, the nucleic acid hybridizes under stringent or very stringent conditions to a nucleic acid complementary to a nucleic acid encoding an enzyme having at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99%, amino acid sequence identity to a parent enzyme. In some embodiments, the nucleic acid hybridizes under stringent or very stringent conditions to a nucleic acid complementary to a nucleic acid having the sequence of a parent enzyme. Such hybridization conditions are described herein but are also well known in the art.

[0098] Nucleic acids may encode a "full-length" ("fl" or "FL") amylase, which includes a signal sequence, only the mature form of an enzyme, which lacks the signal sequence, or a truncated form of an enzyme, which lacks the N or C-terminus of the mature form. Preferably, the nucleic acids are of sufficient length to encode an active enzyme.

[0099] A nucleic acid that encodes an enzyme can be operably linked to various promoters and regulators in a vector suitable for expressing the enzyme in host cells. Exemplary promoters are from *B. licheniformis* amylase (LAT), *B. subtilis* (AmyE or AprE), and *Streptomyces* CelA. Such a nucleic acid can also be linked to other coding sequences, *e.g.*, to encode a chimeric polypeptide.

3. **Production of Enzymes of the Invention**

[00100] The present enzymes can be produced in host cells, for example, by secretion or intracellular expression. A cultured cell material *(e.g., a whole-cell broth)* comprising an enzyme can be obtained following secretion of the enzyme into the cell medium. Optionally, the enzyme can be isolated from the host cells, or even isolated from the cell broth, depending on the desired purity of the final enzyme. A gene encoding an enzyme can be cloned and expressed according to methods well known in the art. Suitable host cells include bacterial, fungal (including yeast and filamentous fungi), and plant cells (including algae). Particularly useful
host cells include *Aspergillus niger*, *Aspergillus oryzae* or *Trichoderma reesei*. Other host cells include bacterial cells, *e.g.*, *Bacillus subtilis* or *B. licheniformis*, as well as *Streptomyces*.

The host cell further may express a nucleic acid encoding a homologous or heterologous glucoamylase, *i.e.*, a glucoamylase that is not the same species as the host cell, or one or more other enzymes. The glucoamylase may be a variant glucoamylase, such as one of the glucoamylase variants disclosed in U.S. Patent No. 8,058,033 (Danisco US Inc.), for example. Additionally, the host may express one or more accessory enzymes, proteins, peptides. These may benefit liquefaction, saccharification, fermentation, SSF, etc., processes. Furthermore, the host cell may produce biochemicals in addition to enzymes used to digest the various feedstock(s). Such host cells may be useful for fermentation or simultaneous saccharification and fermentation processes to reduce or eliminate the need to add enzymes.

3.1. Vectors

A DNA construct comprising a nucleic acid encoding an enzyme can be constructed to be expressed in a host cell. Because of the well-known degeneracy in the genetic code, different polynucleotides that encode an identical amino acid sequence can be designed and made with routine skill. It is also well-known in the art to optimize codon use for a particular host cell. Nucleic acids encoding enzymes can be incorporated into a vector. Vectors can be transferred to a host cell using well-known transformation techniques, such as those disclosed below.

The vector may be any vector that can be transformed into and replicated within a host cell. For example, a vector comprising a nucleic acid encoding an enzyme can be transformed and replicated in a bacterial host cell as a means of propagating and amplifying the vector. The vector also may be transformed into an expression host, so that the encoding nucleic acids can be expressed as a functional enzyme. Host cells that serve as expression hosts can include filamentous fungi, for example. The Fungal Genetics Stock Center (FGSC) Catalogue of Strains lists suitable vectors for expression in fungal host cells. See FGSC, Catalogue of Strains, University of Missouri, at www fgsc net (last modified January 17, 2007). A representative vector is pJG153, a promoterless Cre expression vector that can be replicated in a bacterial host. See Harrison et al. (2011) *Applied Environ. Microbiol.* 77:3916-22. pJG153 can be modified with routine skill to comprise and express a nucleic acid encoding an enzyme variant.

A nucleic acid encoding an enzyme can be operably linked to a suitable promoter, which allows transcription in the host cell. The promoter may be any DNA sequence that shows transcriptional activity in the host cell of choice and may be derived from genes
encoding proteins either homologous or heterologous to the host cell. Exemplary promoters for directing the transcription of the DNA sequence encoding an enzyme, especially in a bacterial host, are the promoter of the lac operon of *E. coli*, the *Streptomyces coelicolor* agarase gene dagA or celA promoters, the promoters of the *Bacillus licheniformis* a-amylase gene (amyL), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (amyM), the promoters of the *Bacillus amyloliquefaciens* α-amylase (amyQ), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral a-amylase, *A. niger* acid stable a-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase, or *A. nidulans* acetamidase. When a gene encoding an enzyme is expressed in a bacterial species such as *E. coli*, a suitable promoter can be selected, for example, from a bacteriophage promoter including a T7 promoter and a phage lambda promoter. Examples of suitable promoters for the expression in a yeast species include but are not limited to the Gal 1 and Gal 10 promoters of *Saccharomyces cerevisiae* and the *Pichia pastoris* AOX1 or AOX2 promoters, *cbhl* is an endogenous, inducible promoter from *T. reesei*. See Liu et al. (2008) "Improved heterologous gene expression in *Trichoderma reesei* by cellobiohydrolase I gene (cbhl) promoter optimization," *Acta Biochim. Biophys. Sin (Shanghai)* 40(2): 158-65.

[00105] The coding sequence can be operably linked to a signal sequence. The DNA encoding the signal sequence may be the DNA sequence naturally associated with the enzyme gene to be expressed or from a different Genus or species. A signal sequence and a promoter sequence comprising a DNA construct or vector can be introduced into a fungal host cell and can be derived from the same source. For example, the signal sequence is the *cbhl* signal sequence that is operably linked to a *cbhl* promoter.

[00106] An expression vector may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably linked to the DNA sequence encoding an enzyme. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

[00107] The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell. Examples of such sequences are the origins of replication of plasmids pUC19, pACYCIW, pUBHO, pE194, pAMBI, and pIJ702.

[00108] The vector may also comprise a selectable marker, e.g., a gene the product of which complements a defect in the isolated host cell, such as the *dal* genes from *B. subtilis* or *B.
licheniformis, or a gene that confers antibiotic resistance such as, e.g., ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Furthermore, the vector may comprise Aspergillus selection markers such as amdS, argB, niaD and xxsC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, such as known in the art.

See e.g., International PCT Application WO 91/17243.

[00109] Intracellular expression may be advantageous in some respects, e.g., when using certain bacteria or fungi as host cells to produce large amounts of enzyme for subsequent enrichment or purification. Extracellular secretion of enzyme into the culture medium can also be used to make a cultured cell material comprising the isolated enzyme.

[00110] The expression vector typically includes the components of a cloning vector, such as, for example, an element that permits autonomous replication of the vector in the selected host organism and one or more phenotypically detectable markers for selection purposes. The expression vector normally comprises control nucleotide sequences such as a promoter, operator, ribosome binding site, translation initiation signal and optionally, a repressor gene or one or more activator genes. Additionally, the expression vector may comprise a sequence coding for an amino acid sequence capable of targeting the enzyme to a host cell organelle such as a peroxisome, or to a particular host cell compartment. Such a targeting sequence includes but is not limited to the sequence, SKL. For expression under the direction of control sequences, the nucleic acid sequence of the enzyme is operably linked to the control sequences in proper manner with respect to expression.

[00111] The procedures used to ligate the DNA construct encoding an enzyme, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (see, e.g., Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, 2nd ed., Cold Spring Harbor, 1989, and 3rd ed., 2001).

3.2. Transformation and Culture of Host Cells

[00112] An isolated cell, either comprising a DNA construct or an expression vector, is advantageously used as a host cell in the recombinant production of an enzyme. The cell may be transformed with the DNA construct encoding the enzyme, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage, as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g., by homologous or heterologous recombination.
Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

Examples of suitable bacterial host organisms are Gram positive bacterial species such as *Bacillaceae* including *Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Geobacillus* (formerly *Bacillus*) *stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus lautus, Bacillus megaterium*, and *Bacillus thuringiensis*; *Streptomyces* species such as *Streptomyces murinus*; lactic acid bacterial species including *Lactococcus* sp. such as *Lactococcus lactis; Lactobacillus* sp. including *Lactobacillus reuteri; Leuconostoc* sp.; *Pediococcus* sp.; and *Streptococcus* sp. Alternatively, strains of a Gram negative bacterial species belonging to *Enterobacteriaceae* including *E. coli*, or to *Pseudomonadaceae* can be selected as the host organism.

A suitable yeast host organism can be selected from the biotechnologically relevant yeasts species such as but not limited to yeast species such as *Pichia* sp., *Hansenula* sp., or *Kluyveromyces, Yarrowinia, Schizosaccharomyces* species or a species of *Saccharomyces*, including *Saccharomyces cerevisiae* or a species belonging to *Schizosaccharomyces* such as, for example, *S. pombe* species. A strain of the methylotrophic yeast species, *Pichia pastoris*, can be used as the host organism. Alternatively, the host organism can be a *Hansenula* species. Suitable host organisms among filamentous fungi include species of *Aspergillus*, e.g., *Aspergillus niger, Aspergillus oryzae, Aspergillus tubigensis, Aspergillus awamori*, or *Aspergillus nidulans*. Alternatively, strains of a *Fusarium* species, e.g., *Fusarium oxysporum* or of a *Rhizomucor* species such as *Rhizomucor miehei* can be used as the host organism. Other suitable strains include * Thermomyces* and *Mucor* species. In addition, *Trichoderma* sp., such as *T. reesei* can be used as a host. A suitable procedure for transformation of *Aspergillus* host cells includes, for example, that described in EP 238023. An enzyme expressed by a fungal host cell can be glycosylated, *i.e.*, will comprise a glycosyl moiety. The glycosylation pattern can be the same or different as present in the wild-type enzyme. The type and/or degree of glycosylation may impart changes in enzymatic and/or biochemical properties.

It may be advantageous to delete genes from expression hosts, where the gene deficiency may be cured by the transformed expression vector. Known methods may be used to obtain a fungal host cell having one or more inactivated genes. 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. Any gene from a *Trichoderma* sp. or other filamentous fungal host that has been cloned can be deleted, for example, *cbhl, cbh2, egll*, and *egl2* genes.
Gene deletion may be accomplished by inserting a form of the desired gene to be inactivated into a plasmid by methods known in the art.

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., Sambrook et al. (2001), supra. The expression of heterologous protein in Trichoderma is described, for example, in U.S. Patent No. 6,022,725. Reference is also made to Cao et al. (2000) Science 9:991-1001 for transformation of Aspergillus strains. Genetically stable transformants can be constructed with vector systems whereby the nucleic acid encoding an enzyme is stably integrated into a host cell chromosome. Transformants are then selected and purified by known techniques.

The preparation of Trichoderma sp. for transformation, for example, may involve the preparation of protoplasts from fungal mycelia. See Campbell et al. (1989) Curr. Genet. 16: 53-56. The mycelia can be obtained from germinated vegetative spores. The mycelia are treated with an enzyme that digests the cell wall, resulting in protoplasts. The protoplasts are protected by the presence of an osmotic stabilizer in the suspending medium. These stabilizers include sorbitol, mannitol, potassium chloride, magnesium sulfate, and the like. Usually the concentration of these stabilizers varies between 0.8 M and 1.2 M, e.g., a 1.2 M solution of sorbitol can be used in the suspension medium.

Uptake of DNA into the host Trichoderma sp. strain depends upon the calcium ion concentration. Generally, between about 10-50 mM CaCl₂ is used in an uptake solution. Additional suitable compounds include a buffering system, such as TE buffer (10 mM Tris, pH 7.4; 1 mM EDTA) or 10 mM MOPS, pH 6.0 and polyethylene glycol. The polyethylene glycol is believed to fuse the cell membranes, thus permitting the contents of the medium to be delivered into the cytoplasm of the Trichoderma sp. strain. This fusion frequently leaves multiple copies of the plasmid DNA integrated into the host chromosome.

Usually transformation of Trichoderma sp. uses protoplasts or cells that have been subjected to a permeability treatment, typically at a density of 10⁵ to 10⁷/mL, particularly 2×10⁶/mL. A volume of 100 μL of these protoplasts or cells in an appropriate solution (e.g., 1.2 M sorbitol and 50 mM CaCl₂) may be mixed with the desired DNA. Generally, a high concentration of PEG is added to the uptake solution. From 0.1 to 1 volume of 25% PEG 4000 can be added to the protoplast suspension; however, it is useful 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 to facilitate transformation.

Similar procedures are available for other fungal host cells. See, e.g., U.S. Patent No. 6,022,725.

3.3. Expression

[00120] A method of producing an enzyme may comprise cultivating a host cell as described above under conditions conducive to the production of the enzyme and recovering the enzyme from the cells and/or culture medium.

[00121] The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of an enzyme. Suitable media and media components are available from commercial suppliers or may be prepared according to published recipes (e.g., as described in catalogues of the American Type Culture Collection).

[00122] An enzyme secreted from the host cells can be used in a whole broth preparation. In the present methods, the preparation of a spent whole fermentation broth of a recombinant microorganism can be achieved using any cultivation method known in the art resulting in the expression of an enzyme. Fermentation may, therefore, be understood as comprising shake flask cultivation, small- or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the enzyme to be expressed or isolated. The term "spent whole fermentation broth" is defined herein as unfractionated contents of fermentation material that includes culture medium, extracellular proteins (e.g., enzymes), and cellular biomass. It is understood that the term "spent whole fermentation broth" also encompasses cellular biomass that has been lysed or permeabilized using methods well known in the art.

[00123] An enzyme secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulfate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

[00124] The polynucleotide encoding an enzyme in a vector can be operably linked to a control sequence that is capable of providing for expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The control sequences may be modified, for example by the addition of further transcriptional regulatory elements to make the level of transcription directed by the control sequences more responsive to transcriptional modulators. The control sequences may in particular comprise promoters.
Host cells may be cultured under suitable conditions that allow expression of an enzyme. Expression of the enzymes may be constitutive such that they are continually produced, or inducible, requiring a stimulus to initiate expression. In the case of inducible expression, protein production can be initiated when required by, for example, addition of an inducer substance to the culture medium, for example dexamethasone or IPTG or Sophorose. Polypeptides can also be produced recombinantly in an *in vitro* cell-free system, such as the TNT™ (Promega) rabbit reticulocyte system.

An expression host also can be cultured in the appropriate medium for the host, under aerobic conditions. Shaking or a combination of agitation and aeration can be provided, with production occurring at the appropriate temperature for that host, *e.g.*, from about 25°C to about 75°C (*e.g.*, 30°C to 45°C), depending on the needs of the host and production of the desired enzyme. Culturing can occur from about 12 to about 100 hours or greater (and any hour value there between, *e.g.*, from 24 to 72 hours). Typically, the culture broth is at a pH of about 4.0 to about 8.0, again depending on the culture conditions needed for the host relative to production of an enzyme.

3.4. Identification of Enzyme Activity

To evaluate the expression of an enzyme in a host cell, assays can measure the expressed protein, corresponding mRNA, or enzyme activity. For example, suitable assays include Northern blotting, reverse transcriptase polymerase chain reaction, and *in situ* hybridization, using an appropriately labeled hybridizing probe. Suitable assays also include measuring enzyme activity in a sample, for example, by assays directly measuring products in the culture media. For example, glucose concentration may be determined using glucose reagent kit No. 15-UV (Sigma Chemical Co.) or an instrument, such as Technicon Autoanalyzer. α-Amylase activity also may be measured by any known method, such as the PAHBAH or ABTS assays, described below. Assays are also known in the art to measure other enzyme activities.

3.5. Methods for Enriching and Purifying Enzymes of the Invention

Fermentation, separation, and concentration techniques are well known in the art and conventional methods can be used in order to prepare a concentrated enzyme polypeptide-containing solution.

After fermentation, a fermentation broth is obtained, the microbial cells and various suspended solids, including residual raw fermentation materials, are removed by conventional separation techniques in order to obtain an enzyme solution. Filtration,
centrifugation, microfiltration, rotary vacuum drum filtration, ultrafiltration, centrifugation followed by ultrafiltration, extraction, or chromatography, or the like, are generally used. 

[00130] It is desirable to concentrate an enzyme polypeptide-containing solution in order to optimize recovery. Use of unconcentrated solutions requires increased incubation time in order to collect the enriched or purified enzyme precipitate.

[00131] The enzyme containing solution is concentrated using conventional concentration techniques until the desired enzyme level is obtained. Concentration of the enzyme containing solution may be achieved by any of the techniques discussed herein. Exemplary methods of enrichment and purification include but are not limited to rotary vacuum filtration and/or ultrafiltration.

[00132] The enzyme solution is concentrated into a concentrated enzyme solution until the enzyme activity of the concentrated enzyme polypeptide-containing solution is at a desired level.

[00133] During fermentation, an enzyme polypeptide accumulates in the culture broth. For the isolation, enrichment, or purification of the desired enzyme, the culture broth is centrifuged or filtered to eliminate cells, and the resulting cell-free liquid is used for enzyme enrichment or purification. In one embodiment, the cell-free broth is subjected to salting out using ammonium sulfate at about 70% saturation; the 70% saturation-precipitation fraction is then dissolved in a buffer and applied to a column such as a Sephadex G-100 column, and eluted to recover the enzyme-active fraction. For further enrichment or purification, a conventional procedure such as ion exchange or affinity chromatography may be used.

[00134] For production scale recovery, enzyme polypeptides can be enriched or partially purified as generally described above by removing cells via flocculation with polymers. Alternatively, the enzyme can be enriched or purified by microfiltration followed by concentration by ultrafiltration using available membranes and equipment. However, for some applications, the enzyme does not need to be enriched or purified, and whole broth culture can be lysed and used without further treatment. The enzyme can then be processed, for example, into granules.

4. Compositions and Uses of Proteases

[00135] The present proteases are useful for a variety of industrial applications. For example, proteases are useful in a starch conversion process, particularly in a saccharification process of a starch that has undergone liquefaction. The desired end-product may be any product that may be produced by the enzymatic conversion of the starch substrate. For example, the desired product may be a syrup rich in glucose and maltose, which can be used in other
processes, such as the preparation of HFCS, or which can be converted into a number of other useful products, such as ascorbic acid intermediates (e.g., gluconate; 2-keto-L-gulonic acid; 5-keto-gluconate; and 2,5-diketogluconate); ethanol, butanol, 1,3-propanediol; aromatic amino acids (e.g., tyrosine, phenylalanine and tryptophan); organic acids (e.g., lactate, pyruvate, succinate, citrate, isocitrate, gluconic acid, and oxaloacetate); amino acids (e.g., serine, lysine, glutamic acid, and glycine); antibiotics; antimicrobials; enzymes; vitamins; and hormones. The starch conversion process may be a precursor to, or simultaneous with, a fermentation process designed to produce alcohol for fuel or drinking (i.e., potable alcohol). One skilled in the art is aware of various fermentation conditions that may be used in the production of these end-products. Proteases are also useful in compositions and methods of food preparation. These various uses of proteases are described in more detail below.

4.1. Preparation of Starch Substrates

Those of general 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 may be obtained from corn, cobs, wheat, barley, rye, triticale, milo, sago, millet, 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 70-72% starch. Specifically contemplated starch substrates are corn starch and wheat 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 also be highly refined raw starch or feedstock from starch refinery processes. Various starches also are commercially available. For example, corn starch is available from Cerestar, Sigma, and Katayama Chemical Industry Co. (Japan); wheat starch is available from Sigma; sweet potato starch is available from Wako Pure Chemical Industry Co. (Japan); and potato starch is available from Nakaari Chemical Pharmaceutical Co. (Japan).

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 or grinding. In wet milling, whole grain is 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 is especially suitable for production of syrups. In dry milling or grinding, whole kernels are ground into a fine powder and often processed without fractionating the grain into its component parts. In some cases, oils from the kernels are recovered. Dry ground grain thus will comprise...
significant amounts of non-starch carbohydrate compounds, in addition to starch. Dry grinding
of the starch substrate can be used for production of ethanol and other biochemicals. The starch
to be processed may be a highly refined starch quality, for example, at least 90%, at least 95%,
at least 97%, or at least 99.5% pure.

4.2. Gelatinization and Liquefaction of Starch

As used herein, the term "liquefaction" or "liquefy" means a process by which
starch is converted to less viscous and shorter chain dextrins. Generally, this process involves
gelatinization of starch simultaneously with or followed by the addition of an a-amylase,
although additional liquefaction-inducing enzymes optionally may be added. In some
embodiments, the starch substrate prepared as described above is slurred 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%. a-Amylase (EC 3.2.1.1) may be added to the
slurry, with a metering pump, for example. The a-amylase typically used for this application is
a thermally stable, bacterial a-amylase, such as a Geobacillus stearothermophilus a-amylase.
The a-amylase is usually supplied, for example, at about 1500 units per kg dry matter of starch.
To optimize a-amylase stability and activity, the pH of the slurry typically is adjusted to about
pH 5.5-6.5 and about 1 mM of calcium (about 40 ppm free calcium ions) can also be added.
Geobacillus stearothermophilus variants or other a-amylases may require different conditions.
Bacterial a-amylase remaining in the slurry following liquefaction may be deactivated via a
number of methods, including lowering the pH in a subsequent reaction step or by removing
calcium from the slurry in cases where the enzyme is dependent upon calcium.

The slurry of starch plus the a-amylase may be pumped continuously through a
jet cooker, which is steam heated to 105°C. Gelatinization occurs 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 is brief. The partly
gelatinized starch may be passed into a series of holding tubes maintained at 105-1 10°C and
held for 5-8 min. to complete the gelatinization process ("primary liquefaction"). Hydrolysis to
the required DE is completed in holding tanks at 85-95°C or higher temperatures for about 1 to 2
hours ("secondary liquefaction"). These tanks may contain baffles to discourage back mixing.
As used herein, the term "minutes of secondary liquefaction" refers to the time that has elapsed
from the start of secondary liquefaction to the time that the Dextrose Equivalent (DE) is
measured. The slurry is then allowed to cool to room temperature. This cooling step can be 30
minutes to 180 minutes, or more. The liquefied starch typically is in the form of a slurry having
a dry solids content (w/w) of about 10-50%; about 10-45%; about 15-40%; about 20-40%; about 25-40%; or about 25-35%.

[00141] Liquefaction with amylases advantageously can be conducted at low pH, eliminating the requirement to adjust the pH to about pH 5.5-6.5. Proteases can be used for liquefaction at a pH range of 2 to 7, e.g., pH 3.0 - 7.5, pH 4.0 - 6.0, or pH 4.5 - 5.8. Amylases can maintain liquefying activity at a temperature range of about 85°C - 95°C, e.g., 85°C, 90°C, or 95°C. For example, liquefaction can be conducted with an amylase in a solution of 25% DS corn starch for 10 min at pH 5.8 and 85°C, or pH 4.5 and 95°C, for example. Liquefying activity can be assayed using any of a number of known viscosity assays in the art.

[00142] In particular embodiments, starch liquefaction is performed at a temperature range of 90-115°C, for the purpose of producing high-purity glucose syrups, HFCS, maltodextrins, etc.

4.3. Saccharification

[00143] The liquefied starch can be saccharified into a syrup rich in lower DP (e.g., DPI + DP2) saccharides, using α-amylases, optionally in the presence of other enzyme(s). The exact composition of the products of saccharification depends on the combination of enzymes used, as well as the type of granular starch processed. Advantageously, the syrup may contain a weight percent of DP2 of the total oligosaccharides in the saccharified starch exceeding 30%, e.g., 45% - 65% or 55% - 65%. The weight percent of (DPI + DP2) in the saccharified starch may exceed about 70%, e.g., 75% - 85% or 80% - 85%. The present amylases also produce a relatively high yield of glucose, e.g., DPI > 20%, in the syrup product.

[00144] Whereas liquefaction is generally run as a continuous process, saccharification is often conducted as a batch process. Saccharification typically is most effective at temperatures of about 60-65°C and a pH of about 4.0-4.5, e.g., pH 4.3, necessitating cooling and adjusting the pH of the liquefied starch. Saccharification may be performed, for example, at a temperature between about 40°C, about 50°C, or about 55°C to about 60°C or about 65°C. Saccharification is normally conducted in stirred tanks, which may take several hours to fill or empty. Enzymes typically are added either at a fixed ratio to dried solids as the tanks are filled or added as a single dose at the commencement of the filling stage. A saccharification reaction to make a syrup typically is run over about 24-72 hours, for example, 24-48 hours. When a maximum or desired DE has been attained, the reaction is stopped by heating to 85°C for 5 min., for example. Further incubation will result in a lower DE, eventually to about 90 DE, as accumulated glucose re-polymerizes to isomaltose and/or other reversion products via an enzymatic reversion reaction and/or with the approach of thermodynamic equilibrium. When using an amylase,
saccharification optimally is conducted at a temperature range of about 30°C to about 75°C, e.g., 45°C - 75°C or 47°C - 74°C. The saccharifying may be conducted over a pH range of about pH 3 to about pH 7, e.g., pH 3.0 - pH 7.5, pH 3.5 - pH 5.5, pH 3.5, pH 3.8, or pH 4.5.

An amylase may be added to the slurry in the form of a composition. Amylase can be added to a slurry of a granular starch substrate in an amount of about 0.6 - 10 ppm ds, e.g., 2 ppm ds. An amylase can be added as a whole broth, clarified, enriched, partially purified, or purified enzyme. The specific activity of the amylase may be about 300 U/mg of enzyme, for example, measured with the PAHBAH assay. The amylase also can be added as a whole broth product.

An amylase may be added to the slurry as an isolated enzyme solution. For example, an amylase can be added in the form of a cultured cell material produced by host cells expressing an amylase. An amylase may also be secreted by a host cell into the reaction medium during the fermentation or SSF process, such that the enzyme is provided continuously into the reaction. The host cell producing and secreting amylase may also express an additional enzyme, such as a glucoamylase. For example, U.S. Patent No. 5,422,267 discloses the use of a glucoamylase in yeast for production of alcoholic beverages. For example, a host cell, e.g., *Trichoderma reesei oxAspergillus niger*, may be engineered to co-express an amylase and a glucoamylase, e.g., HgGA, TrGA, or a TrGA variant, during saccharification. The host cell can be genetically modified so as not to express its endogenous glucoamylase and/or other enzymes, proteins or other materials. The host cell can be engineered to express a broad spectrum of various saccharolytic enzymes. For example, the recombinant yeast host cell can comprise nucleic acids encoding a glucoamylase, an alpha-glucosidase, an enzyme that utilizes pentose sugar, an a-amylase, a pullulanase, an isoamylase, and/or an isopullulanase. See, e.g., WO 2011/153516 A2.

4.4. Fermentation

The soluble starch hydrolysate, particularly a glucose rich syrup, can be fermented by contacting the starch hydrolysate with a fermenting organism typically at a temperature around 32°C, such as from 30°C to 35°C for alcohol-producing yeast. The temperature and pH of the fermentation will depend upon the fermenting organism. EOF products include metabolites, such as citric acid, lactic acid, succinic acid, monosodium glutamate, gluconic acid, sodium gluconate, calcium gluconate, potassium gluconate, itaconic acid and other carboxylic acids, glucono delta-lactone, sodium erythorbate, lysine and other amino acids, omega 3 fatty acid, butanol, isoprene, 1,3-propanediol and other biomaterials.
Ethanologenic microorganisms include yeast, such as *Saccharomyces cerevisiae* and bacteria, *e.g.*, *Zymomonas mobilis*, expressing alcohol dehydrogenase and pyruvate decarboxylase. The ethanologenic microorganism can express xylose reductase and xylitol dehydrogenase, which convert xylose to xylulose. Improved strains of ethanologenic microorganisms, which can withstand higher temperatures, for example, are known in the art and can be used. See Liu *et al.* (2011) *Sheng Wu Gong Cheng Xue Bao* 27: 1049-56.

Commercial sources of yeast include ETHANOL RED® (LeSaffre); THERMOSACC® (Lallemand); RED STAR® (Red Star); FERMIOL® (DSM Specialties); and SUPERSTART® (Alltech). Microorganisms that produce other metabolites, such as citric acid and lactic acid, by fermentation are also known in the art. See, *e.g.*, Papagianni (2007) *Biotechnol. Adv.* 25:244-63; John *et al.* (2009) *Biotechnol. Adv.* 27:145-52.

The saccharification and fermentation processes may be carried out as an SSF process. Fermentation may comprise subsequent enrichment, purification, and recovery of ethanol, for example. During the fermentation, the ethanol content of the broth or "beer" may reach about 8-18% v/v, *e.g.*, 14-15% v/v. The broth may be distilled to produce enriched, *e.g.*, 96% pure, solutions of ethanol. Further, CO$_2$ generated by fermentation may be collected with a CO$_2$ scrubber, compressed, and marketed for other uses, *e.g.*, carbonating beverage or dry ice production. Solid waste from the fermentation process may be used as protein-rich products, *e.g.*, livestock feed.

As mentioned above, an SSF process can be conducted with fungal cells that express and secrete amylase continuously throughout SSF. The fungal cells expressing amylase also can be the fermenting microorganism, *e.g.*, an ethanologenic microorganism. Ethanol production thus can be carried out using a fungal cell that expresses sufficient amylase so that less or no enzyme has to be added exogenously. The fungal host cell can be from an appropriately engineered fungal strain. Fungal host cells that express and secrete other enzymes, in addition to amylase, also can be used. Such cells may express one or more glucoamylase and/or a pullulanase, phytase, *alpha*-glucosidase, isoamylase, beta-amylase cellulase, xylanase, other hemicellulases, protease, *beta*-glucosidase, pectinase, esterase, redox enzymes, transferase, or other enzyme.

A variation on this process is a "fed-batch fermentation" system, where the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression may inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. The actual substrate concentration in fed-batch systems is estimated by the changes of measurable factors such as pH, dissolved oxygen.
and the partial pressure of waste gases, such as CO₂. Batch and fed-batch fermentations are common and well known in the art.

Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor, and an equal amount of conditioned medium is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth. Continuous fermentation permits modulation of cell growth and/or product concentration. For example, a limiting nutrient such as the carbon source or nitrogen source is maintained at a fixed rate and all other parameters are allowed to moderate. Because growth is maintained at a steady state, cell loss due to medium being drawn off should be balanced against the cell growth rate in the fermentation. Methods of optimizing continuous fermentation processes and maximizing the rate of product formation are well known in the art of industrial microbiology.

### 4.6. Compositions

Proteases may be combined with a glucoamylase (EC 3.2.1.3), e.g., a *Trichoderma* glucoamylase or variant thereof. An exemplary glucoamylase is *Trichoderma reesei* glucoamylase (TrGA) and variants thereof that possess superior specific activity and thermal stability. See U.S. Published Applications Nos. 2006/0094080, 2007/0004018, and 2007/0015266 (Danisco US Inc.). Suitable variants of TrGA include those with glucoamylase activity and at least 80%, at least 90%, or at least 95% sequence identity to wild-type TrGA. α-amylases advantageously increase the yield of glucose produced in a saccharification process catalyzed by TrGA.

Alternatively, the glucoamylase may be another glucoamylase derived from plants (including algae), fungi, or bacteria. For example, the glucoamylases may be *Aspergillus niger* G1 or G2 glucoamylase or its variants (e.g., Boel et al. (1984) *EMBO J.* 3:1097-1102; WO 92/00381; WO 00/04136 (Novo Nordisk A/S)); and *A. awamori* glucoamylase (e.g., WO 84/02921 (Cetus Corp.)). Other contemplated *Aspergillus* glucoamylase include variants with enhanced thermal stability, e.g., G137A and G139A (Chen et al. (1996) *Prot. Eng.* 9:499-505); D257E and D293E/Q (Chen et al. (1995) *Prot. Eng.* 8:575-582); N182 (Chen et al. (1994) *Biochem. J.* 301:275-281); A246C (Fierobe et al. (1996) *Biochemistry,* 35: 8698-8704); and variants with Pro residues in positions A435 and S436 (Li et al. (1997) *Protein Eng.* 10:1 199-1204). Other contemplated glucoamylases include *Talaromyces* glucoamylases, in particular derived from *T. emersonii* (e.g., WO 99/28448 (Novo Nordisk A/S)), *T. leycettanus* (e.g., U.S. Patent No. RE 32,153 (CPC International, Inc.)), *T. duponti*, or *T. thermophilus* (e.g., U.S. Patent No. 4,587,215). Contemplated bacterial glucoamylases include glucoamylases from the...
genus *Clostridium*, in particular *C. thermoamylolyticum* (e.g., EP 135138 (CPC International, Inc.) and *C. thermohydrosulfuricum* (e.g., WO 86/01831 (Michigan Biotechnology Institute)). Suitable glucoamylases include the glucoamylases derived from *Aspergillus oryzae*, such as a glucoamylase shown in WO 00/04136 (Novo Nordisk A/S). Also suitable are commercial glucoamylases, such as AMG 200L; AMG 300 L; SAN™ SUPER and AMG™ E (Novozymes); OPTIDEX® 300 and OPTIDEX L-400 (Danisco US Inc.); AMIGASE™ and AMIGASE™ PLUS (DSM); G-ZYME® G900 (Enzyme Bio-Systems); and G-ZYME® G990 ZR (A. *niger* glucoamylase with a low protease content). Still other suitable glucoamylases include *Aspergillus fumigatus* glucoamylase, *Talaromyces* glucoamylase, *Thielaviella* glucoamylase, *Trametes* glucoamylase, *Thermomyces* glucoamylase, *Athelia* glucoamylase, or *Humicola* glucoamylase (e.g., HgGA). Glucoamylases typically are added in an amount of about 0.1 - 2 glucoamylase units (GAU)/g ds, e.g., about 0.16 GAU/g ds, 0.23 GAU/g ds, or 0.33 GAU/g ds.

[00155] Optionally, OPTIMASH™ BG may be used which is an enzyme preparation intended for the fuel alcohol industry. This product is capable of reducing viscosity of barley and wheat mashes. OPTIMASH™ BG enzyme contains a combination of enzymes, including but not limited to, a beta glucanase and a xylanase, which effectively modify and digest non-starch carbohydrates, the structural material of plant cells. OPTIMASH™ BG is produced by subraergedfermentation of a genetically modified strain of *Trichoderma reesei*. Alternately, or in combination, OPTIMASH™ TBG may be used, which is an enzyme blend that is heat stable, food grade preparation of the enzyme cellulase, EC 3.2.1.4. The product has been specifically formulated for use in the fermentation ethanol and starch processing industries for the breakdown of the non-starch polysaccharides of barley and wheat. It is produced by the fermentation of a non-genetically modified strain of *Geosmithia emersonii*, also known as *Talaromyces emersonii*. The major enzyme activity of OPTIMASH™ TBG enzyme is a component, endo-1,3(4)- B-glucanase (systematic name: (1,3-1,3;L4)-a-D-glucan 3(4)-glucanohydrolase), which catalyses the endohydrolysis of 1,3- or 1,4-linkages in β-D-glucans.

[00156] Other suitable enzymes that can be used include a phytase, protease, pullulanase, β-amylase, isoamylase, a different a-amylase, alpha-glucosidase, cellulase, xylanase, other hemicellulases, beta-glucosidase, transferase, pectinase, lipase, cutinase, esterase, redox enzymes, or a combination thereof. For example, a debranching enzyme, such as an isoamylase (EC 3.2.1.68), may be added in effective amounts well known to the person skilled in the art. A pullulanase (EC 3.2.1.41), e.g., PROMOZYME®, is also suitable. Pullulanase typically is added at 100 U/kg ds. Further suitable enzymes include proteases, such as fungal and bacterial...
proteases. Fungal proteases include those obtained from Aspergillus, such as A. niger, A. awamori, A. oryzae; Mucor (e.g., M. miehei); Rhizopus; and Trichoderma.

β-Amylases (EC 3.2.1.2) are exo-acting maltogenic amylases, which catalyze the hydrolysis of 1,4-a-glucosidic linkages into amylpectin and related glucose polymers, thereby releasing maltose. β-Amylases have been isolated from various plants and microorganisms. See Fogarty *et al.* (1979) in *PROGRESS IN INDUSTRIAL MICROBIOLOGY*, Vol. 15, pp. 112-115. These β-Amylases have optimum temperatures in the range from 40°C to 65°C and optimum pH in the range from about 4.5 to about 7.0. Contemplated β-amylases include, but are not limited to, β-amylases from barley SPEZYME® BBA 1500, SPEZYME® DBA, OPTIMALT™ ME, OPTIMAL™ BBA (Danisco US Inc.); and NOVOZYM™ WBA (Novozymes A/S).

Compositions comprising the present proteases may be aqueous or non-aqueous formulations, granules, powders, gels, slurries, pastes, etc., which may further comprise any one or more of the additional enzymes listed, herein, along with buffers, salts, preservatives, water, co-solvents, surfactants, and the like. Such compositions may work in combination with endogenous enzymes or other ingredients already present in a slurry, water bath, washing machine, food or drink product, etc, for example, endogenous plant (including algal) enzymes, residual enzymes from a prior processing step, and the like.

**EXPERIMENTAL**

**Example 1**

**Study the effect on ethanol production for Proteinase T, NprE and FNA protease addition during liquefaction step of the dry grind ethanol process for corn as a substrate**

This experiment was performed to study the effect of the proteases addition during the liquefaction unit operation in the dry grind process on the ethanol yield for the corn as substrate. The corn flour was mixed with DI water to prepare 25% ds slurry. The unadjusted pH of the slurry was at ~pH 6.0 and was left unchanged. Enzyme solutions containing SPEZYME® CL alpha amylase at 2.5 AAUs/g ds was dispensed in the slurry. This slurry was mixed at room temperature to blend the added enzymes. A 35 g aliquot with 4 replications for each treatment was weighed into LABOMAT steel beakers. Following the weighing process the proteases were added as such 1) no protease 2) Proteinase T at 0.05mg/g ds, 3) FNA at 0.05mg/g ds and 4) NprE at 0.05mg/g ds. The metal beakers were fitted back into the LABOMAT for liquefaction unit operation at 60°C for 45 min followed by 85°C for 90 min with clockwise and counterclockwise constant mixing at 60rpm.

After the liquefaction the liquefied mash for each treatment was mixed together
for pH adjustment and fermentation preparation. The pH was adjusted to pH 4.5 using 4N sulfuric acid. ADY at 3.57 mg/g ds, urea at 600 ppm and GA at 0.325 GAUs /g ds were added to each flask. A 50 g of slurry was thereafter transferred to 125 ml flask for fermentation. The flasks were incubated at 32°C for 54 hrs. The samples were drawn at regular intervals for ethanol measurements.

[00161] The ethanol data from the table below shows that for the corn fermentation the protease addition during liquefaction helped the ethanol production rate and the final ethanol concentration for the corn slurry treated with Proteinase T, NprE and FNA compared to the control.

Table 2. Effect of protease addition during liquefaction on the ethanol yield during fermentation of corn substrate

<table>
<thead>
<tr>
<th>Enzyme treatments</th>
<th>Ethanol (% v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
</tr>
<tr>
<td>Spezyme CL + Optimash BG</td>
<td>0.29</td>
</tr>
<tr>
<td>Spezyme CL + Optimash BG + Proteinase T</td>
<td>0.34</td>
</tr>
<tr>
<td>Spezyme CL + Optimash BG + NprE</td>
<td>0.44</td>
</tr>
<tr>
<td>Spezyme CL + Optimash BG + FNA</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Example 2

[00162] The objective of this experiment is to determine effects of protease addition on viscosity changes during corn liquefaction by alpha amylases.

Methods:

[00163] In a Rapid Visco Analyzer (RVA) sample cup, 9.1 grams of ground corn flour was mixed with 23.6 grams of water and pH was adjusted to 5.8 with IN H₂SO₄. Then, 15 µgrams of amylase (e.g., Cytophaga spp. Amylase 1777dRG) was added with and without Proteinase T (Product: 4.9 mg/mL, specific protein) or NprE (purified: 22.3 mg/mL, specific protein). Changes in viscosity were monitored using RVA at constant temperature set at 85 °C.

[00164] Titration of Proteinase T with 15 µgrams of Cytophaga spp alpha amylase (1777dRG) shows progressive reduction of the peak and final viscosities during CF slurry gelatinization and pasting steps. The liquefaction reactions were carried out with corn flour (25% DS) at pH 5.8 with the rapid temperature ramp and held at 85 °C. Without protease addition, the viscosity rises above a point at which the instrument torques out and stops collecting data.

[00165] Addition of protease (1mg purified NprE) to 15 µgrams of Cytophaga spp alpha
Amylase (1777 dRG) reduces the peak viscosity by -30% while addition of buffer control does not significantly change. Reactions were carried out in RVA with corn flour (25% DS) at pH 5.8 with the rapid temperature ramp and held at 85 °C. Without protease addition, the viscosity rises above a point at which the instrument torques out and stops collecting data.

Dose dependent reduction of peak and final viscosity values. Percent reduction shown list changes in viscosity relative to no protease addition control. The final viscosity value for no protease addition was estimated from previous experiments. The data are presented as Figures 1 to 3.

Example 3

Study the addition of Proteinase T and SPEZYME FAN during liquefaction on ethanol production in SSF.

Materials:

- 32% ds ground corn from ADM in Cedar Rapids, IA
- Thin stillage from Lincolnway, IA (used at 30% of diluant)
- Big River Dyersville liquefact
- Urea from JT Baker, USP grade (Lot number G05583)
- Yeast from Lincolnway Ethanol Red (Sticker # 201 L-0365)
- 6N HCl, 25 % v/v NH₄OH, 1N H₂SO₄

Enzymes used in liquefaction and/or fermentation are shown in Table 3.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme activity or concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPEZYME CL</td>
<td>15,064 AAUs/g</td>
</tr>
<tr>
<td>TrGA (GC 321)</td>
<td>373 GAUs/g</td>
</tr>
<tr>
<td>AkAA (GC 626)</td>
<td>10,562 SSU/g</td>
</tr>
<tr>
<td>Fermgen</td>
<td>1,046 SAPU/g</td>
</tr>
<tr>
<td>Proteinase T</td>
<td>165 SAPU/g</td>
</tr>
<tr>
<td>SPEZYME FAN</td>
<td>3,000 SAPU/g</td>
</tr>
</tbody>
</table>

Liquefaction process - Addition of Proteases. Whole corn ground slurry of 800 g at 32% ds containing 30% thin stillage was used. The slurry was then mixed well and the pH was adjusted to 5.8 using 25 % v/v NH₄OH. The slurry was placed in a 70°C (158.0°F) water...
bath and continuously mixed for 90 seconds. SPEZYME CL was added into the slurry with the dosage of 0.75 AAUs/g ds. After the 30 minute mark, the corn slurry was taken to a 99°C water bath and continuously mixed for 10 minutes to imitate a jet cooker. In addition, approximately 1 gram sample of the slurry was taken at 30 minutes for DE determination as described in GPA 502. The slurry was then brought back to an 85°C (185.0°F) water bath and continuously mixed for the remainder of the experiment (160 minutes total). The secondary dosage of SPEZYME CL at 1.0 AAUs/g ds was added at the 45 minute mark. Samples of ~1 gram were taken for DE determination at 50 and 160 minutes. This liquefaction procedure was repeated two more times under the same conditions but with proteases added in the slurry in addition to the SPEZYME CL dose: 1) 0.75 AAUs/g ds CL + 1.0 kg/MT ds SPEZYME FAN in the slurry ++ 1.0 AAUs/g ds CL in secondary liquefaction, and 2) 0.75 AAUs/g ds CL + 1.0 kg/MT ds Proteinase T in the slurry ++ 1.0 AAUs/g ds CL in the secondary liquefaction. The moisture content was determined at the end of liquefaction by using a Mettler Toledo HR83 moisture balance and the amount of moisture lost for each of these cooks was calculated. The amount of water lost during liquefaction was added back in at the end using DI water and the pH was then brought back down to 4.5 using 6 N HCl.

[00171] Fermentation process - Proteases used in liquefaction. The three separate liquefacts were thawed at 60°C and then cooled to room temperature, at which point 400 ppm of urea was added to each liquefact. The pH was then adjusted to 4.5 using 6N sulfuric acid. Samples of the initial liquefacts were measured into separate 2.0 mL centrifuge tubes and centrifuged for approximately 3 minutes at 13.2k rpm. After centrifugation, the samples were prepared for HPLC analysis, where 0.5 mL of supernatant was acidified with 50 µL of IN sulfuric acid for 5 minutes, diluted with 4.45 mL of RO water, and then filtered through a 3 mL syringe with a 0.45 µm GHP membrane. The samples were placed in HPLC vials and went through the organic acid column. For each of the three separate liquefacts, a sample of 100.00 grams (+0.05g) was weighed into 4 separate 250 mL wide-mouthed Erlenmeyer flasks. Thus, a total of 12 flasks were used. The dose used for TrGA (GC 321), AkAA (GC 626), and Fermgen are shown in Table 4. As shown, the enzymes were added in duplicate. Dosing of fermentation enzymes
Table 4

<table>
<thead>
<tr>
<th>Liquefact</th>
<th>Enzymes and dosages</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPEZYME CL</td>
<td>TrGA+AkAA</td>
</tr>
<tr>
<td>SPEZYME CL</td>
<td>TrGA + AkAA + 0.02 kg/MT ds Fermgen</td>
</tr>
<tr>
<td>SPEZYME CL + SPEZYME FAN</td>
<td>TrGA+AkAA</td>
</tr>
<tr>
<td>SPEZYME CL + SPEZYME FAN</td>
<td>TrGA+AkAA + 0.02 kg/MT ds Fermgen</td>
</tr>
<tr>
<td>SPEZYME CL + Proteinase T</td>
<td>TrGA+AkAA</td>
</tr>
<tr>
<td>SPEZYME CL + Proteinase T</td>
<td>TrGA+AkAA + 0.02 kg/MT ds Fermgen</td>
</tr>
</tbody>
</table>

[00172] A 20% w/v yeast solution was prepared by adding 5 grams of Ethanol Red yeast in a 50 mL conical centrifuge tube with the addition of DI water up to 25 mL mark. The yeast solution was mixed for 10 minutes at 150 rpm and each fermentation flask was dosed with 500 µl of yeast, which yielded a concentration of 0.1% w/w. Each flask was then covered with a rubber stopper with a hole to vent CO₂. The 12 flasks were incubated at 32°C at 150 rpm for 72 hours. Samples were taken at 5, 18, 24, 42, 48, 66, and 72 hours for ethanol concentrations. A sample was measured into a 2.0 mL centrifuge tube and centrifuged for approximately 3 minutes at 13.2k rpm and prepared for HPLC analysis, where 0.5 mL of supernatant was acidified with 50 µl of 1N sulfuric acid for 5 minutes, diluted with 4.45 mL of RO water, and then filtered through a 3 mL syringe with a 0.45 µm GHP membrane. The samples were placed in HPLC vials and went through the organic acid column.

[00173] As Proteinase T and FAN are thermostable at 70°C, liquefaction was performed with slurry at 70°C for 30 min followed by jet simulation (boil step) which was followed with secondary liquefaction at 85°C for 120 min. In liquefacts Proteinase T and FAN were added in slurry and not in secondary liquefaction. So the liquefacts were incubated with Proteinase T and FAN only for 30 min at 70°C.

[00174] Increase in ethanol by 0.5 to 0.8% v/v when seen using Proteinase T with SPEZYME CL in liquefaction compared to control when only SPEZYME CL was used. The fermentation with liquefact from SPEZYME CL and Proteinase T and TRGA+AKAA+Fermgen almost reached 17.5% v/v whereas the fermentation with liquefact from only SPEZYME CL and TRGA+AKAA+Fermgen reached 16.47% w/w. Ethanol increase was also seen with use of SPEZYME FAN and Fermgen in liquefaction.

[00175] During the replication, the fermentation was allowed for 72 hrs and the ethanol started decreasing as the yeast started to metabolize the ethanol. This data indicates that adding the Proteinase T and FAN during liquefaction increased starch availability and ethanol fermentation rate. The differentiation in ethanol production rate is seen as early as 16-18 hr.
Higher Proteinase T and FAN dosages were added as they are neutral pH proteases and their activity may be reduced during SSF at pH 4.5. No difference in % ethanol was detected (Figures 4, 5 and 6). The IRS (insoluble residual starch) tests were negative for all the treatment as AKAA was included in the SSF.

Example 4

Analysis of Residual Starch, Free Amino Nitrogen (FAN) and Citric Acid Production in Fermentation on Application of FERMGEN in Low Temperature Liquefaction

In citric acid industry, after filtration, high residual starch in filtrate cake was the big waste for the corn consumption. A new process was developed using the FERMGEN protease addition in the slurry tank before liquefaction, in order to get better liquefaction and filtration performance.

The benchmark was conducted with Liquezyme Supra at 95°C.

Materials: Corn flour, Liquezyme Supra, FERMGEN, 95°C, 55°C water bath, 60°C Oven, Muller, pH6.0 PB buffer, pH4.2 Acetic acid buffer, Spezyme Fred, AnGA.

Prepare DS=20% of corn flour slurry, and adjust pH to 5.7. Record the total weight of baker and mash for evaporation adjustment after liquefaction. For FERMGEN process, add FERMGEN at 0.2kg/tlds into the slurry and put it in 55°C water bath for 0.5hr to do pretreatment process, then add Supra at 0.7kg/tds and transferred to 95°C water bath for 1.5hr to do liquefaction process. For blank group, add Supra at 0.7kg/tds and put it into 95°C water bath for 1.5hr to do liquefaction process. After the liquefaction process, the slurry was filtrated and collected the cake, which was then dried in 60°C oven overnight. The cake was crashed to fine powder and check residual starch in it.

In another set of experiments, after the liquefaction process, the slurry was filtrated and collected the filtrate, which was then analyzed for the FAN concentration with ninhydrin method.

In another set of experiments, after the liquefaction process, the slurry was filtrated and the filtrate was collected. Liquefaction slurry and filtrate were mixed together according to the C/N. A strain of Aspergillus niger was grown on potato dextrose agar (PDA) slant at 55°C for 5-7d, then the spores were washed with sterilized water, and spores suspension was inoculated into fermentation medium to keep every bottle at the same conditions. The fermentation was performed in the shaker with 300rpm/min, 35 °C, 96hr. Ending the fermentation, the broth was filtrated through filter paper, and the filtrate was used for analysis. The citric acid concentration and DPI, DP2, DP3, DP4+ concentration was analyzed by HPLC.
In another set of experiments, Proteinase T was used to increase citric acid fermentation (Table 4.4).

Table 4.1 The effect of FERMGEN on residual starch in the filtration cake

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Liquefaction</th>
<th>Residual Starch (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><a href="mailto:FERMGEN@0.2kg">FERMGEN@0.2kg</a>/tds, 55°C, 0.5hr</td>
<td><a href="mailto:Supra@0.7kg">Supra@0.7kg</a>/tds, 95°C, 1.5hr</td>
<td>29.8±0</td>
</tr>
<tr>
<td>-</td>
<td><a href="mailto:Supra@0.7kg">Supra@0.7kg</a>/tds, 95°C, 1.5hr</td>
<td>33.31±0.59</td>
</tr>
</tbody>
</table>

Table 4.2 The effect of FERMGEN on FAN concentration in the filtrate

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Liquefaction</th>
<th>FAN(mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><a href="mailto:FERMGEN@0.2kg">FERMGEN@0.2kg</a>/tds, 55°C, 0.5hr</td>
<td><a href="mailto:Supra@0.7kg">Supra@0.7kg</a>/tds, 95°C, 1.5hr</td>
<td>56.16±0.25</td>
</tr>
<tr>
<td>-</td>
<td><a href="mailto:Supra@0.7kg">Supra@0.7kg</a>/tds, 95°C, 1.5hr</td>
<td>46.69±0.2</td>
</tr>
</tbody>
</table>

Table 4.3 The effect of FERMGEN on citric acid production

<table>
<thead>
<tr>
<th>Process</th>
<th>DP1(g/L)</th>
<th>DP2(g/L)</th>
<th>DP3(g/L)</th>
<th>DP4+(g/L)</th>
<th>Citric Acid(g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FERMGEN</td>
<td>0.501±1.44</td>
<td>7.34±0.5</td>
<td>3.85±0.08</td>
<td>3.86±0.16</td>
<td>115.28±0.22</td>
</tr>
<tr>
<td>Blank</td>
<td>0.897±0.83</td>
<td>7.32±0.5</td>
<td>3.83±0.1</td>
<td>4.8±0.05</td>
<td>107.95±0.26</td>
</tr>
</tbody>
</table>

Table 4.4 The effect of Proteinase T on citric acid production and residual Sugars

<table>
<thead>
<tr>
<th>Process</th>
<th>DP1(g/L)</th>
<th>DP2(g/L)</th>
<th>DP3(g/L)</th>
<th>DP4+(g/L)</th>
<th>Citric Acid(g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinase T</td>
<td>0.51±0.01</td>
<td>3.94±0.48</td>
<td>2.56±0.13</td>
<td>2.95±0.05</td>
<td>103.04±0.64</td>
</tr>
<tr>
<td>Blank</td>
<td>0.52±0.02</td>
<td>4.36±0.54</td>
<td>2.8±0.09</td>
<td>3.09±0.01</td>
<td>101.46±1.25</td>
</tr>
</tbody>
</table>

The data showed that FERMGEN addition before liquefaction gave a reduction of residual starch (Table 4.1), increased FAN (Table 4.2) and increased citric acid production (Table 4.3, and Table 4.4 for Proteinase T). FERMGEN and Proteinase T application may have increased the small peptides, amino acids and other nitrogen species, which can be directly used for the fermentation. The proteases may make the starch granules bound with protein to increased dissolution. Additionally, it could reduce the viscosity of the fermentation broth and improve oxygen transfer efficiency.
Example 5

Analysis of Residual Starch, Free Amino Nitrogen and Effect of Calcium on Application of Proteinase T in Low Temperature Liquefaction

In this set of experiments, for low temperature liquefaction using Spezyme Alpha as S-type AA, we explored Proteinase T process, which was conducted at 85°C along with Alpha liquefaction process.

Materials: Corn flour, Spezyme Alpha, Proteinase T, 90°C, 85°C water bath, 60°C Oven, Muller pH6.0 PB buffer, pH4.2 Acetic acid buffer, Spezyme Fred AnGA.

Prepare DS=20% of corn flour slurry, and adjust pH to 5.7. Record the total weight of baker and mash for evaporation adjustment after liquefaction. For Proteinase T process, add Proteinase T at 0.2kg/tds and Spezyme Alpha at 0.3kg/tds into the slurry and put it in 85°C water bath for 1.5hr to do liquefaction process. In some experiments, Ca²⁺ is added at 200 ppm. For blank group, add Alpha at 0.3kg/tds (and Proteinase T for Ca²⁺ experiments) and put it into 90°C water bath for 1.5hr to do liquefaction process. In one set of experiments, after the liquefaction process, the slurry was filtrated and collected the cake, which was then dried in 60°C oven overnight. The cake was crushed to fine powder and check residual starch in it. Analysis conditions: Agilent 1260 HPLC, RID Detector, Rezex-ROA-Organic acid (Phenomenex), Mobile Phase: 5mM H₂SO₄, Column: 60°C, Pump: 0.6ml/min, injection: 20µL.

In another set of experiments, after the liquefaction process, the slurry was filtrated and collected the filtrate, which was then analyzed for the FAN concentration with ninhydrin method.

Table 5.1 The effect on residual starch in the filtration cake

<table>
<thead>
<tr>
<th>Liquefaction</th>
<th>Residual Starch(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinase <a href="mailto:T@0.2kg">T@0.2kg</a>/tds+Alpha@0.3kg/tds, 85°C for 1.5hr</td>
<td>25.5±1.47</td>
</tr>
<tr>
<td><a href="mailto:Alpha@0.3kg">Alpha@0.3kg</a>/tds, 90°C, 1.5hr</td>
<td>31.1±0.28</td>
</tr>
</tbody>
</table>

Table 5.2 The effect on FAN concentration in the filtrate

<table>
<thead>
<tr>
<th>Liquefaction</th>
<th>FAN(mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinase <a href="mailto:T@0.2kg">T@0.2kg</a>/tds+Alpha@0.3kg/tds, 85°C for 1.5hr</td>
<td>49.09±0.56</td>
</tr>
<tr>
<td><a href="mailto:Alpha@0.3kg">Alpha@0.3kg</a>/tds, 90°C, 1.5hr</td>
<td>43.4±0.52</td>
</tr>
</tbody>
</table>
Table 5.3 The effect Calcium on FAN in the filtrate

<table>
<thead>
<tr>
<th>Liquefaction</th>
<th>Ca+ (ppm)</th>
<th>FAN(mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinase <a href="mailto:T@0.2kg">T@0.2kg</a>/tds+Alpha@0.3kg/tds, 85°C for 1.5hr</td>
<td>200</td>
<td>65.93±0.41</td>
</tr>
<tr>
<td>Proteinase <a href="mailto:T@0.2kg">T@0.2kg</a>/tds+Alpha@0.3kg/tds, 85°C for 1.5hr</td>
<td>-</td>
<td>49.09±0.56</td>
</tr>
</tbody>
</table>

[00191] The data showed that Proteinase T process gave a reduction of residual starch, which could make the starch granules bound with protein to dissolution due to its dominant thermostability.

[00192] The Ninhydrin analysis showed that Proteinase T process could increase the FAN concentration.

[00193] Addition of 200 ppm Ca\(^{2+}\) increased FAN concentrations as compared to controls with no added Ca\(^{2+}\).

Example 6

[00194] Use of FERMGEN in Lactic Acid Fermentation

[00195] Materials and Methods: Strain of Bacillus coagulans 20138 was obtained from CICC (China Center of Industrial Culture Collection).

[00196] Seed media: 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, K2HP04 2.0 g, MgSO4-7H2O 0.2 g, MnSO4.H2O 0.05 g. Distilled water 1.0L, pH6.8; for inoculum media additional 20g agar were added, Sterilization® 121°C,20min.

[00197] Fermentation medium: whole ground Corn liquefact, one treated with additional Protease like FERMGEN, another run as control without protease.

[00198] Casein, Beef extract, Yeast extract, Glucose, Sodium acetate, diammonium hydrogen citrate, Tween 80, K2HP04, MgSO4.7H2O, MnSO4.H2O, Agar powder, NH4OH, calcium hydroxide were analytical grade from local supplier. Whole ground corn was from Yixin Xielian Citric acid factory. FERMGEN, SPEZYME ALPHA, TrGA was from Genencor/DuPont.

[00199] Prepare DS=16% of whole ground corn slurry, and adjust pH to 5.6. Record the total weight of baker and mash for evaporation adjustment after liquefaction, for "pretreatment using protease", add FERMGEN at 0.5kg/tds and SPEZYME ALPHA at 0.3kg/tds into the slurry and put it in 60°C water bath holding 30min, and then transferred to 87°C water bath for 1.5hr for liquefaction, for control group "without protease pretreatment", add only SPEZYME ALPHA at 0.3kg/tds and put it in 60°C water bath holding 30min, and then transferred to 87°C
water bath for 1.5hr for liquefaction. After the liquefaction, the slurry was filtrated and collected the filtrated as fermentation medium.

Lactic acid fermentation was carried out using *Bacillus coagulans* strain. The inoculums of *Bacillus coagulans* was transferred to each 100mL seed culture and cultivated at 50°C, 12~24hrs, 200rpm. The cultivation was controlled based on OD₆₀₀ value around 0.5. 500 mL filtrate was used as fermentation medium, Treesei GlucoAmylase (TrGA) was dosed at 0.75 GAU/g for SSF process, then 50mL seed culture was added to each fermentor to start fermentation. Fermentation pH was maintained at pH 6.5 using 15% Ca(OH)₂ and the temperature was set at 55°C, and the agitation was 300rpm. Samples were taken at 18h, 25h, 44h, 50h. Lactic acid concentration of the fermentation broth was given in Table 6.1.

<table>
<thead>
<tr>
<th>Time(hrs)</th>
<th>Lactic acid (m/v%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>liquefact Control</td>
<td>18</td>
</tr>
<tr>
<td>liquefact plus protease</td>
<td>18</td>
</tr>
<tr>
<td>liquefact Control</td>
<td>25</td>
</tr>
<tr>
<td>liquefact plus protease</td>
<td>25</td>
</tr>
<tr>
<td>liquefact Control</td>
<td>44</td>
</tr>
<tr>
<td>liquefact plus protease</td>
<td>44</td>
</tr>
<tr>
<td>liquefact Control</td>
<td>50</td>
</tr>
<tr>
<td>liquefact plus protease</td>
<td>50</td>
</tr>
</tbody>
</table>

While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein can be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.
Claims

1. A method for producing a fermentation product from corn-like grain, comprising:
   a) liquifying the corn-like grain slurry at a pH of about 4.0 to about 7.0 at a temperature in the range from about 25°C to about 65°C using:
   an alpha amylase, a protease; and optionally a beta-glucanase;
   b) saccharifying and fermenting the slurry using enzymes, including a glucoamylase and a fermenting organism to produce the fermentation product.

2. A method for reducing viscosity of a corn-like grain slurry during a liquefaction process, comprising:
   a) liquifying the corn-like grain slurry at a pH of about 4.0 to about 7.0 at a temperature in the range from about 25°C to about 65°C using:
      an alpha amylase, a protease; and optionally a beta-glucanase;
      b) saccharifying and fermenting the slurry using enzymes, including a glucoamylase and a fermenting organism to produce ethanol, wherein the yield and/or rate of ethanol production is greater than the rate observed without the addition of protease.

3. A method for increasing yield and/or rate of an organic acid production from corn-like grain, comprising:
   a) liquifying the corn-like grain slurry at a pH of about 4.0 to about 7.0 at a temperature in the range from about 25°C to about 65°C using:
      an alpha amylase, a protease; and optionally a beta-glucanase;
      b) saccharifying and fermenting the slurry using enzymes, including a glucoamylase and a fermenting organism to produce the organic acid, wherein the yield and/or rate of the organic acid production is greater than the rate observed without the addition of protease.

4. A method for reducing viscosity of a corn-like grain slurry during a fermentation, comprising:
   a) liquifying the corn-like grain slurry at a pH of about 4.0 to about 7.0 at a temperature in the range from about 25°C to about 65°C using an alpha amylase, a protease; and optionally a beta-glucanase;
   b) saccharifying and fermenting the slurry using enzymes, including a glucoamylase and a fermenting organism to produce the fermentation product, wherein the viscosity of the slurry is reduced by at least 20% compared to a slurry not treated with a protease.

5. The method of claim 4, wherein the viscosity is reduced by about 30%.

6. The method of any one of claim 4 or 5, wherein the viscosity is reduced by about 50%.

7. The method of any one of claim 4 to 6, wherein the viscosity is reduced by about 60%.

8. A method for reducing viscosity of a corn-like grain slurry during a liquefaction process,
comprising:
a) liquifying the corn-like gain slurry at a pH of about 4.0 to about 7.0 at a temperature in
the range from about 25°C to about 65°C using an alpha amylase, a protease; and optionally a
beta-glucanase; and

wherein the viscosity of the slurry is reduced by at least 20% compared to a slurry not
treated with a protease.

9. The method of claim 8, wherein the viscosity is reduced by about 30%.
10. The method of any one of claim 8 or 9, wherein the viscosity is reduced by about 50%.
11. The method of any one of claim 8 to 10, wherein the viscosity is reduced by about 60%.

12. A method for reducing viscosity of a corn-like grain slurry during a liquefaction process,
comprising:
liquifying the corn-like gain slurry at a pH of about 4.0 to about 7.0 at a temperature in the range
from about 25°C to about 90°C using:
an alpha amylase, a protease; and optionally a beta-glucanase; and

wherein the viscosity of the slurry is reduced by at least 20% compared to a slurry not
treated with a protease.

13. The method of claim 12, wherein the viscosity is reduced by about 30%.
14. The method of any one of claim 12 or 13, wherein the viscosity is reduced by about 50%.
15. The method of any one of claim 12 to 14, wherein the viscosity is reduced by about 60%.

16. A method for reducing foam in a corn-like grain slurry during a liquefaction process,
comprising:
liquifying the corn-like gain slurry at a pH of about 4.0 to about 7.0 at a temperature in the range
from about 25°C to about 90°C using:
an alpha amylase, a protease; and optionally a beta-glucanase; and

wherein the foam in the slurry is reduced by at least 20% compared to a slurry not treated
with a protease.

17. The method of claim 16, wherein the foam is reduced by about 30%.
18. The method of any one of claim 16 or 17, wherein the foam is reduced by about 50%.
19. The method of any one of claim 16 to 18, wherein the foam is reduced by about 60%.

20. A method for reducing amount of residual starch after a liquefaction process of a corn-like
grain slurry, comprising:
liquifying the corn-like gain slurry at a pH of about 4.0 to about 7.0 at a temperature in the range
from about 25°C to about 90°C using:
an alpha amylase, a protease; and optionally a beta-glucanase; and
wherein the amount of residual starch of the slurry is reduced by at least 20% compared to a slurry not treated with a protease.

21. The method of claim 20, wherein the amount of residual starch is reduced by about 30%.

22. The method of any one of claim 20 or 21, wherein the amount of residual starch is reduced by about 50%.

23. The method of any one of claim 20 to 22, wherein the amount of residual starch is reduced by about 60%.

24. The method of any one of claims above, wherein the protease is proteinase T.

25. The method of any one of claims above, wherein the protease is NprE.

26. The method of any one of claims above, wherein the protease is proteinase T variant.

27. The method of any one of claims above, wherein the protease is proteinase T variant Q17I.

28. The method of any one of claims above, wherein the protease is proteinase T variant Q17V.

29. The method of any one of claims above, wherein the protease is FNA.

30. The method of any one of claims above, wherein the protease is FERMGEN.

31. The method of claim 1, wherein the fermentation product is an alcohol, an organic acid, an amino acid, a sugar, an antibiotic, an enzyme, a vitamin or a hormone.
FIG. 1

Titration of Proteinase T with 15 μgrams of Cytophaga spp Alpha Amylase.

FIG. 2

Addition of Protease (1 mg Purified NprE) to 15 μgrams of Cytophaga spp Alpha Amylase.
Dose Dependent Reduction of Peak and Final Viscosity Values.

FIG. 3
3 / 4

EOF Ethanol Levels (48 Hours)

The Final Ethanol Yield at 48 Hr for Different Liquefaction and SSF Treatments.

**FIG. 4**

EOF Ethanol Levels (48 Hours)

The Final Ethanol Yield at 48 Hr for Different Liquefaction and SSF Treatments.

**FIG. 5**
The Final Ethanol Yield at 48 Hr for Both Proteinase T and FAN Used in SSF with Fermgen.

**FIG. 6**
### A. CLASSIFICATION OF SUBJECT MATTER

**INV.** C12P19/14  C12P7/06  C12P7/40

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 practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE, FSTA

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of Box C.  
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**Date of the actual completion of the international search:** 14 January 2015  
**Date of mailing of the international search report:** 26/01/2015

Name and mailing address of the ISA:
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NL-2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016

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