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(54) **USE OF GLUCOAMYLASE AND BUTTIAUXELLA PHYTASE DURING SACCHARIFICATION**

VERWENDUNG VON GLUCOAMYLASE UND BUTTIAUXELLA PHYTASE WÄHREND DER VERZUCKERUNG

UTILISATION DE GLUCOAMYLASE ET PHYTASE DE BUTTIAUXIELLA DURANT LA SACCHARIFICATION

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Description

TECHNICAL FIELD

[0001] The described methods relate to the use of glucoamylase and a *Buttiauxella* spp. phytase in a starch conversion processes, *e.g.*, for the production of DDGS for animal feed or in fermentation processes for producing organic compounds such as ethanol.

BACKGROUND

[0002] Industrial fermentation methods predominantly use glucose as a feedstock for the production of a multitude of end-products, including enzymes, proteins, amino acids, organic acids, sugar alcohols, pharmaceuticals and other biochemicals. In many applications glucose is produced from the enzymatic conversion of substrates comprising starch and cellulose (*e.g.*, whole milled cereal grains). The processing of starch to produce glucose generally involves two steps, namely liquefaction of granular starch and saccharification of the liquefied starch to produce glucose. Further steps may include purification and isomerization, *e.g.*, when the desired end-product is a purified dextrose or fructose, or fermentation and distillation, *e.g.*, when the desired end-product is an alcohol (*e.g.*, ethanol).

[0003] Liquifaction converts a slurry of starch polymer granules into a solution of shorter chain length dextrins of lower viscosity. The saccharification step further converts those shorter-chain dextrins into glucose. Commonly, the starch is liquefied by exposure to an elevated temperature and enzymatic bioconversion. A common enzymatic liquefaction process involves adding a thermostable bacterial alpha (α)-amylase (*e.g.,* SPEZYME® FRED or SPEZYME® XTRA (Danisco US, Inc, Genencor Division) or TERMAMYL® SC or TERMAMYL™ 120L (Novozymes)) to a slurry comprising a substrate that includes granular starch. The pH is adjusted to between 5.5 to 6.5 and the temperature is elevated to greater than 90°C. The starch is first gelatinized and then exposed to the saccharifying enzymes. Typically, saccharification takes place in the presence of glucoamylase enzymes such as glucoamylase from *Aspergillus niger* (*e.g.,* OPTIDEX® L-400 (Danisco US, Inc. Genencor Division)) at a more acidic pH than that used in the liquefaction step. The pH of a typical saccharification step is around pH 4.0 to 5.0. The resulting sugars are then fermented to provide the desired end-products (*i.e.,* ethanol). In the process of producing ethanol, side-products and waste-products such as distillers dried grains and solubles (DDGS) are produced and used for feed. Further, the resulting liquid from the process (*i.e.,* the thin stillage) is recycled by mixing it with slurry.

[0004] A number of variations exist for the liquefaction and saccharification of a starch substrate. For example, WO 2008/097620 (August 2008) describes methods for starch hydrolysis employing an alpha-amylase and a phytase. Specific phytases, and their use in starch hydrolysis, are described in WO 2008/097619 (August 2008). However, a need continues to exist for advances in starch liquefaction, saccharification, and fermentation. Shi et al, Aquaculture, 275 (January 2008), 70-75, disclose phytases from Buttiauxella, isolated from grass carp intestine.

BRIEF SUMMARY

[0005] Described are methods involving the use of a glucoamylase in combination with a phytase in a starch conversion process. Such a process may be used for the production of organic compounds such as ethanol, the production of DDGS for animal feed, or both. In some embodiments, the methods comprise adding an enzyme blend comprising a glucoamylase, and phytase to a starch conversion processes during pre-saccharification, saccharification, and/or combined saccharification/fermentation. Such methods provide certain advantages over the use of a glucoamylase alone.

[0006] The invention provides a method of producing an alcohol, comprising:

(a) contacting a slurry comprising a starch substrate with at least one α-amylase producing oligosaccharides;
(b) contacting the oligosaccharides with at least one glucoamylase and at least one phytase, wherein the phytase has at least 90% sequence identity to the sequence of SEQ ID NO: 5, to produce fermentable sugars;
(c) fermenting the fermentable sugars in the presence of a fermenting organism to produce alcohol; and optionally
(d) recovering the alcohol and/or DDGS. In some embodiments, the temperature can be raised above the gelatinization temperature of the starch substrate after step (a) and before step (b).

[0007] In some embodiments, the starch substrate is a milled grain and the milled grain is chosen from maize, barley, wheat, rice, sorghum, rye, millet, and/or triticale. In some embodiments, the phytase has an alanine at amino acid 92 and/or at least one of the following amino acids: a threonine at position 89, an isoleucine at position 134, a serine at position 164, a lysine at position 176, a proline at position 178, a glutamic acid at position 207, a serine at position 209, a leucine at position 248, a tyrosine at position 256, a glutamic acid at position 261, and a lysine at position 269. In some embodiments, the phytase has at least one of the following amino acid changes: A89T, D92A, T134I, F164S, T176K,

A178P, K207E, A209S, S248L, Q256Y, A261E, and N269K. In some embodiments, the phytase is wild-type *Buttiauxella* spp., phytase (BP-WT) or a variant selected from BP-11 and BP-17. In some embodiments, the phytase has the amino acid sequence set forth in SEQ ID NO: 5 or SEQ ID NO: 8.

[0008] In some embodiments, the method also includes contacting the oligosaccharides with at least one other/additional enzyme chosen from an α-amylase, a second glucoamylase, a second phytase, a cellulose, a pullulanase, a protease, and/or a laccase. The alcohol can be ethanol.

[0009] The invention also provides a method for reducing phytic acid during ethanol fermentation comprising:

(a) contacting a slurry including a starch substrate, with at least one α-amylase to produce a liquefact;
(b) contacting the liquefact with at least one glucoamylase and at least one phytase, wherein the phytase has at least 90% amino acid sequence identity to SEQ ID NO: 5 and wherein the phytase has an alanine at position 92, under conditions such that fermentable sugars are produced;
(c) fermenting the fermentable sugars in the presence of a fermenting organism under conditions such that ethanol and/or DDGS are produced; and optionally
(d) recovering the ethanol and/or DDGS. In some embodiments, the method includes raising the temperature above the liquefaction temperature for the starch substrate. In some embodiments, contacting the liquefact with at least one glucoamylase and at least one phytase and fermenting the fermentable sugars in the presence of a fermenting organism occur simultaneously.

[0010] In some embodiments, the glucoamylase is from a filamentous fungus chosen from *Trichoderma, Penicillium, Taleromyces, Aspergillus,* and/or *Humicola.* In some embodiments, the *Trichodema* is *Trichoderma reesei.* In some embodiments, the phytase is BP-17. In some embodiments, the DDGS have active residual phytase. The DDGS can be blended into a feed. In some embodiments, when the DDGS are blended with grains or feed to produce an animal feed, the active phytase reduces the phytic acid in the feed. In some embodiments, the starch substrate is a milled grain, such as maize, barley, millet, wheat, rice, sorghum, rye, and/or triticale.

[0011] The invention also provides a method of reducing phytic acid in DDGS, comprising

(a) contacting a slurry comprising a starch substrate with at least one α-amylase to produce a liquefact;
(b) contacting the liquefact with at least one *Trichoderma reesei* glucoamylase (TrGA) and at least one phytase, wherein the phytase has at least 90% amino acid sequence identity to SEQ ID NO: 5 under conditions such that fermentable sugars are produced;
(c) and fermenting the fermentable sugars in the presence of a fermenting organism to produce ethanol and/or DDGS. In some embodiments, contacting the starch substrate with at least one glucoamylase and at least one phytase and fermenting the fermentable sugars in the presence of a fermenting organism occur simultaneously.

In some embodiments, the phytase has at least 95% sequence identity with the phytase of SEQ ID NO: 5 and has an alanine at amino acid 92. In some embodiments, the phytase is wild-type *Buttiauxella* spp., phytase (BP-WT) or a variant selected from BP-11 and BP-17.

[0012] The methods of the invention may make use of a single composition comprising a blend of a glucoamylase in combination with a phytase. Such a blend may be added during the pre-saccharification, saccharification, and/or combined saccharification/fermentation steps of a starch conversion process.

[0013] These and other aspects of the present compositions and methods are described in greater detail, below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figure 1 shows the free phosphorus content in DDGS obtained using an enzyme composition including BP-17 phytase at different levels in a yeast fermentation of corn.

BRIEF DESCRIPTION OF THE SEQUENCES

[0015]

SEQ ID NO: 1 is the amino acid sequence of *Trichoderma reesei* glucoamylase (TrGA).
SEQ ID NO: 2 is the amino acid sequence of the catalytic domain of TrGA corresponding to residues 1-453.
SEQ ID NO: 3 is the amino acid sequence of the linker region of TrGA corresponding to residues 453-491.
SEQ ID NO: 4 is the amino acid sequence of the starch binding domain of TrGA corresponding to residues 492-599.
SEQ ID NO: 5 is the amino acid sequence of the mature protein sequence of *Buttiauxella* phytase.
SEQ ID NO: 6 is the amino acid sequence of the mature protein sequence of *Buttiauxella* phytase variant D92A.

SEQ ID NO: 7 is the amino acid sequence of the mature protein sequence of *Buttiauxella* phytase variant BP-11.
SEQ ID NO: 8 is the amino acid sequence of the mature protein sequence of *Buttiauxella* phytase variant BP-17.

DETAILED DESCRIPTION

I. Introduction

[0016] Described herein are compositions and methods which relate to an enzyme blend including a glucoamylase in combination with at least one phytase for use in a starch conversion processes, *e.g.*, for the production of DDGS for animal feed or in a fermentation processes for producing organic compounds such as ethanol. The compositions and methods may be used for the reduction of phytic acid during saccharification, fermentation and/or simultaneous saccharification and fermentation (SSF), resulting in a reduction in phytic acid in the products or by-products of the fermentation (*e.g.*, the DDGS and the thin stillage).

[0017] The phytase used in the methods of the invention has at least 90% sequence identity to SEQ ID NO: 5, including at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and up to and including 100%. In some embodiments, the phytase further includes an alanine at position 92. In some embodiments, the phytase includes an alanine at position 92, and additionally has at least one of the following amino acid sequence features: a threonine at position 89, an isoleucine at position 134, a serine at position 164, a lysine at position 176, a proline at position 178 , a glutamic acid at position 207, a serine at position 209, a leucine at position 248, a tyrosine at position 256, a glutamic acid at position 261, and a lysine at position 269. In some embodiments, the phytase has at least one of the following amino acid substitutions: A89T, T134I, F164S, T176K, A178P, K207E, A209S, S248L, Q256Y, A261E, and N269K, with or without the additional substitution D92A.

[0018] In some embodiment, the glucoamylase is obtained from a filamentous fungus. In particular embodiments, the glucoamylase has at least about 90% sequence identity to *Trichoderma reesei* glucoamylase (TrGA; SEQ ID NO: 1), including at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and up to 100%.

[0019] These and other features of the present methods are described in more detail, below.

II. Definitions

[0020] Unless otherwise indicated, the present methods involve conventional techniques commonly used in molecular biology, protein engineering, recombinant DNA techniques, microbiology, cell biology, cell culture, transgenic biology, immunology, and protein purification. Such techniques are known to those with skill in the art and are described in numerous texts and reference works. Although particular methods and materials are exemplified, similar or equivalent methods and materials can be used. Unless defined otherwise, all technical and scientific terms should be accorded their ordinary meaning. The following terms are defined for clarity:

"Alpha amylases" are α-1,4-glucan-4-glucanohydrolases (E.C. 3.2.1.1) having the ability to cleave or hydrolyze internal α-1,4 -glycosidic linkages in starch *(e.g.,* amylopectin or amylose polymers).

[0021] The terms "granular starch hydrolyzing (GSH) enzyme" and "enzymes having granular starch hydrolyzing (GSH) activity" refer to enzymes that have the ability to hydrolyze starch in granular form.

[0022] The term "functional equivalent" refers to a molecule, *e.g.*, an enzyme, that has the same functional characteristics (such as enzymatic activity) of another molecule. The term "variant," when used with reference to an enzyme (*e.g.*, an α-amylase, a glucoamylase, an acid fungal protease, a phytase, or the like), refers to an enzyme derived from a parent enzyme but having a substitution, insertion, or deletion of one or more amino acids as compared to the parent enzyme. The term also includes hybrid forms of the enzyme, wherein, for example, the enzyme may have a C-terminus derived from one *Bacillus* spp. (*e.g., B. licheniformis*) and an N- terminus derived from a different *Bacillus* spp. (*e.g., B. stearothermophilus*), or vice versa. A variant may have one or more altered properties compared to the parent enzyme such as increased thermal stability, increased proteolytic stability, increase specific activity, broader substrate specificity, broader activity over a pH range, resistance to inhibition (*e.g.*, substrate), or combinations thereof. A "parent enzyme" refers to an enzyme that is used as a starting point for modifications. A parent enzyme may be a naturally-occurring or "wild-type" enzyme.

[0023] As used herein "liquefaction" or "to liquefy" refers to a process by which starch is converted to shorter-chain, less-viscous dextrins.

[0024] As used herein, "dextrins" refer to short chain polymers of glucose *(e.g.,* 2 to 10 units).

[0025] As used herein, the term "starch" refers to any material comprised of the complex polysaccharide carbohydrates (amylose and amylopectin) having the formula $(C_6H_{10}O_5)_x$, wherein x is any number.

[0026] As used herein, the term "granular starch" means raw starch, *i.e.,* starch that has not been subject to a temperature at which gelatinization occurs.

[0027] As used herein, the terms "saccharifying enzyme" and "glucoamylase" are used interchangeably and refer to any enzyme that is capable of catalyzing the release of D-glucose from the non-reducing ends of starch and related oligosaccharides and polysaccharides.

[0028] As used herein, the term "oligosaccharide" refers to molecules having 2 to 10 monosaccharide units joined in glycosidic linkages. The monosaccharides may be glucose and/or other sugars. Oligosaccharides include dextrins and starch.

[0029] As used herein, the term "fermentable sugars" refers to sugars that are capable of being fermented by a fermenting organism. Fermentable sugars include, but are not limited to, oligosaccharides and dextrins.

[0030] As used herein, the term "dextrose equivalent" or "DE" refers to an industry standard for measuring the concentration of total reducing sugars, calculated as D-glucose on a dry weight basis. Unhydrolyzed granular starch has a DE that is essentially 0 and D-glucose has a DE of 100.

[0031] As used herein, the term "total sugar content" refers to the total sugar content present in a starch composition. The "total sugar content" can be measured at various times or points in a process.

[0032] As used herein, the term "dry solids" or "ds" refers to the total solids within a slurry expressed as a percentage on a dry weight basis.

[0033] As used herein, "percent (%) sequence identity" with respect to an amino acid or nucleotide sequence refers to the percentage of amino acid residues or nucleotides in a one sequence that are identical to the amino acid residues or nucleotides in another sequence, as determined by aligning the sequences and introducing gaps, where necessary, to achieve the best alignment (*i.e.*, maximum percent sequence identity), and not considering conservative substitutions in determining sequence identity. Methods for performing sequence alignment and determining sequence identity are known and can be performed without undue experimentation to obtain definitive values. A number of algorithms are available for aligning sequences and determining sequence identity, including but not limited to: the homology alignment algorithm of Needleman et al., (1970) J. Mol. Biol. 48:443; the local homology algorithm of Smith et al., (1981) Adv. Appl. Math. 2:482; the search for similarity method of Pearson et al. (1988) Proc. Natl. Acad. Sci. USA 85:2444; the Smith-Waterman algorithm (1997) Meth. Mol. Biol. 70:173-187; BLASTP, BLASTN, and BLASTX algorithms (see Altschul et al., (1990) J. Mol. Biol. 215:403-410). Computerized programs using these algorithms are also available, and include, but are not limited to: ALIGN or Megalign (DNASTAR) software, or WU-BLAST-2 (see, *e.g.,* Altschul et al. (1996) Meth. Enzym. 266:460-480); or GAP, BESTFIT, BLAST (*e.g.,* Altschul *et al., supra,* FASTA, and TFASTA, available in the Genetics Computing Group (GCG) package, Version 8, Madison, Wis., USA); and CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, CA, USA.

[0034] Those skilled in the art know how to determine appropriate parameters for measuring alignment, including algorithms needed to achieve maximal alignment over the length of the sequences being compared. In some embodiments, the sequence identity is determined using the default parameters determined by the program. In some embodiments, sequence identity can be determined by the Smith-Waterman homology search algorithm (see *e.g.,* (1997) Meth. Mol. Biol. 70:173-187) as implemented in MSPRCH program (Oxford Molecular, Accelrys Ltd., Oxford England) using an affine gap search with the following search parameters: gap open penalty of 12, and gap extension penalty of 1. In some embodiments, paired amino acid comparisons can be carried out using the GAP program of the GCG sequence analysis software package of Genetics Computer Group, Inc., Madison, Wis., employing the blosum62 amino acid substitution matrix, with a gap weight of 12 and a length weight of 2. In some embodiments, with respect to optimal alignment of two amino acid sequences, the contiguous segment of the variant amino acid sequence may have at least one additional amino acid residue or at least one deleted amino acid residue with respect to the reference amino acid sequence. The contiguous segment used for comparison to the reference amino acid sequence includes at least about 20 contiguous amino acid residues and can include at least about 30, at least about 40, at least about 50 or more amino acid residues. Corrections for increased sequence identity associated with inclusion of gaps in the derivative's amino acid sequence can be made by assigning gap penalties.

[0035] Sequence searches are typically carried out using the BLASTN program when evaluating a given nucleic acid sequence relative to nucleic acid sequences in the GENBANK® DNA Sequence database and other public databases. The BLASTX program is preferred for searching nucleic acid sequences that have been translated in all reading frames against amino acid sequences in the GENBANK®Protein Sequences and other public databases. Both BLASTN and BLASTX are typically run using default parameters of an open gap penalty of 11.0, and an extended gap penalty of 1.0, and utilize the BLOSUM-62 matrix (see, *e.g.,* Altschul *et al.* (1997)).

[0036] Alignments of selected sequences find use in determining % identity (a term that is used interchangeably herein with the term % homology) between two or more sequences. The CLUSTAL-W program in MacVector version 6.5, operated with default parameters, including an open gap penalty of 10.0, an extended gap penalty of 0.1, and a BLOSUM 30 similarity matrix can be used.

[0037] As used herein, the term "milled" refers to plant material that has been reduced in size, *e.g.,* by grinding,

crushing, fractionating or any other means of particle size reduction or selection. The term encompasses dry and wet milling. "Dry milling" refers to the milling of whole dry grain, while "wet milling" refers to a process whereby grain is first soaked (*i.e.,* steeped) in water to soften the grain.

[0038] As used herein, the term "gelatinization" refers to the solubilization of a starch molecule, generally by cooking at an elevated temperature, to form a viscous suspension.

[0039] As used herein, the term "gelatinization temperature" refers to the temperature at which gelatinization of a starch-containing substrate begins. In some embodiments, this is the lowest temperature at which gelatinization of a starch containing substrate begins. The exact temperature of gelatinization depends on the specific form of starch present and may vary depending on factors such as plant species, environmental conditions, growth conditions, and other parameters.

[0040] As used herein, the term "below the gelatinization temperature" refers to a temperature that is less than the gelatinization temperature.

[0041] As used herein, the term "slurry" refers to an aqueous mixture comprising insoluble solids (*e.g.*, granular starch).

[0042] As used herein, the term "fermentation" refers to the enzymatic breakdown of organic substances by microorganisms to produce simpler organic compounds. While fermentation occurs under anaerobic conditions it is not intended that the term be solely limited to strict anaerobic conditions, as fermentation also occurs in the presence of oxygen (*e.g.*, under microaerophilic and other conditions).

[0043] As used herein, the phrase "simultaneous saccharification and fermentation" or "SSF" refers to a process in the production of an end-product in which a fermenting organism, such as an ethanol producing microorganism, and at least one enzyme, such as a saccharifying enzyme, are combined in the same process step in the same vessel.

[0044] As used herein, the term "thin stillage" means the liquid portion of stillage separated from the solids *(e.g.,* by screening or centrifugation) which contains suspended fine particles and dissolved material. The term "backset" is generally used to mean recycled thin stillage.

[0045] As used herein, the term "end-product" refers to a carbon-source derived product which is enzymatically converted from a fermentable substrate. In some embodiments, the end-product is an alcohol (*e.g.*, ethanol).

[0046] As used herein, the term "derived from" encompasses the terms "originated from," "obtained from," "obtainable from," and "isolated from."

[0047] As used herein the term "fermenting organism" refers to a microorganism or cell that is suitable for use in fermentation methods for directly or indirectly producing an end-product. In some embodiments, the fermentating organism is eukaryotic (*e.g.*, fungi), while in others it is prokaryotic (*e.g.*, bacteria).

[0048] As used herein the term "ethanol producer" or ethanol producing microorganism" refers to a fermenting organism that is capable of producing ethanol from a mono- or oligosaccharide.

[0049] As used herein, the terms "recovered," "isolated," and "separated" refer to a protein, cell, nucleic acid, or amino acid that is removed from at least one component with which it is naturally associated.

[0050] As used herein, the terms "protein" and "polypeptide" are used interchangeably to refer to a series of amino acid residue linked via peptide bonds. Both the conventional one-letter and three-letter codes for amino acid residues are used. The 3-letter code is in conformity with the IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). It is understood that a polypeptide can be encoded by more than one nucleotide sequence due to the degeneracy of the genetic code. Unless otherwise indicated amino acids are written left to right in amino to carboxy orientation.

[0051] As used herein, the term "phytase" refers to an enzyme which is capable of catalyzing the hydrolysis of esters of phosphoric acid, including phytate, and releasing inorganic phosphate and inositol. In some embodiments, in addition to phytate, the phytase is capable of hydrolyzing at least one of the inositol-phosphates of intermediate degrees of phosphorylation.

[0052] As used herein, the term "wild-type" refers to a naturally-occurring (native) polypeptide or polynucleotide. The term wild-type may, in some cases, be used interchangeably with the terms "parent" or "parent sequence."

[0053] As used herein, the terms "contacting" and "exposing" refer to placing at least one enzyme in sufficient proximity to its cognate substrate to enable the enzyme to convert the substrate to at least one end-product. The end-product may be a "product of interest" (*i.e.*, an end-product that is the desired outcome of the fermentation reaction). "Contacting" includes mixing a solution comprising an enzyme with the cognate substrate.

[0054] As used herein, the singular terms "a," "an," and "the" includes the plural unless the context clearly indicates otherwise. Thus, for example, reference to a composition containing "a compound" includes a mixture of two or more compounds. The term "or" generally means "and/or," unless the content clearly dictates otherwise.

[0055] Numeric ranges are inclusive of the numbers defining the range. Where a range of values is provided, it is understood that each intervening value between the upper and lower limits of that range is also specifically disclosed, to a tenth of the unit of the lower limit (unless the context clearly dictates otherwise). The upper and lower limits of smaller ranges may independently be included or excluded in the range..

III. Exemplary Embodiments

A. Glucoamylases

[0056] Various glucoamylases (GA) (E.C. 3.2.1.3) may be used in the invention. In some embodiments, the GA are endogenously expressed by bacteria, plants, and/or fungi, while in other embodiments the GA are heterologous to the host cells (*e.g.*, bacteria, plants and/or fungi). In some embodiments, the GA are produced by strains of filamentous fungi and yeast, *e.g.*, commercially available GA produced by strains of *Aspergillus* and *Trichoderma.* Suitable GA include naturally occurring wild-type enzymes as well as variant and genetically engineered mutant enzyme, such as hybrid GA. Hybrid GA include those having a catalytic domain from a GA from one organism (*e.g.*, Talaromyces GA) and a starch binding domain (SBD) from a GA from a different organism (*e.g., Trichoderma* GA). In some embodiments, the linker is included with the starch binding domain (SBD) or the catalytic domain. The following are exemplary GA suitable for use as described: *Aspergillus niger* G1 and G2 GA (see *e.g.,* Boel et al. (1984) EMBO J. 3:1097-1102; WO 92/00381, WO 00/04136 and USP 6,352,851); *Aspergillus awamori* GA (see *e.g.,* WO 84/02921); *Aspergillus oryzae* GA (see *e.g.,* Hata et al. (1991) Agric. Biol. Chem. 55:941-949), and *Aspergillus shirousami* GA (see *e.g.,* Chen et al. (1996) Prot. Eng. 9:499-505; Chen et al. (1995) Prot. Eng. 8:575-582; and Chen et al. (1994) Biochem J. 302:275-281).

[0057] Additional GA include those obtained from strains of *Talaromyces* (*e.g., T. emersonii, T. leycettanus, T. duponti* and *T. thermophilus* (see *e.g.,* WO 99/28488; U.S. Pat. No. RE 32,153; U.S. Pat. No. 4,587,215); strains of *Trichoderma* (*e.g., T. reesei*); strains of *Rhizopus,* (*e.g., R. niveus and R. oryzae*); strains of *Mucor* and strains of *Humicola,* (*e.g., H. grisea* (see, *e.g.,* Boel et al. (1984) EMBO J. 3:1097-1102; WO 92/00381; WO 00/04136; Chen et al. (1996) Prot. Eng. 9:499-505; Taylor et al. (1978) Carbohydrate Res. 61:301-308; U.S. Pat. Nos. 4,514,496, 4,092,434, and 4,618,579; Jensen et al. (1988) Can. J. Microbiol. 34:218-223; and SEQ ID NO: 3 of WO 2005/052148); as well as GA having at least about 80%, at least about 85%, at least about 90%, or at least about 95% sequence identity to SEQ ID NO: 4 disclosed in U.S. Pat. Pub. No. 2006-0094080.

[0058] In some embodiments, the GA has at least about 85%, at least about 90%, at least about 92%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% and even at least about 99% sequence identity to the amino acid sequence of SEQ ID NO: 3 of WO 05/052148. Other GA useful in the present invention include those obtained from *Athelia rolfsii* and variants thereof (see, *e.g.,* WO 04/111218) and *Penicillium* spp. (see, *e.g., Penicillium chrysogenum*).

[0059] Commercially available GA suitable for use as described include but are not limited to DISTILLASE®, OPTI-DEX® L-400 and G ZYME® G990 4X, GC480, G-ZYME 480, FERMGEN® 1-400 (Danisco US, Inc, Genencor Division) CU.CONC® (Shin Nihon Chemicals, Japan), GLUCZYME (Amano Pharmaceuticals, Japan (see *e.g.* Takahashi et al. (1985) J. Biochem. 98:663-671)). Additional enzymes for use as described include three forms of GA (E.C.3.2.1.3) produced by a *Rhizopus* spp., namely "Gluc1" (MW 74,000), "Gluc2" (MW 58,600), and "Gluc3" (MW 61,400). Generally, any suitable GA can be used in accordance with the present composition and methods.

[0060] The mature amino acid sequence (SEQ ID NO: 1) of the *Trichoderma reesei* GA (TrGA) is shown below. The sequence has 599 amino acids, the catalytic domain (SEQ ID NO: 2) is underlined and corresponds to residues 1-453; the linker region (SEQ ID NO: 3) corresponds to residues 453-491; and the starch binding domain SEQ ID NO: 4 (in italics) corresponds to residues 492-599.

Mature protein sequence of *Trichoderma reesei* glucoamylase (TrGA) (SEQ ID NO: 1)

1 SVDDFISTET PIALNNLLCN VGPDGCRAFG TSAGAVIASP STIDPDYYYM
51 WTRDSALVFK NLIDRFTETY DAGLQRRIEQ YITAQVTLQG LSNPSGSLAD
101 GSGLGEPKFE LTLKPFTGNW GRPQRDGPAL RAIALIGYSK WLINNNYQST
151 VSNVIWPIVR NDLNYVAQYW NQTGFDLWEE VNGSSFFTVA NQHRALVEGA
201 TLAATLGQSG SAYSSVAPQV LCFLQRFWVS SGGYVDSNIN TNEGRTGKDV
251 NSVLTSIHTF DPNLGCDAGT FQPCSDKALS NLKVVVDSFR SIYGVNKGIP
301 AGAAVAIGRY AEDVYYNGNP WYLATFAAAE QLYDAIYVWK KTGSITVTAT
351 SLAFFQELVP GVTAGTYSSS SSTFTNIINA VSTYADGFLS EAAKYVPADG
401 SLAEQFDRNS GTPLSALHLT WSYASFLTAT ARRAGIVPPS WANSSASTIP
451 STCSGASVVG SYSRPTATSF PPSQTPKPGV PSGTPYTPLP *CATPTSVAVT*
501 *FHELVSTQFG QTVKVAGNAA ALGNWSTSAA VALDAVNYAD NHPLWIGTVN*
551 LEAGDVVEYK YINVGQDGSV TWESDPNHTY TVPAVACVTQ VVKEDTWQS

[0061] In some embodiments, the GA has at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% and even at least about 99% sequence identity to the amino acid sequence of SEQ ID NOs: 1 or 2. In some embodiments, the GA is the TrGA disclosed in U.S. Pat. No. 7,413,887.

B. Phytases

[0062] Various phytases as defined in the claims may be used in the invention. Useful phytases include those capable of hydrolyzing phytic acid under the defined conditions of saccharification, fermentation and/or simultaneous saccharification and fermentation described herein. The methods involve the addition of at least one phytase to a saccharification and/or SSF and the phytase is capable of liberating at least one inorganic phosphate from an inositol hexaphosphate (*e.g.*, phytic acid). As applicable, the phytase is obtained from a *Buttiauxiella* spp, such as *B. agrestis, B. brennerae, B. ferragutiase, B. gaviniae, B. izardii, B. noackiae,* or *B. warmboldiae.* Strains of *Buttiauxella* spp. are available from DSMZ, the German National Resource Center for Biological Material (Inhoffenstrabe 7B, 38124 Braunschweig, Germany) and other repositories. The phytase has at least 90%, e.g. at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% and even at least about 99% sequence identity to *Buttiauxiella* spp. phytase, having the amino acid sequence set forth in SEQ ID NO: 5.

Mature protein sequence of *Buttiauxella* phytase (SEQ ID NO: 5)

```
NDTPASGYQV EKVVILSRHG VRAPTKMTQT MRDVTPNTWP EWPVKLGYIT
PRGEHLISLM GGFYRQKFQQ QGILSQGSCP TPNSIYVWAD VDQRTLKTGE
AFLAGLAPQC GLTIHHQQNL EKADPLFHPV KAGTCSMDKT QVQQAVEKEA
QTPIDNLNQH YIPFLALMNT TLNFSTSAWC QKHSADKSCD LGLSMPSKLS
IKDNGNKVAL DGAIGLSSTL AEIFLLEYAQ GMPQAAWGNI HSEQEWASLL
KLHNVQFDLM ARTPYIARHN GTPLLQAISN ALNPNATESK LPDISPDNKI
LFIAGHDTNI ANIAGMLNMR WTLPGQPDNT PPGGALVFER LADKSGKQYV
SVSMVYQTLE QLRSQTPLSL NQPAGSVQLK IPGCNDQTAE GYCPLSTFTR
VVSQSVEPGC QLQ
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[0063] A variant of *Buttiauxiella* spp. phytase having an alanine at amino acid 92 is set forth in SEQ ID NO: 6.

Mature protein sequence of *Buttiauxella* phytase variant D92A (SEQ ID NO: 6)

```
NDTPASGYQV EKVVILSRHG VRAPTKMTQT MRDVTPNTWP EWPVKLGYIT
PRGEHLISLM GGFYRQKFQQ QGILSQGSCP TPNSIYVWAD VAQRTLKTGE
AFLAGLAPQC GLTIHHQQNL EKADPLFHPV KAGTCSMDKT QVQQAVEKEA
QTPIDNLNQH YIPFLALMNT TLNFSTSAWC QKHSADKSCD LGLSMPSKLS
IKDNGNKVAL DGAIGLSSTL AEIFLLEYAQ GMPQAAWGNI HSEQEWASLL
KLHNVQFDLM ARTPYIARHN GTPLLQAISN ALNPNATESK LPDISPDNKI
LFIAGHDTNI ANIAGMLNMR WTLPGQPDNT PPGGALVFER LADKSGKQYV
SVSMVYQTLE QLRSQTPLSL NQPAGSVQLK IPGCNDQTAE GYCPLSTFTR
VVSQSVEPGC QLQ
```

[0064] In some embodiments, the phytase is *Buttiauxella* phytase variant BP-11, having the amino acid sequence set forth in SEQ ID NO: 7, and which includes substitutions at amino acid residues A89, T134, F164, T176, A178, K207, A209, S248, Q256, A261, and N269, relative to the sequence of the wild-type enzyme (SEQ ID NO: 5). The particular substitutions are A89T, T134I, F164S, T176K, A178P, K207E, A209S, S248L, Q256Y, A261E, and N269K.

Mature protein sequence of *Buttiauxella* phytase variant BP-11 (SEQ ID NO: 7)

```
NDTPASGYQV EKVVILSRHG VRAPTKMTQT MRDVTPNTWP EWPVKLGYIT
PRGEHLISLM GGFYRQKFQQ QGILSQGSCP TPNSIYVWTD VDQRTLKTGE
AFLAGLAPQC GLTIHHQQNL EKADPLFHPV KAGICSMDKT QVQQAVEKEA

QTPIDNLNQH YIPSLALMNT TLNFSKSPWC QKHSADKSCD LGLSMPSKLS
IKDNGNEVSL DGAIGLSSTL AEIFLLEYAQ GMPQAAWGNI HSEQEWALLL
KLHNVYFDLM ERTPYIARHK GTPLLQAISN ALNPNATESK LPDISPDNKI
LFIAGHDTNI ANIAGMLNMR WTLPGQPDNT PPGGALVFER LADKSGKQYV
SVSMVYQTLE QLRSQTPLSL NQPAGSVQLK IPGCNDQTAE GYCPLSTFTR
VVSQSVEPGC QLQ
```

[0065] In some embodiments, the phytase has an alanine at position 92 (as set forth in SEQ ID NO: 6), and at least

one of the following amino acids: a threonine at position 89, an isoleucine at position 134, a serine at position 164, a lysine at position 176, a proline at position 178, a glutamic acid at position 207, a serine at position 209, a leucine at position 248, a tyrosine at position 256, a glutamic acid at position 261, and a lysine at position 269.

[0066] In some embodiments, the phytase has at least one of the following amino acid changes: A89T, D92A, T134I, F164S, T176K, A178P, K207E, A209S, S248L, Q256Y, A261E, and N269K.

[0067] In some embodiments, the phytase is *Buttiauxella* phytase variant BP-17, having the amino acid sequence set forth in SEQ ID NO: 8, and which includes substitutions at amino acid residues A89, D92, T134, F164, T176, A178 , K207, A209, S248, Q256, A261, and N269, relative to the sequence of the wild-type enzyme (SEQ IFD NO: 5).

[0068] The particular substitutions are A89T, D92A, T134I, F164S, T176K, A178P, K207E, A209S, S248L, Q256Y, A261E, and N269K.

Mature protein sequence of *Buttiauxella* phytase variant BP-17 (SEQ ID NO: 8)

NDTPASGYQV EKVVILSRHG VRAPTKMTQT MRDVTPNTWP EWPVKLGYIT
PRGEHLISLM GGFYRQKFQQ QGILSQGSCP TPNSIYVW**T**D V**A**QRTLKTGE
AFLAGLAPQC GLTIHHQQNL EKADPLFHPV KAG**I**CSMDKT QVQQAVEKEA
QTPIDNLNQH YIP**S**LALMNT TLNFS**K**S**P**WC QKHSADKSCD LGLSMPSKLS
IKDNGN**E**V**S**L DGAIGLSSTL AEIFLLEYAQ GMPQAAWGNI HSEQEWA**L**LL
KLHNV**Y**FDLM **E**RTPYIARH**K** GTPLLQAISN ALNPNATESK LPDISPDNKI
LFIAGHDTNI ANIAGMLNMR WTLPGQPDNT PPGGALVFER LADKSGKQYV
SVSMVYQTLE QLRSQTPLSL NQPAGSVQLK IPGCNDQTAE GYCPLSTFTR
VVSQSVEPGC QLQ

[0069] Methods for identification of suitable phytases, as defined in the claims, including those from *Buttiauxella* spp. are known in the art (see, *e.g.,* WO 06/043178).

C. Secondary Enzymes and components

[0070] The methods described herein may optionally include other enzymes, including but are not limited to α-amylases, acid fungal proteases, other GA, other phytases, cellulases, hemicellulases, xylanases, proteases, pullulanases, beta amylases, lipases, cutinases, pectinases, β-glucosidases, galactosidases, esterases, cyclodextrin transglycosyltransferases (CGTases), oxido-reductases, esterases, β-amylases, and combinations thereof. In some embodiments, the secondary enzyme is a second GA, including any GA mentioned, above. In some embodiments the additional enzyme is a second phytase, including any bacterial or fungal phytase, such as those mentioned, above. In the methods of the invention, a (first) α-amylase is always present in step (a). In some embodiments, the additional enzyme is an α-amylase, such as an acid stable α-amylase which, when added in an effective amount, has activity in the pH range of about 3.0 to about 7.0, including from about 3.5 to about 6.5. α-amylases that find use in the present invention include but are not limited to, fungal α-amylases or bacterial α-amylases. In some embodiments, the α-amylase is a wild-type α-amylase, a variant or fragment thereof, or a hybrid α-amylase that is derived from, for example, a catalytic domain from one enzyme and a starch binding domain from another. α-amylases include acid stable α-amylases and α-amylases having granular starch hydrolyzing activity (GSHE).

[0071] In some embodiments, α-amylases include those obtained from filamentous fungal strains including but not limited to strains such as *Aspergillus* (*e.g., A. niger, A. kawachi,* and *A. oryzae*); *Trichoderma* spp., *Rhizopus* spp., *Mucor* spp., and *Penicillium* spp. In some embodiments, the α-amylase is obtained from a strain of *Aspergillus kawachi* or a strain of *Trichoderma reesei.* In some embodiments, the α-amylase is a GSHE such as TrAA or AkAA. In some embodiments, the α-amylase is a hybrid enzyme comprising a fragments derived from enzymes obtained from A. *kawachi* and *A. niger.*

[0072] Additional α-amylases useful as secondary enzymes include those obtained from bacteria such as *Bacillus* (*e.g., B. licheniformis, B. lentus, B. coagulans, B. amyloliquefaciens, B. stearothermophilus, B subtilis,* and hybrids, mutants and variants thereof (see, *e.g.,* U.S. Pat Nos. 5,763,385, 5,824,532, 5,958,739, 6,008,026, and 6,361,809). Some of these amylases are commercially available, *e.g.*, TERMAMYL®, LIQUEZYME® SC, and SUPRA® available from Novo Nordisk A/S, ULTRATHIN® from Diversa, and SPEZYME® FRED, SPEZYME® XTRA, and GZYME® G997 available from Danisco US, Inc, Genencor Division.

[0073] In some embodiments, the secondary enzyme is a cellulase. Cellulases are enzymes that hydrolyze cellulose (β-1, 4-D-glucan linkages) and/or derivatives thereof, such as phosphoric acid swollen cellulose. Cellulases include exocellobiohydrolases (CBH), endoglucanases (EG) and β-glucosidases (BG) (EC3.2.191, EC3.2.1.4 and EC3.2.1.21). Examples of suitable cellulases include, but are not limited to, those from *Penicillium, Trichoderma, Humicola, Fusarium, Thermomonospora, Cellulomonas, Clostridium,* and *Aspergillus.* Commercially available cellulases sold for feed appli-

cations include β-glucanases such as ROVABIO® (Adisseo), NATUGRAIN® (BASF), MULTIFECT® BGL (Danisco Genencor), and ECONASE® (AB Enzymes).

[0074] In some embodiments, the secondary enzyme is a xylanase. Xylanases (*e.g.* endo-β-xylanases (E.C. 3.2.1.8)) hydrolyze xylan backbone chains. Suitable xylanases include those obtained from bacterial sources (*e.g., Bacillus, Streptomyces, Clostridium, Acidothermus, Microtetrapsora,* and *Thermonospora*), and from fungal sources (*e.g., Aspergillus, Trichoderma, Neurospora, Humicola, Penicillium,* and *Fusarium* (see, *e.g.,* EP 473 545, U.S. Pat No. 5,612,055, WO 92/06209, and WO 97/20920). Commercial preparations include MULTIFECT® and FEEDTREAT® Y5 (Danisco US, Inc. Genencor Division), RONOZYME® WX (Novozymes A/S), and NATUGRAIN® WHEAT (BASF).

[0075] In some embodiments, the secondary enzyme is a protease. In some embodiments, the protease is obtained from *Bacillus* (*e.g., B. amyloliquefaciens, B. lentus, B. licheniformis,* and *B. subtilis*). These enzymes include subtilisins (see, *e.g.,* U.S. Pat. No. 4,760,025). Suitable commercial protease include MULTIFECT® P 3000 (Danisco US, Inc. Genencor Division) and SUMIZYME® FP (Shin Nihon). In some embodiments, the protease is derived from a fungal source (*e.g., Trichoderma* NSP-*24, Aspergillus, Humicola,* and *Penicillium*). In some embodiments, the protease is an acid fungal protease (AFP) including but not limited to those obtained from *Aspergillus, Trichoderma, Mucor* and *Rhizopus,* such as *A. niger, A. awamori, A. oryzae,* and *M. miehei.* Proteases can be obtained from the heterologous or endogenous protein expression of bacteria, plants, and fungi sources. Proteases include naturally occurring wild-type proteases as well as variant and genetically engineered mutant proteases, including those described in U.S. Pat. No. 7,429,476. In some embodiments, the secondary component is at least one fermenting organism.

IV. Compositions for use in the methods of the invention

[0076] The methods of the invention may employ a composition comprising blended or formulated enzymes, including at least one glucoamylase and at least one phytase. The phytase is a *Buttiauxella* spp. phytase as defined in the claims. In particular embodiments, the phytase is a BP-WT, BP-11, or BP-17 phytase.

[0077] In some embodiments, the phytase has an alanine at position 92. The phytase may further include other mutations described herein.

[0078] In some embodiments of the methods, the enzyme components of the composition are a blended formulation comprising at least the two enzyme components mixed together. In some embodiments, the compositions comprise a GA and phytase formulated in a sutable enzyme formulation. In some embodiments, the formulated enzyme composition provides a specific preselected ratio of GA and phytase and, optionally, other secondary enzymes. In some embodiments, the enzyme components are individually added during one or more process steps to produce a composition comprising the two enzymes. This may involve adding the separate components of the composition in a time or step-wise manner such that a ratio is maintained, or adding the components simultaneously.

[0079] In some embodiments, the amount of phytase used is from about 0.01 to about 10 FTU/g, including about 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.11, 0.12, 0.13, 0.14, 0.15, 0.16, 0.17, 0.18, 0.19, 0.2, 0.25, 0.28, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, 0.95, 1.0, 1.1, 1.5, 1.8, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20. Larger amounts of phytase can also be used. In some embodiments, the about of phytase used is from about 0.01 to about 1.0 FTU/g. In some embodiments, the amount of phytase used is at least about 0.01 FTU/g, including at least about 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.11, 0.12, 0.13, 0.14, 0.15, 0.16, 0.17, 0.18, 0.19, 0.2, 0.25, 0.28, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, 0.7, 0.75, 0.8 FTU/g.

V. Methods of Use

[0080] The methods described use a glucoamylase in combination with a phytase during saccharification, fermentation, and/or SSF in a starch conversion processes, resulting in a process that produces less phytic acid end-products and/or by-products than are produced using conventional methods. In some embodiments, the process results in DDGS with reduced phytic acid compared to conventional methods. In some embodiments, the process results in ethanol and provides thin stillage with reduced phytic acid compared to conventional methods.

[0081] Various types of plant material can be used with the present methods. In some embodiments, the plant material is grain. In some embodiments, the plant material is obtained from wheat, corn, rye, sorghum (*e.g.*, milo), rice, millet, barley, triticale, cassava (*e.g.*, tapioca), potato, sweet potato, sugar beets, sugarcane, and legumes such as soybean and peas, and combinations thereof. Plant materials include hybrid varieties and genetically modified varieties (*e.g.*, transgenic corn, barley, or soybeans comprising heterologous genes). Any part of the plant can be used as a substrate, including but not limited to, leaves, stems, hulls, husks, tubers, cobs, grains, and the like. In some embodiments, essentially the entire plant is used, for example, the entire corn stover. In some embodiments, whole grain is used as a source of granular starch. Whole grains include corn, wheat, rye, barley, sorghum, and combinations thereof. In other embodiments, granular starch is obtained from fractionated cereal grains including fiber, endosperm, and/or germ com-

ponents. Methods for fractionating plant material, such as corn and wheat, are known in the art. In some embodiments, plant material obtained from different sources is mixed (*e.g.* corn and milo or corn and barley). The plant material is prepared by means such as milling. Two general milling processes are wet milling and dry milling. In dry milling, the whole grain is milled and used, while in wet milling, the grain is separated (*e.g.*, the germ from the meal). Means of milling whole cereal grains are known and include the use of hammer mills and roller mills. Reference is made TO THE ALCOHOL TEXTBOOK: A REFERENCE FOR THE BEVERAGE, FUEL AND INDUSTRIAL ALCOHOL INDUSTRIES 3rd ED. K.A. Jacques et al., Eds, (1999) Nottingham University Press. See, Chapters 2 and 4. The plant material containing a starch substrate is hydrolyzed and/or liquefied using an α-amylase to produce oligosaccharides. In some embodiments, an alpha α-amylase is added to a slurry of milled starch substrate (*e.g.*, milled grain) to produce a liquefact containing dextrins and/or oligosaccharides. The skilled person will be able to determine the effective dosage, pH, and contact time of α-amylase to be used in the processes. The optimal usage level in a liquifaction depends upon processing parameters such as type of plant material, viscosity, processing time, pH, temperature and ds.

VI. Sequential and Simultaneous Saccharification and Fermentation

[0082] The liquefact containing dextrins and/or oligosaccharides from the liquefaction is subjected to saccharification, fermentation, and/or simultaneous saccharification and fermentation (SSF). Saccharification further reduces the sugars in a liquefact containing dextrins, and/or oligosaccharides to fermentable sugars. The fermentable sugars are then converted by fermenting microorganisms to obtain end-products such as alcohols and DDGS, which can be recovered using a suitable method.

[0083] In some embodiments, saccharification and fermentation occur simultaneously, in a process called simultaneous saccharification fermentation (SSF). In some embodiments the saccharification and fermentation occur separately. In some embodiments, the GA/phytase enzyme composition is added during a pre-saccharification step, during the saccharification process, during the fermentation process, or during the SSF process.

[0084] In some embodiments, the saccharification process lasts for 12 to 120 hours. However, it is common to perform a pre-saccharification step for 30 minutes to 2 hours (including for example, 30 to 60 minutes) and then to complete saccharification during fermentation. Saccharification is commonly carried out at temperatures of 30 to 65°C and typically at pH of 4.0 to 5.0. Where a pre-saccharification step is included, the phytase is added during the pre-saccharification step.

[0085] Any of the GA described herein find use as saccharfying enzymes. In some embodiments, the enzyme compositions are added at the beginning of the saccharification step as a GA/phytase blend. In other embodiments, the GA and phytase are added separately. In some embodiments, the GA is added at the beginning of the saccharification step and the phytase is added later but before the fermentation step is completed. In some embodiments, saccharification and fermentation are conducted simultaneously and a GA/phytase blend is added during simultaneous saccharification/fermentation (SSF). The resulting fermentable sugars are subjected to fermentation with fermenting microorganisms. In some embodiments, the contacting step and the fermenting step are performed simultaneously in the same reaction vessel. In other embodiments, these steps are performed sequentially. Fermentation processes are generally described in The Alcohol Textbook 3rd ED, A Reference for the Beverage, Fuel and Industrial Alcohol Industries, Eds. Jacques et al., (1999) Nottingham University Press, UK.

[0086] The fermentable sugars or dextrins (*e.g.* glucose) resulting from the saccharification are used as a fermentation feedstock in microbial fermentations under suitable conditions to obtain end-products, such as alcohol (*e.g.,* ethanol), organic acids (*e.g.,* succinic acid, lactic acid), sugar alcohols (*e.g.*, glycerol), ascorbic acid intermediates (*e.g.,* gluconate, DKG, KLG) amino acids (*e.g.,* lysine), and/or proteins (*e.g.,* antibodies and fragment thereof).

[0087] The fermentable sugars may be fermented with yeast at temperatures in the range of about 15 to about 40°C, about 20 to about 38°C, and even about 25 to about 35°C; at a pH range of about 3.0 to about 6.5; about 3.0 to about 6.0; about 3.0 to about 5.5, about 3.5 to about 5.0, and even about 3.5 to about 4.5; and for a period of time of about 5 hrs to about 120 hours, including about 12 to about 120 and from about 24 to about 90 hours, to produce an alcohol product, such as ethanol.

[0088] Yeast cells may be provided in an amount of about 10^4 to about 10^{12} cells, or from about 10^7 to about 10^{10} viable yeast cells per ml of fermentation broth. The fermentation process may include the addition of raw materials, such as nutrients, acids, and additional enzymes, as well as supplements such as vitamins (*e.g.*, biotin, folic acid, nicotinic acid, riboflavin), cofactors, macro-nutrients, micro-nutrients and salts (*e.g.*, $(NH4)_2SO_4$; K_2HPO_4; NaCl; $MgSO_4$ H_3BO_3; $ZnCl_2$; and $CaCl_2$).

VII. Fermenting Organisms

[0089] Any suitable fermenting organism may be used with the present methods. Examples of suitable fermenting organisms are ethanologenic microorganisms or ethanol producing microorganisms such as ethanologenic bacteria which express alcohol dehydrogenase and pyruvate dehydrogenase, which can be obtained from *Zymomonas moblis*

(see *e.g.,* U.S. Pat. Nos. 5,000,000, 5,028,539, 5,424,202, 5,514,583, and 5,554,520). The ethanologenic microorganisms may express xylose reductase and xylitol dehydrogenase, which are enzymes that convert xylose to xylulose. Alternatively or additionally, xylose isomerase is used to convert xylose to xylulose. A microorganism capable of fermenting both pentoses and hexoses to ethanol may be utilized. The microorganism can be a naturally-occurring or non-genetically engineered microorganism or an engineered or recombinant microorganism. Fermenting microorganisms include bacterial strains from *Bacillus, Lactobacillus, E. coli, Erwinia, Pantoea* (*e.g., P. citrea*), *Pseudomonas* and *Klebsiella* (*e.g., K. oxytoca*) (see *e.g.* U.S. Pat. Nos. 5,028,539 and 5,424,202 and WO 95/13362). The fermenting microorganism selected depends on the end-product to be produced.

[0090] The ethanol-producing microorganism may be a fungal microorganism, such as a *Saccharomyces* strain including but not limited to *S. cerevisiae* (see, *e.g.,* U.S. Pat. No. 4,316,956). A variety of *S. cerevisiae* are commercially available and include but are not limited to FALI® (Fleischmann's Yeast), SUPERSTART® (Alltech), FERMIOL® (DSM Specialties), RED STAR® (Lesaffre), and ANGEL ALCOHOL YEAST® (Angel Yeast Company, China).

VIII. Recovery of Alcohol, DDGS and Other End-products

[0091] The end product of the fermentation process is an alcohol (*e.g.*, ethanol or butanol), which can be separated and/or purified from the fermentation media. Methods for separation and purification are known in the art and include methods such as subjecting the media to extraction, distillation, column chromatography, molecular sieve dehydration, or ultra filtration. The end-product may be identified directly by submitting the media to high-pressure liquid chromatography (HPLC) or gas chromatography (CG) analysis.

[0092] Where the end-product is ethanol, it may be used for fuel, cleaning, or chemical synthesis, or injected as a beverage. Fermentation co-products such as distillers dried grains (DDG) and distiller's dried grain plus solubles (DDGS) can be used as an animal feed.

[0093] The present methods can reduce the phytic acid content of the fermentation broth, the thin stillage and/or the co-products of the fermentation such as distillers dried grains (DDG); distillers dried grains with solubles (DDGS); distillers wet grains (DWG), and distillers wet grains with solubles (DWGS). For example, the compositions and methods can reduce the phytic acid content of fermentation filtrate by at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85% and even at least about 90% or more as compared to essentially the same process but without the phytase. The amount of phytate found in the DDGS can be reduced by at least about 50%, at least about 70%, at least about 80% and at least about 90% as compared to the phytate content in DDGS from a corresponding process which is essentially the same as the claimed process but without a phytase pretreatment incubation. For example, while the % phytate content in commercial samples of DDGS may vary, a general range of % phytate is be from about 1% to about 3% or higher. In comparison, the % phytate in the DDGS obtained using the current process is less than about 1.0%, less than about 0.8% and even less than about 0.5%. DDGS can be added to an animal feed before or after pelletization and may include active phytase

[0094] In some industrial ethanol processes, ethanol is distilled from the filtrate resulting in a thin stillage portion that is suitable for recycling into the fermentation stream. Using the present compositions and methods, the thin stillage has a lower phytic acid content compared to that obtained using a onventional method. The reduction in phytic acid may result from the addition of phytase during a pretreatment step, during saccharification, during saccharification/fermentation, or a combination, thereof. The reduction in phytic acid content of the thin stillage may be at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85% or even at least about 90% or more, compared to essentially the same process but without the phytase. Similarly, the amount of phytate found in the thin stillage can be reduced by at least about 50%, at least about 60%, at least about 70%, at least about 80% or even at least about 90% compared to the phytate content in thin stillage resulting from an otherwise similar process that lacks a phytase.

EXAMPLES

[0095] The following examples are offered to illustrate, but not to limit the methods.

[0096] In the disclosure and experimental section that follows, the following abbreviations apply: wt% (weight percent); °C (degrees Centigrade); H_2O (water); dH_2O (deionized water); dIH_2O (deionized water, Milli-Q filtration); g or gm (grams); μg (micrograms); mg (milligrams); kg (kilograms); μL (microliters); ml and mL (milliliters); mm (millimeters); μm (micrometer); M (molar); mM (millimolar); μM (micromolar); U (units); MW (molecular weight); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); ds (dry solids); DO (dissolved oxygen); W/V (weight to volume); W/W (weight to weight); V/V (volume to volume); Genencor (Danisco US, Inc., Genencor Division, Palo Alto, CA); IKA (IKA Works Inc. 2635 North Chase Parkway SE, Wilmington, NC); MT (Metric ton); Ncm (Newton centimeter); GAU (glucoamylase activity unit; FTU (phytase activity unit) and ETOH (ethanol).

EXAMPLE 1: Viscosity Measurements

[0097] A glass cooker -viscometer, LR-2.ST system IKA was used to determine viscosity. In brief, the viscometer consists of a 2,000 ml double-walled glass vessel with an anchor mixer that is stirred by a Eurostar Labortechnik power control-viscometer. The viscosity range of the viscometer is 0-600 Ncm.

[0098] In general, a slurry comprising a starch substrate and an appropriate amount of enzyme was poured into the viscometer vessel. The temperature and viscosity were recorded during heating to 85°C and incubation was continued for additional 60 to 120 mins. Viscosity was measured in Ncm and recorded at intervals.

EXAMPLE 2: Use of a Glucoamylase and a Phytase in Ethanol Fermentation

[0099] This example shows the efficacy of glucoamylase (GA) and phytase in ethanol fermentation for producing DDGS and thin stillage with a lower phytate content than obtained using a conventional method. The GA used was the *Trichoderma reesei* GA (TrGA) corresponding to SEQ ID NO: 1 (see *e.g.,* U.S. Pat Nos. 7,354,752 and 7,413,887). The phytase used was *Buttiauxella* phytase, BP-17 corresponding to SEQ ID NO: 8.

[0100] Glucoamylase activity units (GAU) were defined as the amount of enzyme required to produce 1 g of reducing sugar calculated as glucose per hour from a soluble starch substrate at pH 4.2 and 60°C. The PNPG assay is used to measure the activity of glucoamylase.

[0101] Phytase activity (FTU) was measured by the release of inorganic phosphate, which forms a yellow complex with acidic molybdate/vandate reagent that can be measured at a wavelength of 415 nm in a spectrophometer. The released inorganic phosphate was quantified with a phosphate standard curve. One unit of phytase (FTU) was defined as the amount of enzyme that releases 1 micromole of inorganic phosphate from phytate per minute under the reaction conditions given in the European Standard (CEN/TC 327, 2005-TC327WI 003270XX).

[0102] To measure phytic acid content, phytic acid was extracted from a sample by adjusting the pH of a 5% slurry (for dry samples) to pH 10.0, and then using an HPLC ion exchange column to bind the phytic acid, which was eluted from the column using a NaOH gradient system. The phytic acid content in the liquid was calculated by comparing phytic acid to a standard.

EXAMPLE 3: Use of TrGA and BP17 in yeast fermentation - effect on DDGS

[0103] DDGS (Distillers Dry Grain Solids with Solubles), a component in some animal feeds that is derived from ethanol processing plants, contains phytic acid that is non-digestible by non-ruminants like poultry, fish and pigs. The phytic acid then is discharged through manure resulting in a phosphate pollution. As shown in this example, adding a phytic acid hydrolyzing enzyme like phytase in combination with glucoamylase during the simultaneous saccharification/fermentation process reduces the levels of phytic acid in DDGS.

[0104] A liquefact from a conventional starch liquefaction process using corn as the feedstock was prepared and frozen for use in the experiment. The liquefact was thawed, 200 ppm urea was added, and the solids were adjusted to 32.9% ds prior to adjusting the pH to 4.2 with 6N sulfuric acid. Fermentations were conducted in 250 ml flasks containing an aliquot of 200 gm of mash (*i.e.,* the liquefact-containing mixture). The enzymes were diluted so that 1.0 ml of each at the designated activity was added to the flasks. Each condition was replicated. The flasks were inoculated by adding 1 ml of 10% yeast slurry containing 1% glucose about one hour prior to use. BP-17 phytase was added in the 1.0 ml sample at different levels of activity (0, 0.1, 0.25, 0.5, 1.0, 3.0, and 5.0 FTU/gds corn) during simultaneous saccharification/fermentation. *Trichoderma* GA was also added to hydrolyze the soluble dextrins for providing glucose. After the fermentation, the DDGS was analyzed for free phosphorous/free phosphate. Free phosphate was determined by following the colorimetric method of Fiske-Subbarow (see *e.g.,* Fiske, C.H. and Subbarow, Y. (1925) J. Biol.Chem. 66:375-400). The samples were ground in a Tekmar analytical mill and free phosphate was extracted in water by adding 1 g of sample to a 100 ml volumetric flask containing 80 ml water. A magnetic bar was added to each flask and they were stirred for 1 hour at room temperature. The flasks were then brought to volume with water, mixed well, and filtered through Whatman no. 1 filter paper. The flasks were placed in a 32°C water bath, and occasionally mixed. The filtrates were then assayed for phosphate as follows. To 3.0 ml of sample, 1 ml of acid molybdate reagent was added, followed by 1 ml of reducing reagent, and the absorbance of the color developed at room temperature for 20 minutes was measured at 660 nm. The phosphate level in the samples was then calculated from a phosphate standard curve. The final results were calculated as μg phosphorus per g of sample.

[0105] Figure 1 is a graph showing the effect of BP-17 phytase concentration during yeast fermentation on the phytic acid reductions. In these experiments, 32% whole ground corn containing 50% thin stillage was used at a pH of 4.2 containing 0.325 GAU/gds, GC147. The graph shows that the level of free phosphorous reached a plateau of about 0.75 % free phosphorous with the addition of about 0.7 FTU/g phytase. Thus, the levels of phytic acid were reduced with the addition of a very small amount of phytase, *i.e.,* 0.1 FTU/gds, such that more than 80% of the phytic acid was removed.

Claims

1. A method of producing an alcohol comprising:

    a) contacting a slurry comprising a starch substrate with at least one α-amylase producing oligosaccharides;
    b) contacting the oligosaccharides with at least one glucoamylase and at least one phytase, wherein the phytase has at least 90% sequence identity to the sequence of SEQ ID NO: 5, to produce fermentable sugars;
    c) fermenting the fermentable sugars in the presence of a fermenting organism to produce alcohol; and optionally
    d) recovering the alcohol and/or distillers dried grains and solubles (DDGS).

2. The method of claim 1, further comprising raising the temperature above the gelatinization temperature of the starch substrate after step (a) and before step (b).

3. The method of claim 1, wherein the starch substrate is a milled grain selected from the group consisting of maize, barley, wheat, rice, sorghum, rye, millet, and triticale.

4. The method of claim 1, wherein the phytase has an alanine at amino acid 92 and/or at least one of the following amino acids: a threonine at position 89, an isoleucine at position 134, a serine at position 164, a lysine at position 176, a proline at position 178, a glutamic acid at position 207, a serine at position 209, a leucine at position 248, a tyrosine at position 256, a glutamic acid at position 261, and a lysine at position 269.

5. The method of claim 4 wherein the phytase comprises the sequence of SEQ ID NO: 5 or SEQ ID NO: 8.

6. The method of claim 5 wherein the phytase consists of the sequence of SEQ ID NO: 5 or SEQ ID NO: 8.

7. The method of claim 1, further comprising contacting the oligosaccharides with at least one other enzyme selected from an α-amylase, a second glucoamylase, a second phytase, a cellulase, a pullulanase, a protease, and a laccase.

8. The method of claim 1 wherein the alcohol is ethanol.

9. A method of reducing phytic acid during ethanol fermentation, comprising:

    a) contacting a slurry comprising a starch substrate with at least one α-amylase to produce a liquefact;
    b) contacting the liquefact with at least one glucoamylase and at least one phytase, wherein the phytase has at least 90% amino acid sequence identity to SEQ ID NO:5, and wherein the phytase has an alanine at position 92, under conditions such that fermentable sugars are produced;
    c) fermenting the fermentable sugars in the presence of a fermenting organism under conditions that produce ethanol and/or DDGS; and optionally
    d) recovering the ethanol and/or DDGS.

10. The method of claim 9, further comprising a step of raising the temperature above the liquefaction temperature of the starch substrate.

11. The method of claim 9, wherein the glucoamylase is from a filamentous fungus selected from the group consisting of *Trichoderma, Penicillium, Taleromyces, Aspergillus,* and *Humicola.*

12. The method of claim 11, wherein the *Trichoderma* is *Trichoderma reesei.*

13. The method of claim 9, wherein the phytase comprises the sequence of SEQ ID NO: 8.

14. The method of claim 13, wherein the phytase consists of the sequence of SEQ ID NO: 8.

15. The method of claim 9, wherein the DDGS comprises active phytase.

16. The method of claim 15 wherein, when the DDGS are blended with grains or feed to produce an animal feed, the active phytase reduces the phytic acid in the feed.

17. The method of claim 9, wherein the starch substrate is a milled grain.

18. The method of claim 17, wherein the milled grain is selected from maize, barley, millet, wheat, rice, sorghum, rye and triticale.

19. A method of reducing phytic acid in DDGS, comprising:

a) contacting a slurry comprising a starch substrate with at least one α-amylase to produce a liquefact;
b) contacting the liquefact with at least one *Trichoderma reesei* glucoamylase (TrGA) and at least one phytase, wherein the phytase has at least 90% amino acid sequence identity to SEQ ID NO:5, under conditions such that fermentable sugars are produced; and
c) fermenting the fermentable sugars in the presence of a fermenting organism to produce ethanol and/or DDGS.

20. The method of claim 19, wherein the phytase has at least 95% sequence identity with the amino acid sequence of SEQ ID NO:5 and has an alanine at amino acid 92.

21. The method of claim 19 wherein the phytase comprises the sequence of SEQ ID NO: 8.

22. The method of claim 21 wherein the phytase consists of the sequence of SEQ ID NO: 8.

Patentansprüche

1. Verfahren zur Herstellung eines Alkohols, umfassend:

a) das Kontaktieren einer Aufschlämmung, die ein Stärkesubstrat umfasst, mit mindestens einer Oligosaccharide erzeugenden α-Amylase;
b) das Kontaktieren der Oligosaccharide mit mindestens einer Glucoamylase und mindestens einer Phytase, wobei die Phytase mindestens 90 % Sequenzidentität mit der Sequenz von SEQ ID NO: 5 aufweist, um fermentierbare Zucker zu erzeugen;
c) das Fermentieren der fermentierbaren Zucker in Gegenwart eines Fermentierungsorganismus, um Alkohol zu erzeugen; und wahlweise
d) das Gewinnen des Alkohols und/oder der Trockenschlempe (DDGS).

2. Verfahren nach Anspruch 1, ferner das Erhöhen der Temperatur über die Gelatinisierungstemperatur des Stärkesubstrats nach Schritt (a) und vor Schritt (b) umfassend.

3. Verfahren nach Anspruch 1, wobei das Stärkesubstrat ein gemahlenes Getreide ist ausgewählt aus der Gruppe bestehend aus Mais, Gerste, Weizen, Reis, Sorghum, Roggen, Hirse und Triticale.

4. Verfahren nach Anspruch 1, wobei die Phytase an Aminosäure 92 ein Alanin und/oder mindestens eine der folgenden Aminosäuren aufweist: ein Threonin an Position 89, ein Isoleucin an Position 134, ein Serin an Position 164, ein Lysin an Position 176, ein Prolin an Position 178, eine Glutaminsäure an Position 207, ein Serin an Position 209, ein Leucin an Position 248, ein Tyrosin an Position 256, eine Glutaminsäure an Position 261 und ein Lysin an Position 269.

5. Verfahren nach Anspruch 4, wobei die Phytase die Sequenz von SEQ ID NO: 5 oder SEQ ID NO: 8 umfasst.

6. Verfahren nach Anspruch 5, wobei die Phytase aus der Sequenz von SEQ ID NO: 5 oder SEQ ID NO: 8 besteht.

7. Verfahren nach Anspruch 1, ferner das Kontaktieren der Oligosaccharide mit mindestens einem anderen Enzym umfassend, das unter einer α-Amylase, einer zweiten Glucoamylase, einer zweiten Phytase, einer Cellulase, einer Pullulanase, einer Protease und einer Laccase ausgewählt wird.

8. Verfahren nach Anspruch 1, wobei der Alkohol Ethanol ist.

9. Verfahren zum Reduzieren phytischer Säure während der Ethanolfermentierung, umfassend:

a) das Kontaktieren einer Aufschlämmung, die ein Stärkesubstrat umfasst, mit mindestens einer α-Amylase, um eine verflüssigte Substanz zu erzeugen;

b) das Kontaktieren der verflüssigten Substanz mit mindestens einer Glucoamylase und mindestens einer Phytase, wobei die Phytase mindestens 90 % Aminosäure-Sequenzidentität mit SEQ ID NO: 5 aufweist, und

wobei die Phytase an Position 92 ein Alanin aufweist, unter Bedingungen, derart, dass fermentierbare Zucker erzeugt werden;

c) das Fermentieren der fermentierbaren Zucker in Gegenwart eines Fermentierungsorganismus unter Bedingungen zum Erzeugen von Ethanol und/oder DDGS; und wahlweise
d) das Gewinnen des Ethanols und/oder von DDGS.

10. Verfahren nach Anspruch 9, ferner einen Schritt des Erhöhens der Temperatur über die Verflüssigungstemperatur des Stärkesubstrats umfassend.

11. Verfahren nach Anspruch 9, wobei die Glucoamylase von einem fadenförmigen Pilz ausgewählt aus der Gruppe bestehend aus *Trichoderma, Penicillium, Taleromyces, Aspergillus* und *Humicola* stammt.

12. Verfahren nach Anspruch 11, wobei die *Trichoderma Trichoderma reesei* ist.

13. Verfahren nach Anspruch 9, wobei die Phytase die Sequenz von SEQ ID NO: 8 umfasst.

14. Verfahren nach Anspruch 13, wobei die Phytase aus der Sequenz von SEQ ID NO: 8 besteht.

15. Verfahren nach Anspruch 9, wobei die DDGS aktive Phytase umfasst.

16. Verfahren nach Anspruch 15, wobei die DDGS mit Getreiden oder Futter gemischt werden, um ein Tierfutter herzustellen, wobei die aktive Phytase die phytische Säure in dem Futter reduziert.

17. Verfahren nach Anspruch 9, wobei das Stärkesubstrat ein gemahlenes Getreide ist.

18. Verfahren nach Anspruch 17, wobei das gemahlene Getreide unter Mais, Gerste, Hirse, Weizen, Reis, Sorghum, Roggen und Triticale ausgewählt wird.

19. Verfahren zum Reduzieren phytischer Säure in DDGS, umfassend:

a) das Kontaktieren einer Aufschlämmung, die ein Stärkesubstrat umfasst, mit mindestens einer α-Amylase, um eine verflüssigte Substanz zu erzeugen;
b) das Kontaktieren der verflüssigten Substanz mit mindestens einer von *Trichoderma reesei*-Glucoamylase (TrGA) und mindestens einer Phytase, wobei die Phytase mindestens 90 % Aminosäure-Sequenzidentität mit SEQ ID NO: 5 aufweist, unter Bedingungen, derart, dass fermentierbare Zucker erzeugt werden; und
c) das Fermentieren der fermentierbaren Zucker in Gegenwart eines Fermentierungsorganismus, um Ethanol und/oder DDGS herzustellen.

20. Verfahren nach Anspruch 19, wobei die Phytase mindestens 95 % Sequenzidentität mit der Aminosäuresequenz von SEQ ID NO: 5 und ein Alanin bei Aminosäure 92 aufweist.

21. Verfahren nach Anspruch 19, wobei die Phytase die Sequenz von SEQ ID NO: 8 umfasst.

22. Verfahren nach Anspruch 21, wobei die Phytase aus der Sequenz von SEQ ID NO: 8 besteht.

Revendications

1. Procédé de production d'un alcool comprenant:

a) la mise en contact d'une suspension comprenant un substrat amidon avec au moins une α-amylase produisant des oligosaccharides;
b) la mise en contact des oligosaccharides avec au moins une glucoamylase et au moins une phytase, la phytase ayant au moins 90 % d'identité de séquence vis-à-vis de la séquence de SEQ ID n°: 5, pour produire

des sucres fermentescibles;
c) la fermentation des sucres fermentescibles en présence d'un organisme de fermentation pour produire de l'alcool; et éventuellement
d) la récupération de l'alcool et/ou de drêches séchées et solubles de distillerie (DDGS).

2. Procédé selon la revendication 1, comprenant en outre l'élévation de la température au-dessus de la température de gélatinisation du substrat amidon après l'étape (a) et avant l'étape (b).

3. Procédé selon la revendication 1, dans lequel le substrat amidon est un grain broyé sélectionné dans le groupe constitué du maïs, de l'orge, du blé, du riz, du sorgho, du seigle, du millet, et du triticale.

4. Procédé selon la revendication 1, dans lequel la phytase présente une alanine à l'acide aminé 92 et/ou au moins l'un des acides aminés suivants: une thréonine à la position 89, une isoleucine à la position 134, une sérine à la position 164, une lysine à la position 176, une proline à la position 178, un acide glutamique à la position 207, une sérine à la position 209, une leucine à la position 248, une tyrosine à la position 256, un acide glutamique à la position 261, et une lysine à la position 269.

5. Procédé selon la revendication 4, dans lequel la phytase comprend la séquence de SEQ ID n°: 5 ou SEQ ID n°: 8.

6. Procédé selon la revendication 5, dans lequel la phytase est constituée de la séquence de SEQ ID n°: 5 ou SEQ ID n°: 8.

7. Procédé selon la revendication 1, comprenant en outre la mise en contact des oligosaccharides avec au moins une autre enzyme sélectionnée parmi une α-amylase, une seconde glucoamylase, une seconde phytase, une cellulase, une pullulanase, une protéase, et une laccase.

8. Procédé selon la revendication 1, dans lequel l'alcool est l'éthanol.

9. Procédé de réduction de l'acide phytique durant la fermentation d'éthanol, comprenant:

a) la mise en contact d'une suspension comprenant un substrat amidon avec au moins une α-amylase pour produire un produit de liquéfaction;
b) la mise en contact du produit de liquéfaction avec au moins une glucoamylase et au moins une phytase, la phytase ayant au moins 90 % d'identité de séquence d'acides aminés vis-à-vis de SEQ ID n°: 5, et la phytase ayant une alanine à la position 92, sous des conditions telles que des sucres fermentescibles sont produits;
c) la fermentation des sucres fermentescibles en présence d'un organisme de fermentation sous des conditions de production d'éthanol et/ou de DDGS; et éventuellement
d) la récupération de l'éthanol et/ou des DDGS.

10. Procédé selon la revendication 9, comprenant en outre une étape d'élévation de la température au-dessus de la température de liquéfaction du substrat amidon.

11. Procédé selon la revendication 9, dans lequel la glucoamylase provient d'un champignon filamenteux sélectionné dans le groupe constitué de *Trichoderma, Penicillium, Taleromyces, Aspergillus,* et *Humicola.*

12. Procédé selon la revendication 11, dans lequel *Trichoderma* est *Trichoderma reesei.*

13. Procédé selon la revendication 9, dans lequel la phytase comprend la séquence de SEQ ID n°: 8.

14. Procédé selon la revendication 13, dans lequel la phytase est constituée de la séquence de SEQ ID n°: 8.

15. Procédé selon la revendication 9, dans lequel les DDGS comprennent de la phytase active.

16. Procédé selon la revendication 15 dans lequel, lorsque les DDGS sont mélangées avec des grains ou un aliment pour animaux pour produire un aliment pour animaux, la phytase active réduit l'acide phytique dans l'aliment pour animaux.

17. Procédé selon la revendication 9, dans lequel le substrat amidon est un grain broyé.

18. Procédé selon la revendication 17, dans lequel le grain broyé est sélectionné parmi le maïs, l'orge, le millet, le blé, le riz, le sorgho, le seigle et le triticale.

19. Procédé de réduction de la teneur en acide phytique dans des DDGS, comprenant:

a) la mise en contact d'une suspension comprenant un substrat amidon avec au moins une α-amylase pour produire un produit de liquéfaction;
b) la mise en contact du produit de liquéfaction avec au moins une glucoamylase de *Trichoderma reesei* (TrGA) et au moins une phytase, la phytase présentant au moins 90 % d'identité de séquence d'acides aminés vis-à-vis de SEQ ID n°: 5, sous des conditions telles que des sucres fermentescibles sont produits; et
c) la fermentation des sucres fermentescibles en présence d'un organisme de fermentation pour produire de l'éthanol et/ou des DDGS.

20. Procédé selon la revendication 19, la phytase ayant au moins 95 % d'identité de séquence vis-à-vis de la séquence d'acides aminés de SEQ ID n°: 5 et présente une alanine à l'acide aminé 92.

21. Procédé selon la revendication 19, dans lequel la phytase comprend la séquence de SEQ ID n°: 8.

22. Procédé selon la revendication 21, dans lequel la phytase est constituée de la séquence de SEQ ID n°: 8.

Free Phosphorous Content in DDGS from BP-17 Phytase at Different Levels in Yeast Fermentation: Calorimetric Method (Fiske-Subbarow Method)

Phytase FTU/g	Avg %P
0	0.408
0.10	0.703
0.25	0.724
0.50	0.727
1.00	0.742
3.00	0.755
5.00	0.766

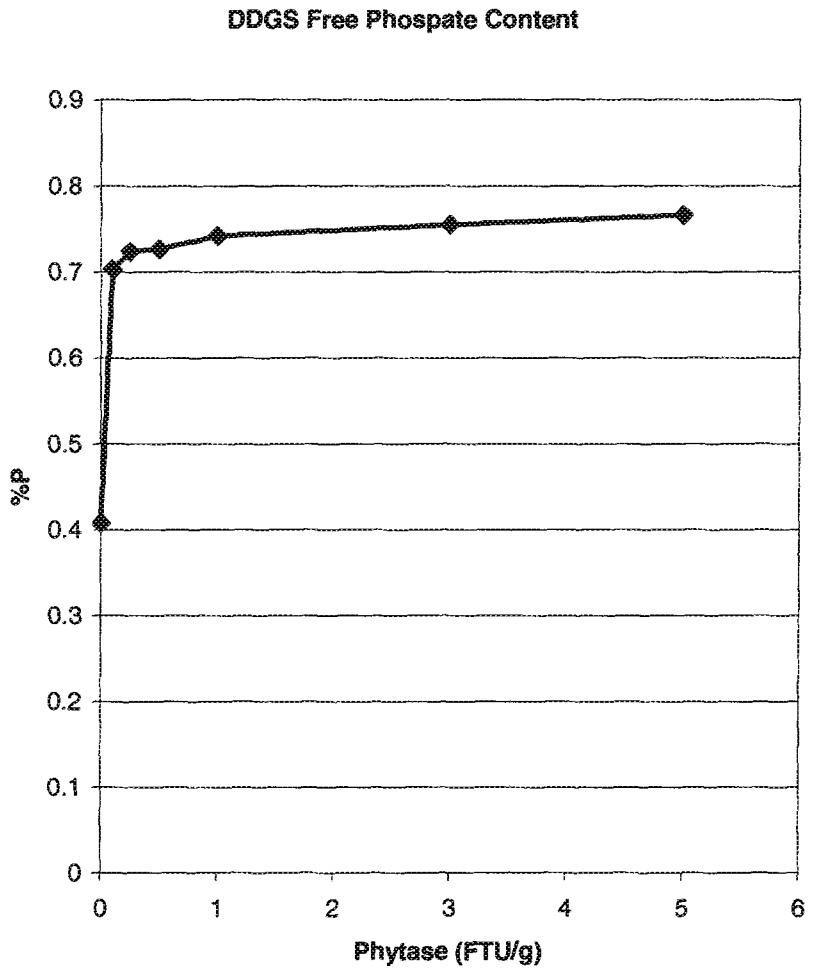

FIGURE 1

REFERENCES CITED IN THE DESCRIPTION

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SZABADALMI IGÉNYPONTOK

1. Eljárás alkohol előállítására, amely tartalmazza:

a) érintkezésbe hozunk iszapot, amely keményítő szubsztrátot tartalmaz, legalább egy α-amiláz termelő oligoszacharidal;

b) érintkezésbe hozzuk az oligoszacharidot legalább egy glukoamilázzal és legalább egy fitázzal, ahol a fitáznak legalább 90% szekvencia azonossága van a szekvenciával SEQ ID NO: 5, hogy fermentálható cukrokat állítsunk elő;

c) fermentáljuk a fermentálható cukrokat fermentáló organizmus jelenlétében, hogy alkoholt állítsunk elő; és opcionálisan

d) visszanyerjük az alkoholt és/vagy száraz cefrét (szeszfőzdei szárított gabonák és oldható anyagok, DDGS).

2. Az 1. igénypont szerinti eljárás,, amely továbbá tartalmazza a hőmérséklet emelését a keményítő szubsztrát kocsonyásodási hőmérséklete fölé lépés (a) után és lépés (b) előtt.

3. Az 1. igénypont szerinti eljárás, ahol a keményítő szubsztrát őrölt gabona, amely ki van választva a következőből álló csoportból: kukorica, árpa, búza, rizs, cirok, rozs, köles, és tritikale.

4. Az 1. igénypont szerinti eljárás, ahol a fitáznak alanínja aminosav 92-ön és/vagy a következő aminosavak legalább egyike: treonin a helyen 89, izoleucin a helyen 134, szerin a helyen 164, lizin a helyen 176, prolin a helyen 178, glutaminsav a helyen 207, szerin a helyen 209, leucine a helyen 248, tirozin a helyen 256, glutaminsav a helyen 261, és lizin a helyen 269.

5. A 4. igénypont szerinti eljárás, ahol a fitáz tartalmazza a szekvenciát SEQ ID NO: 5 vagy SEQ ID NO: 8.

6. Az 5. igénypont szerinti eljárás, ahol a fitáz áll a szekvenciából SEQ ID NO: 5 vagy szekvenciából SEQ ID NO: 8.

7. Az 1. igénypont szerinti eljárás, amely továbbá tartalmazza az oligoszacharidok érintkezésbe hozását legalább egy más enzimmel, amely ki van választva a következők közül: α-amiláz, második glukoamiláz, második fitáz, celluláz, pullulanáz, proteáz, és a lakkáz.

8. Az 1. igénypont szerinti eljárás, ahol az alkohol etanol.

9. Eljárás fitinsav csökkentésére etanol fermantáció alatt, amely tartalmazza:

a) iszap kontaktálását, amely tartalmaz keményítő szubsztrátot, legalább egy α-amilázzal, hogy folyós anyagot állítsunk elő;

b) kontaktáljuk a folyós anyagot legalább egy glukoamilázzal és legalább egy fitázzal,ahol a fitáznak legalább 90% aminosav szekvencia azonossága van SEQ ID NO:5-el, és ahol a fitáznak van alanínja a helyen 92, olyan feltételek mellett, hogy fermentálható cukrok vannak előállítva;

c) fermentáljuk a fermentálható cukrokat fermentáló organizmus jelenlétében olyan feltételek mellett, amely etanolt és/vagy DDGS-t állít elő; és opcionálisan

d) visszanyerjük az etanolt és/vagy DDGS-t.

10. A 9. igénypont szerinti eljárás, amely továbbá tartalmazza a hőmérséklet emelésének lépését a keményítő szubsztrát folyósítási hőmérséklete fölé.

11. A 9. igénypont szerinti eljárás, ahol a glukoamiláz filamentózus gombából van, amely a következőkből álló csoportból van kiválasztva: *Trichoderma, Penicillium, Talaromyces, Aspergillus,* és *Humicola.*

12. A 11. igénypont szerinti eljárás, ahol a *Trichoderma Trichoderma reesei.*

13. A 9. igénypont szerinti eljárás, ahol a fitáz tartalmazza a szekvenciát SEQ ID NO: 8.

14. A 13. igénypont szerinti eljárás, ahol a fitáz áll a szekvenciából SEQ ID NO: 8.

15. A 9. igénypont szerinti eljárás, ahol a DDGS tartalmaz aktív fitázt.

16. A 15. igénypont szerinti eljárás, ahol ha a DDGS keverve van gabonával vagy táppal, hogy állati tápot állítsunk elő, az aktív fitáz csökkenti a fitinsavat a tápban.

17. A 9. igénypont szerinti eljárás,ahol a keményítő szubsztrát őrölt gabona.

18. A 17. igénypont szerinti eljárás, ahol az őrölt gabona ki van választva a következők közül: kukorica, árpa, búza, rizs, cirok, rozs, köles, és triticale.

19. Eljárás fitinsav csökkentésére DDGS-ben, amely tartalmazza:

a) kontaktálunk iszapot, amely tartalmaz keményítő szubsztrátot, legalább egy α-amilázzal, hogy folyós anyagot állítsunk elő;

b) kontaktáljuk a folyós anyagot legalább egy *Trichoderma reesei* glukoamilázzal (TrGA) és legalább egy fitázzal, ahol a fitáznak legalább 90% aminosav szekvencia azonossága van SEQ ID NO:5-el, olyan feltételek mellett, hogy fermentálható cukrok vannak elöállítva; és

c) fementáljuk a fermentálható cukrokat fermentáló organizmus jelenlétében, hogy etanolt és/vagy DDGS-t állítsunk elő.

20. A 19. igénypont szerinti eljárás, ahol a fitáznak legalább 95% szekvencia azonossága van aminosav szekvenciával SEQ ID NO:5 és alaninja van aminosav 92-ön.

21. A 19. igénypont szerinti eljárás, ahol a fitáz tartalmazza a szekvenciát SEQ ID NO: 8.

22. A 21. igénypont szerinti eljárás, ahol a fitáz SEQ ID NO: 8 szekvenciából áll.