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(54) Title: METHODS FOR ENHANCING BY-PRODUCTS FROM FERMENTATION PROCESSES

(57) Abstract: The present invention relates to methods for enhancing yield and/or quality of by-products from fermentation processes used for producing fermentation products. The invention includes a process of fermenting starch-containing material into a fermentation product comprising a fermentation step wherein one or more enzymes are added before or during the fermentation step. More specifically, the present invention provides methods for increasing oil recovery yield from processes for producing fermentation products and further enhancing the quality of DDGS by removing fat content from DDGS that are produced as by-products in processes for producing fermentation products.



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METHODS FOR ENHANCING BY-PRODUCTS FROM FERMENTATION PROCESSES

FIELD OF THE INVENTION

The present invention relates to methods for enhancing the yield of oil and/or the quality
5 of by-products from fermentation processes to produce fermentation products.

BACKGROUND OF THE INVENTION

Due to the limited reserves of fossil fuels and worries about emission of greenhouse
gases there is an increasing focus on using renewable energy sources.

10 Processes for producing fermentation products, such as ethanol, from a starch or
lignocellulose containing material are well known in the art. The preparation of the starch-
containing material such as corn for utilization in such fermentation processes typically begins
with grinding the corn in a dry-grind or wet-milling process. Wet-milling processes involve
fractionating the corn into different components where only the starch fraction enters into the
15 fermentation process. Dry-grind processes involve grinding the corn kernels into meal and
mixing the meal with water and enzymes. Generally two different kinds of dry-grind processes
are used. The most commonly used process, often referred to as a "conventional process,"
includes grinding the starch-containing material and then liquefying gelatinized starch at a high
temperature using typically a bacterial alpha-amylase, followed by simultaneous saccharification
20 and fermentation (SSF) carried out in the presence of a glucoamylase and a fermentation
organism. Another well known process, often referred to as a "raw starch hydrolysis" process
(RSH process), includes grinding the starch-containing material and then simultaneously
saccharifying and fermenting granular starch below the initial gelatinization temperature typically
in the presence of an acid fungal alpha-amylase and a glucoamylase.

25 In a process for producing ethanol from corn, following SSF or the RSH process the
ethanol is distilled from the whole mash after fermentation. The resulting ethanol-free slurry,
usually referred to as whole stillage, is separated into solid and liquid fractions (*i.e.*, wet cake
and thin stillage containing about 35 and 7% solids, respectively). The thin stillage is often
condensed by evaporation into a thick stillage or syrup and recombined with the wet cake and
30 further dried into distillers' dried grains with solubles distillers' dried grain with solubles (DDGS)
for use in animal feed. The oil content of DDGS is sometimes higher than desired and methods
of recovering more oil as a separate by-product for use in biodiesel production or other
biorenewable products are sought.

Much of the work in oil recovery from fermentation processes has focused on improving the extractability of the oil from the whole stillage. Effective removal of oil is often accomplished by hexane extraction. However, the utilization of hexane extraction has not seen widespread application due to the high capital investment required. Therefore, other methods that improve oil extraction from fermentation processes have been explored.

U.S. Patent No. 6,433,146 discloses extracting oil and zein from corn or corn processing by-products using ethanol.

U.S. Patent No. 7,601,858 discloses a method for recovering oil from a concentrated byproduct, such as thin stillage formed during a dry milling process used for producing ethanol. The method includes forming a concentrate from the byproduct, e.g., by evaporating the by-product, and recovering oil from the concentrate.

U.S. Patent No. 7,608,729 discloses a method of freeing the bound oil present in whole stillage and thin stillage by heating the stillage to a temperature sufficient to at least partially separate, or bind, the oil from the stillage.

U.S. Application Publication No. 2010/0058649 discloses a method of separating an oil fraction from a fermentation product, adjusting the pH of the oil fraction, and recovering the oil from the oil fraction.

Wang (2008, *Lipid Technology* 20(9): 203-207) and Wang et al. (2009, *J. Agric. Food Chem.* 57: 2302-2307) have investigated the use of enzymes and different grind and extrusion methods to enhance oil partitioning.

Both whole stillage and thin stillage consist of a large amount of water. Methods for dewatering stillage are known in the art.

It is an object of the present invention to provide improved methods for increasing the amount of recoverable oil, dewatering stillage, and increasing the quality of the DDGS from fermentation processes.

SUMMARY OF THE INVENTION

The present invention relates to a process of fermenting a starch-containing material into a fermentation product comprising a fermentation step in the presence of a hemicellulase(s) and/or an endoglucanase(s).

The present invention also relates to a method of increasing the amount of oil recovered from a process for producing a fermentation product.

The present invention also relates to a method of dewatering stillage obtained from a process for producing a fermentation product.

The present invention also relates to a method of decreasing the amount of fat or oil in the distillers' dried grains with solubles (DDGS) produced in a process for producing a fermentation product.

The present invention also relates to a method of increasing the amount of oil partitioned into the thin stillage produced by a process for producing a fermentation product.

The present invention also relates to a method of dewatering the whole stillage produced by a process for producing a fermentation product.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Acetylxyylan esterase: The term "acetylxyylan esterase" means a carboxylesterase (EC 3.1.1.72) that catalyzes the hydrolysis of acetyl groups from polymeric xylan, acetylated xylose, acetylated glucose, alpha-naphthyl acetate, and *p*-nitrophenyl acetate. For purposes of the present invention, acetylxyylan esterase activity is determined using 0.5 mM *p*-nitrophenylacetate as substrate in 50 mM sodium acetate pH 5.0 containing 0.01% TWEEN™ 20. One unit of acetylxyylan esterase is defined as the amount of enzyme capable of releasing 1 micromole of *p*-nitrophenolate anion per minute at pH 5, 25°C.

Alpha-Amylases: The term "alpha-amylase" means an alpha-1,4-glucan-4-glucanohydrolase (E.C. 3.2.1.1) that catalyzes the hydrolysis of starch and other linear and branched 1,4-glucosidic oligo- and polysaccharides.

Alpha-L-arabinofuranosidase: The term "alpha-L-arabinofuranosidase" means an alpha-L-arabinofuranoside arabinofuranohydrolase (EC 3.2.1.55) that catalyzes the hydrolysis of terminal non-reducing alpha-L-arabinofuranoside residues in alpha-L-arabinosides. The enzyme acts on alpha-L-arabinofuranosides, alpha-L-arabinans containing (1,3)- and/or (1,5)-linkages, arabinoxylans, and arabinogalactans. Alpha-L-arabinofuranosidase is also known as arabinosidase, alpha-arabinosidase, alpha-L-arabinosidase, alpha-arabinofuranosidase, polysaccharide alpha-L-arabinofuranosidase, alpha-L-arabinofuranoside hydrolase, L-arabinosidase, or alpha-L-arabinanase. For purposes of the present invention, alpha-L-arabinofuranosidase activity is determined using 5 mg of medium viscosity wheat arabinoxylan (Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland) per ml of 100 mM sodium acetate pH 5 in a total volume of 200 microliters for 30 minutes at 40°C followed by arabinose analysis by AMINEX® HPX-87H column chromatography (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Alpha-glucuronidase: The term "alpha-glucuronidase" means an alpha-D-glucosiduronate glucuronohydrolase (EC 3.2.1.139) that catalyzes the hydrolysis of an alpha-D-glucuronoside to D-glucuronate and an alcohol. For purposes of the present invention, alpha-glucuronidase activity is determined according to de Vries, 1998, *J. Bacteriol.* 180: 243-249. One unit of alpha-glucuronidase equals the amount of enzyme capable of releasing 1 micromole of glucuronic or 4-O-methylglucuronic acid per minute at pH 5, 40°C.

Beta-glucosidase: The term "beta-glucosidase" means a beta-D-glucoside glucohydrolase (E.C. 3.2.1.21), which catalyzes the hydrolysis of terminal non-reducing beta-D-glucose residues with the release of beta-D-glucose. For purposes of the present invention, beta-glucosidase activity is determined according to the basic procedure described by Venturi *et al.*, 2002, Extracellular beta-D-glucosidase from *Chaetomium thermophilum* var. *coprophilum*: production, purification and some biochemical properties, *J. Basic Microbiol.* 42: 55-66. One unit of beta-glucosidase is defined as 1.0 micromole of *p*-nitrophenolate anion produced per minute at 25°C, pH 4.8 from 1 mM *p*-nitrophenyl-beta-D-glucopyranoside as substrate in 50 mM sodium citrate containing 0.01% TWEEN® 20.

Beta-xylosidase: The term "beta-xylosidase" means a beta-D-xyloside xylohydrolase (E.C. 3.2.1.37) that catalyzes the exo-hydrolysis of short beta (1→4) -xylooligosaccharides, to remove successive D-xylose residues from the non-reducing termini. For purposes of the present invention, one unit of beta-xylosidase is defined as 1.0 micromole of *p*-nitrophenolate anion produced per minute at 40°C, pH 5 from 1 mM *p*-nitrophenyl-beta-D-xyloside as substrate in 100 mM sodium citrate containing 0.01% TWEEN® 20.

Cellobiohydrolase: The term "cellobiohydrolase" means a 1,4-beta-D-glucan cellobiohydrolase (E.C. 3.2.1.91) that catalyzes the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, cellooligosaccharides, or any beta-1,4-linked glucose containing polymer, releasing cellobiose from the reducing or non-reducing ends of the chain (Teeri, 1997, Crystalline cellulose degradation: New insight into the function of cellobiohydrolases, *Trends in Biotechnology* 15: 160-167; Teeri *et al.*, 1998, *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose?, *Biochem. Soc. Trans.* 26: 173-178). Cellobiohydrolase activity is determined according to the procedures described by Lever *et al.*, 1972, *Anal. Biochem.* 47: 273-279; van Tilbeurgh *et al.*, 1982, *FEBS Letters*, 149: 152-156; van Tilbeurgh and Claeysens, 1985, *FEBS Letters*, 187: 283-288; and Tomme *et al.*, 1988, *Eur. J. Biochem.* 170: 575-581. In the present invention, the Tomme *et al.* method can be used to determine cellobiohydrolase activity.

Cellulosic material: The term “cellulosic material” means any material containing cellulose. The predominant polysaccharide in the primary cell wall of biomass is cellulose, the second most abundant is hemicellulose, and the third is pectin. The secondary cell wall, produced after the cell has stopped growing, also contains polysaccharides and is strengthened by polymeric lignin covalently cross-linked to hemicellulose. Cellulose is a homopolymer of anhydrocellobiose and thus a linear beta-(1-4)-D-glucan, while hemicelluloses include a variety of compounds, such as xylans, xyloglucans, arabinoxylans, and mannans in complex branched structures with a spectrum of substituents. Although generally polymorphous, cellulose is found in plant tissue primarily as an insoluble crystalline matrix of parallel glucan chains. Hemicelluloses usually hydrogen bond to cellulose, as well as to other hemicelluloses, which help stabilize the cell wall matrix.

Cellulose is generally found, for example, in the stems, leaves, hulls, husks, and cobs of plants or leaves, branches, and wood of trees. The cellulosic material can be, but is not limited to, herbaceous material (including energy crops), agricultural residue, wood (including forestry residue), municipal solid waste, waste paper, and pulp and paper mill residue (see, for example, Wiseloge *et al.*, 1995, in Handbook on Bioethanol (Charles E. Wyman, editor), pp. 105-118, Taylor & Francis, Washington D.C.; Wyman, 1994, *Bioresource Technology* 50: 3-16; Lynd, 1990, *Applied Biochemistry and Biotechnology* 24/25: 695-719; Mosier *et al.*, 1999, Recent Progress in Bioconversion of Lignocellulosics, in *Advances in Biochemical Engineering/Biotechnology*, T. Scheper, managing editor, Volume 65, pp.23-40, Springer-Verlag, New York). It is understood herein that the cellulose may be in the form of lignocellulose, a plant cell wall material containing lignin, cellulose, and hemicellulose in a mixed matrix. In a preferred aspect, the cellulosic material is any biomass material. In another preferred aspect, the cellulosic material is lignocellulose, which comprises cellulose, hemicelluloses, and lignin.

In one aspect, the cellulosic material is herbaceous material (including energy crops). In another aspect, the cellulosic material is agricultural residue. In another aspect, the cellulosic material is wood (including forestry residue). In another aspect, the cellulosic material is municipal solid waste. In another aspect, the cellulosic material is waste paper. In another aspect, the cellulosic material is pulp and paper mill residue.

In another aspect, the cellulosic material is corn stover. In another aspect, the cellulosic material is wheat straw. In another aspect, the cellulosic material is bagasse. In another aspect, the cellulosic material is corn cob. In another aspect, the cellulosic material is switchgrass. In another aspect, the cellulosic material is corn fiber. In another aspect, the cellulosic material is

rice straw. In another aspect, the cellulosic material is miscanthus. In another aspect, the cellulosic material is orange peel. In another aspect, the cellulosic material is poplar. In another aspect, the cellulosic material is pine. In another aspect, the cellulosic material is willow. In another aspect, the cellulosic material is eucalyptus.

5 In another aspect, the cellulosic material is microcrystalline cellulose. In another aspect, the cellulosic material is bacterial cellulose. In another aspect, the cellulosic material is algal cellulose. In another aspect, the cellulosic material is cotton linter. In another aspect, the cellulosic material is amorphous phosphoric-acid treated cellulose. In another aspect, the cellulosic material is filter paper.

10 In another aspect, the cellulosic material is an aquatic biomass. As used herein the term "aquatic biomass" means biomass produced in an aquatic environment by a photosynthesis process. The aquatic biomass can be algae; submerged plants; emergent plants; and floating-leaf plants.

The cellulosic material may be used as is or may be subjected to pretreatment, using
15 conventional methods known in the art, as described herein. In a preferred aspect, the cellulosic material is pretreated.

Cellulolytic enzyme or cellulase: The term "cellulolytic enzyme" or "cellulase" means one or more (*e.g.*, several) enzymes that hydrolyze a cellulosic material. Such enzymes include endoglucanase(s), cellobiohydrolase(s), beta-glucosidase(s), or combinations thereof. The two
20 basic approaches for measuring cellulolytic activity include: (1) measuring the total cellulolytic activity, and (2) measuring the individual cellulolytic activities (endoglucanases, cellobiohydrolases, and beta-glucosidases) as reviewed in Zhang *et al.*, Outlook for cellulase improvement: Screening and selection strategies, 2006, *Biotechnology Advances* 24: 452-481. Total cellulolytic activity is usually measured using insoluble substrates, including Whatman №1
25 filter paper, microcrystalline cellulose, bacterial cellulose, algal cellulose, cotton, pretreated lignocellulose, *etc.* The most common total cellulolytic activity assay is the filter paper assay using Whatman №1 filter paper as the substrate. The assay was established by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987, Measurement of cellulase activities, *Pure Appl. Chem.* 59: 257-68).

30 For purposes of the present invention, cellulolytic enzyme activity is determined by measuring the increase in hydrolysis of a cellulosic material by cellulolytic enzyme(s) under the following conditions: 1-20 mg of cellulolytic enzyme protein/g of cellulose in PCS for 3-7 days at a suitable temperature, *e.g.*, 50°C, 55°C, or 60°C, compared to a control hydrolysis without addition of cellulolytic enzyme protein. Typical conditions are 1 ml reactions, washed or

unwashed PCS, 5% insoluble solids, 50 mM sodium acetate pH 5, 1 mM MnSO₄, 50°C, 55°C, or 60°C, 72 hours, sugar analysis by AMINEX® HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Coding sequence: The term “coding sequence” means a polynucleotide, which directly specifies the amino acid sequence of its polypeptide product. The boundaries of the coding sequence are generally determined by an open reading frame, which usually begins with the ATG start codon or alternative start codons such as GTG and TTG and ends with a stop codon such as TAA, TAG, and TGA. The coding sequence may be a DNA, cDNA, synthetic, or recombinant polynucleotide.

Control sequence: The term “control sequence” means all components necessary for the expression of a polynucleotide encoding an alpha-amylase of the present invention. Each control sequence may be native or foreign to the polynucleotide encoding the alpha-amylase or native or foreign to each other. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the polynucleotide encoding an alpha-amylase.

Endoglucanase: The term “endoglucanase” means an endo-1,4-(1,3;1,4)-beta-D-glucan 4-glucanohydrolase (E.C. 3.2.1.4), which catalyzes endohydrolysis of 1,4-beta-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxymethyl cellulose and hydroxyethyl cellulose), lichenin, beta-1,4 bonds in mixed beta-1,3 glucans such as cereal beta-D-glucans or xyloglucans, and other plant material containing cellulosic components. Endoglucanase activity can be determined by measuring reduction in substrate viscosity or increase in reducing ends determined by a reducing sugar assay (Zhang *et al.*, 2006, *Biotechnology Advances* 24: 452-481). For purposes of the present invention, endoglucanase activity is determined using carboxymethyl cellulose (CMC) as substrate according to the procedure of Ghose, 1987, *Pure and Appl. Chem.* 59: 257-268, at pH 5, 40°C.

Expression: The term “expression” includes any step involved in the production of the polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

Expression vector: The term “expression vector” means a linear or circular DNA molecule that comprises a polynucleotide encoding a polypeptide of the present invention and is operably linked to additional nucleotides that provide for its expression.

Family 61 glycoside hydrolase: The term “Family 61 glycoside hydrolase” or “Family GH61” or “GH61” means a polypeptide falling into the glycoside hydrolase Family 61 according to Henrissat, 1991, A classification of glycosyl hydrolases based on amino-acid sequence similarities, *Biochem. J.* 280: 309-316, and Henrissat and Bairoch, 1996, Updating the sequence-based classification of glycosyl hydrolases, *Biochem. J.* 316: 695-696. The enzymes in this family were originally classified as a glycoside hydrolase family based on measurement of very weak endo-1,4-beta-D-glucanase activity in one family member. The structure and mode of action of these enzymes are certainly non-canonical and they cannot be considered as bona fide glycosidases. However, they are kept in the CAZy classification on the basis of their capacity to enhance the breakdown of lignocellulose when used in conjunction with a cellulase or a mixture of cellulases.

Ferulic acid esterase or feruloyl esterase: The term “ferulic acid esterase” or “feruloyl esterase” means a 4-hydroxy-3-methoxycinnamoyl-sugar hydrolase (EC 3.1.1.73) that catalyzes the hydrolysis of the 4-hydroxy-3-methoxycinnamoyl (feruloyl) group from an esterified sugar, which is usually arabinose in "natural" substrates, to produce ferulate (4-hydroxy-3-methoxycinnamate). Feruloyl esterase is also known as ferulic acid esterase, hydroxycinnamoyl esterase, FAE-III, cinnamoyl ester hydrolase, FAEA, cinnAE, FAE-I, or FAE-II. For purposes of the present invention, feruloyl esterase activity is determined using 0.5 mM *p*-nitrophenylferulate as substrate in 50 mM sodium acetate pH 5.0. One unit of feruloyl esterase equals the amount of enzyme capable of releasing 1 micromole of *p*-nitrophenolate anion per minute at pH 5, 25°C.

Hemicellulolytic enzyme or hemicellulase: The term “hemicellulolytic enzyme” or “hemicellulase” means one or more (e.g., several) enzymes that hydrolyze a hemicellulosic material. See, for example, Shallom and Shoham, 2003, Microbial hemicellulases, *Current Opinion In Microbiology* 6(3): 219-228. Hemicellulases are key components in the degradation of plant biomass. Examples of hemicellulases include, but are not limited to, an acetylmannan esterase, an acetyxylan esterase, an arabinanase, an arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase. The substrates of these enzymes, the hemicelluloses, are a heterogeneous group of branched and linear polysaccharides that are bound via hydrogen bonds to the cellulose microfibrils in the plant cell wall, crosslinking them into a robust network. Hemicelluloses are also covalently attached to lignin, forming together with cellulose a highly complex structure. The variable structure and organization of hemicelluloses require the concerted action of many enzymes for its complete degradation. The catalytic modules of hemicellulases are either glycoside hydrolases (GHs) that hydrolyze

glycosidic bonds, or carbohydrate esterases (CEs), which hydrolyze ester linkages of acetate or ferulic acid side groups. These catalytic modules, based on homology of their primary sequence, can be assigned into GH and CE families marked by numbers. Some families, with overall similar fold, can be further grouped into clans, marked alphabetically (e.g., GH-A). A most informative and updated classification of these and other carbohydrate active enzymes is available on the Carbohydrate-Active Enzymes (CAZy) database. Hemicellulolytic enzyme activities can be measured according to Ghose and Bisaria, 1987, *Pure & Appl. Chem.* 59: 1739-1752, at a suitable temperature, e.g., 50°C, 55°C, or 60°C.

Host cell: The term "host cell" means any cell type that is susceptible to transformation, transfection, transduction, and the like with a nucleic acid construct or expression vector comprising a polynucleotide of the present invention. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

Nucleic acid construct: The term "nucleic acid construct" means a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or is modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature or which is synthetic. The term nucleic acid construct is synonymous with the term "expression cassette" when the nucleic acid construct contains the control sequences required for expression of a coding sequence.

Operably linked: The term "operably linked" means a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of the polynucleotide sequence such that the control sequence directs the expression of the coding sequence of a polypeptide.

Polypeptide fragment: The term "polypeptide fragment" means a polypeptide having one or more (several) amino acids deleted from the amino and/or carboxyl terminus of a mature polypeptide; wherein the fragment has enzyme activity.

Polypeptide having cellulolytic enhancing activity: The term "polypeptide having cellulolytic enhancing activity" means a GH61 polypeptide that catalyzes the enhancement of the hydrolysis of a cellulosic material by enzyme having cellulolytic activity. For purposes of the present invention, cellulolytic enhancing activity is determined by measuring the increase in reducing sugars or the increase of the total of cellobiose and glucose from the hydrolysis of a cellulosic material by cellulolytic enzyme under the following conditions: 1-50 mg of total protein/g of cellulose in PCS, wherein total protein is comprised of 50-99.5% w/w cellulolytic enzyme protein and 0.5-50% w/w protein of a GH61 polypeptide having cellulolytic enhancing

activity for 1-7 days at a suitable temperature, e.g., 50°C, 55°C, or 60°C, compared to a control hydrolysis with equal total protein loading without cellulolytic enhancing activity (1-50 mg of cellulolytic protein/g of cellulose in PCS). In a preferred aspect, a mixture of CELLUCLAST® 1.5L (Novozymes A/S, Bagsværd, Denmark) in the presence of 2-3% of total protein weight *Aspergillus oryzae* beta-glucosidase (recombinantly produced in *Aspergillus oryzae* according to WO 02/095014) or 2-3% of total protein weight *Aspergillus fumigatus* beta-glucosidase (recombinantly produced in *Aspergillus oryzae* as described in WO 2002/095014) of cellulase protein loading is used as the source of the cellulolytic activity.

The GH61 polypeptides having cellulolytic enhancing activity enhance the hydrolysis of a cellulosic material catalyzed by enzyme having cellulolytic activity by reducing the amount of cellulolytic enzyme required to reach the same degree of hydrolysis preferably at least 1.01-fold, more preferably at least 1.05-fold, more preferably at least 1.10-fold, more preferably at least 1.25-fold, more preferably at least 1.5-fold, more preferably at least 2-fold, more preferably at least 3-fold, more preferably at least 4-fold, more preferably at least 5-fold, even more preferably at least 10-fold, and most preferably at least 20-fold.

Pretreated corn stover: The term “PCS” or “Pretreated Corn Stover” means a cellulosic material derived from corn stover by treatment with heat and dilute sulfuric acid.

Sequence Identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter “sequence identity”.

For purposes of the present invention, the degree of sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Residues} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

For purposes of the present invention, the degree of sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4)

substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Deoxyribonucleotides} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

5 **Xylan-containing material:** The term "xylan-containing material" means any material comprising a plant cell wall polysaccharide containing a backbone of beta-(1-4)-linked xylose residues. Xylans of terrestrial plants are heteropolymers possessing a beta-(1-4)-D-xylopyranose backbone, which is branched by short carbohydrate chains. They comprise D-glucuronic acid or its 4-O-methyl ether, L-arabinose, and/or various oligosaccharides, composed
10 of D-xylose, L-arabinose, D- or L-galactose, and D-glucose. Xylan-type polysaccharides can be divided into homoxylans and heteroxylans, which include glucuronoxylans, (arabino)glucuronoxylans, (glucurono)arabinoxylans, arabinoxylans, and complex heteroxylans. See, for example, Ebringerova *et al.*, 2005, *Adv. Polym. Sci.* 186: 1-67.

In the methods of the present invention, any material containing xylan may be used. In a
15 preferred aspect, the xylan-containing material is lignocellulose.

Xylan degrading activity or xylanolytic activity: The term "xylan degrading activity" or "xylanolytic activity" means a biological activity that hydrolyzes xylan-containing material. The two basic approaches for measuring xylanolytic activity include: (1) measuring the total xylanolytic activity, and (2) measuring the individual xylanolytic activities (e.g., acetylxylan
20 esterases, alpha-glucuronyl esterases, alpha-glucuronidases, arabinofuranosidases, beta-xylosidases, endoxylanases, and feruloyl esterases). Recent progress in assays of xylanolytic enzymes was summarized in several publications including Biely and Puchard, Recent progress in the assays of xylanolytic enzymes, 2006, *Journal of the Science of Food and Agriculture* 86(11): 1636-1647; Spanikova and Biely, 2006, Glucuronoyl esterase - Novel carbohydrate
25 esterase produced by *Schizophyllum commune*, *FEBS Letters* 580(19): 4597-4601; Herrmann *et al.*, 1997, The beta-D-xylosidase of *Trichoderma reesei* is a multifunctional beta-D-xylan xylohydrolase, *Biochemical Journal* 321: 375-381.

Total xylan degrading activity can be measured by determining the reducing sugars formed from various types of xylan, including, for example, oat spelt, beechwood, and
30 larchwood xylans, or by photometric determination of dyed xylan fragments released from various covalently dyed xylans. The most common total xylanolytic activity assay is based on production of reducing sugars from polymeric 4-O-methyl glucuronoxylan as described in Bailey, Biely, Poutanen, 1992, Interlaboratory testing of methods for assay of xylanase activity, *Journal of Biotechnology* 23(3): 257-270. Xylanase activity can also be determined with 0.2% AZCL-

arabinoxylan as substrate in 0.01% TRITON® X-100 and 200 mM sodium phosphate buffer pH 6 at 37°C. One unit of xylanase activity is defined as 1.0 micromole of azurine produced per minute at 37°C, pH 6 from 0.2% AZCL-arabinoxylan as substrate in 200 mM sodium phosphate pH 6 buffer.

5 For purposes of the present invention, xylan degrading activity is determined by measuring the increase in hydrolysis of birchwood xylan (Sigma Chemical Co., Inc., St. Louis, MO, USA) by xylan-degrading enzyme(s) under the following typical conditions: 1 ml reactions, 5 mg/ml substrate (total solids), 5 mg of xylanolytic protein/g of substrate, 50 mM sodium acetate pH 5, 50°C, 24 hours, sugar analysis using *p*-hydroxybenzoic acid hydrazide (PHBAH)
10 assay as described by Lever, 1972, A new reaction for colorimetric determination of carbohydrates, *Anal. Biochem* 47: 273-279.

Xylanase: The term "xylanase" means a 1,4-beta-D-xylan-xylohydrolase (E.C. 3.2.1.8) that catalyzes the endohydrolysis of 1,4-beta-D-xylosidic linkages in xylans. For purposes of the present invention, xylanase activity is determined with 0.2% AZCL-arabinoxylan as substrate in
15 0.01% TRITON® X-100 and 200 mM sodium phosphate buffer pH 6 at 37°C. One unit of xylanase activity is defined as 1.0 micromole of azurine produced per minute at 37°C, pH 6 from 0.2% AZCL-arabinoxylan as substrate in 200 mM sodium phosphate pH 6 buffer.

Processes of the Present Invention

20 One aspect of the present invention relates to a process of producing a fermentation product from a starch-containing material or a lignocellulosic material comprising a fermentation step in the presence of a hemicellulase(s).

In another aspect, the present invention relates to a process of producing a fermentation product from a starch-containing material or a lignocellulosic material comprising a fermentation
25 step in the presence of a hemicellulase(s) and an endoglucanase(s).

In another aspect, the present invention relates to a process of producing a fermentation product from a starch-containing material or a lignocellulosic material comprising a fermentation step in the presence of a hemicellulase(s), an endoglucanase(s), and a GH61 polypeptide(s).

In another aspect, the present invention relates to a process of producing a fermentation
30 product from a starch-containing material or a lignocellulosic material comprising a fermentation step in the presence of an endoglucanase(s) and a beta-glucanase(s).

The methods of the invention can be used in conventional as well as raw starch hydrolysis processes, as well as in a biomass process.

The addition of hemicellulase(s), alone or in combination with other enzymes (e.g., endoglucanase(s)), can enhance dewatering of whole stillage and oil extraction from thin stillage or syrup following fermentation, which can be used in biodiesel or other biorenewable product production. Not being bound by any particular theory, it is believed that the addition of hemicellulase(s), alone or in combination with other enzyme activities, to the fermentation medium during the fermentation step of a process for producing fermentation products may release bound oil in the starch-containing material. Forming more "unbound" oil may allow for the oil to be partitioned into the aqueous phase of the whole stillage following solid/liquid separation into the wet cake and thin stillage. Such oil can then be recovered from the thin stillage or "aqueous" layer of the separated whole stillage by one or more oil extraction methods known in the art.

In an embodiment, the endoglucanase(s) is added in an amount of 0.01-1.0, e.g., 0.02-0.08, 0.025-0.06, 0.025-0.05, or 0.03-0.04 EGU/g dry solids.

In an embodiment, the endoglucanase(s) is added in an amount of 1-30, e.g., 5-30, 7-25, 10-20, 10-17, or 12-15 micrograms/g dry solids.

In an embodiment, the hemicellulase(s) is added in an amount of 0.01-1.0, e.g., 0.015-0.08, 0.015-0.06, 0.015-0.04, or 0.02-0.03 FXU/g dry solids.

In an embodiment, the hemicellulase(s) is added in an amount of 1-30, e.g., 5-30, 7-25, 10-20, 10-17, or 12-15 micrograms/g dry solids.

The instant invention also leads to the enhancement of DDGS by decreasing the fat or oil content in the DDGS thus producing a higher quality DDGS.

Processes for producing fermentation products from a gelatinized starch-containing material

In an embodiment, the present invention relates to a process for producing a fermentation product comprising:

- (a) liquefying a starch-containing material in the presence of an alpha-amylase;
- (b) saccharifying the liquefied material obtained in step (a) using a carbohydrate-source generating enzyme; and
- (c) fermenting using a fermenting organism in the presence of a hemicellulase(s).

In another embodiment, the present invention relates to a process for producing a fermentation product comprising the steps of:

- (a) liquefying the starch-containing material in the presence of an alpha-amylase;
- (b) saccharifying the liquefied material obtained in step (a) using a carbohydrate-source generating enzyme; and

(c) fermenting using a fermenting organism in the presence of an endoglucanase(s) and a hemicellulase(s), wherein the endoglucanase(s) is present in an amount of 0.01-1.0 EGU/g dry solids and in an amount of 1-30 micrograms/g dry solids and the hemicellulase(s) is present in an amount of 0.01-1.0 FXU/g dry solids and in an amount of 1-30 micrograms/g dry solids.

The saccharification and fermentation steps may be carried out either sequentially or simultaneously. The hemicellulase(s) and/or endoglucanase(s) may be added during saccharification and/or fermentation when the process is carried out as a sequential saccharification and fermentation process and before or during fermentation when steps (b) and (c) are carried out simultaneously (SSF process).

The liquefaction is preferably carried out in the presence of an alpha-amylase, preferably a bacterial alpha-amylase or acid fungal alpha-amylase. In an embodiment, a pullulanase, isoamylase, and and/or phytase is added during liquefaction. The fermenting organism is preferably a yeast, e.g., a strain of *Saccharomyces cerevisiae*. Suitable fermenting organisms are listed in the "Fermenting Organisms" section below.

Liquefaction may be carried out as a three-step hot slurry process. The slurry is heated to between 60-95°C, preferably 80-85°C, and an alpha-amylase is added to initiate liquefaction (thinning). Then the slurry may be jet-cooked at a temperature between 95-140°C, preferably 105-125°C, for about 1-15 minutes, preferably for about 3-10 minutes, especially around about 5 minutes. The slurry is cooled to 60-95°C and more alpha-amylase is added to complete the hydrolysis (secondary liquefaction). The liquefaction process is usually carried out at a pH of 4.0 to 6.5, in particular at a pH of 4.5 to 6.

Saccharification may be carried out using conditions well known in the art with a saccharifying enzyme, e.g., beta-amylase, glucoamylase or maltogenic amylase, and optionally a debranching enzyme, such as an isoamylase or a pullulanase. For instance, a full saccharification process may last up to from about 24 to about 72 hours, however, it is common to do a pre-saccharification for typically 40-90 minutes at a temperature between 30-65°C, typically about 60°C, followed by complete saccharification during fermentation in a simultaneous saccharification and fermentation process (SSF process). Saccharification is typically carried out at a temperature from 20-75°C, preferably from 40-70°C, typically around 60°C, and at a pH between 4 and 5, normally at about pH 4.5.

The most widely used process to produce a fermentation product, especially ethanol, is the simultaneous saccharification and fermentation (SSF) process, in which there is no holding stage for the saccharification, meaning that a fermenting organism, such as a yeast, and

enzyme(s), including the hemicellulase(s) and/or endoglucanase(s), may be added together. SSF is typically carried out at a temperature from 25°C to 40°C, such as from 28°C to 35°C, from 30°C to 34°C, preferably around about 32°C. In an embodiment, fermentation is ongoing for 6 to 120 hours, in particular 24 to 96 hours.

5 In a particular embodiment, the process of the invention further comprises, prior to step (a), the steps of:

x) reducing the particle size of the starch-containing material, preferably by milling; and

y) forming a slurry comprising the starch-containing material and water.

10 The aqueous slurry may contain from 10-55 w/w % dry solids (DS), preferably 25-45 w/w % dry solids (DS), more preferably 30-40 w/w % dry solids (DS) of the starch-containing material. The slurry is heated to above the gelatinization temperature and an alpha-amylase, preferably a bacterial and/or acid fungal alpha-amylase, may be added to initiate liquefaction (thinning). The slurry may be jet-cooked to further gelatinize the slurry before being subjected to
15 an alpha-amylase in step (a).

Processes for producing fermentation products from an ungelatinized starch-containing material

In another embodiment, the invention relates to processes for producing a fermentation product from a starch-containing material without gelatinization (*i.e.*, without cooking) of the
20 starch-containing material in the presence of a hemicellulase(s).

In another embodiment, the invention relates to processes for producing a fermentation product from a starch-containing material without gelatinization (*i.e.*, without cooking) of the starch-containing material wherein an endoglucanase(s) and a hemicellulase(s) are present during the raw starch hydrolysis process, wherein the endoglucanase(s) is present in an amount
25 of 0.01-1.0 EGU/g dry solids and in an amount of 1-30 micrograms/g dry solids, the hemicellulase(s) is present in an amount of 0.01-1.0 FXU/g dry solids and in an amount of 1-30 micrograms/g dry solids.

In these embodiments, the desired fermentation product, such as ethanol, is produced from an ungelatinized starch containing material. The process comprises simultaneously
30 saccharifying and fermenting a starch-containing material, *e.g.*, granular starch, using an alpha-amylase, a carbohydrate-source generating enzyme, and a fermenting organism at a temperature below the initial gelatinization temperature of the starch-containing material, in the presence of the endoglucanase(s) and/or hemicellulase(s).

As used herein, the term "initial gelatinization temperature" means the lowest temperature at which starch gelatinization commences. In general, starch heated in water begins to gelatinize between about 50°C and 75°C; the exact temperature of gelatinization depends on the specific starch and can readily be determined by the skilled artisan. Thus, the initial gelatinization temperature may vary according to the plant species, to the particular variety of the plant species as well as with the growth conditions. In the context of this invention the initial gelatinization temperature of a given starch-containing material may be determined as the temperature at which birefringence is lost in 5% of the starch granules using the method described by Gorinstein and Lii, 1992, *Starch/Starke* 44(12): 461-466.

An RSH process is conducted at a temperature below the initial gelatinization temperature, which means that the temperature typically lies in the range between 30-75°C, preferably between 45-60°C. In an embodiment, the process is carried out at a temperature from 25°C to 40°C, such as from 28°C to 35°C, such as from 30°C to 34°C, preferably around 32°C.

A skilled person in the art can easily determine which doses/quantities of enzyme and fermenting organism to use.

In one embodiment, a slurry of a starch-containing material, such as granular starch, having 10-55 w/w % dry solids (DS), preferably 25-45 w/w % dry solids, more preferably 30-40 w/w % dry solids of the starch-containing material may be prepared. The slurry may include water and/or process waters, such as stillage (backset), scrubber water, evaporator condensate or distillate, side-stripper water from distillation, or process water from other fermentation product plants. Because the RSH process of the invention is carried out below the initial gelatinization temperature, and thus no significant viscosity increase takes place, high levels of stillage may be used if desired. In an embodiment the aqueous slurry contains from about 1 to about 70 vol. %, preferably 15-60 vol. %, especially from about 30 to 50 vol. % water and/or process waters, such as stillage (backset), scrubber water, evaporator condensate or distillate, side-stripper water from distillation, or process water from other fermentation product plants, or combinations thereof, or the like.

The starch-containing material may be prepared by reducing the particle size, preferably by dry or wet milling, to 0.05 to 3.0 mm, preferably 0.1-0.5 mm. After being subjected to a process of the invention 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 preferably at least 99% of the dry solids in the starch-containing material are converted into a soluble starch hydrolyzate.

Processes for producing fermentation products from a lignocellulose-containing material

The present invention also relates to processes of producing a fermentation product, comprising the steps of:

- 5 (a) pre-treating a lignocellulose-containing material;
 (b) hydrolyzing the pre-treated lignocellulose-containing material; and
 (c) fermenting using a fermenting organism in the presence of a hemicellulase(s).

The present invention also relates to processes of producing a fermentation product, comprising the steps of:

- 10 (a) pre-treating a lignocellulose-containing material;
 (b) hydrolyzing the pre-treated lignocellulose-containing material; and
 (c) fermenting using a fermenting organism in the presence of an endoglucanase(s)
and a hemicellulase(s), wherein the endoglucanase(s) is present in an amount of 0.01-1.0
EGU/g dry solids and in an amount of 1-30 micrograms/g dry solids, the hemicellulase(s) is
15 present in an amount of 0.01-1.0 FXU/g dry solids and in an amount of 1-30 micrograms/g dry
solids.

Steps (b) and (c) may be performed separately or simultaneously. Alternatively, step (b) may be initiated followed by the continuation of step (b) with step (c), otherwise known as a hybrid hydrolysis and fermentation process.

- 20 The lignocellulose-containing material may be pre-treated before being hydrolyzed and fermented. The goal of pre-treatment is to separate and/or release cellulose, hemicellulose and/or lignin and this way improve the rate of enzymatic hydrolysis.

During the pre-treatment, the lignocellulose-containing material may be present in an amount between 10-80 wt. %, e.g., between 20-50 wt. %.

- 25 The lignocellulose-containing material may be chemically, mechanically and/or biologically pre-treated before hydrolysis and/or fermentation. Mechanical treatment (often referred to as a physical pre-treatment) may be used alone or in combination with subsequent or simultaneous hydrolysis, especially enzymatic hydrolysis, to promote the separation and/or release of cellulose, hemicellulose and/or lignin.

- 30 Preferably, the chemical, mechanical and/or biological pre-treatment is carried out prior to the hydrolysis and/or fermentation. Alternatively, the chemical, mechanical and/or biological pre-treatment is carried out simultaneously with hydrolysis, such as simultaneously with addition of one or more cellulolytic enzymes, or other enzyme activities mentioned below, to release fermentable sugars, such as glucose and/or maltose.

Chemical pre-treatment refers to any chemical treatment which promotes the separation and/or release of cellulose, hemicellulose and/or lignin. Examples of suitable chemical pre-treatment steps include treatment with, for example, dilute acid, lime, alkaline, organic solvent, ammonia, sulphur dioxide, carbon dioxide. Further, wet oxidation and pH-controlled hydrothermolysis are also contemplated chemical pre-treatments.

Preferably, the chemical pre-treatment is acid treatment, more preferably, a continuous dilute and/or mild acid treatment, such as, treatment with sulfuric acid, or another organic acid, such as acetic acid, citric acid, tartaric acid, succinic acid, or a mixture thereof. Other acids may also be used. Mild acid treatment means in the context of the present invention that the treatment pH lies in the range from 1-5, preferably from pH 1-3. In a specific embodiment the acid concentration is in the range from 0.1 to 2.0 wt. % acid, preferably sulphuric acid. The acid may be mixed or contacted with the material to be fermented according to the invention and the mixture may be held at a temperature in the range of 160-220°C, such as 165-195°C, for periods ranging from minutes to seconds, e.g., 1-60 minutes, such as 2-30 minutes or 3-12 minutes. Addition of strong acids, such as sulphuric acid, may be applied to remove hemicellulose. This enhances the digestibility of cellulose.

Cellulose solvent treatment has been shown to convert about 90% of cellulose to glucose. It has also been shown that enzymatic hydrolysis could be greatly enhanced when the lignocellulosic structure is disrupted. Alkaline H₂O₂, ozone, organosolv (uses Lewis acids, FeCl₃, (Al)₂SO₄ in aqueous alcohols), glycerol, dioxane, phenol, or ethylene glycol are among solvents known to disrupt cellulose structure and promote hydrolysis (Mosier et al., 2005, *Bioresource Technology* 96: 673-686).

The pre-treatment may also be an alkaline chemical pre-treatment with base, e.g., NaOH, Na₂CO₃ and/or ammonia or the like. Pre-treatment methods using ammonia are described in, e.g., WO 2006/110891, WO 2006/110899, WO 2006/110900, WO 2006/110901, which are hereby incorporated by reference.

Wet oxidation techniques involve use of oxidizing agents, such as: sulphite based oxidizing agents or the like. Examples of solvent pre-treatments include treatment with DMSO (dimethyl sulfoxide) or the like. Chemical pre-treatment is generally carried out for 1 to 60 minutes, such as from 5 to 30 minutes, but may be carried out for shorter or longer periods of time dependent on the material to be pre-treated.

Other examples of suitable pre-treatment methods are described by Schell et al., 2003, *Appl. Biochem and Biotechn.* 105-108: 69-85, and Mosier et al., 2005, *Bioresource Technology*

96: 673-686, and U.S. application publication no. 2002/0164730, which references are hereby all incorporated by reference.

Mechanical pre-treatment refers to any mechanical or physical pre-treatment which promotes the separation and/or release of cellulose, hemicellulose and/or lignin from lignocellulose-containing material. For example, mechanical pre-treatment includes various types of milling, irradiation, steaming/steam explosion, and hydrothermolysis.

Mechanical pre-treatment includes comminution (mechanical reduction of the particle size). Comminution includes dry milling, wet milling and vibratory ball milling. Mechanical pre-treatment may involve high pressure and/or high temperature (steam explosion). In an embodiment of the invention high pressure means pressure in the range from 300 to 600 psi, preferably 400 to 500 psi, such as around 450 psi. In an embodiment of the invention high temperature means temperatures in the range from about 100 to 300°C, preferably from about 140 to 235°C. In a preferred embodiment mechanical pre-treatment is a batch-process, steam gun hydrolyzer system which uses high pressure and high temperature as defined above. A Sunds Hydrolyzer (available from Sunds Defibrator AB (Sweden) may be used for this.

In an embodiment of the invention both chemical and mechanical pre-treatments are carried out involving, for example, both dilute or mild acid pretreatment and high temperature and pressure treatment. The chemical and mechanical pretreatment may be carried out sequentially or simultaneously, as desired.

Accordingly, in a preferred embodiment, the lignocellulose-containing material is subjected to both chemical and mechanical pre-treatment to promote the separation and/or release of cellulose, hemicellulose and/or lignin.

In a preferred embodiment the pre-treatment is carried out as a dilute and/or mild acid steam explosion step. In another preferred embodiment pre-treatment is carried out as an ammonia fiber explosion step (or AFEX pretreatment step).

Biological pre-treatment refers to any biological pre-treatment which promotes the separation and/or release of cellulose, hemicellulose, and/or lignin from the lignocellulose-containing material. Biological pre-treatment techniques can involve applying lignin-solubilizing microorganisms (see, for example, Hsu, T.A., 1996, Pretreatment of biomass, in *Handbook on Bioethanol: Production and Utilization*, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212; Ghosh and Singh, 1993, Physicochemical and biological treatments for enzymatic/microbial conversion of lignocellulosic biomass, *Adv. Appl. Microbiol.* 39: 295-333; McMillan, 1994, Pretreating lignocellulosic biomass: a review, in *Enzymatic Conversion of Biomass for Fuels Production*, Himmel, M. E., Baker, J. O., and Overend, R. P., eds., ACS

Symposium Series 566, American Chemical Society, Washington, DC, chapter 15; Gong, C. S., Cao, N. J., Du, J., and Tsao, G. T., 1999, Ethanol production from renewable resources, in *Advances in Biochemical Engineering/Biotechnology*, Scheper, T., ed., Springer-Verlag Berlin Heidelberg, Germany, 65: 207-241; Olsson and Hahn-Hagerdal, 1996, Fermentation of lignocellulosic hydrolysates for ethanol production, *Enz. Microb. Tech.* 18: 312-331; and Vallander and Eriksson, 1990, Production of ethanol from lignocellulosic materials: State of the art, *Adv. Biochem. Eng./Biotechnol.* 42: 63-95).

The pre-treated material is hydrolyzed, preferably enzymatically, before and/or during fermentation. The pre-treated lignocellulose-containing material may be hydrolyzed in order to break the lignin seal and disrupt the crystalline structure of cellulose. In a preferred embodiment hydrolysis is carried out enzymatically by one or more hydrolases (class E.C. 3 according to Enzyme Nomenclature), preferably one or more carbohydrases including cellulolytic enzymes and hemicellulolytic enzymes, or a combination thereof. In addition, alpha-amylase, glucoamylase, protease, and/or the like may be present during hydrolysis and/or fermentation as the lignocellulose-containing material may include some, e.g., starchy and/or proteinaceous material.

The enzyme(s) used for hydrolysis are capable of directly or indirectly converting carbohydrate polymers into fermentable sugars, such as glucose and/or maltose, which can be fermented into a desired fermentation product, such as ethanol.

In an embodiment, the carbohydrase(s) has(have) cellulolytic (cellobiohydrolase, endoglucanase and beta-glucosidase) and/or hemicellulolytic (e.g., xylanase) activity.

In an embodiment hydrolysis is carried out using a cellulolytic enzyme preparation further comprising one or more polypeptides having cellulolytic enhancing activity. In a preferred embodiment the polypeptide(s) having cellulolytic enhancing activity is(are) of family GH61A origin. Examples of suitable and preferred cellulolytic enzyme preparations and polypeptides having cellulolytic enhancing activity are described in the "Cellulolytic Enzymes" section and "Cellulolytic Enhancing Polypeptides" sections below.

Suitable enzymes are described in the "Enzymes" section below.

Hemicellulose polymers can be broken down by hemicellulolytic enzymes and/or acid hydrolysis to release its five and six carbon sugar components. The six carbon sugars (hexoses), such as arabinose, galactose, glucose, and mannose, can readily be fermented to fermentation products such as acetone, butanol, citric acid, ethanol, fumaric acid, glycerol, etc. by suitable fermenting organisms including yeast.

Yeast is the preferred fermenting organism for ethanol fermentation. Preferred are strains of *Saccharomyces*, especially strains of *Saccharomyces cerevisiae*, preferably strains which are resistant towards high levels of ethanol, *i.e.*, up to, *e.g.*, about 10, 12, 15 or 20 vol. % or more ethanol.

5 Enzymatic hydrolysis is preferably carried out in a suitable aqueous environment under conditions which can readily be determined by one skilled in the art. In a preferred embodiment hydrolysis is carried out at suitable, preferably optimal, conditions for the enzyme(s) in question.

10 Suitable process time, temperature and pH conditions can readily be determined by one skilled in the art. Preferably, hydrolysis is carried out at a temperature between 25 and 70°C, *e.g.*, between 40 and 60°C, especially around 50°C. The step is preferably carried out at a pH in the range from 3-8, *e.g.*, pH 4-6. Hydrolysis is typically carried out for between 12 and 96 hours, *e.g.*, between 16 to 72 hours, in particular between 24 and 48 hours.

15 In an embodiment, the pre-treated lignocellulose-containing material is washed and/or detoxified before or after hydrolysis step (b). This may improve the fermentability of, *e.g.*, dilute-acid hydrolyzed lignocellulose-containing material, such as corn stover. Detoxification may be carried out in any suitable way, *e.g.*, by steam stripping, evaporation, ion exchange, resin or charcoal treatment of the liquid fraction or by washing the pre-treated material.

Fermentation Medium

20 "Fermentation media" or "fermentation medium" refers to the environment in which fermentation is carried out and which includes the fermentation substrate, that is, the carbohydrate source that is metabolized by the fermenting organism.

25 The fermentation medium may comprise nutrients and growth stimulator(s) for the fermenting organism(s). Nutrient and growth stimulators are widely used in the art of fermentation and include nitrogen sources, such as ammonia; urea, vitamins and minerals, or combinations thereof. The fermentation medium may also include enzymes such as amylases and/or other carbohydrate source generating enzymes, especially wherein the process of the invention is carried out as a simultaneous saccharification and fermentation process or an RSH process.

Fermenting Organisms

30 The phrase "fermenting organism" refers to any organism, including bacterial and fungal organisms, suitable for use in a fermentation process and capable of producing a desired

fermentation product. The fermenting organism may be a C6 or C5 fermenting organism, or a combination thereof. Both C5 and C6 fermenting organisms are well known in the art.

Suitable fermenting organisms are able to ferment, *i.e.*, convert, fermentable sugars, such as arabinose, fructose, galactose, glucose, maltose, mannose, and/or xylose, directly or indirectly into the desired fermentation product.

Examples of fermenting organisms include fungal organisms such as yeast. Preferred yeast includes strains of *Saccharomyces*, in particular strains of *Saccharomyces cerevisiae* or *Saccharomyces uvarum*; a strain of *Pichia*, preferably *Pichia stipitis* such as *Pichia stipitis* CBS 5773 or *Pichia pastoris*; a strain of *Candida*, in particular a strain of *Candida utilis*, *Candida arabinofermentans*, *Candida diddensii*, *Candida sonorensis*, *Candida shehatae*, *Candida tropicalis*, or *Candida boidinii*. Other fermenting organisms include strains of *Hansenula*, in particular *Hansenula polymorpha* or *Hansenula anomala*; *Kluyveromyces*, in particular *Kluyveromyces fragilis* or *Kluyveromyces marxianus*; and *Schizosaccharomyces*, in particular *Schizosaccharomyces pombe*.

Commercially available yeast includes, *e.g.*, RED STAR™ and ETHANOL RED™ yeast (available from Fermentis/Lesaffre, USA), FALI (available from Fleischmann's Yeast, USA), SUPERSTART and THERMOSACC™ fresh yeast (available from Ethanol Technology, WI, USA), BIOFERM AFT and XR (available from NABC - North American Bioproducts Corporation, GA, USA), GERT STRAND (available from Gert Strand AB, Sweden), and FERMIOL (available from DSM Specialties).

Preferred bacterial fermenting organisms include strains of *Escherichia*, in particular *Escherichia coli*, strains of *Zymomonas*, in particular *Zymomonas mobilis*, strains of *Zymobacter*, in particular *Zymobacter palmae*, strains of *Klebsiella* in particular *Klebsiella oxytoca*, strains of *Leuconostoc*, in particular *Leuconostoc mesenteroides*, strains of *Clostridium*, in particular *Clostridium butyricum*, strains of *Enterobacter*, in particular *Enterobacter aerogenes* and strains of *Thermoanaerobacter*, in particular *Thermoanaerobacter* BG1L1 (*Appl. Microbiol. Biotech.* 77: 61-86) and *Thermoanaerobacter ethanolicus*, *Thermoanaerobacter thermosaccharolyticum*, or *Thermoanaerobacter mathranii*. Strains of *Lactobacillus* are also envisioned as are strains of *Corynebacterium glutamicum* R, *Bacillus thermoglucosidasius*, and *Geobacillus thermoglucosidasius*.

In an embodiment the fermenting organism is a C6 sugar fermenting organism, such as a strain of, *e.g.*, *Saccharomyces cerevisiae*.

In connection with fermentation of lignocellulose derived materials, C5 sugar fermenting organisms may be used. Most C5 sugar fermenting organisms also ferment C6 sugars.

Examples of C5 sugar fermenting organisms include strains of *Pichia*, such as of the species *Pichia stipitis*. C5 sugar fermenting bacteria are also known. Also some *Saccharomyces cerevisiae* strains ferment C5 (and C6) sugars. Examples are genetically modified strains of *Saccharomyces spp.* that are capable of fermenting C5 sugars include the ones disclosed in,
5 e.g., Ho et al., 1998, *Applied and Environmental Microbiology* 64: 1852-1859 and Karhumaa et al., 2006, *Microbial Cell Factories* 5:18, and Kuyper et al., 2005, *FEMS Yeast Research* 5: 925-934.

The fermentative performance of certain fermenting organisms may be inhibited by the presence of inhibitors in the fermentation media and thus reduce ethanol production capacity.
10 Compounds in biomass hydrolyzates and high concentrations of ethanol are known to inhibit the fermentative capacity of certain yeast cells. Pre-adaptation or adaptation methods may reduce this inhibitory effect. Typically pre-adaptation or adaptation of yeast cells involves sequentially growing yeast cells, prior to fermentation, to increase the fermentative performance of the yeast and increase ethanol production. Methods of yeast pre-adaptation and adaptation are known in
15 the art. Such methods may include, for example, growing the yeast cells in the presence of crude biomass hydrolyzates; growing yeast cells in the presence of inhibitors such as phenolic compounds, furaldehydes and organic acids; growing yeast cells in the presence of non-inhibiting amounts of ethanol; and supplementing the yeast cultures with acetaldehyde. In one embodiment, the fermenting organism is a yeast strain subject to one or more pre-adaptation or
20 adaptation methods prior to fermentation.

According to the invention the fermenting organism is preferably grown under precise conditions at a particular growth rate. When the fermenting organism is introduced into/added to the fermentation medium the inoculated fermenting organism pass through a number of stages. Initially growth does not occur. This period is referred to as the "lag phase" and may be
25 considered a period of adaptation. During the next phase referred to as the "exponential phase" the growth rate gradually increases. After a period of maximum growth the rate ceases and the fermenting organism enters "stationary phase". After a further period of time the fermenting organism enters the "death phase" where the number of viable cells declines.

In one embodiment the fermenting organism is added to the fermentation medium so
30 that the viable fermenting organism count per mL of fermentation medium is in the range from 10^5 to 10^{12} , preferably from 10^7 to 10^{10} , especially about 5×10^7 .

Starch-Containing Materials

Any suitable starch-containing material may be used in the present invention. The starting material is generally selected based on the desired fermentation product. Examples of starch-containing materials include whole grains, barley, beans, cassava, corn, milo, peas, rice, rye, sago, sorghum, sweet potatoes, tapioca, wheat, or mixtures thereof or starches derived therefrom, or cereals. Contemplated are also waxy and non-waxy types of corn and barley.

The term "granular starch" means raw uncooked starch, *i.e.*, starch in its natural form found in cereal, tubers or grains. Starch is formed within plant cells as tiny granules insoluble in water. When put in cold water, the starch granules may absorb a small amount of the liquid and swell. At temperatures up to 50°C to 75°C the swelling may be reversible. However, at higher temperatures an irreversible swelling called "gelatinization" begins. Granular starch to be processed may be a highly refined starch quality, preferably at least 90%, at least 95%, at least 97% or at least 99.5% pure or it may be a more crude starch-containing material comprising (*e.g.*, milled) whole grains including non-starch fractions such as germ residues and fibers. The raw material, such as whole grains, may be reduced in particle size, *e.g.*, by milling, in order to open up the structure and allow for further processing. Two processes are preferred according to the invention: wet and dry milling. In dry milling whole kernels are milled and used. Wet milling gives a good separation of germ and meal (starch granules and protein) and is often applied at locations where the starch hydrolyzate is used in production of, *e.g.*, syrups. Dry and wet milling are known in the art of starch processing and are equally contemplated for a process of the invention. In an embodiment the particle size is reduced to between 0.05 to 3.0 mm, preferably 0.1-0.5 mm, or so that at least 30%, preferably at least 50%, more preferably at least 70%, even more preferably at least 90% of the starch-containing material fits through a sieve with a 0.05 to 3.0 mm screen, preferably 0.1-0.5 mm screen.

Lignocellulose-Containing Material (Biomass)

Any suitable lignocellulose-containing material may be used in the methods of the present invention. Lignocellulose-containing material may be any material containing lignocellulose. In a preferred embodiment the lignocellulose-containing material contains at least 50 wt. %, preferably at least 70 wt. %, more preferably at least 90 wt. % lignocellulose. It is to be understood that the lignocellulose-containing material may also comprise other constituents such as cellulosic material, such as cellulose, hemicellulose and may also comprise constituents such as sugars, such as fermentable sugars and/or un-fermentable sugars.

Lignocellulose-containing material is generally found, for example, in the stems, leaves, hulls, husks, and cobs of plants or leaves, branches, and wood of trees. Lignocellulosic material can also be, but is not limited to, herbaceous material, agricultural residues, forestry residues, municipal solid wastes, waste paper, and pulp and paper mill residues. It is understood herein that lignocellulose-containing material may be in the form of plant cell wall material containing lignin, cellulose, and hemi-cellulose in a mixed matrix.

In an embodiment the lignocellulose-containing material is selected from one or more of corn fiber, rice straw, pine wood, wood chips, poplar, bagasse, and paper and pulp processing waste.

Other examples of suitable lignocellulose-containing material include corn stover, corn cobs, hard wood such as poplar and birch, soft wood, cereal straw such as wheat straw, switch grass, Miscanthus, rice hulls, municipal solid waste (MSW), industrial organic waste, office paper, or mixtures thereof.

In one embodiment the lignocellulose-containing material is corn stover or corn cobs. In another embodiment, the lignocellulose-containing material is corn fiber. In another embodiment, the lignocellulose-containing material is switch grass. In another embodiment, the lignocellulose-containing material is bagasse.

Fermentation Products

The term "fermentation product" means a product produced by a process including a fermentation step using a fermenting organism. Fermentation products include alcohols (e.g., arabinitol, n-butanol, isobutanol, ethanol, glycerol, methanol, ethylene glycol, 1,3-propanediol [propylene glycol], butanediol, glycerin, sorbitol, and xylitol); organic acids (e.g., acetic acid, acetic acid, adipic acid, ascorbic acid, citric acid, 2,5-diketo-D-gluconic acid, formic acid, fumaric acid, glucaric acid, gluconic acid, glucuronic acid, glutaric acid, 3-hydroxypropionic acid, itaconic acid, lactic acid, malic acid, malonic acid, oxalic acid, oxaloacetic acid, propionic acid, succinic acid, and xylonic acid); ketones (e.g., acetone); amino acids (e.g., aspartic acid, glutamic acid, glycine, lysine, serine, and threonine); an alkane (e.g., pentane, hexane, heptane, octane, nonane, decane, undecane, and dodecane), a cycloalkane (e.g., cyclopentane, cyclohexane, cycloheptane, and cyclooctane), an alkene (e.g. pentene, hexene, heptene, and octene); isoprene; polyketide; gases (e.g., methane, hydrogen (H₂), carbon dioxide (CO₂), and carbon monoxide (CO)); antibiotics (e.g., penicillin and tetracycline); enzymes; vitamins (e.g., riboflavin, B₁₂, beta-carotene); and hormones. In a preferred embodiment the fermentation product is ethanol, e.g., fuel ethanol; drinking ethanol, *i.e.*, potable neutral spirits; or industrial

ethanol or products used in the consumable alcohol industry (e.g., beer and wine), dairy industry (e.g., fermented dairy products), leather industry and tobacco industry. Preferred beer types comprise ales, stouts, porters, lagers, bitters, malt liquors, happoushu, high-alcohol beer, low-alcohol beer, low-calorie beer or light beer. Preferred fermentation processes include alcohol fermentation processes. In an embodiment, the fermentation product is ethanol, which may be used as fuel ethanol or as potable ethanol.

Distillation

Subsequent to fermentation the fermentation product may be separated from the fermentation medium. The slurry may be distilled to extract the desired fermentation product or the desired fermentation product from the fermentation medium by micro or membrane filtration techniques. Alternatively the fermentation product may be recovered by stripping. Methods for recovering fermentation products are well known in the art. Typically, the fermentation product, e.g., ethanol, with a purity of up to, e.g., about 96 vol. % ethanol is obtained.

Following the completion of the fermentation process, the material remaining is considered the whole stillage. As used herein, the term "whole stillage" includes the material that remains at the end of the fermentation process both before and after recovery of the fermentation product, e.g., ethanol. The fermentation product can optionally be recovered by any method known in the art. In one embodiment, the whole stillage is separated or partitioned into a solid and liquid phase by one or more methods for separating the thin stillage from the wet cake. Such methods include, for example, centrifugation and decanting. The fermentation product can be optionally recovered before or after the whole stillage is separated into a solid and liquid phase.

Thus, in one embodiment, the method of the invention further comprises distillation to obtain the fermentation product, e.g., ethanol. The fermentation and the distillation may be carried out simultaneously and/or separately/sequentially; optionally followed by one or more process steps for further refinement of the fermentation product.

In an embodiment of the invention, the aqueous by-product (whole stillage) from the distillation process is separated into two fractions, e.g., by centrifugation: wet grain (solid phase), and thin stillage (supernatant).

In another embodiment, the method of the invention further comprises separation of the whole stillage produced by distillation into wet grain and thin stillage; and recycling thin stillage to the starch containing material prior to liquefaction.

In one embodiment, the thin stillage is recycled to the milled whole grain slurry.

The wet grain fraction may be dried, typically in a drum dryer. The dried product is referred to as distillers dried grains, and can be used, e.g., as animal feed.

The thin stillage fraction may be evaporated providing two fractions (see Fig. 1):

(i) a condensate fraction of 4-6% DS (mainly of starch, proteins, and cell wall components), and

(ii) a syrup fraction, mainly consisting of limit dextrins and non fermentable sugars, which may be introduced into a dryer together with the wet grains (from the whole stillage separation step) to provide a product referred to as distillers dried grain with solubles, which also can be used as animal feed.

Thin stillage is the term used for the supernatant of the centrifugation of the whole stillage. Typically, the thin stillage contains 4-6% DS (mainly starch and proteins) and has a temperature of about 60-90°C.

In another embodiment, the thin stillage is not recycled, but the condensate stream of evaporated thin stillage is recycled to the slurry containing the milled whole grain to be jet cooked.

Distillers' Dried Grains with Solubles

As explained above, stillage is the product which remains after the mash has been converted to sugar, fermented and distilled into ethanol. Stillage can be separated into two fractions, such as, by centrifugation or screening: (1) wet cake (solid phase) and (2) the thin stillage (supernatant). The solid fraction or distillers' wet grain (DWG) can be pressed to remove excess moisture and then dried to produce distillers' dried grains (DDG). After ethanol has been removed from the liquid fraction, the remaining liquid can be evaporated to concentrate the soluble material into condensed distillers' solubles (DS) or dried and ground to create distillers' dried solubles (DDS). DDS is often mixed with DDG to form distillers' dried grain with solubles (DDGS). DDG, DDGS, and DWG are collectively referred to as distillers' grain(s).

DDGS following an ethanol production process from corn typically contains about 13% oil, 31% protein and 56% carbohydrates and other components. Removal of some of the oil from the DDGS will improve the quality of the DDGS for the feed market as many feed producers prefer less oil and fat in the DDGS to make high quality feed.

Oil Extraction and Dewatering

Methods for dewatering stillage and for extracting oil from a fermentation product are known in the art. These methods include decanting or otherwise separating the whole stillage

into wet cake and thin stillage. See, e.g., U.S. Patent Nos. 6,433,146, 7,601,858, and 7,608,729, and U.S. Application Publication No. 2010/0058649. Furthermore, the thin stillage can be evaporated or condensed into syrup or thick stillage from which the oil can be extracted utilizing centrifugation, filtering, heat, high temperature, increased pressure, or a combination of the same. Another way to extract oil is to lower the pH of the thin stillage or syrup. The use of surfactants to break emulsions also enhances oil extraction. Presses can also be used for dewatering.

In one embodiment of the invention, the presence of hemicellulase(s) and/or endoglucanase(s) during fermentation in the processes of the invention increases the amount of oil in the thin stillage and further the syrup or thick stillage as compared to the amount of oil in the thin stillage, syrup or thick stillage when a hemicellulase and/or an endoglucanase are not added to the fermentation process.

Recovery

The fermentation product(s) can be optionally recovered from the fermentation medium using any method known in the art including, but not limited to, chromatography, electrophoretic procedures, differential solubility, distillation, or extraction. For example, alcohol is separated from the fermented cellulosic material and purified by conventional methods of distillation. Ethanol with a purity of up to about 96 vol.% can be obtained, which can be used as, for example, fuel ethanol, drinking ethanol, *i.e.*, potable neutral spirits, or industrial ethanol.

Hemicellulases

Hemicellulose can be broken down by hemicellulases and/or acid hydrolysis to release its five and six carbon sugar components.

Any hemicellulase suitable for use in hydrolyzing hemicellulose, preferably into xylose, may be used. Preferred hemicellulases include acetylxylan esterases, endo-arabinases, exo-arabinases, arabinofuranosidases, feruloyl esterase, endo-galactanases, exo-galactanases, glucuronidases, mannases, xylanases, and mixtures of two or more thereof. Preferably, the hemicellulase for use in the present invention is an exo-acting hemicellulase, and more preferably, the hemicellulase is an exo-acting hemicellulase which has the ability to hydrolyze hemicellulose under acidic conditions of below pH 7, preferably pH 3-7.

In one aspect, the hemicellulase(s) comprises a commercial hemicellulolytic enzyme preparation. Examples of commercial hemicellulolytic enzyme preparations suitable for use in the present invention include, for example, SHEARZYME™ (Novozymes A/S), CELLIC™ HTec

(Novozymes A/S), CELLIC™ HTec2 (Novozymes A/S), VISCOZYME® (Novozymes A/S), ULTRAFLO® (Novozymes A/S), PULPZYME® HC (Novozymes A/S), MULTIFECT® Xylanase (Genencor), ACCELLERASE® XY (Genencor), ACCELLERASE® XC (Genencor), ECOPULP® TX-200A (AB Enzymes), HSP 6000 Xylanase (DSM), DEPOL™ 333P (Biocatalysts Limit, Wales, UK), DEPOL™ 740L (Biocatalysts Limit, Wales, UK), and DEPOL™ 762P (Biocatalysts Limit, Wales, UK).

In an embodiment the hemicellulase is a xylanase. In an embodiment the xylanase may preferably be of microbial origin, such as of fungal origin (e.g., *Aspergillus*, *Fusarium*, *Humicola*, *Meripilus*, *Trichoderma*) or from a bacterium (e.g., *Bacillus*). In a preferred embodiment the xylanase is derived from a filamentous fungus, preferably derived from a strain of *Aspergillus*, such as *Aspergillus aculeatus*; or a strain of *Humicola*, preferably *Humicola lanuginosa*. Examples of xylanases useful in the methods of the present invention include, but are not limited to, *Aspergillus aculeatus* xylanase (GeneSeqP:AAR63790; WO 94/21785), *Aspergillus fumigatus* xylanases (WO 2006/078256), and *Thielavia terrestris* NRRL 8126 xylanases (WO 2009/079210). The xylanase may preferably be an endo-1,4-beta-xylanase, more preferably an endo-1,4-beta-xylanase of GH 10 or GH 11. Examples of commercial xylanases include SHEARZYME™, BIOFEED WHEAT™, HTec and HTec2 from Novozymes A/S, Denmark.

Examples of beta-xylosidases useful in the methods of the present invention include, but are not limited to, *Trichoderma reesei* beta-xylosidase (UniProtKB/TrEMBL accession number Q92458), *Talaromyces emersonii* (SwissProt accession number Q8X212), and *Neurospora crassa* (SwissProt accession number Q7SOW4).

Examples of acetylxylan esterases useful in the methods of the present invention include, but are not limited to, *Hypocrea jecorina* acetylxylan esterase (WO 2005/001036), *Neurospora crassa* acetylxylan esterase (UniProt accession number q7s259), *Thielavia terrestris* NRRL 8126 acetylxylan esterase (WO 2009/042846), *Chaetomium globosum* acetylxylan esterase (Uniprot accession number Q2GWX4), *Chaetomium gracile* acetylxylan esterase (GeneSeqP accession number AAB82124), *Phaeosphaeria nodorum* acetylxylan esterase (Uniprot accession number Q0UHH1), and *Humicola insolens* DSM 1800 acetylxylan esterase (WO 2009/073709).

Examples of ferulic acid esterases useful in the methods of the present invention include, but are not limited to, *Humicola insolens* DSM 1800 feruloyl esterase (WO 2009/076122), *Neurospora crassa* feruloyl esterase (UniProt accession number Q9HGR3), and *Neosartorya fischeri* feruloyl esterase (UniProt Accession number A1D9T4).

Examples of arabinofuranosidases useful in the methods of the present invention include, but are not limited to, *Humicola insolens* DSM 1800 arabinofuranosidase (WO 2009/073383) and *Aspergillus niger* arabinofuranosidase (GeneSeqP accession number AAR94170).

5 Examples of alpha-glucuronidases useful in the methods of the present invention include, but are not limited to, *Aspergillus clavatus* alpha-glucuronidase (UniProt accession number alcc12), *Trichoderma reesei* alpha-glucuronidase (Uniprot accession number Q99024), *Talaromyces emersonii* alpha-glucuronidase (UniProt accession number Q8X211), *Aspergillus niger* alpha-glucuronidase (Uniprot accession number Q96WX9), *Aspergillus terreus* alpha-
10 glucuronidase (SwissProt accession number Q0CJP9), and *Aspergillus fumigatus* alpha-glucuronidase (SwissProt accession number Q4WW45).

Endoglucanases (EG)

The term "endoglucanase" means an endo-1,4-(1,3;1,4)-beta-D-glucan
15 4-glucanohydrolase (E.C. No. 3.2.1.4), which catalyzes endo-hydrolysis of 1,4-beta-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxymethyl cellulose and hydroxyethyl cellulose), lichenin, beta-1,4 bonds in mixed beta-1,3 glucans such as cereal beta-D-glucans or xyloglucans, and other plant material containing cellulosic components. Endoglucanase activity may be determined using carboxymethyl cellulose (CMC) hydrolysis according to the procedure
20 of Ghose, 1987, *Pure and Appl. Chem.* 59: 257-268.

In a preferred embodiment endoglucanases may be derived from a strain of *Trichoderma*, such as a strain of *Trichoderma reesei*; a strain of *Humicola*, such as a strain of *Humicola insolens*; or a strain of *Chrysosporium*, such as a strain of *Chrysosporium lucknowense*.

Enzymes for Hydrolysis of a Lignocellulose-Containing Material

Hemicellulases

Any of the hemicellulases described above can be used for hydrolyzing a lignocelluloses-containing material.

30 The hemicellulase may be added in an amount effective to hydrolyze hemicellulose, such as, in an amount from about 0.001 to 0.5 wt. % of total solids (TS), more preferably from about 0.05 to 0.5 wt. % of TS.

Xylanases may be added in an amount of 0.001-1.0 g/kg DM (dry matter) substrate, preferably in the amount of 0.005-0.5 g/kg DM substrate, and most preferably from 0.05-0.10 g/kg DM substrate.

5 Cellulolytic Activity

The phrase "cellulolytic activity" as used herein includes enzymes having cellobiohydrolase activity (EC 3.2.1.91), e.g., cellobiohydrolase I and cellobiohydrolase II, endoglucanase activity (EC 3.2.1.4) and/or beta-glucosidase activity (EC 3.2.1.21).

At least three categories of enzymes are important for converting cellulose into fermentable sugars: endoglucanases (EC 3.2.1.4) that cut the cellulose chains at random; cellobiohydrolases (EC 3.2.1.91) which cleave cellobiosyl units from the cellulose chain ends and beta-glucosidases (EC 3.2.1.21) that convert cellobiose and soluble cellodextrins into glucose. Among these three categories of enzymes involved in the biodegradation of cellulose, cellobiohydrolases seems to be the key enzymes for degrading native crystalline cellulose.

The cellulolytic activity may, in a preferred embodiment, be in the form of a preparation of enzymes of fungal origin, such as from a strain of *Trichoderma*, preferably a strain of *Trichoderma reesei*; a strain of *Humicola*, such as a strain of *Humicola insolens*; or a strain of *Chrysosporium*, preferably a strain of *Chrysosporium lucknowense*.

In preferred embodiment the cellulolytic enzyme preparation contains one or more of the following activities: cellulase, hemicellulase, cellulolytic enzyme enhancing activity, beta-glucosidase, endoglucanase, cellobiohydrolase, or xylose isomerase.

In a preferred embodiment the cellulase may be a composition as defined in PCT/US2008/065417, which is hereby incorporated by reference. In a preferred embodiment the cellulolytic enzyme preparation comprises a polypeptide having cellulolytic enhancing activity, preferably a family GH61A polypeptide, preferably the one disclosed in WO 2005/074656 (Novozymes). The cellulolytic enzyme preparation may further comprise a beta-glucosidase, such as a beta-glucosidase derived from a strain of *Aspergillus*, *Penicillium*, or *Trichoderma*, including the fusion protein having beta-glucosidase activity disclosed in WO 2008/057637. In a preferred embodiment the cellulolytic enzyme preparation may also comprises a CBH II enzyme, preferably *Thielavia terrestris* cellobiohydrolase II CEL6A. In another preferred embodiment the cellulolytic enzyme preparation may also comprise cellulolytic enzymes, preferably derived from *Trichoderma reesei* or *Humicola insolens*.

The cellulolytic enzyme preparation may also comprise a polypeptide having cellulolytic enhancing activity (GH61A) disclosed in WO 2005/074656; a beta-glucosidase (fusion protein disclosed in WO 2008/057637); and cellulolytic enzymes derived from *Trichoderma reesei*.

In an embodiment the cellulolytic enzyme is the commercially available product
5 CELLUCLAST® 1.5L, CELLUZYME™, CTEC or CTEC2 available from Novozymes A/S, Denmark or ACCELERASE™ 1000 (from Genencor Inc., USA).

A cellulolytic enzyme may be added during fermentation. The cellulolytic enzyme may be dosed in the range from 0.1-100 FPU per gram total solids (TS), preferably 0.5-50 FPU per gram TS, especially 1-20 FPU per gram TS. In another embodiment at least 0.1 mg cellulolytic
10 enzyme per gram total solids (TS), preferably at least 3 mg cellulolytic enzyme per gram TS, such as between 5 and 10 mg cellulolytic enzyme(s) per gram TS is(are) used for hydrolysis.

Endoglucanases (EG)

Any endoglucanase described above can be used for hydrolyzing a lignocellulosic
15 material.

Cellobiohydrolases (CBH)

The term "cellobiohydrolase" means a 1,4-beta-D-glucan cellobiohydrolase (E.C. 3.2.1.91), which catalyzes the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose,
20 celooligosaccharides, or any beta-1,4-linked glucose containing polymer, releasing cellobiose from the reducing or non-reducing ends of the chain.

Examples of cellobiohydrolases include CBH I and CBH II from *Trichoderma reesei*; *Humicola insolens* and CBH II from *Thielavia terrestris* cellobiohydrolase (CELL6A).

Cellobiohydrolase activity may be determined according to the procedures described by
25 Lever *et al.*, 1972, *Anal. Biochem.* 47: 273-279 and by van Tilbeurgh *et al.*, 1982, *FEBS Letters* 149: 152-156; van Tilbeurgh and Claeysens, 1985, *FEBS Letters* 187: 283-288. The Lever *et al.* method is suitable for assessing hydrolysis of cellulose in corn stover and the method of van Tilbeurgh *et al.* is suitable for determining the cellobiohydrolase activity on a fluorescent disaccharide derivative.

Beta-glucosidases

The term "beta-glucosidase" means a beta-D-glucoside glucohydrolase (E.C. 3.2.1.21), which catalyzes the hydrolysis of terminal non-reducing beta-D-glucose residues with the release of beta-D-glucose. For purposes of the present invention, beta-glucosidase activity is

determined according to the basic procedure described by Venturi *et al.*, 2002, *J. Basic Microbiol.* 42: 55-66, except different conditions were employed as described herein. One unit of beta-glucosidase activity is defined as 1.0 micro-mole of p-nitrophenol produced per minute at 50°C, pH 5 from 4 mM p-nitrophenyl-beta-D-glucopyranoside as substrate in 100 mM sodium citrate, 0.01% TWEEN® 20.

In a preferred embodiment the beta-glucosidase is of fungal origin, such as a strain of *Aspergillus*, *Penicillium*, or *Trichoderma*. In a preferred embodiment the beta-glucosidase is derived from *Trichoderma reesei*, such as the beta-glucosidase encoded by the *bgl1* gene (see Fig. 1 of EP 562003). In another preferred embodiment the beta-glucosidase is derived from *Aspergillus oryzae* (recombinantly produced in *Aspergillus oryzae* according to WO 2002/095014), *Aspergillus fumigatus* (recombinantly produced in *Aspergillus oryzae* according to Example 22 of WO 2002/095014) or *Aspergillus niger* (1981, *J. Appl.* 3: 157-163).

Xylose Isomerases

Xylose isomerases (D-xylose ketoisomerase) (E.C. 5.3.1.5) are enzymes that catalyze the reversible isomerization reaction of D-xylose to D-xylulose. Some xylose isomerases also convert the reversible isomerization of D-glucose to D-fructose. Therefore, xylose isomerase is sometimes referred to as "glucose isomerase."

A xylose isomerase used in a method or process of the invention may be any enzyme having xylose isomerase activity and may be derived from any source, preferably bacterial or fungal origin, such as filamentous fungi or yeast. Examples of bacterial xylose isomerases include the ones belonging to *Actinoplanes*, *Bacillus*, *Flavobacterium*, *Streptomyces*, and *Thermotoga*, including *T. neapolitana* (Vieille *et al.*, 1995, *Appl. Environ. Microbiol.* 61(5): 1867-1875) and *T. maritima*.

Examples of fungal xylose isomerases are derived from *Basidiomycetes*.

A preferred xylose isomerase is derived from a strain of *Candida*, preferably a strain of *Candida boidinii*, especially the *Candida boidinii* xylose isomerase disclosed by, e.g., Vongsuvanlert *et al.*, 1988, *Agric. Biol. Chem.* 52(7): 1817-1824. The xylose isomerase may be derived from a strain of *Candida boidinii* (Kloeckera 2201), deposited as DSM 70034 and ATCC 48180, disclosed in Ogata *et al.*, *Agric. Biol. Chem.* 33: 1519-1520 or Vongsuvanlert *et al.*, 1988, *Agric. Biol. Chem.* 52(2): 1519-1520.

In one embodiment the xylose isomerase is derived from a strain of *Streptomyces*, e.g., derived from a strain of *Streptomyces murinus* (U.S. Patent No. 4,687,742); *S. flavovirens*, *S. albus*, *S. achromogenus*, *S. echinatus*, *S. wedmorensis* all disclosed in U.S. Patent No.

3,616,221. Other xylose isomerases are disclosed in U.S. Patent No. 3,622,463, U.S. Patent No. 4,351,903, U.S. Patent No. 4,137,126, U.S. Patent No. 3,625,828, HU patent no. 12,415, DE patent no. 2,417,642, JP patent no. 69,28,473, and WO 2004/044129 each incorporated by reference herein.

5 The xylose isomerase may be either in immobilized or liquid form. Liquid form is preferred.

An example of a commercially available xylose isomerase is SWEETZYME™ T from Novozymes A/S, Denmark.

The xylose isomerase is added to provide an activity level in the range from 0.01-100 IGIU per gram total solids.

GH61 Polypeptides

In the processes of the present invention, any GH61 polypeptide can be used.

In a first aspect, the GH61 polypeptide comprises the following motifs:

15 [ILMV]-P-X(4,5)-G-X-Y-[ILMV]-X-R-X-[EQ]-X(4)-[HNQ] and [FW]-[TF]-K-[AIV], wherein X is any amino acid, X(4,5) is any four or five contiguous amino acids, and X(4) is any four contiguous amino acids.

The GH61 polypeptide comprising the above-noted motifs may further comprise:

H-X(1,2)-G-P-X(3)-[YW]-[AILMV],

20 [EQ]-X-Y-X(2)-C-X-[EHQN]-[FILV]-X-[ILV], or

H-X(1,2)-G-P-X(3)-[YW]-[AILMV] and [EQ]-X-Y-X(2)-C-X-[EHQN]-[FILV]-X-[ILV],

wherein X is any amino acid, X(1,2) is any one or two contiguous amino acids, X(3) is any three contiguous amino acids, and X(2) is any two contiguous amino acids.

In a preferred aspect, the GH61 polypeptide further comprises H-X(1,2)-G-P-X(3)-[YW]-[AILMV]. In another preferred aspect, the GH61 polypeptide further comprises [EQ]-X-Y-X(2)-C-X-[EHQN]-[FILV]-X-[ILV]. In another preferred aspect, the GH61 polypeptide further comprises H-X(1,2)-G-P-X(3)-[YW]-[AILMV] and [EQ]-X-Y-X(2)-C-X-[EHQN]-[FILV]-X-[ILV].

In a second aspect, the GH61 polypeptide comprises the following motif:

[ILMV]-P-x(4,5)-G-x-Y-[ILMV]-x-R-x-[EQ]-x(3)-A-[HNQ],

30 wherein x is any amino acid, x(4,5) is any 4 or 5 contiguous amino acids, and x(3) is any 3 contiguous amino acids. In the above motif, the accepted IUPAC single letter amino acid abbreviation is employed.

In a third aspect, the GH61 polypeptide comprises an amino acid sequence that has a degree of identity to the mature polypeptide of SEQ ID NO: 1 (*Thielavia terrestris*), SEQ ID

NO: 2 (*Thielavia terrestris*), SEQ ID NO: 3 (*Thielavia terrestris*), SEQ ID NO: 4 (*Thielavia terrestris*), SEQ ID NO: 5 (*Thielavia terrestris*), SEQ ID NO: 6 (*Thielavia terrestris*), SEQ ID NO: 7 (*Thermoascus aurantiacus*), SEQ ID NO: 8 (*Trichoderma reesei*), SEQ ID NO: 9 (*Myceliophthora thermophila*), SEQ ID NO: 10 (*Myceliophthora thermophila*), SEQ ID NO: 11 (*Myceliophthora thermophila*), SEQ ID NO: 12 (*Myceliophthora thermophila*), SEQ ID NO: 13 (*Myceliophthora thermophila*), SEQ ID NO: 14 (*Thermoascus aurantiacus*), SEQ ID NO: 15 (*Aspergillus fumigatus*), SEQ ID NO: 16 (*Penicillium pinophilum*), SEQ ID NO: 17 (*Thermoascus sp.*), SEQ ID NO: 18 (*Penicillium sp.*), SEQ ID NO: 19 (*Thielavia terrestris*), SEQ ID NO: 20 (*Thielavia terrestris*), SEQ ID NO: 21 (*Thielavia terrestris*), SEQ ID NO: 22 (*Thielavia terrestris*), SEQ ID NO: 23 (*Thielavia terrestris*), SEQ ID NO: 24 (*Thielavia terrestris*), SEQ ID NO: 25 (*Thielavia terrestris*), SEQ ID NO: 26 (*Thielavia terrestris*), SEQ ID NO: 27 (*Thielavia terrestris*), SEQ ID NO: 28 (*Thielavia terrestris*), SEQ ID NO: 29 (*Thielavia terrestris*), SEQ ID NO: 30 (*Thermoascus crustaceus*), SEQ ID NO: 31 (*Thermoascus crustaceus*), SEQ ID NO: 32 (*Thermoascus crustaceus*), SEQ ID NO: 33 (*Aurantiporus alborubescens*), or SEQ ID NO: 34 (*Aurantiporus alborubescens*) of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, or at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100%.

In a sixth aspect, the GH61 polypeptide is an artificial variant comprising a substitution, deletion, and/or insertion of one or more (or several) amino acids of the mature polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, or SEQ ID NO: 32; or a homologous sequence thereof.

Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino

acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 5 1979, *In, The Proteins*, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

Alternatively, the amino acid changes are of such a nature that the physico-chemical properties of the polypeptides are altered. For example, amino acid changes may improve the 10 thermal stability of the polypeptide, alter the substrate specificity, change the pH optimum, and the like.

Essential amino acids in a parent polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine 15 mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for cellulolytic enhancing activity to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, 20 electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Lett.* 309: 59-64. The identities of essential amino acids can also be inferred from analysis of identities with polypeptides that are related to the parent polypeptide.

Single or multiple amino acid substitutions, deletions, and/or insertions can be made and 25 tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR, phage 30 display (e.g., Lowman *et al.*, 1991, *Biochemistry* 30: 10832-10837; U.S. Patent No. 5,223,409; WO 92/06204), and region-directed mutagenesis (Derbyshire *et al.*, 1986, *Gene* 46: 145; Ner *et al.*, 1988, *DNA* 7: 127).

Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host

cells (Ness *et al.*, 1999, *Nature Biotechnology* 17: 893-896). Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide.

5 The total number of amino acid substitutions, deletions and/or insertions of the mature GH61 polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, or SEQ ID NO: 32 is not more than 4, *e.g.*, 1, 2, 3, or 4.

In one aspect, the GH61 polypeptide is used in the presence of a soluble activating divalent metal cation described in WO 2008/151043, *e.g.*, manganese sulfate.

15 In one aspect, the GH61 polypeptide is used in the presence of a dioxy compound, a bicyclic compound, a heterocyclic compound, a nitrogen-containing compound, or a sulfur-containing compound.

The dioxy compound may include any suitable compound containing two or more oxygen atoms. In some aspects, the dioxy compounds contain a substituted aryl moiety as described herein. The dioxy compounds may comprise one or more (several) hydroxyl and/or hydroxyl derivatives, but also include substituted aryl moieties lacking hydroxyl and hydroxyl derivatives. Non-limiting examples of dioxy compounds include pyrocatechol or catechol; caffeic acid; 3,4-dihydroxybenzoic acid; 4-*tert*-butyl-5-methoxy-1,2-benzenediol; pyrogallol; gallic acid; methyl-3,4,5-trihydroxybenzoate; 2,3,4-trihydroxybenzophenone; 2,6-dimethoxyphenol; 25 sinapinic acid; 3,5-dihydroxybenzoic acid; 4-chloro-1,2-benzenediol; 4-nitro-1,2-benzenediol; tannic acid; ethyl gallate; methyl glycolate; dihydroxyfumaric acid; 2-butyne-1,4-diol; (croconic acid; 1,3-propanediol; tartaric acid; 2,4-pentanediol; 3-ethoxy-1,2-propanediol; 2,4,4'-trihydroxybenzophenone; *cis*-2-butene-1,4-diol; 3,4-dihydroxy-3-cyclobutene-1,2-dione; dihydroxyacetone; acrolein acetal; methyl-4-hydroxybenzoate; 4-hydroxybenzoic acid; and 30 methyl-3,5-dimethoxy-4-hydroxybenzoate; or a salt or solvate thereof.

The bicyclic compound may include any suitable substituted fused ring system as described herein. The compounds may comprise one or more (several) additional rings, and are not limited to a specific number of rings unless otherwise stated. In one aspect, the bicyclic compound is a flavonoid. In another aspect, the bicyclic compound is an optionally substituted

isoflavonoid. In another aspect, the bicyclic compound is an optionally substituted flavylium ion, such as an optionally substituted anthocyanidin or optionally substituted anthocyanin, or derivative thereof. Non-limiting examples of bicyclic compounds include epicatechin; quercetin; myricetin; taxifolin; kaempferol; morin; acacetin; naringenin; isorhamnetin; apigenin; cyanidin; cyanin; kuromanin; (keracyanin; or a salt or solvate thereof.

The heterocyclic compound may be any suitable compound, such as an optionally substituted aromatic or non-aromatic ring comprising a heteroatom, as described herein. In one aspect, the heterocyclic is a compound comprising an optionally substituted heterocycloalkyl moiety or an optionally substituted heteroaryl moiety. In another aspect, the optionally substituted heterocycloalkyl moiety or optionally substituted heteroaryl moiety is an optionally substituted 5-membered heterocycloalkyl or an optionally substituted 5-membered heteroaryl moiety. In another aspect, the optionally substituted heterocycloalkyl or optionally substituted heteroaryl moiety is an optionally substituted moiety selected from pyrazolyl, furanyl, imidazolyl, isoxazolyl, oxadiazolyl, oxazolyl, pyrrolyl, pyridyl, pyrimidyl, pyridazinyl, thiazolyl, triazolyl, thienyl, dihydrothieno-pyrazolyl, thianaphthenyl, carbazolyl, benzimidazolyl, benzothienyl, benzofuranyl, indolyl, quinoliny, benzotriazolyl, benzothiazolyl, benzooxazolyl, benzimidazolyl, isoquinoliny, isoindolyl, acridinyl, benzoisazolyl, dimethylhydantoin, pyrazinyl, tetrahydrofuranyl, pyrrolinyl, pyrrolidinyl, morpholiny, indolyl, diazepinyl, azepinyl, thiepinyl, piperidinyl, and oxepinyl. In another aspect, the optionally substituted heterocycloalkyl moiety or optionally substituted heteroaryl moiety is an optionally substituted furanyl. Non-limiting examples of heterocyclic compounds include (1,2-dihydroxyethyl)-3,4-dihydroxyfuran-2(5H)-one; 4-hydroxy-5-methyl-3-furanone; 5-hydroxy-2(5H)-furanone; [1,2-dihydroxyethyl]furan-2,3,4(5H)-trione; α -hydroxy- γ -butyrolactone; ribonic γ -lactone; aldohexuronic acid γ -lactone; gluconic acid δ -lactone; 4-hydroxycoumarin; dihydrobenzofuran; 5-(hydroxymethyl)furfural; furoin; 2(5H)-furanone; 5,6-dihydro-2H-pyran-2-one; and 5,6-dihydro-4-hydroxy-6-methyl-2H-pyran-2-one; or a salt or solvate thereof.

The nitrogen-containing compound may be any suitable compound with one or more nitrogen atoms. In one aspect, the nitrogen-containing compound comprises an amine, imine, hydroxylamine, or nitroxide moiety. Non-limiting examples of nitrogen-containing compounds include acetone oxime; violuric acid; pyridine-2-aldoxime; 2-aminophenol; 1,2-benzenediamine; 2,2,6,6-tetramethyl-1-piperidinyloxy; 5,6,7,8-tetrahydrobiopterin; 6,7-dimethyl-5,6,7,8-tetrahydropterine; and maleamic acid; or a salt or solvate thereof.

The quinone compound may be any suitable compound comprising a quinone moiety as described herein. Non-limiting examples of quinone compounds include 1,4-benzoquinone; 1,4-

naphthoquinone; 2-hydroxy-1,4-naphthoquinone; 2,3-dimethoxy-5-methyl-1,4-benzoquinone or coenzyme Q₀; 2,3,5,6-tetramethyl-1,4-benzoquinone or duroquinone; 1,4-dihydroxyanthraquinone; 3-hydroxy-1-methyl-5,6-indolinedione or adrenochrome; 4-tert-butyl-5-methoxy-1,2-benzoquinone; pyrroloquinoline quinone; or a salt or solvate thereof.

5 The sulfur-containing compound may be any suitable compound comprising one or more sulfur atoms. In one aspect, the sulfur-containing comprises a moiety selected from thionyl, thioether, sulfinyl, sulfonyl, sulfamide, sulfonamide, sulfonic acid, and sulfonic ester. Non-limiting examples of sulfur-containing compounds include ethanethiol; 2-propanethiol; 2-propene-1-thiol; 2-mercaptoethanesulfonic acid; benzenethiol; benzene-1,2-dithiol; cysteine; methionine; 10 glutathione; cystine; or a salt or solvate thereof.

In an embodiment, the GH61 polypeptide is present in the amount of 2-1000 micrograms/g dry solids (DS), e.g., 5-100, 10-40, or 20-40 micrograms/g DS.

Enzymes for use in a Conventional Process and Raw Starch Hydrolysis Process

15 Alpha-Amylases

Any alpha-amylase may be used to convert a starch-containing material to dextrans. Preferred alpha-amylases are of microbial, such as bacterial or fungal origin. The most suitable alpha-amylase depends on the process conditions but can easily be determined by one skilled in the art.

20 In one embodiment the preferred alpha-amylase is an acid alpha-amylase, e.g., fungal acid alpha-amylase or bacterial acid alpha-amylase. The phrase "acid alpha-amylase" means an alpha-amylase (E.C. 3.2.1.1) which has optimum activity at a pH in the range of 3 to 7, preferably from 3.5 to 6, or more preferably from 4-5.

25 Bacterial Alpha-Amylases

In another preferred embodiment the alpha-amylase is of *Bacillus* origin. The *Bacillus* alpha-amylase may preferably be derived from a strain of *B. amyloliquefaciens*, *B. licheniformis*, *B. stearothermophilus*, or *B. subtilis*, but may also be derived from other *Bacillus* sp. Specific examples of contemplated alpha-amylases include the *Bacillus licheniformis* alpha-amylase 30 shown in SEQ ID NO: 4 in WO 99/19467, the *Bacillus amyloliquefaciens* alpha-amylase SEQ ID NO: 5 in WO 99/19467 and the *Bacillus stearothermophilus* alpha-amylase shown in SEQ ID NO: 3 in WO 99/19467 (all sequences are hereby incorporated by reference). In an embodiment of the invention the alpha-amylase may be an enzyme having a degree of identity of at least 60%, preferably at least 70%, more preferred at least 80%, even more preferred at least 90%,

such as at least 95%, at least 96%, at least 97%, at least 98% or at least 99% to any of the sequences shown in SEQ ID NO: 3, 4 or 5, respectively, in WO 99/19467.

The *Bacillus* alpha-amylase may also be a variant and/or hybrid, especially one described in any of WO 96/23873, WO 96/23874, WO 97/41213, WO 99/19467, WO 00/60059, and WO 02/10355 (all documents are hereby incorporated by reference). Specifically contemplated alpha-amylase variants are disclosed in U.S. Patent No. 6,093,562, 6,297,038 or 6,187,576 (hereby incorporated by reference) and include *Bacillus stearothermophilus* alpha-amylase (BSG alpha-amylase) variants having a deletion of one or two amino acids in positions R179 to G182, preferably a double deletion disclosed in WO 96/23873 - see e.g., page 20, lines 1-10 (hereby incorporated by reference), preferably corresponding to delta(181-182) compared to the wild-type BSG alpha-amylase amino acid sequence set forth in SEQ ID NO: 3 disclosed in WO 99/19467 or deletion of amino acids R179 and G180 using SEQ ID NO: 3 in WO 99/19467 for numbering (which reference is hereby incorporated by reference). Even more preferred are *Bacillus* alpha-amylases, especially *Bacillus stearothermophilus* alpha-amylase, which have a double deletion corresponding to delta(181-182) and further comprise a N193F substitution (also denoted 1181* + G182* + N193F) compared to the wild-type BSG alpha-amylase amino acid sequence set forth in SEQ ID NO: 3 disclosed in WO 99/19467.

Bacterial Hybrid Alpha-Amylases

A hybrid alpha-amylase specifically contemplated comprises 445 C-terminal amino acid residues of the *Bacillus licheniformis* alpha-amylase (shown in SEQ ID NO: 4 of WO 1999/19467) and the 37 N-terminal amino acid residues of the alpha-amylase derived from *Bacillus amyloliquefaciens* (shown in SEQ ID NO: 5 of WO 99/19467), with one or more, especially all, of the following substitutions: G48A+T49I+G107A+H156Y+A181T+N190F+1201F+A209V+Q264S (using the *Bacillus licheniformis* numbering in SEQ ID NO: 4 of WO 99/19467). Also preferred are variants having one or more of the following mutations (or corresponding mutations in other *Bacillus* alpha-amylase backbones): H154Y, A181T, N190F, A209V and Q264S and/or a deletion of two residues between positions 176 and 179, preferably a deletion of E178 and G179 (using the SEQ ID NO: 5 numbering of WO 99/19467).

Fungal Alpha-Amylases

Fungal alpha-amylases include alpha-amylases derived from a strain of *Aspergillus*, such as, *Aspergillus kawachii*, *Aspergillus niger*, and *Aspergillus oryzae*.

A preferred acidic fungal alpha-amylase is a Fungamyl-like alpha-amylase which is derived from a strain of *Aspergillus oryzae*. According to the present invention, the phrase "Fungamyl-like alpha-amylase" indicates an alpha-amylase which exhibits a high identity, *i.e.*, more than 70%, more than 75%, more than 80%, more than 85% more than 90%, more than 95%, more than 96%, more than 97%, more than 98%, more than 99% or even 100% identity to the mature part of the amino acid sequence shown in SEQ ID NO: 10 in WO 96/23874.

Another preferred acidic alpha-amylase is derived from a strain of *Aspergillus niger*. In a preferred embodiment the acid fungal alpha-amylase is the one from *A. niger* disclosed as "AMYA_ASPNG" in the Swiss-prot/TrEMBL database under the primary accession no. P56271 and described in WO 89/01969 (Example 3). A commercially available acid fungal alpha-amylase derived from *Aspergillus niger* is SP288 (available from Novozymes A/S, Denmark).

Other contemplated wild-type alpha-amylases include those derived from a strain of the genera *Rhizomucor* and *Meripilus*, preferably a strain of *Rhizomucor pusillus* (WO 2004/055178 incorporated by reference) or *Meripilus giganteus*.

In a preferred embodiment the alpha-amylase is derived from *Aspergillus kawachii* and disclosed by Kaneko *et al.*, 1996, *J. Ferment. Bioeng.* 81: 292-298, "Molecular-cloning and determination of the nucleotide-sequence of a gene encoding an acid-stable alpha-amylase from *Aspergillus kawachii*"; and further as EMBL:#AB008370.

The fungal alpha-amylase may also be a wild-type enzyme comprising a starch-binding domain (SBO) and an alpha-amylase catalytic domain (*i.e.*, non-hybrid), or a variant thereof. In an embodiment the wild-type alpha-amylase is derived from a strain of *Aspergillus kawachii*.

Fungal Hybrid Alpha-Amylases

In a preferred embodiment the fungal acid alpha-amylase is a hybrid alpha-amylase. Preferred examples of fungal hybrid alpha-amylases include the ones disclosed in WO 2005/003311 or U.S. Application Publication No. 2005/0054071 (Novozymes) or U.S. application no. 60/638,614 (Novozymes), which are hereby incorporated by reference. A hybrid alpha-amylase may comprise an alpha-amylase catalytic domain (CO) and a carbohydrate-binding domain/module (CBM), such as a starch binding domain, and optionally a linker.

Specific examples of contemplated hybrid alpha-amylases include those disclosed in Tables 1 to 5 of the examples in US patent application no. 60/638,614, including Fungamyl variant with catalytic domain JA118 and the *Athelia rolfsii* SBD (SEQ ID NO: 100 in U.S. application no. 60/638,614), *Rhizomucor pusillus* alpha-amylase with *Athelia rolfsii* AMG linker and SBD (SEQ ID NO: 101 in U.S. Application No. 60/638,614), *Rhizomucor pusillus* alpha-

amylase with *Aspergillus niger* glucoamylase linker and SBD (which is disclosed in Table 5 as a combination of amino acid sequences SEQ ID NO: 20, SEQ ID NO: 72 and SEQ ID NO: 96 in U.S. application no. 11/316,535) or as V039 in Table 5 in WO 2006/069290, and *Meripilus giganteus* alpha-amylase with *Athelia rolfsii* glucoamylase linker and SBD (SEQ ID NO: 102 in U.S. application no. 60/638,614). Other specifically contemplated hybrid alpha-amylases are any of the ones listed in Tables 3, 4, 5, and 6 in Example 4 in U.S. application no. 11/316,535 and WO 2006/069290, each hereby incorporated by reference.

Other specific examples of contemplated hybrid alpha-amylases include those disclosed in U.S. application publication no. 2005/0054071, including those disclosed in Table 3 on page 15, such as *Aspergillus niger* alpha-amylase with *Aspergillus kawachii* linker and starch binding domain.

Contemplated are also alpha-amylases which exhibit a high identity to any of the above-mentioned alpha-amylases, *i.e.*, more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 95%, more than 96%, more than 97%, more than 98%, more than 99% or even 100% identity to the mature enzyme sequences.

An acid alpha-amylases may according to the invention be added in an amount of 0.1 to 10 AFAU/g DS, preferably 0.10 to 5 AFAU/g DS, especially 0.3 to 2 AFAU/g DS.

Commercial Alpha-Amylase Products

Preferred commercial compositions comprising alpha-amylase include MYCOLASE from DSM, BANTM, TERMAMYLTM SC, FUNGAMYLTM, LIQUOZYMETM X, SANTM SUPER, and SANTM EXTRA L (Novozymes A/S) and CLARASETM L-40,000, DEX-LOTM, SPEZYMETM FRED, SPEZYMETM AA, SPEZYMETM, DELTA AA GC358 and Clearflow AA (Genencor Int.), and the acid fungal alpha-amylase sold under the trade name SP288 (available from Novozymes A/S, Denmark).

Phytases

Any phytase may be used in a process of the present invention. Phytases are enzymes that degrade phytates and/or phytic acid by specifically hydrolyzing the ester link between inositol and phosphorus. Phytase activity is credited with phosphorus and ion availability in many ingredients. In some embodiments, the phytase is capable of liberating at least one inorganic phosphate from an inositol hexaphosphate (*e.g.*, phytic acid). Phytases can be grouped according to their preference for a specific position of the phosphate ester group on the

phytate molecule at which hydrolysis is initiated (e.g., 3-phytase (EC 3.1.3.8) or 6-phytase (EC 3.1.3.26)). An example of phytase is myo-inositol-hexakiphosphate-3-phosphohydrolase.

Phytases can be obtained from microorganisms such as fungal and bacterial organisms. For example, the phytase may be obtained from filamentous fungi such as *Aspergillus* (e.g., *A. ficuum*, *A. fumigatus*, *A. niger*, and *A. terreus*), *Cladosporium*, *Mucor* (e.g., *Mucor piriformis*), *Myceliophthora* (e.g., *M. thermophila*), *Penicillium* (e.g., *P. hordei* (ATCC No. 22053)), *P. piceum* (ATCC No. 10519), or *P. brevi-compactum* (ATCC No. 48944), *Talaromyces* (e.g., *T. thermophilus*), *Thermomyces* (WO 99/49740), and *Trichoderma spp.* (e.g., *T. reesei*).

In an embodiment, the phytate-degrading enzyme is obtained from yeast (e.g., *Arxula adenivorans*, *Pichia anomala*, *Schwanniomyces occidentalis*), gram-negative bacteria (e.g., *Escherichia coli*, *Klebsiella spp.*, *Pseudomonas spp.*), and gram-positive bacteria (e.g., *Bacillus spp.* such as *Bacillus subtilis*).

The phytase also may be obtained from *Citrobacter*, *Enterbacter*, or *Peniophora*.

In an embodiment, the phytase is derived from *Buttiauxiella spp.* such as *B. agrestis*, *B. brennerae*, *B. ferragutiase*, *B. gaviniae*, *B. izardii*, *B. noackiae*, and *B. warmboldiae*. In some embodiments, the phytase is a phytase disclosed in WO 2006/043178 or U.S. application no. 11/714,487.

In one preferred embodiment, the phytase has at least 75%, at least 80%, at least 85%, at least 88%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98% and at least 99% sequence identity to the amino acid sequence set forth in SEQ ID NO: 31 of U.S. Application No. 12/263,886.

Commercially-available phytases are NATUPHOS (BASF), RONOZYME P (Novozymes A/S), PHZYME (Danisco A/S, Diversa) and FINASE (AB Enzymes). The method for determining microbial phytase activity and the definition of a phytase unit is disclosed in Engelen *et al.*, 1994, *Journal of AOAC International* 77: 760-764. The phytase may be a wild-type phytase, an active variant or active fragment thereof.

Pullulanases

Any pullulanase may be used in a process of the present invention. In an embodiment, the pullulanase is a GH57 pullulanase, e.g., a pullulanase obtained from a strain of *Thermococcus*, including *Thermococcus sp.* AM4, *Thermococcus sp.* HJ21, *Thermococcus barophilus*, *Thermococcus gammatolerans*, *Thermococcus hydrothermalis*; *Thermococcus kodakarensis*, *Thermococcus litoralis*, and *Thermococcus onnurineus*; or from a strain of *Pyrococcus*, such as *Pyrococcus abyssi* and *Pyrococcus furiosus*.

Carbohydrate-Source Generating Enzymes

The phrase "carbohydrate-source generating enzyme" includes glucoamylase (a glucose generator), beta-amylase and maltogenic amylase (both maltose generators) and also pullulanase and alpha-glucosidase. A carbohydrate-source generating enzyme is capable of producing a carbohydrate that can be used as an energy-source by the fermenting organism(s) in question, for instance, when used in a process for producing a fermentation product such as ethanol. The generated carbohydrate may be converted directly or indirectly to the desired fermentation product, preferably ethanol. According to the invention a mixture of carbohydrate-source generating enzymes may be present. Especially contemplated mixtures are mixtures of at least a glucoamylase and an alpha-amylase, especially an acid amylase, even more preferred an acid fungal alpha-amylase. The ratio between acidic fungal alpha-amylase activity (AFAU) per glucoamylase activity (AGU) (AFAU per AGU) may be at least 0.1, in particular at least 0.16, such as in the range from 0.12 to 0.50 or greater.

Glucoamylases

A glucoamylase may be derived from any suitable source, e.g., derived from a microorganism or a plant. Preferred glucoamylases are of fungal or bacterial origin selected from the group consisting of *Aspergillus* glucoamylases, in particular *A. niger* G1 or G2 glucoamylase (Boel *et al.*, 1984, *EMBO J.* 3(5): 1097-1102), and variants thereof, such as those disclosed in WO 92/00381, WO 2000/104136 and WO 2001/04273 (from Novozymes, Denmark); the *A. awamori* glucoamylase disclosed in WO 84/02921, *A. oryzae* glucoamylase (*Agric. Biol. Chem.* 55(4): 941-949 (1991)), and variants or fragments thereof. Other *Aspergillus* glucoamylase variants include variants with enhanced thermal stability: 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); disulphide bonds, A246C (Fierobe *et al.*, 1996, *Biochemistry* 35: 8698-8704; and introduction of Pro residues in positions A435 and S436 (Li *et al.*, 1997, *Protein Eng.* 10: 1199-1204).

Other glucoamylases include *Athelia rolfsii* (previously denoted *Corticium rolfsii*) glucoamylase (see U.S. Patent No. 4,727,026 and Nagasaka *et al.*, 1998, "Purification and properties of the raw-starch-degrading glucoamylases from *Corticium rolfsii*, *Appl. Microbiol. Biotechnol.* 50:323-330), *Talaromyces* glucoamylases, in particular derived from *Talaromyces emersonii* (WO 99/28448), *Talaromyces leycettanus* (U.S. Patent No. Re. 32,153), *Talaromyces duponti*, and *Talaromyces thermophilus* (U.S. Patent No. 4,587,215).

Bacterial glucoamylases include glucoamylases from *Clostridium*, in particular *C. thermoamylolyticum* (EP 135,138), and *C. thermohydrosulfuricum* (WO 86/01831) and *Trametes cingulata* disclosed in WO 2006/069289 (which are hereby incorporated by reference).

Hybrid glucoamylases may also be used in a process of the invention. Examples of hybrid glucoamylases are disclosed in WO 2005/045018. Specific examples include the hybrid glucoamylase disclosed in Tables 1 and 4 of Example 1 of WO 2005/045018, which is hereby incorporated by reference to the extent it teaches hybrid glucoamylases.

Contemplated are also glucoamylases which exhibit a high identity to any of above-mentioned glucoamylases, *i.e.*, more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 95%, more than 96%, more than 97%, more than 98%, more than 99% or even 100% identity to the mature enzymes sequences.

Commercially available compositions comprising glucoamylase include AMG 200L; AMG 300 L; SANTM SUPER, SANTM EXTRA L, SPIRIZYMETM PLUS, SPIRIZYMETM FUEL, SPIRIZYMETM B4U and AMGTM E (from Novozymes A/S); OPTIDEXTM 300 (from Genencor Int.); AMIGASETM and AMIGASETM PLUS (from DSM); G-ZYMETM G900, G-ZYMETM, GC480, GC148, GC019 and G990 ZR (from Genencor Int.).

Glucoamylases may be added in an amount of 0.02-20 AGU/g DS, preferably 0.1-10 AGU/g DS, especially between 1-5 AGU/g DS, such as 0.5 AGU/g DS.

Beta-amylases

The term "beta-amylase" (E.C 3.2.1.2) is the name traditionally given to exo-acting maltogenic amylases, which catalyze the hydrolysis of 1,4-alpha-glucosidic linkages in amylose, amylopectin and related glucose polymers. Maltose units are successively removed from the non-reducing chain ends in a step-wise manner until the molecule is degraded or, in the case of amylopectin, until a branch point is reached. The maltose released has the beta anomeric configuration, hence the name beta-amylase.

Beta-amylases have been isolated from various plants and microorganisms (Fogarty and Kelly, 1979, *Progress in Industrial Microbiology* 15: 112-115). These beta-amylases are characterized by having optimum temperatures in the range from 40°C to 65°C and optimum pH in the range from 4.5 to 7. A commercially available beta-amylase from barley is NOVOZYMTM WBA from Novozymes A/S, Denmark and SPEZYMETM BBA 1500 from Genencor Int., USA.

Maltogenic amylases

The amylase may also be a maltogenic alpha-amylase. A maltogenic alpha-amylase (glucan 1,4-alpha-maltohydrolase, E.C. 3.2.1.133) is able to hydrolyze amylose and amylopectin to maltose in the alpha-configuration. A maltogenic amylase from *Bacillus stearothermophilus* strain NCIB 11837 is commercially available from Novozymes A/S. Maltogenic alpha-amylases are described in U.S. Patent Nos. 4,598,048, 4,604,355 and 6,162,628, which are hereby incorporated by reference.

The maltogenic amylase may be added in an amount of 0.05-5 mg total protein/gram DS or 0.05-5 MANU/g DS.

Proteases

A protease may be added during saccharification, fermentation, simultaneous saccharification and fermentation. The protease may be added to deflocculate the fermenting organism, especially yeast, during fermentation. The protease may be any protease. In a preferred embodiment the protease is an acid protease of microbial origin, preferably of fungal or bacterial origin. An acid fungal protease is preferred, but also other proteases can be used.

Suitable proteases include microbial proteases, such as fungal and bacterial proteases. Preferred proteases are acidic proteases, *i.e.*, proteases characterized by the ability to hydrolyze proteins under acidic conditions below pH 7.

Contemplated acid fungal proteases include fungal proteases derived from *Aspergillus*, *Candida*, *Coriolus*, *Endothia*, *Enthomophtra*, *Irpex*, *Mucor*, *Penicillium*, *Rhizopus*, *Sclerotium* and *Torulopsis*. Especially contemplated are proteases derived from *Aspergillus niger* (see, *e.g.*, Koaze *et al.*, 1964, *Agr. Biol. Chem. Japan* 28: 216), *Aspergillus saitoi* (see, *e.g.*, Yoshida, 1954, *J. Agr. Chem. Soc. Japan* 28: 66), *Aspergillus awamori* (Hayashida *et al.*, 1977, *Agric. Biol. Chem.* 42(5): 927-933), *Aspergillus aculeatus* (WO 95/02044), or *Aspergillus oryzae*, such as the pepA protease; and acidic proteases from *Mucor pusillus* or *Mucor miehei*.

Contemplated are also neutral or alkaline proteases, such as a protease derived from a strain of *Bacillus*. A particular protease contemplated for the invention is derived from *Bacillus amyloliquefaciens* and has the sequence obtainable at Swissprot as Accession No. P06832.

Also contemplated are the proteases having at least 90% identity to the amino acid sequence obtainable at Swissprot as Accession No. P06832 such as at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or particularly at least 99% identity.

Further contemplated are the proteases having at least 90% identity to amino acid sequence disclosed as SEQ ID NO:1 in WO 2003/048353 such as at 92%, at least 95%, at least 96%, at least 97%, at least 98%, or particularly at least 99% identity.

Also contemplated are papain-like proteases such as proteases within E.C. 3.4.22.* (cysteine protease), such as EC 3.4.22.2 (papain), EC 3.4.22.6 (chymopapain), EC 3.4.22.7 (asclapain), EC 3.4.22.14 (actinidain), EC 3.4.22.15 (cathepsin L), EC 3.4.22.25 (glycyl endopeptidase) and EC 3.4.22.30 (caricain).

In an embodiment the protease is a protease preparation derived from a strain of *Aspergillus*, such as *Aspergillus oryzae*. In another embodiment the protease is derived from a strain of *Rhizomucor*, preferably *Rhizomucor meihei*. In another contemplated embodiment the protease is a protease preparation, preferably a mixture of a proteolytic preparation derived from a strain of *Aspergillus*, such as *Aspergillus oryzae*, and a protease derived from a strain of *Rhizomucor*, preferably *Rhizomucor meihei*.

Aspartic acid proteases are described in, for example, Handbook of Proteolytic Enzymes, Edited by A.J. Barrett, N.D. Rawlings and J.F. Woessner, Academic Press, San Diego, 1998, Chapter 270). Suitable examples of aspartic acid protease include, e.g., those disclosed in Berka *et al.*, 1990, *Gene* 96: 313; Berka *et al.*, 1993, *Gene* 125: 195-198; and Gomi *et al.*, 1993, *Biosci. Biotech. Biochem.* 57: 1095-1100, which are hereby incorporated by reference.

Commercially available products include ALCALASE®, ESPERASE™, FLAVOURZYME™, PROMIX™, NEUTRASE®, RENNILASE®, NOVOZYM™ FM 2.0L, and NOVOZYM™ 50006 (available from Novozymes A/S, Denmark) and GC106™ and SPEZYME™ FAN from Genencor Int., Inc., USA.

The protease may be present in an amount of 0.0001-1 mg enzyme protein per 9 DS, preferably 0.001 to 0.1 mg enzyme protein per 9 DS. Alternatively, the protease may be present in an amount of 0.0001 to 1 LAPU/g DS, preferably 0.001 to 0.1 LAPU/g DS and/or 0.0001 to 1 mAU-RH/g DS, preferably 0.001 to 0.1 mAU-RH/g DS.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention as well as combinations of one or more of the embodiments. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties. The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

5 MATERIALS & METHODS

Methods

Measurement of cellulase activity using filter paper assay (FPU assay)

1. Source of Method

1.1 The method is disclosed in "Measurement of Cellulase Activities" by Adney and Baker, 10 1996, Laboratory Analytical Procedure, LAP-006, National Renewable Energy Laboratory (NREL), which is based on the IUPAC method for measuring cellulase activity (Ghose, 1987, Measurement of Cellulase Activities, *Pure & Appl. Chem.* 59: 257-268.

2. Procedure

2.1 The method is carried out as described by Adney and Baker, 1996, *supra*, except for the 15 use of a 96 well plate to read the absorbance values after color development, as described below.

2.2 Enzyme Assay Tubes:

2.2.1 A rolled filter paper strip (#1 Whatman; 1 x 6 cm; 50 mg) is added to the bottom of a test tube (13 X 100 mm).

20 2.2.2 To the tube is added 1.0 ml of 0.05 M sodium citrate buffer (pH 4.80).

2.2.3 The tubes containing filter paper and buffer are incubated 5 minutes at 50°C ($\pm 0.1^\circ\text{C}$) in a circulating water bath.

2.2.4 Following incubation, 0.5 ml of enzyme dilution in citrate buffer is added to the tube. Enzyme dilutions are designed to produce values slightly above and below the target value of 25 2.0 mg glucose.

2.2.5 The tube contents are mixed by gently vortexing for 3 seconds.

2.2.6 After vortexing, the tubes are incubated for 60 minutes at 50°C ($\pm 0.1^\circ\text{C}$) in a circulating water bath.

2.2.7 Immediately following the 60 minute incubation, the tubes are removed from the water 30 bath, and 3.0 ml of dinitrosalicylic acid (DNS) reagent is added to each tube to stop the reaction. The tubes are vortexed 3 seconds to mix.

2.3 Blank and Controls

2.3.1 A reagent blank is prepared by adding 1.5 ml of citrate buffer to a test tube.

2.3.2 A substrate control is prepared by placing a rolled filter paper strip into the bottom of a test tube, and adding 1.5 ml of citrate buffer.

2.3.3 Enzyme controls are prepared for each enzyme dilution by mixing 1.0 ml of citrate buffer with 0.5 ml of the appropriate enzyme dilution.

5 2.3.4 The reagent blank, substrate control, and enzyme controls are assayed in the same manner as the enzyme assay tubes, and done along with them.

2.4 *Glucose Standards*

2.4.1 A 100 ml stock solution of glucose (10.0 mg/ml) is prepared, and 5 ml aliquots are frozen. Prior to use, aliquots are thawed and vortexed to mix.

10 2.4.2 Dilutions of the stock solution are made in citrate buffer as follows:

G1 = 1.0 ml stock + 0.5 ml buffer = 6.7 mg/ml = 3.3 mg/0.5 ml

G2 = 0.75 ml stock + 0.75 ml buffer = 5.0 mg/ml = 2.5 mg/0.5 ml

G3 = 0.5 ml stock + 1.0 ml buffer = 3.3 mg/ml = 1.7 mg/0.5 ml

G4 = 0.2 ml stock + 0.8 ml buffer = 2.0 mg/ml = 1.0 mg/0.5 ml

15 2.4.3 Glucose standard tubes are prepared by adding 0.5 ml of each dilution to 1.0 ml of citrate buffer.

2.4.4 The glucose standard tubes are assayed in the same manner as the enzyme assay tubes, and done along with them.

2.5 *Color Development*

20 2.5.1 Following the 60 minute incubation and addition of DNS, the tubes are all boiled together for 5 minutes in a water bath.

2.5.2 After boiling, they are immediately cooled in an ice/water bath.

2.5.3 When cool, the tubes are briefly vortexed, and the pulp is allowed to settle. Then each tube is diluted by adding 50 microliters from the tube to 200 microliters of double distilled water
25 in a 96-well plate. Each well is mixed, and the absorbance is read at 540 nm.

2.6 *Calculations (examples are given in the NREL document)*

2.6.1 A glucose standard curve is prepared by graphing glucose concentration (mg/0.5 ml) for the four standards (G1-G4) vs. absorbance at 540 nm. This is fitted using a linear regression (Prism Software), and the equation for the line is used to determine the glucose produced for
30 each of the enzyme assay tubes.

2.6.2 A plot of glucose produced (mg/0.5 ml) vs. total enzyme dilution is prepared, with the Y-axis (enzyme dilution) being on a log scale.

2.6.3 A line is drawn between the enzyme dilution that produced just above 2.0 mg glucose and the dilution that produced just below that. From this line, it is determined the enzyme dilution that would have produced exactly 2.0 mg of glucose.

2.6.4 The Filter Paper Units/ml (FPU/ml) are calculated as follows:

5 FPU/mL = 0.37 enzyme dilution producing 2.0 mg glucose.

EGU assay

10 Ten liters of a 0.1 M phosphate pH 6.0 buffer is prepared and is subsequently used to prepare 1 liter of 35 g/l, pH 6.0 carboxymethyl cellulose (CMC, Aqualon 7LF) reagent. An EGU standard is allowed to stand 15 minutes at room temperature before it is weighed out. Approximately 1 g of this standard is weighed out into a 50 ml volumetric flask and filled to the mark with phosphate buffer. This stock solution is then stirred for 15 minutes. Working standards are prepared as described in the table below:

Standard no.	Dilution ratio	Obtain dilution ratio by dilution as:		Concentration (EGU/mL)
		Enzyme stock solution	Diluent	
1	0	0	1000	0.000
2	20	50	950	0.270
3	14	71	928	0.386
4	10	100	900	0.540
5	8	125	875	0.675
6	7	142	857	0.772
7	5	200	800	1.080

15

The sample is prepared for the assay by allowing it to warm to room temperature. The sample is weighed out, diluted with 0.1 M phosphate pH 6.0 buffer and stirred for a minimum of 15 minutes with a maximum stir time of 30 minutes. The enzyme samples are diluted so that the activity of the final dilution is approximately in the middle of the curve. All weights and dilutions are recorded. Samples can be analyzed robotically using a ZYMATE II ROBOT (Zymark, USA), or manually according to the following specifications: A 0.375 ml volume of phosphate buffer is pipetted into a test tube. Next, 0.125 ml of the standard or sample is pipetted into the test tube with the phosphate buffer. It is important that the solutions must be freshly stirred when they are pipetted out. Four ml of CFC substrate is preheated to 25°C, and mixed on Whirl mixer for 25-30 seconds. The sample in the test tube is incubated in a 40°C water bath for 30 minutes. After

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wiping the test tube it is placed in the viscometer's test tube holder so the vibrating spindle is in the center of the test tube, and left for 20 seconds. The mV signal is read and recorded. The measurements of the enzyme standards are used to plot a standard curve, with enzyme activity as the x-axis and the corresponding mV signal as the y-axis. The standard curve is fitted by regression to a 3rd degree polynomial. The mV values for the different dilutions of the samples are then used to read off the corresponding enzyme activity values from the standard curve. The activity of each sample (EGU/g) is then computed as follows:

$$\text{Sample Activity} = (\text{C} \times \text{Flask} \times \text{Dilution}) / (\text{Weight})$$

wherein:

- | | | |
|----|----------|--|
| 10 | C | = enzyme activity calculated via line of regression (EGU/mL) |
| | Flask | = volume of solution flask (mL) |
| | Dilution | = further dilution of the sample |

FXU assay

- 15 Two liters of 15% (w/v) BRIJ 35 working solution is prepared from a 30% (w/v) BRIJ 35 (Sigma B4184-1L) stock solution by diluting with demineralized water. Five liters of sample diluents (50 mM ACES buffer with 225 mg BRIJ/L, pH 6.0) is prepared by weighing out 45.55 g $\pm$ 0.005 g ACES and transferring the salt to a 5000 mL graduated flask. Approximately 4550 ml demineralized water is added and mixed with a magnetic stirrer for minimum 20 minutes or until
- 20 dissolved. 7.50 ml of Brij 35 stock solution 15% (w/v) is pipetted into the solution. The pH is adjusted with sodium hydroxide to 6.00 ± 0.03 at room temperature and the volume is adjusted to 5.0 l.

- 25 250 ml of 50 mM XYL-BUF/S: MES buffer, pH 6.0 is prepared by weighing out 2.441 g MES $\pm$ 0.005 g and transferred to a 250 mL graduated flask. Approximately 240 ml demineralized water is added and mixed with a magnetic stirrer for minimum 20 minutes or until dissolved. The pH is adjusted with sodium hydroxide to 6.00 ± 0.03 at room temperature and the volume is adjusted to 250 ml. 50 ml of XYL-SUB/S: Substrate wheat arabinoxylan 5.60 g/l in MES buffer 50 mM, pH 6.0 is prepared by weighing out of 0.2800 g $\pm$ 0.001 g of wheat arabinoxylan in a 100 ml beaker. The solution is stirred with heat in the beaker to approximately
- 30 55°C for 30 minutes or until dissolved. After cooling, the pH is adjusted with sodium hydroxide to 6.00 ± 0.03 at room temperature and the volume is adjusted to 50 ml. 50 ml of 500 mM NaOH is prepared by weighing 4 beads (pellets: fresh, not caked together) of NaOH (e.g., Merck 1.06498) to a 50 ml volumetric flask. The actual weight is noted of the 4 beads (approximately 0.8 g) = W. Multiply W, weight with 50 (= approximately 40 g), and the result is noted. The flask

is filled up to 50*W+/- 0.001 g weight with demineralized water and stirred with a magnetic stirrer for 5 minutes. 25 mL of p-hydroxy benzoic acid hydrazide (PAHBAH) PAHBAH/S: PAHBAH/Bismuth/Tartrate solution is prepared by weighing out 0.1380 ± 0.0001 g of Bismuth (III)-acetate (e.g., Aldrich 401587). Then, 0.5000 ± 0.001 g of PAHBAH is weighed out (Sigma H-9882, 152.2 g/mol, 4 kg aliquoted in 2.5 g glass containers). A 1.2500 ± 0.001 g quantity of potassium sodium tartrate, tetrahydrate is weighed out (Merck 1.08087, 282.2 g/mol). The powders are transferred to a 25 ml graduated flask. The flask is filled up to the mark with 500 mM NaOH and wrapped immediately in aluminium foil to protect from light or poured into a brown bottle. The sample is stirred for 15 minutes with a magnetic stirrer.

An FXU standard is prepared by weighing out 0.4520 g (± 0.0005 g) and dissolved in sample diluent in a 250 ml volumetric flask and stirred for 15 minutes. A standard curve is prepared with a 7 point curve with a factor 4.0 between lowest and highest standard point, the recommended volume 1200 microliters. The standard solutions are prepared by further diluting the stock solution with sample diluent on a diluter into the sample cups, according to the table below:

Standard no.	Example		Dilution Ratio	Concentration (FXU/mL)
	Stock solution (microliters)	Diluent (microliters)		
1	30	1170	40	0.1563
2	40	1160	30	0.2084
3	50	1150	24	0.2605
4	60	1140	20	0.3126
5	80	1120	15	0.4168
6	100	1100	12	0.5210
7	120	1080	10	0.6252

A sample is weighed out and dissolved in sample diluent. The stock solution is stirred for 15 minutes. The working solution is diluted with the stock solution to lie within the range of the standard curve with sample diluent. The activity assay is carried out using a Konelab 30 analyzer (ThermoFischer Scientific). All reagents, standards and samples are placed in the analyzer. The enzyme activity of the diluted samples is read from the standard curve. The results can be calculated automatically. Calculation of activity of a sample in FXU/g is performed according to the formula:

$$\text{FXU/g} = (\text{S} \times \text{V} \times \text{F}) / \text{W}$$

wherein

S = Reading from the standard curve in FXU/mL

V = Volume of the measuring flask used in mL

5 F = Dilution factor for second dilution

W = Weight of sample (g)

Glucoamylase activity

Glucoamylase activity may be measured in AGI units or in Glucoamylase Units (AGU).

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Glucoamylase activity (AGI)

Glucoamylase (equivalent to amyloglucosidase) converts starch into glucose. The amount of glucose is determined by the glucose oxidase method for the activity determination. The method described in the section 76-11 Starch-Glucoamylase Method with Subsequent
 15 Measurement of Glucose with Glucose Oxidase in "Approved methods of the American Association of Cereal Chemists". Vol. 1-2 MCC, from American Association of Cereal Chemists, 2000; ISBN: 1-891127-12-8.

One glucoamylase unit (AGI) is the quantity of enzyme which will form 1 micromole of glucose per minute under the standard conditions of the method.

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<u>Standard conditions/reaction conditions:</u>	
Substrate	Soluble starch, concentration approx. 16 g dry matter/L
Buffer	Acetate, approx. 0.04 M, pH=4.3
pH	4.3
Incubation Temperature	60°C
Reaction Time	15 minutes
Termination of the Reaction	NaOH to a concentration of approximately 0.2 g/L (about pH 9)
Enzyme Concentration	0.15-0.55 AAU/mL

The starch should be Lintner starch, which is a thin-boiling starch used in the laboratory as a colorimetric indicator. Lintner starch is obtained by dilute hydrochloric acid treatment of native starch so that it retains the ability to color blue with iodine.

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Glucoamylase activity (AGU)

The Novo Glucoamylase Unit (AGU) is defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute under the standard conditions 37°C, pH 4.3, substrate: 23.2 mM maltose, buffer: acetate 0.1 M, reaction time 5 minutes.

- 5 An autoanalyzer system may be used. Mutarotase is added to the glucose dehydrogenase reagent so that any alpha-D-glucose present is turned into beta-D-glucose. Glucose dehydrogenase reacts specifically with beta-D-glucose in the reaction mentioned above, forming NADH which is determined using a photometer at 340 nm as a measure of the original glucose concentration.

<u>AMG incubation:</u>	
Substrate:	23.2 mM maltose
Buffer:	0.1 M acetate
pH	4.30
Incubation temperature:	37°C ± 1
Reaction time:	5 minutes
Enzyme working range:	0.5-4.0 AGU/mL

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<u>Color reaction:</u>	
GlucDH:	430 U/L
Mutarotase:	9 U/L
NAD	0.21 mM
Buffer:	0.12 M phosphate; 0.15 M NaCl
pH:	7.60 ± 0.05
Incubation temperature:	37°C ± 1
Reaction time:	5 minutes
Wavelength:	340 nm

Alpha-amylase activityAlpha-amylase activity (KNU)

- 15 The alpha-amylase activity may be determined using potato starch as substrate. This method is based on the degradation of modified potato starch by the enzyme, and the reaction is followed by mixing samples of the starch/enzyme solution with an iodine solution. Initially, a blackish-blue color is formed, but during the degradation of the starch the blue color gets

weaker and gradually turns into a reddish-brown, which is compared to a colored glass standard.

One Kilo Novo alpha-amylase Unit (KNU) is defined as the amount of enzyme which, under standard conditions (*i.e.*, at 37°C +/- 0.05; 0.0003 M Ca²⁺; and pH 5.6) dextrinizes 5260 mg starch dry substance Merck Amylum soluble.

Acid alpha-amylase activity

When used according to the present invention the activity of any acid alpha-amylase may be measured in AFAU (Acid Fungal Alpha-amylase Units). Alternatively the activity of an acid alpha-amylase may be measured in AAU (Acid Alpha-amylase Units).

Acid alpha-amylase units (AAU)

The acid alpha-amylase activity can be measured in AAU (Acid Alpha-amylase Units), which is an absolute method. One Acid Amylase Unit (AAU) is the quantity of enzyme converting 1 g of starch (100% of dry matter) per hour under standardized conditions into a product having a transmission at 620 nm after reaction with an iodine solution of known strength equal to the one of a color reference.

Standard conditions/reaction conditions:

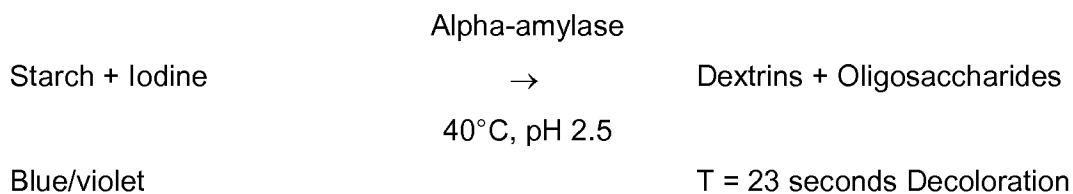
Substrate:	Soluble starch. Concentration approx. 20 g DS/L.
Buffer:	Citrate, approx. 0.13 M, pH=4.2
Iodine solution:	40.176 g potassium iodide + 0.088 g iodine/L
City water	15-20°dH (German degree hardness)
pH:	4.2
Incubation temperature:	30°C
Reaction time:	11 minutes
Wavelength:	620 nm
Enzyme concentration:	0.13-0.19 AAU/mL
Enzyme working range:	0.13-0.19 AAU/mL

The starch should be Lintner starch. Further details can be found in EP 0140410, which is hereby incorporated by reference.

Acid alpha-amylase activity (AFAU)

Acid alpha-amylase activity may be measured in AFAU (Acid Fungal Alpha-amylase Units), which is determined relative to an enzyme standard. One AFAU is defined as the amount of enzyme which degrades 5.260 mg starch dry matter per hour under the below mentioned standard conditions.

Acid alpha-amylase, an endo-alpha-amylase (1,4-alpha-D-glucan-glucanohydrolase, E.C. 3.2.1.1) hydrolyzes alpha-1,4-glucosidic bonds in the inner regions of the starch molecule to form dextrans and oligosaccharides with different chain lengths. The intensity of color formed with iodine is directly proportional to the concentration of starch. Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under the specified analytical conditions.

Standard conditions/reaction conditions:

Substrate:	Soluble starch, approx. 0.17 g/l
Buffer:	Citrate, approx. 0.03 M
Iodine (12):	0.03 g/l
CaCl ₂ :	1.85 mM
pH:	2.50 ± 0.05
Incubation temperature:	40°C
Reaction time:	23 seconds
Wavelength:	590 nm
Enzyme concentration:	0.025 AFAU/ml
Enzyme working range:	0.01-0.04 AFAU/ml

Xylose/glucose isomerase assay (IGIU)

One IGIU is the amount of enzyme which converts glucose to fructose at an initial rate of 1 micromole per minute at standard analytical conditions.

Standard Conditions:

Glucose concentration:	45% w/w
pH:	7.5
Temperature:	60°C
5 Mg ²⁺ concentration:	99 mg/l (1.0 g/l MgSO ₄ * 7 H ₂ O)
Ca ²⁺ concentration	< 2 ppm
Activator, SO ₂ concentration:	100 ppm (0.18 g/l Na ₂ S ₂ O ₅)
Buffer, Na ₂ CO ₃ , concentration:	2 mM Na ₂ CO ₃

10 Protease activityProtease assay method (LAPU)

One Leucine Amino Peptidase Unit (LAPU) is the amount of enzyme which decomposes 1 micromole substrate per minute at the following conditions: 26 mM of L-leucine-p-nitroanilide as substrate, 0.1 M Tris buffer (pH 8.0), 40°C, 10 minute reaction time.

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Protease assay method AU(RH)

The proteolytic activity may be determined with denatured hemoglobin as substrate. In the Anson-Hemoglobin method for the determination of proteolytic activity denatured hemoglobin is digested, and the undigested hemoglobin is precipitated with trichloroacetic acid (TCA). The amount of TCA soluble product is determined with phenol reagent, which gives a blue color with tyrosine and tryptophan.

20

One Anson Unit (AU(RH)) is defined as the amount of enzyme which under standard conditions (*i.e.*, 25°C, pH 5.5 and 10 minute reaction time) digests hemoglobin at an initial rate such that there is liberated per minute an amount of TCA soluble product which gives the same color with phenol reagent as one milliequivalent of tyrosine.

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Determination of maltogenic amylase activity (MANU)

One MANU (Maltogenic Amylase Novo Unit) may be defined as the amount of enzyme required to release one micromole of maltose per minute at a concentration of 10 mg of maltotriose (Sigma M 8378) substrate per ml of 0.1 M citrate buffer, pH 5.0 at 37°C for 30 minutes.

30

Materials

Spirizyme Excel - a *T. cingulata* and *T. emersonii* glucoamylase blend (Novozymes A/S, Denmark))

CELLIC™ CTec – a *Trichoderma reesei* cellulase composition supplemented with GH61 polypeptide and beta-glucosidase fusion protein (Novozymes A/S, Denmark).

CELLIC™ HTec – xylanase (Novozymes A/S, Denmark).

CELLIC™ CTec2 – a blend of (a) a *Trichoderma reesei* cellulase composition supplemented with GH61 polypeptide and beta-glucosidase and (b) CELLIC™ HTec2 (Novozymes A/S, Denmark).

CELLIC™ HTec2 – xylanase (Novozymes A/S, Denmark).

Glucoamylase A (AMG A): Glucoamylase derived from *Trametes cingulata* disclosed in SEQ ID NO: 2 in WO 2006/069289 (Novozymes A/S).

Alpha-Amylase A (AAA): a hybrid alpha-amylase consisting of *Rhizomucor pusillus* alpha-amylase with *Aspergillus niger* glucoamylase linker and SBD disclosed as V039 in Table 5 in WO 2006/069290 (Novozymes A/S).

Celluclast 1.5L – a *Trichoderma reesei* cellulase composition with xylanase activity.

Ultraflo – *Humicola insolens* beta-glucanase with ferulic acid esterase and xylanase activity.

Shearzyme 2x – *Aspergillus aculeatus* xylanase.

Shearzyme Plus – *Aspergillus aculeatus* xylanase.

EXAMPLES

Example 1

Unfermented corn mash was used to prepare fermentation samples. Urea and penicillin were added to a final concentration of 1000 ppm and 3 mg/L respectively. The pH of the mash was adjusted to pH 5. Approximately 620 g mash was placed into LR-2.ST 1 L reactor vessels (IKA, Wilmington, NC) which were held in a 32°C water bath with constant mixing throughout the 54 hour fermentation.

Spirizyme Excel was dosed at a final concentration of 0.06% (% w/w of corn). CELLIC™ CTec, CELLIC™ HTec and *Aspergillus fumigatus* GH61 polypeptide were each equally dosed as 33.3 micrograms enzyme protein/g DS. Fermentations were initiated by adding 0.68 g of dry Ethanol Red yeast (RedStar Yeast Co., Milwaukee, WI) directly to the reactor.

After 54 hours, the fermentations were stopped by adding 10 microliters of 40% (v/v) H₂SO₄/g mash and stored in the glass reactors overnight at 4°C. Each treatment was then transferred to three 250 ml Nalgene bottles, capped, and centrifuged for 15 minutes at 3500 x g

at approximately 30°C in a F14BCI-6x250cy rotor in an Avanti J-E centrifuge (Beckman Coulter, Brea, CA). The supernatants from each treatment were recombined, allowed to cool to room temperature, and the oil was recovered according to the following method:

Supernatant from each treatment was combined in a 250 ml separatory funnel. The Nalgene bottles in which the samples were centrifuged were rinsed with a small amount of hexane (<5 ml), followed by extraction of the supernatant (three extractions, 40 ml hexane per extraction). The organic phase was collected in a pre-weighed round-bottomed flask, transferred to 50 ml tubes for centrifugation at 11,000 x g for 15 minutes then transferred back to the round-bottomed flask. The organic layer was distilled off under vacuum using a R-205 rotary evaporator (Buchi, Flawil, Switzerland) at 240 rpm with a water bath set to 85°C and the round-bottomed flask was reweighed to obtain the final oil weight.

In order to accurately compare oil yields, oil recovered was reported as a mass percent of the initial mash used in the treatment. For SSF treatments, the recorded initial mash weight was used for the calculation. To calculate the initial mash used for each sample, the amount of mash aliquoted into the large-scale fermentation was measured. The oil yield was calculated based on a percent weight basis of oil obtained to starting corn mash used (Table 1).

Table 1: Oil Yield

Treatment	% oil (w/w)
Spirizyme Excel	0.60%
Spirizyme Excel, CELLIC™ CTec, CELLIC™ HTec and GH61 polypeptide	0.86%

The results show that the addition of a blend of CELLIC™ CTec, CELLIC™ HTec, and GH61 polypeptide significantly increased the amount of corn oil extracted by 43% compared to the control sample.

Example 2

Unfermented industrially produced mash was blended in a blender for 5 minutes. This served to increase available surface area for enzymatic activity and possibly to further disrupt the oil-containing germ. A 100 g sample of this corn mash was aliquoted into plastic 250 mL Erlenmeyer® flasks with filter-equipped caps. Saccharification was performed using AAA + AMG A (10.5 AGU/FAU-F) at a dose of 0.04 wt. %. CELLIC™ CTec2 was dosed at 12 mg product/g DS.

Rehydrated RED STAR™ yeast (Fermentis/Lesaffre, USA) was used in the fermentation, 3.3 g of yeast suspended in 50 mL of 32°C tap water for 30 minutes before pitching 2 mL of this culture into each mash. SSF was allowed to proceed for 72 hours in a reciprocal/orbital shaking air incubator at 32°C. After 72 hours, the fermentations were stopped
5 by adding 10 microliters of 40% (v/v) H₂SO₄/g mash.

In order to measure the amount of oil captured in the aqueous and oil phases of the samples, a hexanes-based extractive assay was developed. The fermented samples were decanted into two 50 mL conical centrifuge tubes. The wall of the fermentation flask was rinsed well with deionized water and decanted into a third 50 ml centrifuge tube. All tubes were
10 centrifuged in an Allegra 6R benchtop centrifuge with rotor GH-3.8 (Beckmen Coulter, Brea, CA) at 1462 x g for 10 minutes. The supernatant was decanted into a fresh 250 mL volumetric flask. Using a small amount (<5 ml) of hexane, residual oil was rinsed from the centrifuge tube walls and added to the volumetric flask. Deionized water was added to resuspend the pellet in the two centrifuge tubes and combined into one tube and centrifuged again. The supernatant was
15 poured into the volumetric flask and the sides of the tubes were rinsed again using a small amount of hexane. The supernatant in the flask was poured into a 250 ml separatory funnel, followed by 2 g of NaCl, and inverted several times to mix. Then, 15 ml of hexane were added to the funnel and mixed thoroughly with venting, and then allowed to separate in order to decant the aqueous layer into a beaker. The hexane layer was transferred into a suitable vessel. The
20 aqueous layer was added back to the funnel and washed with hexane and collected a second time. A third wash was performed in the funnel with hexane leaving the aqueous layer out and only washing the walls of the funnel. All of the hexane layers were poured back into the funnel for a fourth time and rinsed with 100 ml of hot tap water. After the phases separated, the aqueous phase was decanted and the hexane layer was transferred into a preweighed round
25 bottom flask. The organic layer was distilled off under vacuum at 240 rpm with a water bath set to 85°C using an R-205 rotary evaporator (Buchi, Flawil, Switzerland) and the round bottom flask was re-weighed to obtain the final oil weight.

In order to accurately compare oil yields, oil recovered was reported as a mass percent of the initial mash used in the treatment. For SSF treatments, the recorded initial mash weight
30 was used for the calculation. To calculate the initial mash used for each sample, the amount of mash aliquoted into the large-scale fermentation was measured. The oil yield was calculated based on a percent weight basis of oil obtained to starting corn mash used (Table 2).

Table 2: Oil Yield

Treatment	% oil (w/w)
1. AAA + AMG A (10.5 AGU/FAU-F)	0.48%
2. AAA + AMG A (10.5 AGU/FAU-F) + CELLIC™ CTec2	1.48%

The results show that the addition of CELLIC™ CTec2 significantly increased the amount of corn oil extracted by 208% compared to the control sample.

5

Example 3

Industrially produced corn mash was used for all assay validation experiments. A 5 gallon container containing the liquefied mash was thawed, partitioned into smaller quantities (1 liter screw top Nalgene bottles), and refrozen. One liter of this mash was thawed for approximately 4 hours prior to starting this study. The mash was supplemented with 3 ppm penicillin and 1000 ppm urea using solutions of 1 g/l penicillin and 200 g/l urea, and adjusted to pH 5.0 with 40% (v/v) H₂SO₄. The dry solids content of the mash was measured on a halogen lamp moisture balance model HB43-S (Mettler-Toledo, Toledo, Ohio) with a resulting value of approximately 30% DS. Approximately 25 g of the industrial mash were added to 50 ml conical centrifuge tubes with screw caps. A total of 24 fermentations were run.

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Spirizyme Ultra was dosed at a final concentration of 0.056% (% w/w of corn). Celluclast 1.5L, Shearzyme 2x, CELLIC™ Ctec2, CELLIC™ Htec2, and Shearzyme Plus were each dosed as 10 or 100 micrograms enzyme protein/g DS.

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Water was dosed into each 25 g sample such that the total added volume of enzyme and water was normalized to each sample in the set. A total of 5.5 g of Fermentis Ethanol Red yeast was rehydrated in 100 mL of 35°C tap water by incubating at 32°C for 30 minutes. Each 25 g sample was then dosed with a 250 microliter aliquot of the rehydrated yeast solution. Tubes were weighed at time 0 and then fermented for 70 hours at 32°C at 150 rpm in an incubated orbital shaker model SHKE4000-5 (ThermoScientific, Waltham, MA). After 52 or 70 hours, tubes were weighed and ethanol concentration was calculated using the equation:

25

$$\text{g ethanol/g DS} = \frac{\text{g CO}_2 \text{ weight loss} \times \frac{1 \text{ mol CO}_2}{44.0098 \text{ g CO}_2} \times \frac{1 \text{ mol ethanol}}{1 \text{ mol CO}_2} \times \frac{46.094 \text{ g ethanol}}{1 \text{ mol ethanol}}}{\text{g mash in tube} \times \% \text{DS}}$$

Distillation

A Büchi Multivapor evaporation system was used for all distillations (Büchi, Flawil, Switzerland). The unit distilled 12 samples at a time. The parameters tested for wet cake dewatering processing are shown in Table 3 and the parameters for corn oil extraction processing are shown in Table 4:

Table 3. Distillation parameters for wet cake dewatering

Time	54 min
Temperature	75°C
Vacuum	275 – 185 mBar (35 min)
	185 – 175 mBar (15 min)
	175 mBar (14 min)
RPM	8

Table 4. Distillation parameters for corn oil extraction

Time	45 min
Temperature	75°C
Vacuum	450 - 200 mBar
RPM	8

Backend Processing (Wet cake dewatering)

After distillation, the whole stillage samples were centrifuged at 1,400 x g for 10 minutes in an Avanti J-E series centrifuge with a JS-5.3 rotor (Beckman Coulter, Brea, CA). Following centrifugation the samples were immediately decanted into pre-weighed 15 mL conical tubes. Weights of the resulting wet cake and thin stillage were recorded. Wet cake was scraped out of the tube and placed in pre-weighed drying pans and incubated at 55°C overnight and transferred to 105°C for 2 hours in incubation ovens (VWR Models: 1545 and 1370FM, respectively). Weights were recorded before and after each incubation. These values were used to calculate dry solids values using the following formula:

$$\%DS = \frac{\text{g dry sample}}{\text{g wet sample}} * 100\%$$

Backend Processing (Corn Oil Extraction)

Hexane was added to each whole stillage sample at a dose of 0.125 mL hexane/g starting material. Each tube was sealed to prevent sample leakage, and mixed thoroughly. Tubes were centrifuged at 3,000 x g for 10 minutes in an Avanti JE Series centrifuge with a JS-5.3 rotor. After centrifugation, the oil/hexane layer (supernatant) was removed using a positive displacement pipette, transferred to a pre-weighed 5 ml flip-top tube, and reweighed. The density of the sample was measured using a research analytical density meter model DDM 2911 (Rudolph Research Analytical, Hackettstown, NJ). The density of the supernatant was then inserted into the standard curve equation to find the percent oil in the supernatant. From this value the total percent oil in the starting material was derived.

The results are shown below in Tables 5-13.

Table 5

<i>T. aurantiacus</i> GH61A	Dose (micrograms EP/g DS)	Dose (Activity/g DS)	% Change relative to control			
			Wet cake	Thin Stillage	Dry Solids	Oil
	10	n/a	-2.0	1.5	2.5	1.8
	100	n/a	-1.4	1.1	1.7	2.7

Table 6

CELLIC™ CTec	Dose (micrograms EP/g DS)	Dose (Activity/g DS)	% Change relative to control			
			Wet cake	Thin Stillage	Dry Solids	Oil
	10	0.0578 EGU	-2.4	1.5	2.5	1.8
	100	0.578 EGU	1.9	-1.4	1.3	7.6

Table 7

Celluclast 1.5L	Dose (micrograms EP/g DS)	Dose (Activity/g DS)	% Change relative to control			
			Wet cake	Thin Stillage	Dry Solids	Oil
	10	0.04 EGU	-3.9	2.7	1.3	-1.2
	100	0.4 EGU	-4.8	3.5	3.8	2.3

Table 8

Shearzyme 2X	Dose (micrograms EP/g DS)	Dose (Activity/g DS)	% Change relative to control			
			Wet cake	Thin Stillage	Dry Solids	Oil
	10	0.3 FXU	-1.6	1.2	0.3	1.6
	100	3.0 FXU	-2.4	1.4	0.04	8.9

5

Table 9

CELLIC™ HTec2	Dose (micrograms EP/g DS)	Dose (Activity/g DS)	% Change relative to control			
			Wet cake	Thin Stillage	Dry Solids	Oil
	10	0.011 EGU, 0.2173 FXU	-2.5	1.9	0.5	-1.8
	100	0.11 EGU, 2.173 FXU	-2.3	1.7	0.6	3.0

Table 10

Shearzyme Plus	Dose (micrograms EP/g DS)	Dose (Activity/g DS)	% Change relative to control			
			Wet cake	Thin Stillage	Dry Solids	Oil
	10	0.0365 EGU, 0.026 FXU	-5.5	4.0	4.3	4.7
	100	0.365 EGU, 0.26 FXU	-11.9	8.5	5.6	18.7

Table 11

CELLIC™ CTec2	Dose (micrograms EP/g DS)	Dose (Activity/g DS)	% Change relative to control			
			Wet cake	Thin Stillage	Dry Solids	Oil
	10	0.076 EGU, 0.0147 FXU	-3.1	2.2	2.3	9.7
	100	0.76 EGU, 0.147 FXU	-9.6	6.6	4.8	7.1

- 5 The results indicate that the combination of cellulase, xylanase, and GH61 activities increased the efficacy of oil extraction and dewatering in a synergistic fashion. Furthermore, the greater the ratio between cellulase activity and xylanase activity when these activities were combined, led to a greater enhancement of the corn oil extraction and dewatering effects. On their own, none of the activities (cellulase, xylanase and GH61) contributed significantly to enhancing oil extraction or dewatering, unless they were used in uneconomically high doses.
- 10

Example 4

- Industrially produced corn mash was used for all samples. A 5 gallon container containing the liquefied mash was thawed, partitioned into smaller quantities (1 liter screw top Nalgene bottles) and refrozen. One liter of this mash was thawed for about 4 hours prior to starting this study. The mash was supplemented with 3 ppm penicillin and 1000 ppm urea using solutions of 1 g/l penicillin and 200 g/L urea, respectively, and adjusted to pH 5.0 with 40%
- 15

H₂SO₄. The dry solids content of the mash was measured on a Mettler-Toledo moisture balance (HB43-S halogen), with a resulting value of 30.00% DS. Approximately 5 g of the industrial mash was added to 15 ml conical centrifuge tubes with flip tops (NUNC #362694). A total of 126 fermentations were run. Enzymes were dosed according to the product specifications in the following table and the volume of stock solution to add to fermentation was determined using the equation:

$$\text{Enz. dose (ml)} = \frac{\text{Final enz. dose (mg EP/g DS)} \times \text{Mash weight (g)} \times \text{Solid content (\%DS)}}{\text{Conc. enzyme (mg EP/ml)}}$$

	Enzyme	Dose	Units
1	Spirizyme Ultra (Control)	0.500	AGU/g DS
2	CELLIC™ CTEC2	100.000	micrograms EP/g DS
3	CELLIC™ CTEC2	1000.000	micrograms EP/g DS
4	Celluclast 1.5L	100.000	micrograms EP/g DS
5	Shearzyme Plus	10.000	micrograms EP/g DS
6	Shearzyme Plus	100.000	micrograms EP/g DS

Water was dosed into each sample such that the total added volume of enzyme and water was approximately 70 microliters/25 g sample. Rehydrated yeast (5.5 g Fermentis Ethanol Red yeast in 100 mL 35°C tap water incubated at 32°C for 30 minutes) was dosed at 50 microliters of yeast solution in each 5 g sample. Tubes were weighed at time 0 and then fermented for 52 or 70 hours at 32°C, monitoring weight loss throughout fermentation and back calculating to determine the amount of ethanol produced according to the following equation:

$$\text{g ethanol/g DS} = \frac{\text{g CO}_2 \text{ weight loss} \times \frac{1 \text{ mol CO}_2}{44.0098 \text{ g CO}_2} \times \frac{1 \text{ mol ethanol}}{1 \text{ mol CO}_2} \times \frac{46.094 \text{ g ethanol}}{1 \text{ mol ethanol}}}{\text{g mash in tube} \times \%DS}$$

HPLC analysis

At the end of the fermentation time point, 50 microliters of 40% H₂SO₄ was added to each sample and vortexed to ensure mixing. Samples were centrifuged at 1570 x g (3000 rpm) for 10 minutes in a Beckman Coulter benchtop centrifuge (Allegra 6R) with rotor GH3.8 and then filtered into HPLC vials through 0.45 micron syringe filters (Whatman) prior to submission for HPLC analysis.

HPLC system: Agilent's 1100/1200 series with Chem station software

Degasser

Quadratic Pump

Auto-Sampler

Column Compartment /w Heater

Refractive Index Detector (RI)

Column: Bio-Rad HPX- 87H Ion Exclusion Column 300 mm x 7.8 mm parts# 125-0140
Bio-Rad guard cartridge cation H parts# 125-0129, Holder parts# 125-0131

Method: 0.005 M H₂SO₄ mobile phase
Flow rate of 0.6 ml/min
Column temperature - 65°C
RI detector temperature - 55°C

The method quantifies several analytes using calibration standards for dextrans (DP4+), maltotriose, maltose, glucose, fructose, acetic acid, lactic acid, glycerol and ethanol. A 4 point calibration including the origin is used.

The results of ethanol yield and increases over the control at 52 and 70 hours of fermentation are summarized in Table 12.

Table 12 - Comparison of ethanol yields after 52 and 70 hour fermentation time

Sample	52 hour fermentation		70 hour fermentation	
	Ethanol (g/L)	% Increase	Ethanol (g/L)	% Increase
Control	133.30		135.29	
CELLIC™ CTec2 (100 micrograms EP/g DS)	134.64	1.01%	136.88	1.17%
CELLIC™ CTec2 (1 mg EP/g DS)	137.26	2.97%	138.74	2.55%
Celluclast 1.5L (100 micrograms EP/g DS)	134.42	0.84%	135.89	0.44%
Shearzyme Plus (10 micrograms EP/g DS)	133.90	0.45%	135.51	0.17%
Shearzyme Plus (100 micrograms EP/g DS)	133.50	0.15%	135.76	0.35%

The results show that after 52 hours, there are two samples that have significant increases in ethanol, CELLIC™ CTec2 dosed at 100 micrograms EP/g DS (1.01% increase) and at 1 mg EP/g DS (2.97% increase). After 70 hours, the same results were observed with CELLIC™ CTec2 the only sample having a significant increase over the control when dosed at 100 micrograms EP/g DS (1.17% increase) and 1 mg EP/g DS (2.55% increase). No other sample indicated a significant increase over the control even though these too are cellulose and hemicellulose degrading enzymes (cellulases and xylanase). This suggests that a combination of cellulase and xylanase degrading enzymes is needed in order to enhance ethanol yield in the ratio contained in CELLIC™ CTec2. This optimal ratio is supported by the fact that Shearzyme Plus also contains cellulases and xylanase degrading enzymes but at a much higher amount of xylanase the results in yield are not as high as CELLIC™ CTec2.

Example 5

A portion of corn was milled dry using a Fitzpatrick Hammer mill with a screen no. 7. The dry matter content of the milled product was measured gravimetrically to 88.0% w/w. A mash containing 30% dry matter was made using 681.8 g of milled corn and 1318.2 g of city water. 0.11 g of Termamyl SC-L (alpha-amylase) was added in a dosage that was equivalent to 20 KNU/kg milled corn. The mash was heated from 55°C to 90°C during 60 minutes under stirring and then cooled to 32°C and divided in 240 g portions for the simultaneous saccharification and

fermentation trials that were carried in 500 ml blue cap flask with an air lock placed in the rubber stopper.

After the liquefaction of the corn mash using Termamyl SC-L the enzyme classes and products shown in the Table 13 were used:

5

Table 13

Enzyme classes	Enzyme products and dosages (% of corn dry matter)
Glucoamylase	Spirizyme Plus 0.1%
Glucoamylase + cellulase complex with xylanase activity (family GH3)	Spirizyme Plus 0.1% + Celluclast 1.5L 1%
Glucoamylase + cellulase complex with xylanase activity (family GH3) + ferulic acid esterase and beta-glucanase with xylanase activity (family GH3)	Spirizyme Plus 0.1% + Celluclast 1.5L 0.5% + Ultraflo L 0.5%
Glucoamylase + ferulic acid esterase and beta-glucanase with xylanase activity (family GH3)	Spirizyme Plus 0.1% + Ultraflo L 1%

After 30 minutes conditioning with the enzymes and dosages provided in Table 13, 0.20 g of fresh Baker's yeast (Danish Distillers A/S) was added to each flask. The simultaneous saccharification and fermentation trials were carried out in a thermostatted chamber with shaking table at 32°C at 150 rpm in an Innova® 44 shaker. The fermentation time was 67 hours and at the end of the fermentation period all samples were analyzed for content of ethanol and residual soluble sugars on the HPLC.

For fat/oil determination all the samples were centrifuged and the supernatants and the sediment were separated and freeze dried in aluminum trays. The oil content in the sediment was analyzed on an ASE 300 accelerated solvent extractor (Dionex Corporation).

The oil from the supernatant was diluted in hexane and filtrated. The hexane was evaporated in a fume cupboard and the oil was weighed.

The results from the ethanol determinations after 67 hours were in the interval of 11–13% w/w resulting in alcohol yields in the interval of 27–34 kg ethanol/100 kg dry matter based on milled corn. These yields were in the normal range for laboratory trials.

Table 14 shows the actual oil recovery data and average yields of oil to the supernatant (stillage) is calculated as well as the extra yields obtained according to the invention. The yield calculation was based on an oil content of 10.50% w/w found in the milled corn product.

Table 14 - Oil Recovery

Enzyme products and dosages (% of corn dry matter)	Mass of DDG, g	Mass of oil in DDG, g	Yield of oil (to supernatant),%	Average Yield of oil (to supernatant),%	% Extra yield according to the invention
Spirizyme Plus 0.1%	30.86	3.76	52.8	59.5	0
	31.35	2.68	66.3		
Spirizyme Plus 0.1% + Celluclast 1.5 L 1%	27.98	2.04	74.3	74.0	24
	28.59	2.10	73.6		
Spirizyme Plus 0.1% + Celluclast 1.5 L 0.5% + Ultraflo L 0.5%	24.50	1.90	76.1	80.1	35
	23.54	1.26	84.2		
Spirizyme Plus 0.1% + Ultraflo L 1%	22.84	2.45	69.2	69.1	16
	23.40	2.46	69.1		

The results show that there is a synergistic effect of cellulases, xylanases and ferulic esterase resulted in an extra yield of 35% oil. Almost the double mass of oil could be extracted by applying the synergistically working cell wall degrading enzymes (Celluclast + Ultraflo). It is believed that the higher the concentration of ethanol in the thin stillage, the greater the amount of oil (including phospholipids) that can be extracted.

No special pre-treatment of the corn meal other than milling was made.

A fine milling is recommended, either dry or wet at a suitable step in the process to further improve the yields.

The invention is further defined by the following paragraphs:

1. A process of recovering oil, comprising
 - (a) converting a starch-containing material into dextrins with an alpha-amylase;
 - 5 (b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;
 - (c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s);
 - (d) distilling the fermentation product to form a whole stillage;
 - 10 (e) separating the whole stillage into thin stillage and wet cake; and
 - (f) recovering the oil from the thin stillage.
2. A process of recovering oil, comprising
 - (a) saccharifying a cellulosic material with an enzyme composition;
 - 15 (b) fermenting the saccharified cellulosic material into a fermentation product a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s);
 - (c) distilling the fermentation product to form a whole stillage;
 - (d) separating the whole stillage into thin stillage and wet cake; and
 - (e) recovering the oil from the thin stillage.
- 20 3. A process of recovering oil, comprising
 - (a) converting a starch-containing material into dextrins with an alpha-amylase;
 - (b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;
 - 25 (c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s) and an endoglucanase(s);
 - (d) distilling the fermentation product to form a whole stillage;
 - (e) separating the whole stillage into thin stillage and wet cake; and
 - 30 (f) recovering the oil from the thin stillage.

4. A process of recovering oil, comprising
- (a) saccharifying a cellulosic material with an enzyme composition;
 - (b) fermenting the saccharified cellulosic material into a fermentation product with fermenting organism, wherein the fermentation medium comprises a hemicellulase(s) and an endoglucanase(s);
 - (c) distilling the fermentation product to form a whole stillage;
 - (d) separating the whole stillage into thin stillage and wet cake; and
 - (e) recovering the oil from the thin stillage.
5. A process of recovering oil, comprising
- (a) converting a starch-containing material into dextrins with an alpha-amylase;
 - (b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;
 - (c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s), an endoglucanase(s), and a GH61 polypeptide;
 - (d) distilling the fermentation product to form a whole stillage;
 - (e) separating the whole stillage into thin stillage and wet cake; and
 - (f) recovering the oil from the thin stillage.
6. A process of recovering oil, comprising
- (a) saccharifying a cellulosic material with an enzyme composition;
 - (b) fermenting the saccharified cellulosic material into a fermentation product with a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s), an endoglucanase(s), and a GH61 polypeptide;
 - (c) distilling the fermentation product to form a whole stillage;
 - (d) separating the whole stillage into thin stillage and wet cake; and
 - (e) recovering the oil from the thin stillage.

7. A process of recovering oil, comprising

(a) converting a starch-containing material into dextrins with an alpha-amylase;

(b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;

(c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises an endoglucanase(s) and a beta-glucanase(s);

(d) distilling the fermentation product to form a whole stillage;

(e) separating the whole stillage into thin stillage and wet cake; and

(f) recovering the oil from the thin stillage.

8. A process of recovering oil, comprising

(a) saccharifying a cellulosic material with an enzyme composition;

(b) fermenting the saccharified cellulosic material into a fermentation product with a fermenting organism, wherein the fermentation medium comprises an endoglucanase(s) and a beta-glucanase(s);

(c) distilling the fermentation product to form a whole stillage;

(d) separating the whole stillage into thin stillage and wet cake; and

(e) recovering the oil from the thin stillage.

9. A process of dewatering a whole stillage, comprising

(a) converting a starch-containing material into dextrins with an alpha-amylase;

(b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;

(c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s);

(d) distilling the fermentation product to form the whole stillage; and

(e) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

10. A method for dewatering a whole stillage, comprising:

(a) saccharifying a cellulosic material with an enzyme composition;

(b) fermenting the saccharified cellulosic material with a fermenting organism to produce a fermentation product, wherein the fermentation medium comprises a hemicellulase(s);

(c) distilling the fermentation product to form the whole stillage; and

(d) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

11. A process of dewatering a whole stillage, comprising

(a) converting a starch-containing material into dextrins with an alpha-amylase;

(b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;

(c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s) and an endoglucanase(s);

(d) distilling the fermentation product to form the whole stillage; and

(e) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

12. A method for dewatering a whole stillage, comprising:

(a) saccharifying a cellulosic material with an enzyme composition;

(b) fermenting the saccharified cellulosic material with a fermenting organism to produce a fermentation product, wherein the fermentation medium comprises a hemicellulase(s) and an endoglucanase(s);

(c) distilling the fermentation product to form the whole stillage; and

(d) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

13. A process of dewatering a whole stillage, comprising
(a) converting a starch-containing material into dextrins with an alpha-amylase;
(b) saccharifying the dextrins using a carbohydrate source generating enzyme to
form a sugar;

5 (c) fermenting the sugar in a fermentation medium into a fermentation product using
a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s), an
endoglucanase(s), and a GH61 polypeptide(s);

(d) distilling the fermentation product to form the whole stillage; and

(e) separating the whole stillage into thin stillage and wet cake, whereby more liquid
10 phase is transferred to the thin stillage.

14. A method for dewatering a whole stillage, comprising:

(a) saccharifying a cellulosic material with an enzyme composition;

(b) fermenting the saccharified cellulosic material with a fermenting organism to
15 produce a fermentation product, wherein the fermentation medium comprises a hemicellulase(s)
, an endoglucanase(s), and a GH61 polypeptide(s);

(c) distilling the fermentation product to form the whole stillage; and

(d) separating the whole stillage into thin stillage and wet cake, whereby more liquid
phase is transferred to the thin stillage.

20 15. A process of dewatering a whole stillage, comprising

(a) converting a starch-containing material into dextrins with an alpha-amylase;

(b) saccharifying the dextrins using a carbohydrate source generating enzyme to
form a sugar;

25 (c) fermenting the sugar in a fermentation medium into a fermentation product using
a fermenting organism, wherein the fermentation medium comprises an endoglucanase and a
beta-glucanase(s);

(d) distilling the fermentation product to form the whole stillage; and

(e) separating the whole stillage into thin stillage and wet cake, whereby more liquid
30 phase is transferred to the thin stillage.

16. A method for dewatering a whole stillage, comprising:

- (a) saccharifying a cellulosic material with an enzyme composition;
- (b) fermenting the saccharified cellulosic material with a fermenting organism to produce a fermentation product, wherein the fermentation medium comprises an endoglucanase and a beta-glucanase(s);
- (c) distilling the fermentation product to form the whole stillage; and
- (d) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

17. A process of producing a feed co-product, comprising

- (a) converting a starch-containing material into dextrins with an alpha-amylase;
- (b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;
- (c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s);
- (d) distilling the fermentation product to form a whole stillage;
- (e) separating the whole stillage into thin stillage and wet cake;
- (f) de-oiling the thin stillage to form soluble with less oil; and
- (g) recovering the feed co-product from the wet cake and soluble with less oil.

18. A process of producing a feed co-product, comprising

- (a) saccharifying a cellulosic material with an enzyme composition;
- (b) fermenting the saccharified cellulosic material into a fermentation product with a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s);
- (c) distilling the fermentation product to form a whole stillage;
- (d) separating the whole stillage into thin stillage and wet cake;
- (e) de-oiling the thin stillage to form soluble with less oil; and
- (f) recovering the feed co-product from the wet cake and soluble with less oil.

19. A process of producing a feed co-product, comprising
- (a) converting a starch-containing material into dextrins with an alpha-amylase;
 - (b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;
 - 5 (c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s) and an endoglucanase(s);
 - (d) distilling the fermentation product to form a whole stillage;
 - (e) separating the whole stillage into thin stillage and wet cake;
 - 10 (f) de-oiling the thin stillage to form soluble with less oil; and
 - (g) recovering the feed co-product from the wet cake and soluble with less oil.
20. A process of producing a feed co-product, comprising
- (a) saccharifying a cellulosic material with an enzyme composition;
 - 15 (b) fermenting the saccharified cellulosic material into a fermentation product with a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s) and an endoglucanase(s);
 - (c) distilling the fermentation product to form a whole stillage;
 - (d) separating the whole stillage into thin stillage and wet cake;
 - 20 (e) de-oiling the thin stillage to form soluble with less oil; and
 - (f) recovering the feed co-product from the wet cake and soluble with less oil.
21. A process of producing a feed co-product, comprising
- (a) converting a starch-containing material into dextrins with an alpha-amylase;
 - 25 (b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;
 - (c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s), an endoglucanase(s), and a GH61 polypeptide;
 - 30 (d) distilling the fermentation product to form a whole stillage;
 - (e) separating the whole stillage into thin stillage and wet cake;
 - (f) de-oiling the thin stillage to form soluble with less oil; and
 - (g) recovering the feed co-product from the wet cake and soluble with less oil.

22. A process of producing a feed co-product, comprising

(a) saccharifying a cellulosic material with an enzyme composition;

(b) fermenting the saccharified cellulosic material into a fermentation product with a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s), an endoglucanase(s), and a GH61 polypeptide;

(c) distilling the fermentation product to form a whole stillage;

(d) separating the whole stillage into thin stillage and wet cake;

(e) de-oiling the thin stillage to form soluble with less oil; and

(f) recovering the feed co-product from the wet cake and soluble with less oil.

23. A process of producing a feed co-product, comprising

(a) converting a starch-containing material into dextrins with an alpha-amylase;

(b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;

(c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises an endoglucanase(s) and a beta-glucanase(s);

(d) distilling the fermentation product to form a whole stillage;

(e) separating the whole stillage into thin stillage and wet cake;

(f) de-oiling the thin stillage to form soluble with less oil; and

(g) recovering the feed co-product from the wet cake and soluble with less oil.

24. A process of producing a feed co-product, comprising

(a) saccharifying a cellulosic material with an enzyme composition;

(b) fermenting the saccharified cellulosic material into a fermentation product with a fermenting organism, wherein the fermentation medium comprises an endoglucanase(s) and a beta-glucanase(s);

(c) distilling the fermentation product to form a whole stillage;

(d) separating the whole stillage into thin stillage and wet cake;

(e) de-oiling the thin stillage to form soluble with less oil; and

(f) recovering the feed co-product from the wet cake and soluble with less oil.

25. The process of any of paragraphs 17-24, wherein the feed co-product is distillers' dry grains (DDG).

26. The process of any of paragraphs 17-24, wherein the feed co-product is distillers' dry grains with solubles (DDGS).

5 27. The process of any of paragraphs 17-24, wherein the feed co-product is distillers' dry soluble (DDS).

28. The process of any of paragraphs 17-24, wherein the feed co-product is distillers' wet grains (DWG).

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29. The process of any of paragraphs 1-6, 9-14, 17-22, and 25-28, wherein the hemicellulase(s) is selected from the group consisting of acetylxy lan esterase, arabinofuranosidase, feruloyl esterase, glucuronidase, xylanase, and xylosidase.

15 30. The process of paragraph 29, wherein the hemicellulase(s) is a xylanase.

31. The process of any of paragraphs 1-30, wherein the fermentation medium further comprises a cellobiohydrolase(s).

20 32. The process of any of paragraphs 1-31, wherein the fermentation medium further comprises a beta-glucosidase(s).

33. The process of any of paragraphs 1-32, wherein the fermentation medium further comprises a protease(s).

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34. The process of any of paragraphs 1-33, wherein the fermentation medium further comprises a GH61 polypeptide(s).

30 35. The process of any of paragraphs 1-34, wherein the fermentation medium further comprises a beta-glucanase(s).

36. The process of any of paragraphs 1-35, wherein the fermentation medium further comprises a ferulic acid esterase(s).

37. The process of any of paragraphs 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24-36, wherein the cellulosic material is pretreated.

38. The process of any of paragraphs 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24-37, wherein the enzyme composition comprises one or more enzymes selected from the group consisting of a cellulase, a GH61 polypeptide having cellulolytic enhancing activity, a hemicellulase, an expansin, an esterase, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.

39. The process of paragraph 38, wherein the enzyme composition comprises an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

40. The process of paragraph 38 or 39, wherein the enzyme composition comprises one or more enzymes selected from the group consisting of acetyxylan esterase, arabinofuranosidase, feruloyl esterase, glucuronidase, xylanase, and xylosidase.

41. The process of any of paragraphs 3-8, 11-16, and 19-40, wherein the endoglucanase(s) is added in an amount of 0.01-1.0 EGU/g, e.g. 0.02-0.08, 0.025-0.06, 0.025-0.05, or 0.03-0.04 EGU/g dry solids.

42. The process of any of paragraphs 3-8, 11-16, and 19-41, wherein the endoglucanase(s) is added in an amount of 1-30, e.g., 5-30, 7-25, 10-20, 10-17, or 12-15 micrograms/g dry solids.

43. The process of any of paragraphs 1-6, 9-14, 17-22, and 25-42, wherein the hemicellulase(s) is added in an amount of 0.01-1.0, e.g., 0.015-0.08, 0.015-0.06, 0.015-0.04, or 0.02-0.03 FXU/g dry solids.

44. The process of any of paragraphs 1-6, 9-14, 17-22, and 25-43, wherein the hemicellulase(s) is added in an amount of 1-30, e.g., 5-30, 7-25, 10-20, 10-17, or 12-15 micrograms/g dry solids.

45. A process of recovering oil, comprising
- (a) converting a starch-containing material into dextrins with an alpha-amylase;
 - (b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;
 - 5 (c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a GH61 polypeptide;
 - (d) distilling the fermentation product to form a whole stillage;
 - (e) separating the whole stillage into thin stillage and wet cake; and
 - (f) recovering the oil from the thin stillage.

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46. The process of paragraph 45, wherein the fermentation medium further comprises a hemicellulase(s) selected from the group consisting of acetyxylan esterase, arabinofuranosidase, feruloyl esterase, glucuronidase, xylanase, and xylosidase.

- 15 47. The process of paragraph 46, wherein the hemicellulase(s) is a xylanase.

48. The process of any of paragraphs 45-47, wherein the fermentation medium further comprises an endoglucanase.

- 20 49. The process of any of paragraphs 45-48, wherein the fermentation medium further comprises one or more cellobiohydrolases and/or beta-glucosidases.

50. The process of any of paragraphs 45-49, wherein the fermentation medium further comprises one or more proteases.

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51. A process for recovering oil, comprising:
- (a) saccharifying a cellulosic material with an enzyme composition;
 - (b) fermenting the saccharified cellulosic material with one or more fermenting microorganisms to produce a fermentation product, wherein the fermentation medium
 - 30 comprises a GH61 polypeptide;
 - (c) distilling the fermentation product to form a whole stillage;
 - (d) separating the whole stillage into thin stillage and wet cake; and
 - (e) recovering the oil from the thin stillage.

52. The process of paragraph 51, wherein the cellulosic material is pretreated.

53. The process of paragraph 51 or 52, wherein the enzyme composition comprises one or more enzymes selected from the group consisting of a cellulase, a GH61 polypeptide having cellulolytic enhancing activity, a hemicellulase, an expansin, an esterase, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.

54. The process of paragraph 53, wherein the cellulase is one or more enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

55. The process of paragraph 53 or 54, wherein the hemicellulase is one or more enzymes selected from the group consisting of acetyxylan esterase, arabinofuranosidase, feruloyl esterase, glucuronidase, xylanase, and xylosidase.

56. The process of any of paragraphs 51-55, wherein steps (a) and (b) are performed simultaneously in a simultaneous saccharification and fermentation.

57. A process of dewatering a whole stillage, comprising

(a) converting a starch-containing material into dextrins with an alpha-amylase;

(b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;

(c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a GH61 polypeptide;

(d) distilling the fermentation product to form the whole stillage;

(e) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

58. The process of paragraph 57, wherein the hemicellulase(s) is selected from the group consisting of acetyxylan esterase, arabinofuranosidase, feruloyl esterase, glucuronidase, xylanase, and xylosidase.

59. The process of paragraph 58, wherein the hemicellulase(s) is a xylanase.

60. The process of any of paragraphs 57-59, wherein the fermentation medium further comprises one or more cellobiohydrolases and/or beta-glucosidases.

61. The process of any of paragraphs 57-60, wherein the fermentation medium further
5 comprises one or more proteases.

62. The process of any of paragraphs 57-61, wherein the fermentation medium further comprises a GH61 polypeptide.

10 63. A process for dewatering a whole stillage, comprising:

(a) saccharifying a cellulosic material with an enzyme composition;

(b) fermenting the saccharified cellulosic material with a fermenting organism to produce a fermentation product, wherein the fermentation medium comprises a GH61 polypeptide;

15 (c) distilling the fermentation product to form the whole stillage;

(d) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

64. The process of paragraph 63, wherein the cellulosic material is pretreated.

20 65. The process of paragraph 63 or 64, wherein the enzyme composition comprises one or more enzymes selected from the group consisting of a cellulase, a GH61 polypeptide having cellulolytic enhancing activity, a hemicellulase, an expansin, an esterase, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.

25 66. The process of paragraph 65, wherein the cellulase is one or more enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

30 67. The process of paragraph 65 or 66, wherein the hemicellulase is one or more enzymes selected from the group consisting of acetyxylan esterase, arabinofuranosidase, feruloyl esterase, glucuronidase, xylanase, and xylosidase.

68. The process of any of paragraphs 1-67, wherein the fermentation product is an alcohol, an organic acid, a ketone, an amino acid, an alkane, a cycloalkane, an alkene, isoprene, polyketide, or a gas.

5 69. The process of paragraph 68, wherein the fermentation product is ethanol.

70. The process of any of paragraphs 1-69, further comprising recovering the fermentation product.

10 71. A process of producing a fermentation product, comprising
(a) liquefying a starch-containing material into dextrins with an alpha-amylase;
(b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;
(c) fermenting the sugar in a fermentation medium into a fermentation product using
15 a fermenting organism, wherein the fermentation medium comprises a GH61 polypeptide.

72. The process of paragraph 71, wherein the fermentation medium further comprises a cellulase(s) (cellobiohydrolase, endoglucanase, and/or beta-glucosidase) and a hemicellulase(s) (e.g., ferulic acid esterase and/or xylanase).

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73. The process of any of paragraphs 1-72, wherein the fermentation medium further comprises a glucoamylase(s).

73. A feed co-product produced by a process of any of paragraphs 17-44.

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74. The feed co-product of paragraph 73, which is distillers' dry grains (DDG).

75. The feed co-product of paragraph 73, which is distillers' dry grains with solubles (DDGS).

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76. The feed co-product of paragraph 73, which is distillers' dry soluble (DDS).

77. The feed co-product of paragraph 73, which is distillers' wet grains (DWG).

78. An animal feed comprising a feed co-product of any of paragraphs 73-77.

CLAIMS

1. A process of recovering oil, comprising
 - (a) converting a starch-containing material into dextrins with an alpha-amylase;
 - 5 (b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;
 - (c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s);
 - (d) distilling the fermentation product to form a whole stillage;
 - 10 (e) separating the whole stillage into thin stillage and wet cake; and
 - (f) recovering the oil from the thin stillage.
2. A process of recovering oil, comprising
 - (a) saccharifying a cellulosic material with an enzyme composition;
 - 15 (b) fermenting the saccharified cellulosic material into a fermentation product with a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s);
 - (c) distilling the fermentation product to form a whole stillage;
 - (d) separating the whole stillage into thin stillage and wet cake; and
 - (e) recovering the oil from the thin stillage.
- 20 3. A process of recovering oil, comprising
 - (a) converting a starch-containing material into dextrins with an alpha-amylase;
 - (b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;
 - 25 (c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s) and an endoglucanase(s);
 - (d) distilling the fermentation product to form a whole stillage;
 - (e) separating the whole stillage into thin stillage and wet cake; and
 - 30 (f) recovering the oil from the thin stillage.

4. A process of recovering oil, comprising
- (a) saccharifying a cellulosic material with an enzyme composition;
 - (b) fermenting the saccharified cellulosic material into a fermentation product with a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s) and an endoglucanase(s);
 - (c) distilling the fermentation product to form a whole stillage;
 - (d) separating the whole stillage into thin stillage and wet cake; and
 - (e) recovering the oil from the thin stillage.
5. A process of recovering oil, comprising
- (a) converting a starch-containing material into dextrins with an alpha-amylase;
 - (b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;
 - (c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s), an endoglucanase(s), and a GH61 polypeptide;
 - (d) distilling the fermentation product to form a whole stillage;
 - (e) separating the whole stillage into thin stillage and wet cake; and
 - (f) recovering the oil from the thin stillage.
6. A process of recovering oil, comprising
- (a) saccharifying a cellulosic material with an enzyme composition;
 - (b) fermenting the saccharified cellulosic material into a fermentation product with a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s), an endoglucanase(s), and a GH61 polypeptide;
 - (c) distilling the fermentation product to form a whole stillage;
 - (d) separating the whole stillage into thin stillage and wet cake; and
 - (e) recovering the oil from the thin stillage.

7. A process of recovering oil, comprising

(a) converting a starch-containing material into dextrins with an alpha-amylase;

(b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;

(c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises an endoglucanase(s) and a beta-glucanase(s);

(d) distilling the fermentation product to form a whole stillage;

(e) separating the whole stillage into thin stillage and wet cake; and

(f) recovering the oil from the thin stillage.

8. A process of recovering oil, comprising

(a) saccharifying a cellulosic material with an enzyme composition;

(b) fermenting the saccharified cellulosic material into a fermentation product with a fermenting organism, wherein the fermentation medium comprises an endoglucanase(s) and a beta-glucanase(s);

(c) distilling the fermentation product to form a whole stillage;

(d) separating the whole stillage into thin stillage and wet cake; and

(e) recovering the oil from the thin stillage.

9. A process of dewatering a whole stillage, comprising

(a) converting a starch-containing material into dextrins with an alpha-amylase;

(b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;

(c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s);

(d) distilling the fermentation product to form the whole stillage; and

(e) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

10. A method for dewatering a whole stillage, comprising:

(a) saccharifying a cellulosic material with an enzyme composition;

(b) fermenting the saccharified cellulosic material with a fermenting organism to produce a fermentation product, wherein the fermentation medium comprises a hemicellulase(s);

(c) distilling the fermentation product to form the whole stillage; and

(d) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

11. A process of dewatering a whole stillage, comprising

(a) converting a starch-containing material into dextrins with an alpha-amylase;

(b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;

(c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s) and an endoglucanase(s);

(d) distilling the fermentation product to form the whole stillage; and

(e) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

12. A method for dewatering a whole stillage, comprising:

(a) saccharifying a cellulosic material with an enzyme composition;

(b) fermenting the saccharified cellulosic material with a fermenting organism to produce a fermentation product, wherein the fermentation medium comprises a hemicellulase(s) and an endoglucanase(s);

(c) distilling the fermentation product to form the whole stillage; and

(d) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

13. A process of dewatering a whole stillage, comprising

(a) converting a starch-containing material into dextrins with an alpha-amylase;

(b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;

(c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s), an endoglucanase(s), and a GH61 polypeptide(s);

(d) distilling the fermentation product to form the whole stillage; and

(e) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

14. A method for dewatering a whole stillage, comprising:

(a) saccharifying a cellulosic material with an enzyme composition;

(b) fermenting the saccharified cellulosic material with a fermenting organism to produce a fermentation product, wherein the fermentation medium comprises a hemicellulase(s), an endoglucanase(s), and a GH61 polypeptide(s);

(c) distilling the fermentation product to form the whole stillage; and

(d) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

15. A process of dewatering a whole stillage, comprising

(a) converting a starch-containing material into dextrins with an alpha-amylase;

(b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;

(c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises an endoglucanase and a beta-glucanase(s);

(d) distilling the fermentation product to form the whole stillage; and

(e) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

16. A method for dewatering a whole stillage, comprising:

- (a) saccharifying a cellulosic material with an enzyme composition;
- (b) fermenting the saccharified cellulosic material with a fermenting organism to produce a fermentation product, wherein the fermentation medium comprises an endoglucanase and a beta-glucanase(s);
- (c) distilling the fermentation product to form the whole stillage; and
- (d) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

17. A process of producing a feed co-product, comprising

- (a) converting a starch-containing material into dextrins with an alpha-amylase;
- (b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;
- (c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s);
- (d) distilling the fermentation product to form a whole stillage;
- (e) separating the whole stillage into thin stillage and wet cake;
- (f) de-oiling the thin stillage to form soluble with less oil; and
- (g) recovering the feed co-product from the wet cake and soluble with less oil.

18. A process of producing a feed co-product, comprising

- (a) saccharifying a cellulosic material with an enzyme composition;
- (b) fermenting the saccharified cellulosic material into a fermentation product with a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s);
- (c) distilling the fermentation product to form a whole stillage;
- (d) separating the whole stillage into thin stillage and wet cake;
- (e) de-oiling the thin stillage to form soluble with less oil; and
- (f) recovering the feed co-product from the wet cake and soluble with less oil.

19. A process of producing a feed co-product, comprising
- (a) converting a starch-containing material into dextrins with an alpha-amylase;
 - (b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;
 - 5 (c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s) and an endoglucanase(s);
 - (d) distilling the fermentation product to form a whole stillage;
 - (e) separating the whole stillage into thin stillage and wet cake;
 - 10 (f) de-oiling the thin stillage to form soluble with less oil; and
 - (g) recovering the feed co-product from the wet cake and soluble with less oil.
20. A process of producing a feed co-product, comprising
- (a) saccharifying a cellulosic material with an enzyme composition;
 - 15 (b) fermenting the saccharified cellulosic material into a fermentation product with a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s) and an endoglucanase(s);
 - (c) distilling the fermentation product to form a whole stillage;
 - (d) separating the whole stillage into thin stillage and wet cake;
 - 20 (e) de-oiling the thin stillage to form soluble with less oil; and
 - (f) recovering the feed co-product from the wet cake and soluble with less oil.
21. A process of producing a feed co-product, comprising
- (a) converting a starch-containing material into dextrins with an alpha-amylase;
 - 25 (b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;
 - (c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s), an endoglucanase(s), and a GH61 polypeptide;
 - 30 (d) distilling the fermentation product to form a whole stillage;
 - (e) separating the whole stillage into thin stillage and wet cake;
 - (f) de-oiling the thin stillage to form soluble with less oil; and
 - (g) recovering the feed co-product from the wet cake and soluble with less oil.

22. A process of producing a feed co-product, comprising

- (a) saccharifying a cellulosic material with an enzyme composition;
- (b) fermenting the saccharified cellulosic material into a fermentation product with a fermenting organism, wherein the fermentation medium comprises a hemicellulase(s), an endoglucanase(s), and a GH61 polypeptide;
- (c) distilling the fermentation product to form a whole stillage;
- (d) separating the whole stillage into thin stillage and wet cake;
- (e) de-oiling the thin stillage to form soluble with less oil; and
- (f) recovering the feed co-product from the wet cake and soluble with less oil.

23. A process of producing a feed co-product, comprising

- (a) converting a starch-containing material into dextrins with an alpha-amylase;
- (b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;
- (c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises an endoglucanase(s) and a beta-glucanase(s);
- (d) distilling the fermentation product to form a whole stillage;
- (e) separating the whole stillage into thin stillage and wet cake;
- (f) de-oiling the thin stillage to form soluble with less oil; and
- (g) recovering the feed co-product from the wet cake and soluble with less oil.

24. A process of producing a feed co-product, comprising

- (a) saccharifying a cellulosic material with an enzyme composition;
- (b) fermenting the saccharified cellulosic material into a fermentation product with a fermenting organism, wherein the fermentation medium comprises an endoglucanase(s) and a beta-glucanase(s);
- (c) distilling the fermentation product to form a whole stillage;
- (d) separating the whole stillage into thin stillage and wet cake;
- (e) de-oiling the thin stillage to form soluble with less oil; and
- (f) recovering the feed co-product from the wet cake and soluble with less oil.

25. A process of recovering oil, comprising

(a) converting a starch-containing material into dextrins with an alpha-amylase;

(b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;

5 (c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a GH61 polypeptide;

(d) distilling the fermentation product to form a whole stillage;

(e) separating the whole stillage into thin stillage and wet cake; and

(f) recovering the oil from the thin stillage.

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26. A process for recovering oil, comprising:

(a) saccharifying a cellulosic material with an enzyme composition;

(b) fermenting the saccharified cellulosic material with a fermenting organism to produce a fermentation product, wherein the fermentation medium comprises a GH61 polypeptide;

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(c) distilling the fermentation product to form a whole stillage;

(d) separating the whole stillage into thin stillage and wet cake; and

(e) recovering the oil from the thin stillage.

20 27. A process of dewatering a whole stillage, comprising

(a) converting a starch-containing material into dextrins with an alpha-amylase;

(b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;

(c) fermenting the sugar in a fermentation medium into a fermentation product using

25 a fermenting organism, wherein the fermentation medium comprises a GH61 polypeptide;

(d) distilling the fermentation product to form the whole stillage;

(e) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

28. A process for dewatering a whole stillage, comprising:

(a) saccharifying a cellulosic material with an enzyme composition;

(b) fermenting the saccharified cellulosic material with a fermenting organism to produce a fermentation product, wherein the fermentation medium comprises a GH61 polypeptide;

(c) distilling the fermentation product to form the whole stillage;

(d) separating the whole stillage into thin stillage and wet cake, whereby more liquid phase is transferred to the thin stillage.

29. A process of producing a fermentation product, comprising

(a) liquefying a starch-containing material into dextrins with an alpha-amylase;

(b) saccharifying the dextrins using a carbohydrate source generating enzyme to form a sugar;

(c) fermenting the sugar in a fermentation medium into a fermentation product using a fermenting organism, wherein the fermentation medium comprises a GH61 polypeptide.