(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 September 2015 (24.09.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/143144 A1

(51) International Patent Classification:
C12P 19/02 (2006.01) *C12P 19/14* (2006.01)
C12P 19/12 (2006.01)

(21) International Application Number:
PCT/US2015/021447

(22) International Filing Date:
19 March 2015 (19.03.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/955,372 19 March 2014 (19.03.2014) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2015/143144 A1

(54) **Title**: METHOD FOR ENHANCING ACTIVITY OF AN X143 POLYPEPTIDE

(57) **Abstract**: he present invention relates to the use of a cofactor, L-ascorbic acid, for enhancing the boosting effect of adding an X143 polypeptide to a saccharification process. Thus the present invention relates to a method for saccharifying starch comprising: contacting the starch with at least a glucoamylase an acidic alpha-amylase, an X143 polypeptide and L-ascorbic acid.

METHOD FOR ENHANCING ACTIVITY OF AN X143 POLYPEPTIDE

Reference to a Sequence Listing

This application contains a Sequence Listing in computer readable form, which is incorporated herein by reference.

Background of the Invention

Field of the Invention

The present invention relates to the field of starch saccharification, and in particular to methods of enhancing the activity of alpha-amylases and glucoamylases.

Description of the Related Art

Saccharification of starch by amylolytic enzymes, such as alpha-amylases and glucoamylases, is well known in the art. Starch processing usually is either performed on geltized starch, in which a starch slurry has been heated to a temperature above the gelatinization temperature (e.g. typically between 80-90°C) or on ungelatinized starch in which case the starch slurry is maintained at temperatures below the gelatinization temperature (e.g. typically between 50-65°C). Both types of processes require the action of alpha-amylases and glucoamylases. The present invention relates to improved processes for saccharification of starch. In WO 2010/059413 a polypeptide isolated from *Aspergillus nidulans*, termed X143, was shown to have a boosting effect on starch saccharification when used in combination with amylolytic enzymes such as alpha-amylases and glucoamylases. The present invention relates to the finding that the effect of adding an X143 polypeptide can be further improved by adding a cofactor.

Summary of the Invention

In a first aspect the present invention relates to a method for saccharifying starch comprising: contacting the starch with at least a glucoamylase, an acidic alpha-amylase, an X143 polypeptide and L-ascorbic acid.

In a second aspect the invention relates to a method of saccharifying a starch containing material, comprising:

(a) liquefying the starch containing material in the presence of at least an acid alpha amylase;

(b) saccharifying the starch containing material in the presence of a glucoamylase, an X143 polypeptide, and L-ascorbic acid.

In a third aspect the present invention relates to a use of an X143 polypeptide in combination with at least an acid alpha amylase, a glucoamylase and L-ascorbic acid for saccharifying starch.

Definitions

cDNA: The term "cDNA" means a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic cell. cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps, including splicing, before appearing as mature spliced mRNA.

Coding sequence: The term “coding sequence” means a polynucleotide, which directly specifies the amino acid sequence of a polypeptide. 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. In one embodiment the coding sequence consist of positions 1 to 102 joined to positions 162 to 1217 of SEQ ID NO: 1.

Control sequences: The term “control sequences” means all components necessary for the expression of a polynucleotide encoding a polypeptide. Each control sequence may be native or foreign to the polynucleotide encoding the polypeptide 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 a polypeptide.

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 and is operably linked to additional nucleotides that provide for its expression.

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.

Isolated: The term "isolated" means a substance in a form or environment that does not occur in nature. Non-limiting examples of isolated substances include (1) any non-naturally occurring substance, (2) any substance including, but not limited to, any enzyme, variant, nucleic acid, protein, peptide or cofactor, that is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature; (3) any substance modified by the hand of man relative to that substance found in nature; or (4) any substance modified by increasing the amount of the substance relative to other components with which it is naturally associated (*e.g.*, recombinant production in a host cell; multiple copies of a gene encoding the substance; and use of a stronger promoter than the promoter naturally associated with the gene encoding the substance).

Mature polypeptide: The term "mature polypeptide" is defined herein as a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. In one aspect, the mature polypeptide is amino acids 19 to 385 of SEQ ID NO: 2.

Mature polypeptide coding sequence: The term "mature polypeptide coding sequence" is defined herein as a nucleotide sequence that encodes a mature polypeptide having retrograded starch activity. In one aspect, the mature polypeptide coding sequence is nucleotides 55 to 1214 of SEQ ID NO: 1 and nucleotides 1 to 54 of SEQ ID NO: 1 encode a signal peptide.

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 of the present invention.

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 a polynucleotide such that the control sequence directs the expression of the coding sequence.

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

Retrogradation is a reaction that takes place in gelatinized starch when the amylose and amylopectin chains realign themselves, causing the liquid to gel.

When native starch is heated and dissolves in water, the crystalline structure of amylose and amylopectin molecules are lost and they hydrate to form a viscous solution. If the viscous solution is cooled or left at lower temperature for long enough period, the linear molecules, amylose, and linear parts of amylopectin molecules retrograde and rearrange themselves again to a more crystalline structure.

Retrograded starch or resistant Starch (RS) is a less digestible form of starch and is formed by heating, e.g. during liquefaction, and subsequent cooling.

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 5.0.0, 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:

(Identical Residues x 100)/(Length of Alignment – 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 5.0.0 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:

(Identical Deoxyribonucleotides x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

Subsequence: The term "subsequence" means a polynucleotide having one or more (*e.g.*, several) nucleotides deleted from the 5' and/or 3' end of a mature polypeptide coding sequence; wherein the subsequence encodes a fragment having biological activity.

Variant: The term "variant" means a polypeptide having cellulolytic enhancing activity comprising an alteration, *i.e.*, a substitution, insertion, and/or deletion of one or more (*e.g.*, several) amino acid residues at one or more (*e.g.*, several) positions. A substitution means a replacement of an amino acid occupying a position with a different amino acid; a deletion means removal of an amino acid occupying a position; and an insertion means adding one or more (*e.g.*, several) amino acids, *e.g.*, 1-5 amino acids, adjacent to an amino acid occupying a position.

Cellulolytic enzyme, cellulolytic composition, or cellulase: The term "cellulolytic enzyme", "cellulolytic composition", 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 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 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).

Cellulolytic enzyme activity is determined by measuring the increase in hydrolysis of a cellulosic material by cellulolytic enzyme(s) under the following conditions: 1-50 mg of cellulolytic enzyme protein/g of cellulose in Pretreated Corn Stover ("PCS") (or other pretreated cellulosic material) 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 $MnSO_4$, 50°C, 55°C, or 60°C, 72 hours, sugar analysis by AMINEX® HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

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 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.

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, and pH, *e.g.*, 5.0 or

5.5, 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 an 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 02/095014) of cellulase protein loading is used as the source of the cellulolytic activity.

The GH61 polypeptide 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, *e.g.*, at least 1.05-fold, at least 1.10-fold, at least 1.25-fold, at least 1.5-fold, at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold, at least 10-fold, or at least 20-fold.

Beta-glucosidase: The term "beta-glucosidase" means a beta-D-glucoside glucohydrolase (E.C. 3.2.1.21) that 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 using *p*-nitrophenyl-beta-D-glucopyranoside as substrate according to the procedure of 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 µmole 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 (polyoxyethylene sorbitan monolaurate).

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 Claeyssens, 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.

Endoglucanase: The term "endoglucanase" means an endo-1,4-(1,3;1,4)-beta-D-glucan 4-glucanohydrolase (E.C. 3.2.1.4) that 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.

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 acetylxylan 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. Some families, with an 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 in 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, and pH, e.g., 5.0 or 5.5.

Alpha-Amylases (alpha-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) are a group of enzymes, which catalyze the hydrolysis of starch and other linear and branched 1,4-glucosidic oligo- and polysaccharides.

Glucoamylases (glucan 1,4-alpha-glucosidase, EC 3.2.1.3) are a group of enzymes, which catalyze the hydrolysis of terminal (1→4)-linked α-D-glucose residues successively from non-reducing ends of the chains with release of beta-D-glucose.

Detailed Description of the Invention

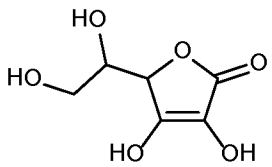
In WO 2010/059413 a polypeptide isolated from *Aspergillus nidulans*, termed X143, was shown to have a boosting effect on starch saccharification when used in combination with amylolytic enzymes such as alpha-amylases and glucoamylases. The present invention relates to the finding that the effect of adding an X143 polypeptide, can be further improved by adding a cofactor.

The present invention provides in a first aspect a method for saccharifying starch comprising: contacting the starch with at least a glucoamylase an acidic alpha-amylase, an X143 polypeptide and a heterocyclic compound, in particular L-ascorbic acid.

Heterocyclic Compounds

In the methods and compositions of the present invention, the heterocyclic compound may be any suitable compound, such as an optionally substituted aromatic or non-aromatic ring comprising a heteroatom.

In one aspect, the heterocyclic compound is selected from of:



(I-1): (1,2-Dihydroxyethyl)-3,4-dihydroxyfuran-2(5H)-one (L-ascorbic acid)

The effective amount of the ascorbic acid compound can depend on one or more (*e.g.*, several) factors including, but not limited to, the mixture of component amylolytic enzymes, the starch substrate, the concentration of starch substrate, the pretreatment(s) of the starch substrate, temperature, and reaction time.

The ascorbic acid compound is preferably present in an amount that is not limiting with regard to the X143 polypeptide having retrograded starch degrading activity, amylolytic enzyme(s), and starch.

In one aspect, an effective amount of the ascorbic acid compound is about 0.1 µM to about 1 M, *e.g.*, about 0.5 µM to about 0.75 M, about 0.75 µM to about 0.5 M, about 1 µM to about 0.25 M, about 1 µM to about 0.1 M, about 5 µM to about 50 mM, about 10 µM to about 25 mM, about 50 µM to about 25 mM, about 10 µM to about 10 mM, about 5 µM to about 5 mM, or about 0.1 mM to about 1 mM. In another aspect, an effective amount of the heterocyclic compound is about 0.1 µM to about 1 M. In another aspect, an effective amount of the heterocyclic compound is about 0.5 µM to about 0.75 M. In another aspect, an effective amount of the heterocyclic compound is about 0.75 µM to about 0.5 M. In another aspect, an effective amount of the heterocyclic compound is about 1 µM to about 0.25 M. In another aspect, an effective amount of the heterocyclic compound is about 1 µM to about 0.1 M. In another aspect, an effective amount of the heterocyclic compound is about 5 µM to about 50 mM. In another

aspect, an effective amount of the heterocyclic compound is about 10 µM to about 25 mM. In another aspect, an effective amount of the heterocyclic compound is about 50 µM to about 25 mM. In another aspect, an effective amount of the heterocyclic compound is about 10 µM to about 10 mM. In another aspect, an effective amount of the heterocyclic compound is about 5 µM to about 5 mM. In another aspect, an effective amount of the heterocyclic compound is about 0.1 mM to about 10 mM, particularly from 0.2 mM to 5.0 mM, more particularly from 0.25 mM to 0.75 mM, such as around 0.5 mM.

In another aspect of the present invention, the ascorbic acid compound may be recycled from a completed saccharification or completed saccharification and fermentation to a new saccharification. The ascorbic acid compound(s) can be recovered using standard methods in the art, *e.g.*, filtration/centrifugation pre- or post-distillation, to remove residual solids, cellular debris, *etc.* and then recirculated to the new saccharification.

X143 Polypeptides and Polynucleotides encoding the X143 polypeptide

An X143 polypeptide to be applied in the methods and compositions of the present invention relates to isolated polypeptides comprising amino acid sequences having a degree of sequence identity to the mature polypeptide of SEQ ID NO: 2 of preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 96%, at least 97%, at least 98%, or at least 99%, which have retrograded starch activity (hereinafter "homologous polypeptides"). In a preferred aspect, the homologous polypeptides comprise amino acid sequences that differ by ten amino acids, preferably by five amino acids, more preferably by four amino acids, even more preferably by three amino acids, most preferably by two amino acids, and even most preferably by one amino acid from the mature polypeptide of SEQ ID NO: 2.

An X143 polypeptide preferably comprises the amino acid sequence of SEQ ID NO: 2 or an allelic variant thereof. In a preferred aspect, the polypeptide comprises the amino acid sequence of SEQ ID NO: 2. In another preferred aspect, the polypeptide comprises the mature polypeptide of SEQ ID NO: 2. In another preferred aspect, the polypeptide comprises amino acids 19 to 385 of SEQ ID NO: 2, or an allelic variant thereof. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 2 or an allelic variant thereof. In another preferred aspect, the polypeptide consists of the mature polypeptide of SEQ ID NO: 2. In another preferred aspect, the polypeptide consists of amino acids 19 to 385 of SEQ ID NO: 2.

An X143 polypeptide is encoded by polynucleotides that hybridize under preferably very low stringency conditions, more preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained

in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) a full-length complementary strand of (i) or (ii) (J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York).

The nucleotide sequence of SEQ ID NO: 1; or a subsequence thereof; as well as the amino acid sequence of SEQ ID NO: 2; or a fragment thereof; may be used to design nucleic acid probes to identify and clone DNA encoding polypeptides having retrograded starch degrading activity from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 14, preferably at least 25, more preferably at least 35, and most preferably at least 70 nucleotides in length. It is, however, preferred that the nucleic acid probe is at least 100 nucleotides in length. For example, the nucleic acid probe may be at least 200 nucleotides, preferably at least 300 nucleotides, more preferably at least 400 nucleotides, or most preferably at least 500 nucleotides in length. Even longer probes may be used, *e.g.*, nucleic acid probes that are preferably at least 600 nucleotides, more preferably at least 700 nucleotides, even more preferably at least 800 nucleotides, or most preferably at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with ^{32}P, ^{3}H, ^{35}S, biotin, or avidin). Such probes are encompassed by the present invention.

A genomic DNA or cDNA library prepared from such other strains may, therefore, be screened for DNA that hybridizes with the probes described above and encodes a polypeptide having amylolytic enhancing activity. Genomic or other DNA from such other strains may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA that is homologous with SEQ ID NO: 1, or a subsequence thereof, the carrier material is preferably used in a Southern blot.

For purposes of the present invention, hybridization indicates that the nucleotide sequence hybridizes to a labeled nucleic acid probe corresponding to the mature polypeptide coding sequence of SEQ ID NO: 1; the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1; its full-length complementary strand; or a subsequence thereof; under very low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions can be detected using, for example, X-ray film.

Preferably, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 1. In another preferred aspect, the nucleic acid probe is nucleotides 55 to 1214 of SEQ ID NO: 1. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that

encodes the polypeptide of SEQ ID NO: 2, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 1.

For long probes of at least 100 nucleotides in length, very low to very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 μg/ml sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures for 12 to 24 hours optimally.

For long probes of at least 100 nucleotides in length, the carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS preferably at 45°C (very low stringency), more preferably at 50°C (low stringency), more preferably at 55°C (medium stringency), more preferably at 60°C (medium-high stringency), even more preferably at 65°C (high stringency), and most preferably at 70°C (very high stringency).

For short probes of about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as prehybridization, hybridization, and washing post-hybridization at about 5°C to about 10°C below the calculated T_m using the calculation according to Bolton and McCarthy (1962, *Proceedings of the National Academy of Sciences USA* 48:1390) in 0.9 M NaCl, 0.09 M Tris-HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1X Denhardt's solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per ml following standard Southern blotting procedures for 12 to 24 hours optimally.

For short probes of about 15 nucleotides to about 70 nucleotides in length, the carrier material is washed once in 6X SCC plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6X SSC at 5°C to 10°C below the calculated T_m.

Isolated X143 polypeptides are encoded by polynucleotides comprising or consisting of nucleotide sequences having a degree of sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1 of preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 96%, at least 97%, at least 98%, or at least 99%, which encode a polypeptide having retrograded starch degrading activity. See polynucleotide section herein.

In another preferred aspect, the nucleotide sequence comprises or consists of SEQ ID NO: 1. In another preferred aspect, the nucleotide sequence comprises or consists of the mature polypeptide coding sequence of SEQ ID NO: 1. In another preferred aspect, the nucleotide sequence comprises or consists of nucleotides 55 to 1214 of SEQ ID NO: 1.

In a further embodiment the nucleotide sequences encode polypeptides comprising or consisting of the amino acid sequence of SEQ ID NO: 2 or the mature polypeptide thereof,

which differ from SEQ ID NO: 1 or the mature polypeptide coding sequence thereof by virtue of the degeneracy of the genetic code.

In another aspect, the X143 polypeptide comprises a substitution, deletion, and/or insertion of one or more (or several) amino acids of the mature polypeptide of SEQ ID NO: 2, 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, 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 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 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, 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 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 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.

The total number of amino acid substitutions, deletions and/or insertions of the mature polypeptide of SEQ ID NO: 2, is not more than 10, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8 or 9.

Compositions

In a particular embodiment the composition comprises (1,2-dihydroxyethyl)-3,4-dihydroxyfuran-2(5H)-one (ascorbic acid) and an X143 polypeptide, particularly an X143 polypeptide from *Aspergillus*, more particularly from *A. nidulans*.

In one embodiment the composition further comprises one or more additional enzymes, *e.g.*, amylolytic enzymes.

Also contemplated are compositions for starch conversion purposes, which besides the X143 polypeptide and ascorbic acid, may also comprise a glucoamylase, and an acid alpha-amylases, and optinally a pullulanase.

The composition may also further comprise one or more (*e.g.*, several) proteins selected from the group consisting of a protease, and a phytase. In one aspect, the composition comprises or further comprises one or more (*e.g.*, several) proteins selected from the group consisting of a cellulase, a GH61 polypeptide having cellulolytic enhancing activity, a hemicellulase, an esterase, an expansin, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin. In another aspect, the cellulase is preferably one or more (*e.g.*, several) enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase. In another aspect, the hemicellulase is preferably one or more (*e.g.*, several) enzymes selected from the group consisting of an acetylmannan esterase, an acetylxylan 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.

In another aspect, the composition comprises one or more (*e.g.*, several) cellulolytic enzymes. In another aspect, the composition comprises or further comprises one or more (*e.g.*, several) hemicellulolytic enzymes. In another aspect, the enzyme composition comprises one or more (*e.g.*, several) cellulolytic enzymes and one or more (*e.g.*, several) hemicellulolytic enzymes. In another aspect, the composition comprises one or more (*e.g.*, several) enzymes selected from the group of cellulolytic enzymes and hemicellulolytic enzymes. In another aspect, the enzyme composition comprises an endoglucanase. In another aspect, the enzyme composition comprises a cellobiohydrolase. In another aspect, the enzyme composition comprises a beta-glucosidase. In another aspect, the enzyme composition comprises a polypeptide having cellulolytic enhancing activity. In another aspect, the enzyme composition comprises an endoglucanase and a polypeptide having cellulolytic enhancing activity. In another aspect, the enzyme composition comprises a cellobiohydrolase and a polypeptide having cellulolytic enhancing activity. In another aspect, the enzyme composition comprises a beta-glucosidase and a polypeptide having cellulolytic enhancing activity. In another aspect, the enzyme composition comprises an endoglucanase and a cellobiohydrolase. In another aspect, the enzyme composition comprises an endoglucanase and a beta-glucosidase. In another aspect, the enzyme composition comprises a cellobiohydrolase and a beta-glucosidase. In another aspect, the enzyme composition comprises an endoglucanase, a cellobiohydrolase, and a polypeptide having cellulolytic enhancing activity. In another aspect, the enzyme composition comprises an endoglucanase, a beta-glucosidase, and a polypeptide having cellulolytic enhancing activity. In another aspect, the enzyme composition comprises a cellobiohydrolase, a beta-glucosidase, and a polypeptide having cellulolytic enhancing activity. In another aspect, the enzyme composition comprises an endoglucanase, a cellobiohydrolase, and a beta-glucosidase. In another aspect, the enzyme composition comprises an endoglucanase, a cellobiohydrolase, a beta-glucosidase, and a polypeptide having cellulolytic enhancing activity.

One or more (*e.g.*, several) components of the enzyme composition may be wild-type proteins, recombinant proteins, or a combination of wild-type proteins and recombinant proteins. For example, one or more (*e.g.*, several) components may be native proteins of a cell, which is used as a host cell to express recombinantly one or more (*e.g.*, several) other components of the composition. One or more (*e.g.*, several) components of the composition may be produced as monocomponents, which are then combined to form the enzyme composition. The enzyme composition may be a combination of multicomponent and monocomponent protein preparations.

The polypeptides used in the methods of the present invention may be in any form suitable for use, such as, for example, a crude fermentation broth with or without cells removed, a cell lysate with or without cellular debris, a semi-purified or purified enzyme preparation, or a host cell as a source of the enzymes. The composition may be a dry powder or granulate, a

non-dusting granulate, a liquid, a stabilized liquid, or a stabilized protected enzyme. Liquid enzyme preparations may, for instance, be stabilized by adding stabilizers such as a sugar, a sugar alcohol or another polyol, and/or lactic acid or another organic acid according to established processes.

In one aspect, the one or more (*e.g.*, several) amylolytic enzymes comprise a commercial amylolytic enzyme preparation.

Amylolytic enzymes useful in the methods and compositions according to the invention

The enzyme(s) and polypeptides described below are to be used in an "effective amount" in processes of the present invention.

Alpha-Amylases

Any alpha-amylase may be used, such as of fungal, bacterial or plant origin. In a preferred embodiment the alpha-amylase is an acid alpha-amylase, *e.g.*, acid fungal or acid bacterial alpha-amylase. The term "acid alpha-amylase" means an alpha-amylase (EC 3.2.1.1) which added in an effective amount has activity optimum at a pH in the range of 3 to 7, preferably from 3.5 to 6, or more preferably from 4-5.

Bacterial Alpha-Amylases

An alpha-amylase for use in the present invention may be a bacterial alpha-amylase, *e.g.*, derived from *Bacillus*. In a preferred embodiment the *Bacillus* alpha-amylase is derived from a strain of *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus stearothermophilus*, or *Bacillus subtilis*, but may also be derived from other *Bacillus* sp.

Specific examples of alpha-amylases include the *Bacillus amyloliquefaciens* alpha-amylase of SEQ ID NO: 5 in WO 99/19467, the *Bacillus licheniformis* alpha-amylase of SEQ ID NO: 4 in WO 99/19467, and the *Bacillus stearothermophilus* alpha-amylase of SEQ ID NO: 3 in WO 99/19467 (all sequences are hereby incorporated by reference). In an embodiment the alpha-amylase may be an enzyme having a degree of identity of at least 60%, *e.g.*, at least 70%, at least 80%, at least 90%, 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 NOS: 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). Specific alpha-amylase variants are disclosed in U.S. Patent Nos. 6,093,562, 6,187,576, and 6,297,038 (hereby incorporated by reference) and include *Bacillus stearothermophilus* alpha-amylase (BSG alpha-amylase) variants having a deletion of one or two amino acids at 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 amino acid sequence of *Bacillus stearothermophilus* alpha-amylase set forth in SEQ ID NO: 3 disclosed in WO 99/19467 or the 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-amylases, which have a double deletion corresponding to delta(181-182) and further comprise a N193F substitution (also denoted I181* + 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

The alpha-amylase may be a hybrid alpha-amylase, *e.g.*, an alpha-amylase comprising 445 C-terminal amino acid residues of the *Bacillus licheniformis* alpha-amylase (shown in SEQ ID NO: 4 of WO 99/19467) 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+I201F+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-amylases): H154Y, A181T, N190F, A209V and Q264S and/or the deletion of two residues between positions 176 and 179, preferably the deletion of E178 and G179 (using SEQ ID NO: 5 of WO 99/19467 for position numbering).

In an embodiment, the bacterial alpha-amylase is dosed in an amount of 0.0005-5 KNU per g DS (dry solids), preferably 0.001-1 KNU per g DS, such as around 0.050 KNU per g DS.

Fungal Alpha-Amylases

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

A preferred acidic fungal alpha-amylase is an alpha-amylase which exhibits a high identity, *i.e.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 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 acid alpha-amylase is derived from a strain of *Aspergillus niger*. In a preferred embodiment the acid fungal alpha-amylase is an *Aspergillus niger* alpha-amylase disclosed as "AMYA_ASPNG" in the Swiss-prot/TeEMBL database under the primary accession no. P56271 and described in WO 89/01969 (Example 3 - incorporated by reference).

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

In a preferred embodiment the alpha-amylase is derived from *Aspergillus kawachii* (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 (SBD) and an alpha-amylase catalytic domain, or a variant thereof.

Fungal Hybrid Alpha-Amylases

In a preferred embodiment the fungal acid alpha-amylase is a hybrid alpha-amylase. Examples of fungal hybrid alpha-amylases include the ones disclosed in WO 2005/003311, U.S. Patent Application Publication No. 2005/0054071 (Novozymes), and WO 2006/069290 (Novozymes), which are hereby incorporated by reference. A hybrid alpha-amylase may comprise an alpha-amylase catalytic domain (CD) and a carbohydrate-binding domain/module (CBM), such as a starch binding domain (SBD), and optionally a linker.

Examples of hybrid alpha-amylases include those disclosed in Tables 1 to 5 of the examples in WO 2006/069290 including the variant with the catalytic domain JA118 and *Athelia rolfsii* SBD (SEQ ID NO: 100 in WO 2006/069290), *Rhizomucor pusillus* alpha-amylase with *Athelia rolfsii* AMG linker and SBD (SEQ ID NO: 101 in WO 2006/069290), *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 WO 2006/069290). Other hybrid alpha-amylases are listed in Tables 3, 4, 5, and 6 in Example 4 in U.S. application no. 11/316,535 and WO 2006/069290 (which are hereby incorporated by reference).

Other examples of hybrid alpha-amylases include those disclosed in U.S. Patent 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.

In particular embodiments of the invention, the alpha-amylase comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NOs: 5, 6, 7, 8, 9 and 10.

Other alpha-amylases exhibit a high degree of sequence identity to any of above mentioned alpha-amylases, *i.e.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or even 100% identity to the mature enzyme sequences disclosed above.

An acid alpha-amylase may be added in an amount of 0.001 to 10 AFAU/g DS, preferably from 0.01 to 5 AFAU/g DS, especially 0.3 to 2 AFAU/g DS or 0.001 to 1 FAU-F/g DS, preferably 0.01 to 1 FAU-F/g DS.

Commercial Alpha-Amylase Products

Preferred commercial compositions comprising alpha-amylase include MYCOLASE™ (DSM), BAN™, TERMAMYL™ SC, FUNGAMYL™, LIQUOZYME™ X, LIQUOZYME™ SC and SAN™ SUPER, SAN™ EXTRA L, LpHera® (Novozymes A/S) and any blends of these, and CLARASE™ L-40,000, DEX-LO™, SPEZYME™ FRED, SPEZYME™ AA, SPEZYME™ ALPHA, SPEZYME™ DELTA AA, GC358, GC980, SPEZYME™ CL and SPEZYME™ RSL (Danisco A/S), and the acid fungal alpha-amylase from *Aspergillus niger* referred to as SP288 (available from Novozymes A/S, Denmark).

Carbohydrate-Source Generating Enzymes (Saccharifying Enzymes)

The term "carbohydrate-source generating enzyme" includes glucoamylase (a glucose generator), beta-amylase and maltogenic amylase (both maltose generators) and also alpha-glucosidase, isoamylase and pullulanase. 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 of the invention for producing a fermentation product, such as ethanol. The generated carbohydrate may be converted directly or indirectly to the desired fermentation product, preferably ethanol. A mixture of carbohydrate-source generating enzymes may be used. Blends include mixtures comprising at least a glucoamylase and an alpha-amylase, especially an acid amylase, even more preferred an acid fungal alpha-amylase.

The ratio between glucoamylase activity (AGU) and acid fungal alpha-amylase activity (FAU-F) (*i.e.*, AGU per FAU-F) may in a preferred embodiment of the invention be between 0.1 and 100 AGU/FAU-F, in particular between 2 and 50 AGU/FAU-F, such as in the range from 10-40 AGU/FAU-F, especially when performing a one-step fermentation (raw starch hydrolysis - RSH), *i.e.*, when saccharification and fermentation are carried out simultaneously (*i.e.*, without a liquefaction step).

In a conventional starch-to-ethanol process (*i.e.*, including a liquefaction step) the ratio may preferably be as defined in EP 140410, especially when saccharification and fermentation are carried out simultaneously.

Glucoamylases

The term "glucoamylase" (1,4-alpha-D-glucan glucohydrolase, EC 3.2.1.3) is an enzyme, which catalyzes the release of D-glucose from the non-reducing ends of starch or related oligo- and polysaccharide molecules.

The glucoamylase may added in an amount of 0.001 to 10 AGU/g DS, preferably from 0.01 to 5 AGU/g DS, such as around 0.1, 0.3, 0.5, 1 or 2 AGU/g DS, especially 0.1 to 0.5 AGU/g DS or 0.02-20 AGU/g DS, preferably 0.1-10 AGU/g DS.

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 *Aspergillus niger* G1 or G2 glucoamylase (Boel *et al.*, 1984, *EMBO J.* 3(5): 1097-1102), or variants thereof, such as those disclosed in WO 92/00381, WO 00/04136 and WO 01/04273 (from Novozymes, Denmark); the *A. awamori* glucoamylase disclosed in WO 84/02921, *Aspergillus oryzae* glucoamylase (Hata et al., 1991, *Agric. Biol. Chem.* 55(4): 941-949), or 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, *Prot. 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, *Appl. Microbiol. Biotechnol.* 50: 323-330), *Talaromyces* glucoamylases, in particular derived from *Talaromyces duponti*, *Talaromyces emersonii* (WO 99/28448), *Talaromyces leycettanus* (U.S. Patent No. Re. 32,153), and *Talaromyces thermophilus* (U.S. Patent No. 4,587,215). *Gloeophyllum* sp. (US 2012/0214196).

Bacterial glucoamylases include glucoamylases from *Clostridium*, in particular *C. thermoamylolyticum* (EP 135138) and *C. thermohydrosulfuricum* (WO 86/01831), *Trametes cingulata*, *Pachykytospora papyracea,* and *Leucopaxillus giganteus*, all disclosed in WO 2006/069289; or *Peniophora rufomarginata* disclosed in PCT/US2007/066618; or a mixture thereof. A hybrid glucoamylase may be used in the present 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 (which hybrids are hereby incorporated by reference).

The glucoamylase may have a high degree of sequence identity to any of above mentioned glucoamylases, *i.e.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or even 100% identity to the mature enzymes sequences mentioned above.

Commercially available glucoamylase compositions include AMG 200L; AMG 300L; SAN™ SUPER, SAN™ EXTRA L, SPIRIZYME™ PLUS, SPIRIZYME™ FUEL, SPIRIZYME™ B4U, SPIRIZYME ULTRA™, AMG™NC, AMG™NC and AMG™ E (from Novozymes A/S, Denmark); OPTIDEX™ 300, GC480™ and GC147™ (from Genencor Int., USA); AMIGASE™

and AMIGASE™ PLUS (from DSM); OPTIMAX® 4060, G-ZYME™ G900, G-ZYME™ and G990 ZR (from Genencor Int.).

In particular embodiments of the invention, the gluco-amylase comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NOs: 11, 12 and 13.

Methods for Processing Starch Material

The composition according to the invention may be used for starch processes, in particular starch conversion, especially saccharification of starch.

Further, the compositions according to the invention are particularly useful in the production of sweeteners and ethanol (see, e.g., U.S. Patent No. 5,231,017, which is hereby incorporated by reference), such as fuel, drinking and industrial ethanol, from starch or whole grains.

The compositions and methods of the present invention can be used to hydrolyse starch and improve yield during saccharification of a starch substrate to fermentable sugars and convert the fermentable sugars to many useful substances, *e.g.*, fuel, potable ethanol, and/or fermentation products (*e.g.*, acids, alcohols, ketones, gases, and the like).

The present invention therefore relates to methods for saccharifying starch comprising: contacting the starch with at least a glucoamylase, an acidic alpha-amylase, an X143 polypeptide and L-ascorbic acid.

In particular the X143 polypeptide is from Aspergillus sp, more particular from A. nidulans or A. oryzae. In one particular embodiment the X143 polypeptide is selected from (i) a polypeptide having at least 75% sequence identity to the mature polypeptide of SEQ ID NO: 2; (ii) a polypeptide encoded by a polynucleotide having at least 75% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

In another specific embodiment the X143 polypeptide is selected from the group consisting of: a polypeptide having at least 80%, at least 85%, 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%, at least 99% or 100% sequence identity to the mature polypeptide of SEQ ID NO: 2.

In a particular aspect the saccharification is performed on ungelatinized starch. In this aspect the invention relates to saccharification methods or processes from starch-containing material without gelatinization of the starch-containing material (*i.e.*, uncooked starch-containing material). In one embodiment a process of the invention includes saccharifying (milled) starch-containing material, especially granular starch, below the initial gelatinization temperature, preferably in the presence of a carbohydrate-source generating enzyme, preferably a glucoamylase, to produce sugars that can optionally be fermented into the desired fermentation product by a suitable fermenting organism.

The term “below the initial gelatinization temperature” means below the lowest temperature where gelatinization of the starch commences. Starch heated in water typically

begins to gelatinize between 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 is the temperature at which birefringence is lost in 5% of the starch granules using the method described by Gorinstein and Lii, 1992, *Starch/Stärke* 44(12): 461-466.

The saccharification is preferably performed at a temperature in the range from 50-67°C, particularly from 55-66°C, more particularly from 58-62°C, such as 60°C.

In another particular aspect the the saccharification is performed on gelatinized starch. In this aspect the present invention relates to a method of saccharifying a starch containing material, comprising:

(a) liquefying the starch containing material in the presence of at least an acid alpha amylase;

(b) saccharifying the starch containing material in the presence of a glucoamylase, an X143 polypeptide, and L-ascorbic acid.

In a further particular embodiment the alpaha amylase and glucoamylase are selected from SEQ ID NO: 3 and SEQ ID NO: 4.

In a particular embodiment the alpha amylase is selected from the group consisting of: a polypeptide having at least 80%, at least 85%, 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%, at least 99% or 100% sequence identity to the polypeptide of SEQ ID NO: 3.

In a particular embodiment the glucoamylase is selected from the group consisting of: a polypeptide having at least 80%, at least 85%, 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%, at least 99% or 100% sequence identity to the polypeptide of SEQ ID NO: 4.

In a further particular embodiment the alpaha amylase and glucoamylase are selected from a polypeptide having 90% identity to SEQ ID NO: 3, and a polypeptide having 90% identity to SEQ ID NO: 4.

In a further particular embodiment the alpaha amylase and glucoamylase are selected from a polypeptide having 95% identity to SEQ ID NO: 3, and a polypeptide having 95% identity to SEQ ID NO: 4.

In either form of the methods according to the invention the saccharified starch may further be fermented to provide a fermentation product, in particular ethanol.

In one embodiment the saccharification and fermentation is performed simulataneously, also known as SSF.

Starch Processing

Native starch consists of microscopic granules, which are insoluble in water at room temperature. When aqueous starch slurry is heated, the granules swell and eventually burst, dispersing the starch molecules into the solution. At temperatures up to about 50°C to 75°C the swelling may be reversible. However, with higher temperatures an irreversible swelling called "gelatinization" begins. During this "gelatinization" process there is a dramatic increase in viscosity. 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 materials 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 allowing for further processing. 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 the production of, *e.g.*, syrups. Both dry and wet milling are well known in the art of starch processing and may be used in a process of the invention. Methods for reducing the particle size of the starch containing material are well known to those skilled in the art.

As the solids level is 30-40% in a typical industrial process, the starch has to be thinned or "liquefied" so that it can be suitably processed. This reduction in viscosity is primarily attained by enzymatic degradation in current commercial practice.

Liquefaction is carried out in the presence of an alpha-amylase, preferably a bacterial alpha-amylase and/or acid fungal alpha-amylase. In an embodiment, a phytase and/or a protease is also present during liquefaction. In an embodiment, viscosity reducing enzymes such as a xylanase and/or beta-glucanase is also present during liquefaction.

During liquefaction, the long-chained starch is degraded into branched and linear shorter units (maltodextrins) by an alpha-amylase. Liquefaction may be carried out as a three-step hot slurry process. The slurry is heated to between 60-95°C (*e.g.*, 70-90°C, such as 77-86°C, 80-85°C, 83-85°C) and an alpha-amylase is added to initiate liquefaction (thinning).

The slurry may in an embodiment be jet-cooked at between 95-140°C, *e.g.*, 105-125°C, for about 1-15 minutes, *e.g.*, about 3-10 minutes, especially around 5 minutes. The slurry is then cooled to 60-95°C and more alpha-amylase is added to obtain final hydrolysis (secondary liquefaction). The jet-cooking process is carried out at pH 4.5-6.5, typically at a pH between 5 and 6. The alpha-amylase may be added as a single dose, *e.g.*, before jet cooking.

The liquefaction process is carried out at between 70-95°C, such as 80-90°C, such as around 85°C, for about 10 minutes to 5 hours, typically for 1-2 hours. The pH is between 4 and 7, such as between 5.5 and 6.2. In order to ensure optimal enzyme stability under these conditions, calcium may optionally be added (to provide 1-60 ppm free calcium ions, such as

about 40 ppm free calcium ions). After such treatment, the liquefied starch will typically have a "dextrose equivalent" (DE) of 10-15.

Generally liquefaction and liquefaction conditions are well known in the art.

Examples of alpha-amylase are disclosed in the “Alpha-Amylases” section below.

Saccharification may be carried out using conditions well known in the art with a carbohydrate-source generating enzyme, in particular a glucoamylase, or a beta-amylase and optionally a debranching enzyme, such as an isoamylase or a pullulanase. For instance, a full saccharification step may last from about 24 to about 72 hours. However, it is common to do a pre-saccharification of 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 (SSF) process. Saccharification is typically carried out at a temperature in the range of 20-75°C, *e.g.*, 25-65°C and 40-70°C, typically around 60°C, and at a pH between about 4 and 5, normally at about pH 4.5.

In a simultaneous saccharification and fermentation (SSF) process, there is no holding stage for saccharification, rather, the yeast and enzymes are added together. 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 yeast, and enzyme(s), may be added together. SSF may typically be 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 about 32°C. In an embodiment fermentation is ongoing for 6 to 120 hours, in particular 24 to 96 hours.

In a typical saccharification process, maltodextrins produced during liquefaction are converted into dextrose by adding a glucoamylase and a debranching enzyme, such as an isoamylase (U.S. Patent No. 4,335,208) or a pullulanase. The temperature is lowered to 60°C, prior to the addition of the glucoamylase and debranching enzyme. The saccharification process proceeds for 24-72 hours. Prior to addition of the saccharifying enzymes, the pH is reduced to below 4.5, while maintaining a high temperature (above 95°C), to inactivate the liquefying alpha-amylase. This process reduces the formation of short oligosaccharide called "panose precursors," which cannot be hydrolyzed properly by the debranching enzyme. Normally, about 0.2-0.5% of the saccharification product is the branched trisaccharide panose (Glc pα1-6Glc pα1-4Glc), which cannot be degraded by a pullulanase. If active amylase from the liquefaction remains present during saccharification (*i.e.*, no denaturing), the amount of panose can be as high as 1-2%, which is highly undesirable since it lowers the saccharification yield significantly.

Other fermentation products may be fermented at conditions and temperatures well known to persons skilled in the art, suitable for the fermenting organism in question.

The fermentation product may be recovered by methods well known in the art, *e.g.*, by distillation. Examples of carbohydrate-source generating enzymes, including glucoamylases, are disclosed in the “Enzymes” section below.

In an embodiment, the starch-containing material is milled to reduce the particle size. In an embodiment the particle size is reduced to between 0.05-3.0 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 0.05-3.0 mm screen, preferably 0.1-0.5 mm screen.

The aqueous slurry may contain from 10-55 wt. % dry solids (DS), preferably 25-45 wt. % dry solids (DS), more preferably 30-40 wt. % dry solids (DS) of starch-containing material.

Conventional starch-conversion processes, such as liquefaction and saccharification processes are described, *e.g.*, in U.S. Patent No. 3,912,590, EP 252730 and EP 063909, which are incorporated herein by reference.

In an embodiment, the conversion process degrading starch to lower molecular weight carbohydrate components such as sugars or fat replacers includes a debranching step.

In the case of converting starch into a sugar, the starch is depolymerized. Such a depolymerization process consists of, *e.g.*, a pre-treatment step and two or three consecutive process steps, *i.e.*, a liquefaction process, a saccharification process, and depending on the desired end-product, an optional isomerization process.

When the desired final sugar product is, *e.g.*, high fructose syrup the dextrose syrup may be converted into fructose. After the saccharification process, the pH is increased to a value in the range of 6-8, *e.g.*, pH 7.5, and the calcium is removed by ion exchange. The dextrose syrup is then converted into high fructose syrup using, *e.g.*, an immobilized glucose isomerase.

The compositions according to the invention are useful in conventional starch processes comprising liquefaction and saccharification.

Production of Fermentation Products

Fermentable sugars (*e.g.*, dextrins, monosaccharides, particularly glucose) are produced from enzymatic saccharification. These fermentable sugars may be further purified and/or converted to useful sugar products. In addition, the sugars may be used as a fermentation feedstock in a microbial fermentation process for producing end-products, such as alcohol (*e.g.*, ethanol,and butanol), organic acids (*e.g.*, succinic acid, 3-HP and lactic acid), sugar alcohols (*e.g.*, glycerol), ascorbic acid intermediates (*e.g.*, gluconate, 2-keto-D-gluconate, 2,5-diketo-D-gluconate, and 2-keto-L-gulonic acid), amino acids (*e.g.*, lysine), proteins (*e.g.*, antibodies and fragment thereof).

In an embodiment, the fermentable sugars obtained during the saccharificationprocess are used to produce alcohol and particularly ethanol.

The organism used in fermentation will depend on the desired end-product. Typically, if ethanol is the desired end product yeast will be used as the fermenting organism. In some preferred embodiments, the ethanol-producing microorganism is a yeast and specifically *Saccharomyces* such as strains of *S. cerevisiae* (U.S. Patent No. 4,316,956). A variety of *S.*

cerevisiae are commercially available and these include but are not limited to FALI (Fleischmann's Yeast), SUPERSTART (Alltech), FERMIOL (DSM Specialties), RED STAR (Lesaffre) and Angel alcohol yeast (Angel Yeast Company, China). The amount of starter yeast employed in the methods is an amount effective to produce a commercially significant amount of ethanol in a suitable amount of time, (*e.g.*, to produce at least 10% ethanol from a substrate having between 25-40% DS in less than 72 hours). Yeast cells are generally supplied in amounts of about 10^4 to about 10^{12}, and preferably from about 10^7 to about 10^{10} viable yeast count per mL of fermentation broth. After yeast is added to the mash, it is typically subjected to fermentation for about 24-96 hours, *e.g.*, 35-60 hours. The temperature is between about 26-34°C, typically at about 32°C, and the pH is from pH 3-6, *e.g.*, around pH 4-5.

The fermentation may include, in addition to a fermenting microorganisms (*e.g.*, yeast), nutrients, and additional enzymes, including phytases. The use of yeast in fermentation is well known in the art.

In further embodiments, use of appropriate fermenting microorganisms, as is known in the art, can result in fermentation end product including, *e.g.*, glycerol, 1,3-propanediol, gluconate, 2-keto-D-gluconate, 2,5-diketo-D-gluconate, 2-keto-L-gulonic acid, succinic acid, lactic acid, amino acids, and derivatives thereof. More specifically when lactic acid is the desired end product, a *Lactobacillus* sp. (*L. casei*) may be used; when glycerol or 1,3-propanediol are the desired end-products *E. coli* may be used; and when 2-keto-D-gluconate, 2,5-diketo-D-gluconate, and 2-keto-L-gulonic acid are the desired end products, *Pantoea citrea* may be used as the fermenting microorganism. The above enumerated list are only examples and one skilled in the art will be aware of a number of fermenting microorganisms that may be used to obtain a desired end product.

The methods of the present invention can be implemented using any conventional starch processing apparatus configured to operate in accordance with the invention.

The term “fermenting organism” refers to any organism, including bacterial and fungal organisms, such as yeast and filamentous fungi, suitable for producing a desired fermentation product. Suitable fermenting organisms are able to ferment, *i.e.*, convert, fermentable sugars, such as arabinose, fructose, glucose, maltose, mannose, or xylose, directly or indirectly into the desired fermentation product.

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

Kluyveromyces fragilis or *Kluyveromyces marxianus*; and strains of *Schizosaccharomyces*, in particular *Schizosaccharomyces pombe*.

Preferred bacterial fermenting organisms include strains of *Escherichia*, in particular *Escherichia coli*, strains of *Zymomonas*, in particular *Zymomonas mobilis*, strains of *Zymobacter*, in particular *Zymobactor 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), *Thermoanarobacter ethanolicus*, *Thermoanaerobacter mathranii*, or *Thermoanaerobacter thermosaccharolyticum*. Strains of *Lactobacillus* are also envisioned as are strains of *Corynebacterium glutamicum R*, *Bacillus thermoglucosidaisus*, 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 one embodiment the fermenting organism is added to the fermentation medium so that the viable fermenting organism, such as yeast, 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 $5x10^7$.

Yeast is the preferred fermenting organism for ethanol fermentation. Preferred are strains of *Saccharomyces*, especially strains of the species *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.

Commercially available yeast include LNF SA-1, LNF BG-1, LNF PE-2,and LNF CAT-1 (available from LNF Brazil), 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).

The fermenting organism capable of producing a desired fermentation product from fermentable sugars 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 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.

It is well known in the art that the organisms described above can also be used to produce other substances, as described herein.

The fermenting microorganism is typically added to the degraded starch or hydrolysate and the fermentation is performed for about 8 to about 96 hours, such as about 24 to about 60 hours. The temperature is typically between about 26°C to about 60°C, in particular about 32°C to 45°C, and at about pH 3 to about pH 8, such as around pH 4-5, 6, or 7.

In a preferred aspect, the yeast and/or another microorganism is applied to the degraded starch material and the fermentation is performed for about 12 to about 96 hours, such as typically 24-60 hours. In a preferred aspect, the temperature is preferably between about 20°C to about 60°C, more preferably about 25°C to about 50°C, and most preferably about 32°C to about 50°C, in particular about 32°C or 50°C, and the pH is generally from about pH 3 to about pH 7, preferably around pH 4-7. However, some fermenting organisms, *e.g.*, bacteria, have higher fermentation temperature optima. Yeast or another microorganism is preferably applied in amounts of approximately 10^5 to 10^{12}, preferably from approximately 10^7 to 10^{10}, especially approximately 2 x 10^8 viable cell count per ml of fermentation broth. Further guidance in respect of using yeast for fermentation can be found in, *e.g.*, "The Alcohol Textbook" (Editors K. Jacques, T.P. Lyons and D.R. Kelsall, Nottingham University Press, United Kingdom 1999), which is hereby incorporated by reference.

For ethanol production, following the fermentation the fermented slurry is distilled to extract the ethanol. The ethanol obtained according to the methods of the invention can be used as, *e.g.*, fuel ethanol, drinking ethanol, *i.e.*, potable neutral spirits, or industrial ethanol.

Starch-Containing Materials

Any suitable starch-containing starting material may be used in a process of the present invention. The starting material is generally selected based on the desired fermentation product. Examples of starch-containing starting materials, suitable for use in the processes of the present invention, include barley, beans, cassava, cereals, corn, milo, peas, potatoes, rice, rye, sago, sorghum, sweet potatoes, tapioca, wheat, and whole grains, or any mixture thereof. The starch-containing material may also be a waxy or non-waxy type of corn and barley. In a preferred embodiment the starch-containing material is corn. In a preferred embodiment the starch-containing material is wheat.

In a particular embodiment the starch is corn.

The present invention is further described by the following examples that should not be construed as limiting the scope of the invention.

Examples

Example 1: Effect of reducing agent (L-ascorbic acid) on *Aspergillus nidulans* X143 polypeptide hydrolysis of starch

Powdered corn starch was combined in a plastic flask with tap water to produce 40.8% solids starch slurry. The slurry was adjusted to a pH of 4.5 using a 0.1M HCl solution. 20 mL aliquots of this slurry were added to 30 mL Nalgene screw top plastic bottles and the weights were recorded. Addition of enzymes and appropriate volume of water were added to the final 35% solids starch slurry. Prior to hydrolysis, copper sulfate was added to *Aspergillus nidulans* X143 polypeptide in 1:1 mol ratio. For the hydrolysis of starch, *Aspergillus nidulans* X143 polypeptide was dosed at 0.05 mg enzyme protein per gram of starch dry substance (mg EP/g DS), with or without reducing agent compound (L-ascorbic acid) at a concentration of 0.25, 0.5 or 1.0 mM. Each of the enzyme-treated bottles was given an alpha amylase (AA) dosage of 0.0375 mg EP/g DS and a glucoamylase (GA) dose of 0.075 mg EP/ g DS. For control, AA and GA were dosed without reducing agent compound (L-ascorbic acid) and without X143 polypeptide. Two bottles were left un-dosed as blank. Once dosed, the bottles were placed into two rotisserie ovens which were set to 60°C.

After 48 hours reaction, 2 mL aliquots were removed from each flask and placed into 15 mL conical spin tubes. Each sample was deactivated with 18 µL of 1M HCl. After deactivation, the samples were vortexed thoroughly. The supernatant in each tube was filtered through a 0.45 µm filter disc into microcentrifuge tubes for HPLC analysis. These microcentrifuge tubes were then placed in boiling water for 10 minutes to completely deactivate any enzyme activity. HPLC samples were prepared in vials by diluting 50 µL of the filtered sample in each Eppendorf tube with 950 µL of 0.005M H2SO4 to yield a 20:1 dilution factor.

HPLC Analysis

HPLC system	Agilent's 1100/1200 series with Chem station software
	Degasser, Quaternary Pump, Auto-Sampler, Column Compartment w/ Heater
	Refractive Index Detector (RI)
Column	Bio-Rad HPX- 87H Ion Exclusion Column, 300mm x 7.8mm, part #125-0140
	Bio-Rad guard cartridge cation H, part #125-0129, Holder part #125-0131
Method	0.005 M H_2SO_4 mobile phase
	Flow rate: 0.6 ml/min
	Column temperature: 65°C
	RI detector temperature: 55°C

The method quantified analytes using calibration standards for DP4+, DP3, DP2, glucose, fructose, acetic acid, lactic acid, glycerol and ethanol (% w/v). A four point calibration including the origin was used for quantification.

Glucose concentrations determined by HPLC were used as a measurement for X143 polypeptide activity on hydrolysis of starch. The resultant glucose production was used to calculate the percentage boosting of activity for each reaction. Percentage boosting of activity was calculated from the ratio of net concentration of glucose after subtracting the background glucose concentration from a control in which no X143 polypeptide and ascorbic acid were added. Data were processed using MICROSOFT EXCEL™ software (Microsoft, Richland, WA, USA).

The reducing agent compound evaluated includes L-ascorbic acid. The compound was obtained from Sigma-Aldrich Co. (St. Louis, Missouri, USA).

As shown in Table 1, addition of X143 polypeptide with the presence of 0.25 and 0.5 mM of L-ascorbic acid enhanced the starch hydrolysis activity of AA and GA. Without the presence of L-ascorbic acid, X143 polypeptide showed much lower boosting of starch hydrolysis activity.

Table 1

	Ascorbic acid (mM)	Glucose (g/L)	Boosting (%)
AA+GA	0.0	276.1	0.0%
AA+GA+X143		286.2	3.7%
AA+GA	0.25	288.5	4.5%
AA+GA+X143		296.2	7.3%
AA+GA	0.5	284.0	2.9%
AA+GA+X143		297.3	7.7%

The invention described and claimed herein is not to be limited in scope by the specific aspects herein disclosed, since these aspects are intended as illustrations of several aspects of the invention. Any equivalent aspects are intended to be within the scope of this invention. Indeed, 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. In the case of conflict, the present disclosure including definitions will control.

Claims

1. A method for saccharifying starch comprising: contacting the starch with at least a glucoamylase, an acidic alpha-amylase, an X143 polypeptide and L-ascorbic acid.

2. The method according to claim 1, wherein the X143 polypeptide is selected from: (i) a polypeptide having at least 75% sequence identity to the mature polypeptide of SEQ ID NO: 2; (ii) a polypeptide encoded by a polynucleotide having at least 75% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

3. The method according to claim 1 or 2, wherein the L-ascorbic acid added in a range from 0.1 mM to to 10.0 mM, particularly from 0.2 mM to 5.0 mM, more particularly from 0.25 mM to 0.75 mM.

4. The methods according to any of claims 1-3, wherein the X143 polypeptide is selected from the group consisting of: a polypeptide having at least 80%, at least 85%, 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%, at least 99% or 100% sequence identity to the mature polypeptide of SEQ ID NO: 2.

5. The method according to any of claims 1-4, wherein the starch is selected from the group consisting of barley, beans, cassava, cereals, corn, milo, peas, potatoes, rice, rye, sago, sorghum, sweet potatoes, tapioca, wheat, and whole grains, or any mixture thereof.

6. The method according to claim 5, wherein the starch is corn starch.

7. The method according to any of claims 1-6, wherein further enzyme activities are added selected from the group consisting of a pullulanase, a protease, a phytase, and optionally one or more proteins selected from the group consisting of a cellulase, a GH61 polypeptide having cellulolytic enhancing activity, a hemicellulase, an esterase, an expansin, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, and a swollenin.

8. The method according to any of claims 1-7, wherein the starch is un-gelatinized.

9. The method according to any of claims 1-8, wherein saccharification is performed at a temperature in the range from 50-67°C, particularly from 55-66°C, more particularly from 58-62°C, such as 60°C.

10. A method of saccharifying a starch containing material, comprising:

(a) liquefying the starch containing material in the presence of at least an acid alpha amylase; and

(b) saccharifying the starch containing material in the presence of a glucoamylase, an X143 polypeptide, and L-ascorbic acid.

11. The method according to claim 8, wherein the X143 polypeptide is selected from: (i) a polypeptide having at least 75% sequence identity to the mature polypeptide of SEQ ID NO: 2; (ii) a polypeptide encoded by a polynucleotide having at least 75% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

12. The method according to claim 10 or 11, wherein the L-ascorbic acid added in a range from 0.1 mM to 10.0 mM, particularly from 0.2 mM to 5.0 mM, , more particularly from 0.25 mM to 0.75 mM.

13. The method according to any of the claims 10-12, wherein the X143 polypeptide is selected from the group consisting of: a polypeptide having at least 80%, at least 85%, 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%, at least 99% or 100% sequence identity to the mature polypeptide of SEQ ID NO: 2.

14. The method according to any of claims 10-13, wherein the starch is selected from the group consisting of barley, beans, cassava, cereals, corn, milo, peas, potatoes, rice, rye, sago, sorghum, sweet potatoes, tapioca, wheat, and whole grains, or any mixture thereof.

15. The method according to any of claims 10-14, wherein further enzyme activities are added selected from the group consisting of a pullulanase, a protease, a phytase, and optionally one or more proteins selected from the group consisting of a cellulase, a GH61 polypeptide having cellulolytic enhancing activity, a hemicellulase, an esterase, an expansin, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, and a swollenin.

16. The method according to any of the preceding claims, wherein the alpaha amylase and glucoamylase are selected from a polypeptide having 90% identity to SEQ ID NO: 3, and a polypeptide having 90% identity to SEQ ID NO: 4.

17. A use of an X143 polypeptide in combination with at least an acid alpha amylase, a glucoamylase and L-ascorbic acid for saccharifying starch.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/021447

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P19/02 C12P19/12 C12P19/14
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, FSTA, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ISAKSEN ET AL: "A C4-oxidizing lytic polysaccharide monooxygenase cleaving both cellulose and cello-oligosaccharides", THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 289, 31 January 2014 (2014-01-31), pages 2632-2642, XP055153074, * See pages 2632-2633 (Abstract/ascorbic acid) *	1-17

Y	DIMAROGONA ET AL: "Recalcitrant polysaccharide degradation by novel oxidative biocatalysts", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 97, 2013, pages 8455-8465, XP035328600, * See page 8457 (right column/ascorbic acid) *	1-17

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 May 2015	26/05/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Korsner, Sven-Erik

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SAYKHEDKAR ET AL: "A time course analysis of the extracellular proteome of Aspergillus nidulans growing on sorghum stover", BIOTECHNOLOGY FOR BIOFUELS, vol. 5, 2012, pages 1-17, XP021133644, * See page 12 (right column/ascorbate) *	1-17
A	DESWAL ET AL: "Optimization of cellulase production by a brown rot fungus Fomitopsis sp. RCK2010 under solid state fermentation", BIORESOURCE TECHNOLOGY, vol. 102, 2011, pages 6065-6072, XP002739502, * See page 6071 (Table 5/ascorbic acid) *	1-17
A	DUBOK CHOI ET AL: "Sugar production from raw seaweed using the enzyme method", JOURNAL OF INDUSTRIAL AND ENGINEERING CHEMISTRY, vol. 15, 2009, pages 12-15, XP026081950, * See page 12 (Abstract) *	1-17
A,P	LEGGIO ET AL: "Structure and boosting activity of a starch-degrading lytic polysaccharide monooxygenase", NATURE COMMUNICATIONS, vol. 6, 22 January 2015 (2015-01-22), pages 1-9, XP002739503, DOI: 10.1038/ncomms6961 * See page 2 (Introduction) and page 3 (Figure 1); early online publication *	1-17
A,P	CHERAS: "Study of the mechanism of catalysis of two new LPMOs for lignocellulose degradation (translated)", National Technical University of Athens Thesis, April 2014 (2014-04), page (cover + iii), XP002739504, Retrieved from the Internet: URL:http://dspace.lib.ntua.gr/bitstream/handle/123456789/38853/cheras_lpmos.pdf?sequence=1 [retrieved on 2015-05-12] * See the Abstract in English (ascorbic acid) *	1-17
Y,P	WO 2014/197705 A1 (NOVOZYMES A/S) 11 December 2014 (2014-12-11) * See page 82 (Table 1) - also present in its priority document *	1-17

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2015/021447

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014197705 A1	11-12-2014	NONE	