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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
31 May 2018 (31.05.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/098381 A1

(51) International Patent Classification:
C12P 7/06 (2006.01) *C12N 1/19* (2006.01)

(21) International Application Number:
PCT/US2017/063159

(22) International Filing Date:
22 November 2017 (22.11.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/426,182 23 November 2016 (23.11.2016) US

(71) Applicants: **NOVOZYMES A/S** [DK/DK]; Krogshoejvej 36, 2880 Bagsvaerd (DK). **MICROBIOGEN PTY. LTD.** [AU/AU]; c/o Peter Hunt & Associates, Level 13, 74 Castlereagh Street, Sydney, New South Wales 2000 (AU).

(72) Inventor; and

(71) Applicant *(for BW only)*: **YAZDI, Hamid, Rismani** [IR/US]; 77 Perry Chapel Church Road, Franklinton, North Carolina 27525 (US).

(72) Inventors: **BELL, Philip, John, Livingstone**; 18 Apps Avenue, Turramurra, New South Wales 2074 (AU). **ATTFIELD, Paul, Victor**; 20 Telopea Street, Mount Colah, New South Wales 2079 (AU). **CATLETT, Michael, Glenn**; 1445 Drew Avenue, Davis, California 95618 (US).

(74) Agent: **FECHTER, Eric** et al.; Novozymes North America, Inc., 77 Perry Chapel Church Road, Franklinton, North Carolina 27525 (US).

(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, DJ, DK, DM, DO,

(54) Title: IMPROVED YEAST FOR ETHANOL PRODUCTION

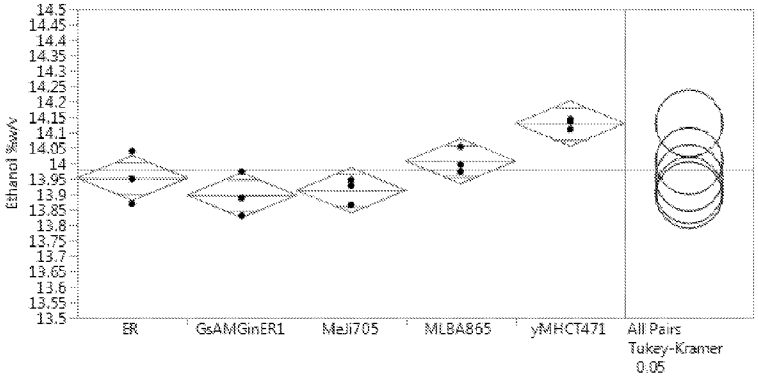


FIG. 2

(57) Abstract: Described herein are processes for producing ethanol from starch-containing material using a *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism strain having properties that are about the same as that of the deposited *Saccharomyces cerevisiae* strain or a derivative of Saccharomyces cerevisiae strain MBG4985 (e.g., a recombinant derivative expressing an alpha-amylase and/or a glucoamylase)having the defining characteristics of strain *Saccharomyces cerevisiae* yeast MBG4985. Also described is *Saccharomyces cerevisiae* strain MBG4985 deposited under the Budapest Treaty and having accession No. NRRL Y67342 or a derivative of strain NRRL Y67342 which exhibits one or more properties or defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985. Also described are compositions comprising the

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DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, 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))*

Saccharomyces yeast and naturally occurring and/or non-naturally occurring components.

IMPROVED YEAST FOR ETHANOL PRODUCTION

REFERENCE TO A SEQUENCE LISTING

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

REFERENCE TO A DEPOSIT OF BIOLOGICAL MATERIAL

This application contains a reference to a deposit of biological material, which is incorporated herein by reference.

FIELD

Described herein are processes, e.g., including a liquefaction step, for producing ethanol from starch-containing material using yeast for converting fermentable sugars into ethanol. Also described are *Saccharomyces* yeast strains having improved ability to ferment sugars to ethanol, methods for the production of *Saccharomyces* yeast strains having improved ability to ferment sugars to ethanol, and the use of *Saccharomyces* yeast strains having improved ability to ferment sugars to ethanol in the production of ethanol. Finally, also described are compositions comprising the *Saccharomyces* yeast strains and naturally occurring and/or non-naturally occurring components.

BACKGROUND

Production of ethanol from starch-containing material is well-known in the art. The production of ethanol as a bio-fuel has become a major industry, with in excess of 24 billion gallons of ethanol being produced worldwide in 2014.

The most commonly industrially used commercial process, often referred to as a "conventional process", includes liquefying gelatinized starch at high temperature (about 85°C) using typically a bacterial alpha-amylase, followed by simultaneous saccharification and fermentation (SSF) carried out anaerobically in the presence of typically a glucoamylase and a *Saccharomyces cerevisae* yeast.

Yeasts which are used for production of ethanol for use as fuel, such as in the corn ethanol industry, require several characteristics to ensure cost effective production of the ethanol. These characteristics include ethanol tolerance, low by-product yield, rapid fermentation, and the ability to limit the amount of residual sugars remaining in the ferment. Such characteristics have a marked effect on the viability of the industrial process.

Yeast of the genus *Saccharomyces* exhibits many of the characteristics required for production of ethanol. In particular, strains of *Saccharomyces cerevisiae* are widely used for the production of ethanol in the fuel ethanol industry. Strains of *Saccharomyces cerevisiae*

that are widely used in the fuel ethanol industry can produce high yields of ethanol under fermentation conditions found in, for example, the fermentation of corn mash. An example of such a strain is the yeast used in commercially available ethanol yeast product called ETHANOL RED™.

Strains of *Saccharomyces cerevisiae* are used in the fuel ethanol industry to ferment sugars such as glucose, fructose, sucrose and maltose to produce ethanol via the glycolytic pathway. These sugars are obtained from sources such as corn and other grains, sugar juice, molasses, grape juice, fruit juices, and starchy root vegetables and may include the breakdown of cellulosic material into glucose.

Although strains of *Saccharomyces cerevisiae* currently used in the fuel ethanol industry are well suited to ethanol production, there is an increasing need for improvements in the efficiency of ethanol production owing to the increased demand for ethanol as a fuel, and the increased availability of starch in new strains of corn.

There is therefore a need for new robust yeast strains of *Saccharomyces* capable of improving the efficiency of ethanol production in industrial scale fermentation.

Further, despite significant improvement of ethanol production processes over the past decade there is still a desire and need for providing processes of producing ethanol from starch-containing material and yeast that can be used in commercial scale ethanol processes.

SUMMARY

Described herein are, *inter alia*, processes for producing ethanol from starch-containing material and yeast suitable for use in such processes.

A first aspect relates to processes for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using an alpha-amylase;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is:

Saccharomyces cerevisiae strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces cerevisiae* strain MBG4985 having defining characteristics of *Saccharomyces cerevisiae* strain MBG4985. In some embodiments, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

As used herein, the terms "properties" and "defining characteristics" of *Saccharomyces cerevisiae* strain MBG4985 include at least increased ethanol boost (i.e., ethanol yield) compared to Ethanol Red™ under the same process conditions. Other "properties" and "defining characteristics" include, *inter alia*, reduced acetaldehyde production, increased temperature tolerance, and decreased glycerol production. A fermenting organism described herein, e.g., used in a process described herein may have one or more the above mentioned "properties" and "defining characteristics".

The fermenting organism organism described herein, especially *Saccharomyces cerevisiae* yeast, having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a derivative of *Saccharomyces cerevisiae* strain MBG4985, having one or more, such as all of the following properties and/or defining characteristics of *Saccharomyces cerevisiae* strain MBG4985:

increased ethanol boost (i.e., ethanol yield) compared to ETHANOL RED™ under the same process conditions;

reduced acetaldehyde production compared to ETHANOL RED™ under the same process conditions;

increased temperature tolerance compared to ETHANOL RED™ under the same process conditions;

decreased glycerol production compared to ETHANOL RED™ under the same process conditions.

A fermenting organism described herein may have one or more, such as all, of the above mentioned "properties" or "defining characteristics".

According to the ethanol production process, liquefaction in step i) is carried out by subjecting starch-containing material at a temperature above the initial gelatinization temperature, typically between 80-90°C, using an alpha-amylase. The pH in liquefaction is preferably between 4.5 and 6.0, such as between 4.8 and 5.8. Examples of alpha-amylase can be found below in the "Alpha-Amylase Present and/or Added During Liquefaction"-section. In one embodiment, the alpha-amylase is a thermostable bacterial alpha-amylase. In one embodiment, the alpha-amylase is from the genus *Bacillus*, such as a strain of *Bacillus stearothermophilus*, in particular a variant of a *Bacillus stearothermophilus* alpha-amylase, such as the one shown in SEQ ID NO: 3 in WO 99/019467 or SEQ ID NO: 1 herein. Examples of suitable *Bacillus stearothermophilus* alpha-amylase variants can be found below in the "Thermostable Alpha-Amylase"-section and include one from the following group of *Bacillus stearothermophilus* alpha-amylase variants with the following mutations: I181*+G182*, and

optionally N193F, and further one of the following substitutions or combinations of substitutions

E129V+K177L+R179E;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

V59A+ Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V;

V59A+ E129V+ K177L+ R179E+ Q254S+ M284V; and

E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 for numbering).

Examples of other suitable *Bacillus stearothermophilus* alpha-amylases having increased thermostability compared to a reference alpha-amylase (*Bacillus stearothermophilus* alpha-amylase with the mutations I181*+G182*, and optionally substitution N193F, truncated at the C-terminal to be 485-495 amino acids long, such as about 491 amino acids long) at pH 4.5 and 5.5, 0.12 mM $CaCl_2$ can be found in WO 2011/082425 hereby incorporated by reference. (See also Example 1 below)

Liquefaction in step i) may be carried out using a combination of alpha-amylase and protease. The protease may be a protease having a thermostability value of more than 20% determined as Relative Activity at 80ºC/70ºC. Examples of suitable proteases are described below in the section “Protease Present and/or Added During Liquefaction”.

The protease may be of fungal origin, such as of filamentous fungus origin. Specific examples of suitable fungal proteases are protease variants of metallo protease derived from a strain of the genus *Thermoascus*, preferably a strain of *Thermoascus aurantiacus*, especially the strain *Thermoascus aurantiacus* CGMCC No. 0670 disclosed as the mature part of SEQ ID NO: 2 disclosed in WO 2003/048353 or the mature part of SEQ ID NO: 1 in WO 2010/008841 or SEQ ID NO: 3 herein with one of the following substitutions or combinations of substitutions:

D79L+S87P+A112P+D142L;

D79L+S87P+D142L; and

A27K+ D79L+ Y82F+S87G+D104P+A112P+A126V+D142L.

Examples of other suitable protease variants can be found in WO 2011/072191 hereby incorporated by reference (See also Example 2 below).

Suitable proteases also include bacterial proteases. A suitable bacterial protease may be derived from a strain of *Pyrococcus*, preferably a strain of *Pyrococcus furiosus*. In one embodiment, the protease is the one shown in SEQ ID NO: 1 in US 6,358,726 or SEQ ID NO: 13 herein.

In one embodiment, 0.5-50 micro gram *Pyrococcus furiosus* protease per gram DS, such as 1-5 micro gram *Pyrococcus furiosus* protease per gram DS, such as about 1.5 or 3

micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step i).

In one embodiment, the alpha-amylase and/or the protease added in the liquefaction step i) is further combined with a glucoamylase. Thus, a glucoamylase may also be present and/or added during liquefaction step i). The glucoamylase may be thermostable. That means that the glucoamylase has a heat stability at 85ºC, pH 5.3, of at least 20%, such as at least 30%, preferably at least 35% determined as described in Example 4 (heat stability). In one embodiment, the glucoamylase present and/or added in liquefaction has a relative activity pH optimum at pH 5.0 of at least 90%, preferably at least 95%, preferably at least 97%. In one embodiment, the glucoamylase has a pH stability at pH 5.0 of at least at least 80%, at least 85%, at least 90% determined as described in Example 4 (pH optimum).

A suitable glucoamylase present and/or added in liquefaction step i) may be derived from a strain of the genus *Penicillium*, such as a strain of *Penicillium oxalicum* disclosed as SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NOs: 9 or 14 herein. In one embodiment, the glucoamylase is a variant of the *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 2 in WO 2011/127802 having a K79V substitution (using the mature sequence shown in SEQ ID NO: 14 for numbering), such as a variant disclosed in WO 2013/053801. In one embodiment, the *Penicillium oxalicum* glucoamylase has a K79V substitution (using SEQ ID NO: 14 for numbering) and further one of the following substitutions or combinations of substitutions:

P11F + T65A + Q327F; and

P2N + P4S + P11F + T65A + Q327F (using SEQ ID NO: 14 for numbering).

Examples of other suitable *Penicillium oxalicum* glucoamylase variants can be found in WO 2013/053801 incorporated by reference (See also Example 15 below).

In one embodiment, the glucoamylase, such as a *Penicillium oxalicum* glucoamylase variant, used in liquefaction has a thermostability determined as DSC Td at pH 4.0 as described in Example 15 of at least 70ºC, preferably at least 75ºC, such as at least 80ºC, such as at least 81ºC, such as at least 82ºC, such as at least 83ºC, such as at least 84ºC, such as at least 85ºC, such as at least 86ºC, such as at least 87%, such as at least 88ºC, such as at least 89ºC, such as at least 90ºC. In one embodiment, the glucoamylase, such as a *Penicillium oxalicum* glucoamylase variant has a thermostability determined as DSC Td at pH 4.0 as described in Example 15 in the range between 70ºC and 95ºC, such as between 80ºC and 90ºC.

In one embodiment, the glucoamylase, such as a *Penicillium oxalicum* glucoamylase variant, used in liquefaction has a thermostability determined as DSC Td at pH 4.8 as described in Example 15 of at least 70ºC, e.g., at least 75ºC, at least 80ºC, at least 81ºC, at least 82ºC, at least 83ºC, at least 84ºC, at least 85ºC, at least 86ºC, at least 87%, at least

88ºC, at least 89ºC, at least 90ºC, or at least 91ºC. In one embodiment, the glucoamylase, such as a *Penicillium oxalicum* glucoamylase variant has a thermostability determined as DSC Td at pH 4.8 as described in Example 15 in the range between 70ºC and 95ºC, such as between 80ºC and 90ºC.

In one embodiment, the glucoamylase, such as a *Penicillium oxalicum* glucoamylase variant, used in liquefaction has a residual activity determined as described in Example 16 of at least 100%, such as at least 105%, at least 110%, at least 115%, at least 120%, or at least 125%. In one embodiment, the glucoamylase, such as a *Penicillium oxalicum* glucoamylase variant has a thermostability determined as residual activity as described in Example 16 in the range between 100% and 130%.

Further, in one embodiment, a pullulanase may be present during liquefaction in combination with an alpha-amylase, a protease and/or a glucoamylase.

In one embodiment, a glucoamylase may be present and/or added in saccharification and/or fermentation or simultaneous saccharification and fermentation. The glucoamylase may not be the same as the thermostable glucoamylase used in liquefaction.

In one embodiment, the glucoamylase expressed by the derivative of *Saccharomyces cerevisiae* strain MBG4985, present and/or added in saccharification and/or fermentation is of fungal origin, such as of filamentous fungus origin. In one embodiment, the glucoamylase is derived from a strain of *Aspergillus*, e.g., *A. niger*, *A. awamori*, or *A. oryzae*; or a strain of *Trichoderma*, e.g., *T. reesei*; or a strain of *Talaromyces*, e.g., *T. emersonii*, or a strain of *Pycnoporus*, or a strain of *Gloeophyllum*, such as *G. serpiarium* or *G. trabeum,* or a strain of *Nigrofomes.*

In one embodiment, the glucoamylase expressed by the derivative of *Saccharomyces cerevisiae* strain MBG4985, present and/or added in saccharification and/or fermentation is derived from *Talaromyces emersonii*, such as the one shown in SEQ ID NO: 19 herein. In another embodiment the glucoamylase is derived from *Gloeophyllum serpiarium*, such as the one shown in SEQ ID NO: 15 herein. In another embodiment the glucoamylase is derived from *Gloeophyllum trabeum* such as the one shown in SEQ ID NO: 17 herein.

In one embodiment, the glucoamylase expressed by the derivative of *Saccharomyces cerevisiae* strain MBG4985, present and/or added in saccharification and/or fermentation is a variant of the *Gloeophyllum trabeum* glucoamylase disclosed in WO 2014/177546 (hereby incorporated by reference), especially a variant having one of the following substitutions or combinations of substitutions: V59A; S95P; A121P; T119W; S95P+A121P; V59A+S95P; S95P+T119W; V59A+S95P+A121P; and S95P+T119W+A121P, especially S95P+A121P (using SEQ ID NO: 17 herein for numbering).

In one embodiment, the glucoamylase is in combination with an alpha-amylase and optionally a protease. The alpha-amylase may be of fungal or bacterial origin.

The alpha-amylase expressed by the derivative of *Saccharomyces cerevisiae* strain MBG4985, present and/or added in combination with a glucoamylase may be derived from a strain of the genus *Rhizomucor*, preferably a strain the *Rhizomucor pusillus,* such as the one shown in SEQ ID NO: 3 in WO 2013/006756, such as a *Rhizomucor pusillus* alpha-amylase hybrid having an *Aspergillus niger* linker and starch-bonding domain, such as the one shown in SEQ ID NO: 16 herein.

In one embodiment, the alpha-amylase expressed by the derivative of *Saccharomyces cerevisiae* strain MBG4985, present and/or added is derived from a strain of *Rhizomucor pusillus*, e.g., with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), such as the one disclosed as SEQ ID NO: 16 herein, and may have one or more of the following substitutions: G128D, D143N, such as G128D+D143N (using SEQ ID NO: 16 for numering).

In one embodiment, a protease is present and/or added in saccharification and/or fermentation, or SSF, which may result in increased ethanol yield. As described, e.g., in US patent No. 5, 231,017 (hereby incorporated by reference) the protease may, e.g., be an acid fungal protease. A protease may also be present and/or added in saccharification and/or fermentation or SSF to improve the oil yield.

In one embodiment, a cellulolytic enzyme composition is present and/or added in saccharification, fermentation or simultaneous saccharification and fermentation (SSF). Examples of such compositions can be found in the “Cellulolytic Enzyme Composition present and/or added during Saccharification and/or Fermentation”-section below. In one embodiment, the cellulolytic enzyme composition is present and/or added together with a glucoamylase, such as one disclosed in the “Glucoamylase Present And/Or Added in Saccharification and/or Fermentation”-section below.

A second aspect relates to processes of producing ethanol from starch-containing material, such as granular starch, comprising:

(i) saccharifying a starch-containing material at a temperature below the initial gelatinization temperature; and

(ii) fermenting using a fermentation organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces cerevisiae* strain MBG4985 having defining characteristics of *Saccharomyces cerevisiae* strain MBG4985. In some embodiments, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, is a process of producing ethanol from starch-containing material, such as granular starch, comprising:

(i) saccharifying a starch-containing material at a temperature below the initial gelatinization temperature; and

(ii) fermenting using a fermentation organism;

wherein saccharification and/or fermentation is done in the presence of the following enzymes: glucoamylase and alpha-amylase, and optionally protease; and

wherein the fermenting organism is a *Saccharomyces* yeast strain providing:

an ethanol yield boost compared to ETHANOL RED™ under the same fermentation conditions;

reduced acetaldehyde production compared to ETHANOL RED™ under the same process conditions;

increased temperature tolerance compared to ETHANOL RED™ under the same process conditions; and/or

decreased glycerol production compared to ETHANOL RED™ under the same process conditions.

In one embodiment, the fermenting organism is a derivative of *Saccharomyces cerevisiae* strain MBG4985 having the defining characteristics (e.g., high ethanol yield boost and/or decreased glycerol production of *Saccharomyces cerevisiae* strain MBG4985). In some embodiments, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

Examples of suitable enzymes used, especially glucoamylases, alpha-amylases, proteases, cellulolytic enzyme compositions etc are described in the “Enzymes And Enzyme Blends Used In A Raw Starch Hydrolysis Process” section below.

A third aspect provides a *Saccharomyces cerevisiae* yeast strain deposited under the Budapest Treaty and having NRRL accession no. NRRL Y67342, or a derivative of *Saccharomyces cerevisiae* strain NRRL Y67342. In some embodiments, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

A fourth aspect provides a method of producing a derivative of strain *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) comprising:

(a) culturing a first yeast strain with a second yeast strain, wherein the second yeast strain is strain *Saccharomyces cerevisiae* strain MBG4985 or a derivative of strain *Saccharomyces cerevisiae* strain MBG4985, under conditions which permit combining of DNA between the first yeast strain and the second yeast strain; and

(b) isolating hybrid strains; and

(c) optionally repeating steps (a) and (b) using a hybrid strain isolated in step (b) as the first yeast strain and/or the second yeast strain. In some embodiments, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

A fifth aspect provides a method of producing a *Saccharomyces* strain having the defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA), comprising:

(a) providing: (i) a first yeast strain; and (ii) a second yeast strain, wherein the second yeast strain is strain *Saccharomyces cerevisiae* strain MBG4985 or a derivative of strain *Saccharomyces cerevisiae* strain MBG4985;

(b) culturing the first yeast strain and the second yeast strain under conditions which permit combining of DNA between the first yeast strain and the second yeast strain;

(c) screening or selecting for a derivative of *Saccharomyces cerevisiae* strain MBG4985;

(d) optionally repeating steps (b) and (c) with the screened or selected strain from step (c) as the first and/or second strain, until a derivative is obtained which exhibits the defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985. In some embodiments, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

A sixth aspect provides a method of producing a recombinant derivative of strain *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) comprising:

(a) transforming *Saccharomyces cerevisiae* strain MBG4985 or a derivative of strain *Saccharomyces cerevisiae* strain MBG4985 with one or more expression vectors encoding a glucoamylase and/or an alpha-amylase; and

(b) isolating the transformed strain.

A seventh aspect provides a *Saccharomyces* strain produced by the method of the fourth, fifth, or sixth aspect.

An eigth aspect provides a *Saccharomyces* strain having the defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA).

A nineth aspect provides a method of producing ethanol, comprising incubating a strain of the first, fourth or fifth aspect with a substrate comprising a fermentable sugar under conditions which promote fermentation of the fermentable sugar to produce ethanol.

A tenth aspect provides use of a strain of the third, sixth or seventh aspect in the production of ethanol.

An eleventh aspect provides a method of producing distiller's grain, comprising:

(a) incubating a *Saccharomyces* strain of the third, seventh or eigth aspect with a substrate comprising fermentable sugar under conditions which allow fermentation of the fermentable sugar to produce ethanol and distiller's grains;

(b) isolating the distiller's grains.

A twelfth aspect provides distiller's grain produced by the method of the tenth aspect.

A thirteenth aspect provides use of a strain of the third, seventh or eigth aspect in the production of distiller's grains.

A fourteenth aspect provides use of a strain of the third, seventh or eigth aspect in the production of a *Saccharomyces* strain which exhibits one or more defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA).

A fifteenth aspect provides a composition comprising a *Saccharomyces* strain of the third, seventh or eigth aspect.

Also described are compositions comprising a *Saccharomyces* yeast strain described herein, e.g., *Saccharomyces cerevisiae* strain MBG4985, or a derivative thereof, and naturally occurring and/or non-naturally occurring components.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a generalized diagram of the expression cassette at the XII-5 integration site. The dominant selection marker was either kanamycin or nourseothricin resistance. The "Gene" is, for example, an alpha-amylase or glucoamylase.

Fig. 2 shows produced ethanol (in percent weight/volume) from triplicate mini-tube fermentation at 54 hours for the strains shown. ER (Ethanol Red) was treated with 0.6 AGU/g-DS Spirizyme Excel since it does not contain the glucoamylase expression cassette, while the remaining strains were treated with 0.3 AGU/g-DS Spirizyme Excel. The top of figure shows the graph of the ethanol concentrations, while the bottom of the figure shows that yMHCT471 produced significantly more ethanol than the other four strains.

Fig. 3 shows produced glycerol (in percent weight/volume) from triplicate mini-tube fermentation at 54 hours for the strains shown. ER (Ethanol Red) was treated with 0.6 AGU/g-DS Spirizyme Excel since it does not contain the glucoamylase expression cassette, while the

remaining strains were treated with 0.3 AGU/g-DS Spirizyme Excel. The top of figure shows the graph of the glycerol concentrations, while the bottom of the figure shows that strains yMHCT471, MLBA865, and MEJI705 produced significantly less ethanol than GsAMGinER1.

Definitions

Allelic variant: The term "allelic variant" means any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

Catalytic domain: The term "catalytic domain" means the region of an enzyme containing the catalytic machinery of the enzyme.

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 or prokaryotic 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 begins with a start codon such as ATG, GTG, or TTG and ends with a stop codon such as TAA, TAG, or TGA. The coding sequence may be a genomic DNA, cDNA, synthetic DNA, or a combination thereof.

Control sequences: The term "control sequences" means nucleic acid sequences necessary for expression of a polynucleotide encoding a mature polypeptide of the present invention. Each control sequence may be native (*i.e.*, from the same gene) or foreign (*i.e.*, from a different gene) 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 a 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 control sequences that provide for its expression.

Fragment: The term “fragment” means a polypeptide having one or more (*e.g.*, several) amino acids absent from the amino and/or carboxyl terminus of a mature polypeptide or domain; wherein the fragment has pullulanas activity.

Host cell: The term "host cell" means any cell type that is susceptible to transformation, transfection, transduction, or 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). An isolated substance may be present in a fermentation broth sample; e.g. a host cell may be genetically modified to express the polypeptide of the invention. The fermentation broth from that host cell will comprise the isolated polypeptide.

Mature polypeptide: The term “mature polypeptide” means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. It is known in the art that a host cell may produce a mixture of two of more different mature polypeptides (*i.e.*, with a different C-terminal and/or N-terminal amino acid) expressed by the same polynucleotide. It is also known in the art that different host cells process polypeptides differently, and thus, one host cell expressing a polynucleotide may produce a different mature polypeptide (*e.g.*, having a different C-terminal and/or N-terminal amino acid) as compared to another host cell expressing the same polynucleotide.

Mature polypeptide coding sequence: The term “mature polypeptide coding sequence” means a polynucleotide that encodes a mature polypeptide having protease activity.

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, which comprises one or more control sequences.

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 expression of the coding sequence.

DETAILED DESCRIPTION

Processes

Described herein are processes for producing ethanol from starch-containing material in a process including liquefaction, saccharification and fermentation. Fermentable sugars generated during saccharification are converted to ethanol during fermentation by yeast. In a first aspect is a process for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using an alpha-amylase;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces cerevisiae* strain MBG4985 having defining characteristics of *Saccharomyces cerevisiae* strain MBG4985. In some embodiments, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

Steps ii) and iii) are carried out either sequentially or simultaneously (SSF). In one embodiment, steps ii) and iii) are carried out simultaneously (SSF).

Nitrogens-Source added During Fermentation

Generally, fermenting organisms such as yeast, including *Saccharomyces cerevisiae* yeast, require an adequate source of nitrogen for propagation and fermentation. Many sources of nitrogen can be used and such sources of nitrogen are well known in the art. The nitrogen source may be organic, such as urea, DDGs, wet cake or corn mash, or inorganic, such as ammonia or ammonium hydroxide. In one embodiment, the nitrogen source is urea.

Liquefaction step i)

According to processes described herein, liquefaction in step i) may be carried out by subjecting starch-containing material at a temperature above the initial gelatinization

temperature to an alpha-amylase and optionally a protease, and/or a glucoamylase. Other enzymes such as a pullulanase and phytase may also be present and/or added in liquefaction.

Liquefaction step i) may be carried out for 0.5-5 hours, such as 1-3 hours, such as typically about 2 hours.

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

Liquefaction is typically carried out at a temperature in the range from 70-100ºC. In one embodiment, the temperature in liquefaction is between 75-95°C, such as between 75-90°C, between 80-90°C, or between 82-88°C, such as about 85ºC.

A jet-cooking step may be carried out prior to liquefaction in step i). The jet-cooking may be carried out at a temperature between 110-145°C, 120-140ºC, 125-135°C, or about 130ºC for about 1-15 minutes, for about 3-10 minutes, or about 5 minutes.

The pH during liquefaction may be between 4 and 7, such as pH 4.5-6.5, pH 5.0-6.5, pH 5.0-6.0, pH 5.2-6.2, or about 5.2, about 5.4, about 5.6, or about 5.8.

In one embodiment, the process further comprises, prior to the step i), the steps of:

a) reducing the particle size of the starch-containing material, preferably by dry milling;

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

The starch-containing starting material, such as whole grains, may be reduced in particle size, e.g., by milling, in order to open up the structure, to increase surface area, and allowing for further processing. Generally, there are two types of processes: wet and dry milling. In dry milling whole kernels are milled and used. Wet milling gives a good separation of germ and meal (starch granules and protein). Wet milling is often applied at locations where the starch hydrolysate is used in production of, e.g., syrups. Both dry milling and wet milling are well known in the art of starch processing. In one embodiment the starch-containing material is subjected to dry milling. In one embodiment, the particle size is reduced to between 0.05 to 3.0 mm, e.g., 0.1-0.5 mm, or so that at least 30%, at least 50%, at least 70%, or at least 90% of the starch-containing material fit through a sieve with a 0.05 to 3.0 mm screen, e.g., 0.1-0.5 mm screen. In another embodiment, at least 50%, e.g., at least 70%, at least 80%, or at least 90% of the starch-containing material fit through a sieve with # 6 screen.

The aqueous slurry may contain from 10-55 w/w-% dry solids (DS), e.g., 25-45 w/w-% dry solids (DS), or 30-40 w/w-% dry solids (DS) of starch-containing material.

The alpha-amylase, optionally a protease, optionally a glucoamylase may initially be added to the aqueous slurry to initiate liquefaction (thinning). In one embodiment, only a portion of the enzymes (e.g., about 1/3) is added to the aqueous slurry, while the rest of the enzymes (e.g., about 2/3) are added during liquefaction step i).

A non-exhaustive list of examples of alpha-amylases can be found below in the "Alpha-Amylase Present and/or Added During Liquefaction"-section. In one embodiment, the alpha-amylase is a bacterial alpha-amylase. Bacterial alpha-amylases are typically thermostable. In one embodiment, the alpha-amylase is from the genus *Bacillus*, such as a strain of *Bacillus stearothermophilus*, in particular a variant of a *Bacillus stearothermophilus* alpha-amylase, such as the one shown in SEQ ID NO: 3 in WO 99/019467 or SEQ ID NO: 1 herein.

In one embodiment, the alpha-amylase has an improved stability compared to a reference alpha-amylase (*Bacillus stearothermophilus* alpha-amylase with the mutations I181*+G182*, optionally with a N193F substitution, truncated to about 491 amino acids, i.e., from 480-495 amino acids, (using SEQ ID NO: 1 herein for numbering) determined by incubating the reference alpha-amylase and variants at pH 4.5 and 5.5 and temperatures of 75°C and 85°C with 0.12 mM $CaCl_2$ followed by residual activity determination using the EnzChek® substrate (EnzChek® Ultra Amylase assay kit, E33651, Molecular Probes). This is described in Example 1.

Examples of suitable *Bacillus stearothermophilus* alpha-amylase variants can be found below in the "Thermostable Alpha-Amylase"-section and include one from the following group of *Bacillus stearothermophilus* alpha-amylase variants with the following mutations: I181*+G182*, and optionally substitution N193F, and further one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

V59A+ Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V;

V59A+ E129V+ K177L+ R179E+ Q254S+ M284V; and

E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 for numbering).

Examples of other suitable *Bacillus stearothermophilus* alpha-amylases having increased thermostability compared to a reference alpha-amylase (*Bacillus stearothermophilus* alpha-amylase with the mutations I181*+G182*, and optionally a N193F substitution, C-terminally truncated to be 485-495 amino acids long, such as about 491 amino acids long) at pH 4.5 and 5.5, 0.12 mM $CaCl_2$ can be found in WO 2011/082425 hereby incorporated by reference. (See also Example 1 below)

Liquefaction in step i) may be carried out using a combination of alpha-amylase and protease. The protease may be a protease having a thermostability value of more than 20% determined as Relative Activity at 80ºC/70ºC determined as described in Example 2 (Relative Activty). Examples of suitable proteases are described below in the section "Protease Present and/or Added During Liquefaction".

The protease may be of fungal origin, such as of filamentous fungus origin. Specific examples of suitable fungal proteases are protease variants of metallo protease derived from a strain of the genus *Thermoascus*, e.g., a strain of *Thermoascus aurantiacus*, such as the strain *Thermoascus aurantiacus* CGMCC No. 0670 disclosed as the mature part of SEQ ID NO. 2 disclosed in WO 2003/048353 or the mature part of SEQ ID NO: 1 in WO 2010/008841 or SEQ ID NO: 3 herein with one of the following substitutions or combinations of substitutions:

D79L+S87P+A112P+D142L;

D79L+S87P+D142L; and

A27K+ D79L+ Y82F+S87G+D104P+A112P+A126V+D142L.

More examples of suitable variants of the *Thermoascus aurantiacus* protease can be found in WO 2011/072191 hereby incorporated by reference (See also Example 2 below).

Suitable proteases also include bacterial proteases. A suitable bacterial protease may be derived from a strain of *Pyrococcus*, e.g., a strain of *Pyrococcus furiosus*. In one embodiment, the protease is the one shown in SEQ ID NO: 1 in US 6,358,726 or SEQ ID NO: 13 herein.

In one embodiment, the alpha-amylase and/or protease, added in the liquefaction step i), is/are further combined with a glucoamylase. Thus, a glucoamylase may also be present and/or added during liquefaction step i). The glucoamylase may be thermostable. This means that the glucoamylase has a heat stability at 85ºC, pH 5.3, of at least 20%, such as at least 30%, preferably at least 35% determined as described in Example 4 (heat stability). In one embodiment, the glucoamylase present and/or added in liquefaction has a relative activity pH optimum at pH 5.0 of at least 90%, e.g., at least 95%, or at least 97%. In one embodiment, the glucoamylase has a pH stability at pH 5.0 of at least at least 80%, e.g., at least 85%, or at least 90% determined as described in Example 4 (pH stability).

A suitable glucoamylase present and/or added in liquefaction step i) may be derived from a strain of the genus *Penicillium*, especially a strain of *Penicillium oxalicum* disclosed as SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NOs: 9 or 14 herein. In one embodiment, the glucoamylase is a variant of the *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 2 in WO 2011/127802 having a K79V substitution (using the mature sequence shown in SEQ ID NO: 14 herein for numbering), such as a variant disclosed in WO 2013/053801. In one embodiment, the *Penicillium oxalicum* glucoamylase has a K79V substitution (using SEQ ID

NO: 14 for numbering) and further one of the following substitutions or combinations of substitutions:

P11F + T65A + Q327F; and

P2N + P4S + P11F + T65A + Q327F (using SEQ ID NO: 14 for numbering).

Examples of other suitable *Penicillium oxalicum* glucoamylase variants can be found in WO 2013/053801 incorporated by reference (see also Examples 10-16 below, such as the *Penicillium oxalicum* glucoamylase variants in Table 15).

Further, according to the processes described herein, a pullulanase may be present during liquefaction in combination with an alpha-amylase, a protease and/or a glucoamylase.

Saccharification and Fermentation

A glucoamylase is expressed by the derivative of *Saccharomyces cerevisiae* strain MBG4985, present and/or added in saccharification step ii) and/or fermentation step iii) or simultaneous saccharification and fermentation (SSF). The glucoamylase expressed by the derivative of *Saccharomyces cerevisiae* strain MBG4985, added in saccharification step ii) and/or fermentation step iii) or simultaneous saccharification and fermentation (SSF) is typically different from the glucoamylase, optionally added in liquefaction step i). In one embodiment, the glucoamylase is express or added together with a fungal alpha-amylase. Examples of glucoamylases can be found in the "Glucoamylases Present and/or Added In Saccharification and/or Fermentation"-section below.

When doing sequential saccharification and fermentation, saccharification step ii) may be carried out under conditions well-known in the art. For instance, saccharification step ii) may last up to from about 24 to about 72 hours. In one embodiment, pre-saccharification is done. Pre-saccharification is typically done for 40-90 minutes at a temperature between 30-65ºC, typically about 60ºC. Pre-saccharification is, in one embodiment, followed by saccharification during fermentation in simultaneous saccharification and fermentation (SSF). Saccharification is typically carried out at temperatures from 20-75ºC, preferably from 40-70ºC, typically about 60ºC, and typically at a pH between 4 and 5, such as about pH 4.5.

Simultaneous saccharification and fermentation ("SSF") is widely used in industrial scale fermentation product production processes, especially ethanol production processes. When doing SSF the saccharification step ii) and the fermentation step iii) are carried out simultaneously. There is no holding stage for the saccharification, meaning that a fermenting organism, such as yeast, and enzyme(s), may be added together. However, it is also contemplated to add the fermenting organism and enzyme(s) separately. SSF is typically 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, or about 32°C. In one embodiment, fermentation is ongoing for 6 to 120 hours, in particular 24 to 96 hours. In one embodiment, the pH is between 4-5.

In one embodiment, a cellulolytic enzyme composition is present and/or added in saccharification, fermentation or simultaneous saccharification and fermentation (SSF). Examples of such cellulolytic enzyme compositions can be found in the "Cellulolytic Enzyme Composition present and/or added during Saccharification and/or Fermentation"-section below. The cellulolytic enzyme composition is present and/or added together with a glucoamylase, such as one disclosed in the "Glucoamylase Present And/Or Added in Saccharification and/or Fermentation"-section below.

Starch-Containing Materials

Any suitable starch-containing starting material may be used with the processes described herein. The starting material is generally selected based on the desired fermentation product, here ethanol. Examples of starch-containing starting materials include cereal, tubers or grains. Specifically, the starch-containing material may be corn, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, oat, rice, peas, beans, or sweet potatoes, or mixtures thereof. Contemplated are also waxy and non-waxy types of corn and barley.

In one embodiment, the starch-containing starting material is corn. In one embodiment, the starch-containing starting material is wheat. In one embodiment, the starch-containing starting material is barley. In one embodiment, the starch-containing starting material is rye. In one embodiment, the starch-containing starting material is milo. In one embodiment, the starch-containing starting material is sago. In one embodiment, the starch-containing starting material is cassava. In one embodiment, the starch-containing starting material is tapioca. In one embodiment, the starch-containing starting material is sorghum. In one embodiment, the starch-containing starting material is rice. In one embodiment, the starch-containing starting material is peas. In one embodiment, the starch-containing starting material is beans. In one embodiment, the starch-containing starting material is sweet potatoes. In one embodiment, the starch-containing starting material is oats.

Fermentation

Fermentation is carried out in a fermentation medium. The fermentation medium includes the fermentation substrate, that is, the carbohydrate source that is metabolized by the fermenting organism. With the processes described herein, the fermentation medium may comprise nutrients and growth stimulator(s) for the fermenting organism(s). Nutrient and growth stimulators are widely used in the art of fermentation and include nitrogen sources, such as ammonia; urea, vitamins and minerals, or combinations thereof.

Fermenting Organisms

Saccharomyces cerevisiae strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain

MBG4985, or a derivative of *Saccharomyces cerevisiae* strain MBG4985 having defining characteristics of strain MBG4985 may be used in a process described herein. In one embodiment, the fermenting organism has properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 as it provides an increase in ethanol yield compared to Ethanol Red™ (ER) under the same process conditions.

In one embodiment, the fermenting organism strain having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 has at least the one or more, such as all of following properties and defining characteristics:

increases ethanol yield compared to ETHANOL RED™ under the same process conditions, e.g., conditions as described herein;

reduces acetaldehyde production compared to ETHANOL RED™ under the same process conditions, e.g., conditions as described herein;

increases temperature tolerance compared to ETHANOL RED™ under the same process conditions, e.g., conditions as described herein; and

decreases glycerol production compared to ETHANOL RED™ under the same process conditions, conditions as described herein.

In one embodiment, the fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 provides an ethanol yield boost over ETHANOL RED™ (ER) of more than 1.0%, e.g., more than 2.0%, such as more than 2.5%, such as about 2.9% using the same process set-up and conditions, e.g., conditions described herein.

In one embodiment, the fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 reduces acetaldehyde production more than 10%, e.g., more than 20%, more than 30%, more than 40%, more than 45%, such as between 5-60%, such as 30-50%, compared to ETHANOL RED™ under the same process conditions, e.g., conditions described herein.

In one embodiment, the fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 increases the temperature tolerance compared to ETHANOL RED™ under the same process conditions, e.g., conditions described herein.

Increased temperature tolerance is an advantage as the fermentation temperature may fluctuate to some degree. In the early part of fermentation plants often do not actively heat the fermentation. The temperature may therefore increase naturally from the yeast's metabolism. The plant may use heat exchangers to control early fermentation temperatures so it does not go too high. During the majority of the year the plants can easily control the early temperature and the peak temperature is typically about 34°C. However, during the summer months the cooling water used in heat exchangers is not cold enough to control the

temperatures. Therefore, in plants that do not have chillers (i.e., a water refrigeration system), the early fermentation temperatures can reach above 36.5°C which stresses the yeast.

In one embodiment, the fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 decreases glycerol production by more than 3%, e.g., more than 4%, more than 5%, more than 6%, more than 7%, such as between 2-15%, such as 5-10%, compared to ETHANOL RED™ under the same process conditions, e.g., conditions described herein.

Recovery

Subsequent to fermentation, e.g., SSF, the ethanol may be separated from the fermentation medium. The slurry may be distilled to recover/extract the desired fermentation product (i.e., ethanol). Alternatively, the desired fermentation product (i.e., ethanol) may be extracted from the fermentation medium by micro or membrane filtration techniques. The fermentation product (i.e., ethanol) may also be recovered by stripping or other method well known in the art.

Alpha-Amylase Present and/or Added In Liquefaction

The alpha-amylase used herein may be present and/or added in liquefaction optionally together with a protease and/or glucoamylase, and/or optional pullulanase, e.g., as disclosed in WO 2012/088303 (Novozymes) or WO 2013/082486 (Novozymes) which references are both incorporated by reference.

The alpha-amylase added in liquefaction step i) may be any alpha-amylase. In one embodiment, the alpha-amylase is a bacterial alpha-amylase, which may be stable at temperature.

Any alpha-amylase described herein, including any bacterial, hybrid and/or thermostable alpha-amylase described below, may be expressed by the *Saccharomyces cerevisiae* strain MBG4985.

Bacterial Alpha-Amylase

The term “bacterial alpha-amylase” means any bacterial alpha-amylase classified under EC 3.2.1.1. A bacterial alpha-amylase used herein may, e.g., be derived from a strain of the genus *Bacillus*, which is sometimes also referred to as the genus *Geobacillus*. In one 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 bacterial alpha-amylases include the *Bacillus stearothermophilus* alpha-amylase (BSG) of SEQ ID NO: 3 in WO 99/19467 or SEQ ID NO: 1 herein, the *Bacillus amyloliquefaciens* alpha-amylase (BAN) of SEQ ID NO: 5 in WO 99/19467, and the *Bacillus licheniformis* alpha-amylase (BLA) of SEQ ID NO: 4 in WO 99/19467 or SEQ ID NO: 21 herein (all sequences are hereby incorporated by reference). In one 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.

In one 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 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% to any of the sequences shown in SEQ ID NO: 3 in WO 99/19467 or SEQ ID NO: 1 herein.

In one embodiment, the alpha-amylase is derived from *Bacillus stearothermophilus.* The *Bacillus stearothermophilus* alpha-amylase may be a mature wild-type or a mature variant thereof. The mature *Bacillus stearothermophilus* alpha-amylases may naturally be truncated during recombinant production. For instance, the *Bacillus stearothermophilus* alpha-amylase may be a truncated at the C-terminal, so that it is from 480-495 amino acids long, such as about 491 amino acids long, e.g., so that it lacks a functional starch binding domain (compared to SEQ ID NO: 3 in WO 99/19467) or SEQ ID NO: 1 herein.

The *Bacillus* alpha-amylase may also be a variant and/or hybrid. Examples of such a variant can be found in any of WO 96/23873, WO 96/23874, WO 97/41213, WO 99/19467, WO 00/60059, and WO 02/10355 (each hereby incorporated by reference). Specific alpha-amylase variants are disclosed in U.S. Patent Nos. 6,093,562, 6,187,576, 6,297,038, and 7,713,723 (hereby incorporated by reference) and include *Bacillus stearothermophilus* alpha-amylase (often referred to as BSG alpha-amylase) variants having a deletion of one or two amino acids at positions R179, G180, I181 and/or G182, preferably a double deletion disclosed in WO 96/23873 – see, e.g., page 20, lines 1-10 (hereby incorporated by reference), such as corresponding to deletion of positions I181 and G182 compared to the amino acid sequence of *Bacillus stearothermophilus* alpha-amylase set forth in SEQ ID NO: 3 disclosed in WO 99/19467 or SEQ ID NO: 1 herein or the deletion of amino acids R179 and G180 using SEQ ID NO: 3 in WO 99/19467 or SEQ ID NO: 1 herein for numbering (which reference is hereby incorporated by reference). In some embodimenst, the *Bacillus* alpha-amylases, such as *Bacillus stearothermophilus* alpha-amylases, have a double deletion corresponding to a deletion of positions 181 and 182 and further optionally 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 or SEQ ID NO: 1 herein. The bacterial alpha-amylase may also have a substitution in a position corresponding to S239 in the *Bacillus licheniformis* alpha-amylase shown in SEQ ID NO: 4 in WO 99/19467 or SEQ ID NO: 21 herein, or a S242 and/or E188P variant of the *Bacillus stearothermophilus* alpha-amylase of SEQ ID NO: 3 in WO 99/19467 or SEQ ID NO: 1 herein.

In one embodiment, the variant is a S242A, E or Q variant, e.g., a S242Q variant, of the *Bacillus stearothermophilus* alpha-amylase (using SEQ ID NO: 1 herein for numbering).

In one embodiment, the variant is a position E188 variant, e.g., E188P variant of the *Bacillus stearothermophilus* alpha-amylase (using SEQ ID NO: 1 herein for numbering).

The bacterial alpha-amylase may, in one embodiment, be a truncated *Bacillus* alpha-amylase. In one embodiment, the truncation is so that, e.g., the *Bacillus stearothermophilus* alpha-amylase shown in SEQ ID NO: 3 in WO 99/19467 or SEQ ID NO: 1 herein, is about 491 amino acids long, such as from 480 to 495 amino acids long, or so it lacks a functional starch bind domain.

Bacterial Hybrid Alpha-Amylases

The bacterial alpha-amylase may also be a hybrid bacterial 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). In one embodiment, this hybrid has 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) or SEQ ID NO: 21 herein. In some embodiments, the variants have 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, e.g., deletion of E178 and G179 (using SEQ ID NO: 5 of WO 99/19467 for position numbering or SEQ ID NO: 21 herein).

In one embodiment, the bacterial alpha-amylase is the mature part of the chimeric alpha-amylase disclosed in Richardson et al. (2002), The Journal of Biological Chemistry, Vol. 277, No 29, Issue 19 July, pp. 267501-26507, referred to as BD5088 or a variant thereof. This alpha-amylase is the same as the one shown in SEQ ID NO: 2 in WO 2007134207. The mature enzyme sequence starts after the initial “Met” amino acid in position 1.

Thermostable Alpha-Amylase

The alpha-amylase may be a thermostable alpha-amylase, such as a thermostable bacterial alpha-amylase, e.g., from *Bacillus stearothermophilus*. In one embodiment, the alpha-amylase used in a process described herein has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$ of at least 10 determined as described in Example 1.

In one embodiment, the thermostable alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$, of at least 15.

In one embodiment, the thermostable alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$, of as at least 20.

In one embodiment, the thermostable alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$, of as at least 25.

In one embodiment, the thermostable alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$, of as at least 30.

In one embodiment, the thermostable alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$, of as at least 40.

In one embodiment, the thermostable alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$, of at least 50.

In one embodiment, the thermostable alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$, of at least 60.

In one embodiment, the thermostable alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$, between 10-70.

In one embodiment, the thermostable alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$, between 15-70.

In one embodiment, the thermostable alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$, between 20-70.

In one embodiment, the thermostable alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$, between 25-70.

In one embodiment, the thermostable alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$, between 30-70.

In one embodiment, the thermostable alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$, between 40-70.

In one embodiment, the thermostable alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$, between 50-70.

In one embodiment, the thermostable alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$, between 60-70.

In one embodiment, the alpha-amylase is a bacterial alpha-amylase, e.g., derived from the genus *Bacillus*, such as a strain of *Bacillus stearothermophilus,* e.g., the *Bacillus stearothermophilus* as disclosed in WO 99/019467 as SEQ ID NO: 3 (SEQ ID NO: 1 herein) with one or two amino acids deleted at positions R179, G180, I181 and/or G182, in particular with R179 and G180 deleted, or with I181 and G182 deleted, with mutations in below list of mutations.

In some embodiment, the *Bacillus stearothermophilus* alpha-amylases have double deletion I181 + G182, and optional substitution N193F, further comprising one of the following substitutions or combinations of substitutions:

V59A+Q89R+G112D+E129V+K177L+R179E+K220P+N224L+Q254S;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+D269E+D281N;

V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+I270L;

V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+H274K;
V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+Y276F;
V59A+E129V+R157Y+K177L+R179E+K220P+N224L+S242Q+Q254S;
V59A+E129V+K177L+R179E+H208Y+K220P+N224L+S242Q+Q254S;
V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+H274K;
V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F;
V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+D281N;
V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+G416V;
V59A+E129V+K177L+R179E+K220P+N224L+Q254S;
V59A+E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
A91L+M96I+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
E129V+K177L+R179E;
E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F+L427M;
E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+N376*+I377*;
E129V+K177L+R179E+K220P+N224L+Q254S;
E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
E129V+K177L+R179E+S242Q;
E129V+K177L+R179V+K220P+N224L+S242Q+Q254S;
K220P+N224L+S242Q+Q254S;
M284V;
V59A+Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V; and
V59A+E129V+K177L+R179E+Q254S+ M284V;

In one embodiment, the alpha-amylase is selected from the group of *Bacillus stearothermophilus* alpha-amylase variants with double deletion I181*+G182*, and optionally substitution N193F, and further one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;
V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
V59A+Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V;
V59A+E129V+K177L+R179E+Q254S+ M284V; and
E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering).

It should be understood that when referring to *Bacillus stearothermophilus* alpha-amylase and variants thereof they are normally produced in truncated form. In particular, the truncation may be so that the *Bacillus stearothermophilus* alpha-amylase shown in SEQ ID NO: 3 in WO 99/19467 or SEQ ID NO: 1 herein, or variants thereof, are truncated in the C-terminal and are typically from 480-495 amino acids long, such as about 491 amino acids long, e.g., so that it lacks a functional starch binding domain.

In one embodiment, the alpha-amylase variant 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 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%, but less than 100% to the sequence shown in SEQ ID NO: 3 in WO 99/19467 or SEQ ID NO: 1 herein.

In one embodiment, the bacterial alpha-amylase, e.g., *Bacillus* alpha-amylase, such as especially *Bacillus stearothermophilus* alpha-amylase, or variant thereof, is dosed to liquefaction in a concentration between 0.01-10 KNU-A/g DS, e.g., between 0.02 and 5 KNU-A/g DS, such as 0.03 and 3 KNU-A, preferably 0.04 and 2 KNU-A/g DS, such as especially 0.01 and 2 KNU-A/g DS. In one embodiment, the bacterial alpha-amylase, e.g., *Bacillus* alpha-amylase, such as especially *Bacillus stearothermophilus* alpha-amylases, or variant thereof, is dosed to liquefaction in a concentration of between 0.0001-1 mg EP (Enzyme Protein)/g DS, e.g., 0.0005-0.5 mg EP/g DS, such as 0.001-0.1 mg EP/g DS.

Protease Present and/or Added In Liquefaction

In the processes described ehrein, the protease may optionally be present and/or added in liquefaction together with alpha-amylase, and an optional glucoamylase, and/or pullulanase.

Proteases are classified on the basis of their catalytic mechanism into the following groups: Serine proteases (S), Cysteine proteases (C), Aspartic proteases (A), Metallo proteases (M), and Unknown, or as yet unclassified, proteases (U), see Handbook of Proteolytic Enzymes, A.J.Barrett, N.D.Rawlings, J.F.Woessner (eds), Academic Press (1998), in particular the general introduction part.

In one embodiment, the thermostable protease used according to a process described herein is a “metallo protease” defined as a protease belonging to EC 3.4.24 (metalloendopeptidases); preferably EC 3.4.24.39 (acid metallo proteinases).

To determine whether a given protease is a metallo protease or not, reference is made to the above “Handbook of Proteolytic Enzymes” and the principles indicated therein. Such determination can be carried out for all types of proteases, be it naturally occurring or wild-type proteases; or genetically engineered or synthetic proteases.

Protease activity can be measured using any suitable assay, in which a substrate is employed, that includes peptide bonds relevant for the specificity of the protease in question.

Assay-pH and assay-temperature are likewise to be adapted to the protease in question. Examples of assay-pH-values are pH 6, 7, 8, 9, 10, or 11. Examples of assay-temperatures are 30, 35, 37, 40, 45, 50, 55, 60, 65, 70 or 80°C.

Examples of protease substrates are casein, such as Azurine-Crosslinked Casein (AZCL-casein). Two protease assays are described below in the "Materials & Methods"-section, of which the so-called "AZCL-Casein Assay" is the preferred assay.

In one embodiment, the thermostable protease has at least 20%, such as at least 30%, such as at least 40%, such as at least 50%, such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 95%, such as at least 100% of the protease activity of the Protease 196 variant or Protease Pfu determined by the AZCL-casein assay described in the "Materials & Methods" section.

There are no limitations on the origin of the protease used in a process described herein as long as it fulfills the thermostability properties defined below.

In one embodiment the protease is of fungal origin.

The protease may be a variant of, e.g., a wild-type protease as long as the protease has the thermostability properties defined herein. In one embodiment, the thermostable protease is a variant of a metallo protease as defined above. In one embodiment, the thermostable protease used in a process described herein is of fungal origin, such as a fungal metallo protease, such as a fungal metallo protease derived from a strain of the genus *Thermoascus*, preferably a strain of *Thermoascus aurantiacus*, especially *Thermoascus aurantiacus* CGMCC No. 0670 (classified as EC 3.4.24.39).

In one embodiment, the thermostable protease is a variant of the mature part of the metallo protease shown in SEQ ID NO: 2 disclosed in WO 2003/048353 or the mature part of SEQ ID NO: 1 in WO 2010/008841 and shown as SEQ ID NO: 3 herein further with one of the following substitutions or combinations of substitutions:

S5*+D79L+S87P+A112P+D142L;
D79L+S87P+A112P+T124V+D142L;
S5*+N26R+D79L+S87P+A112P+D142L;
N26R+T46R+D79L+S87P+A112P+D142L;
T46R+D79L+S87P+T116V+D142L;
D79L+P81R+S87P+A112P+D142L;
A27K+D79L+S87P+A112P+T124V+D142L;
D79L+Y82F+S87P+A112P+T124V+D142L;
D79L+Y82F+S87P+A112P+T124V+D142L;
D79L+S87P+A112P+T124V+A126V+D142L;
D79L+S87P+A112P+D142L;
D79L+Y82F+S87P+A112P+D142L;

S38T+D79L+S87P+A112P+A126V+D142L;
D79L+Y82F+S87P+A112P+A126V+D142L;
A27K+D79L+S87P+A112P+A126V+D142L;
D79L+S87P+N98C+A112P+G135C+D142L;
D79L+S87P+A112P+D142L+T141C+M161C;
S36P+D79L+S87P+A112P+D142L;
A37P+D79L+S87P+A112P+D142L;
S49P+D79L+S87P+A112P+D142L;
S50P+D79L+S87P+A112P+D142L;
D79L+S87P+D104P+A112P+D142L;
D79L+Y82F+S87G+A112P+D142L;
S70V+D79L+Y82F+S87G+Y97W+A112P+D142L;
D79L+Y82F+S87G+Y97W+D104P+A112P+D142L;
S70V+D79L+Y82F+S87G+A112P+D142L;
D79L+Y82F+S87G+D104P+A112P+D142L;
D79L+Y82F+S87G+A112P+A126V+D142L;
Y82F+S87G+S70V+D79L+D104P+A112P+D142L;
Y82F+S87G+D79L+D104P+A112P+A126V+D142L;
A27K+D79L+Y82F+S87G+D104P+A112P+A126V+D142L;
A27K+Y82F+S87G+D104P+A112P+A126V+D142L;
A27K+D79L+Y82F+ D104P+A112P+A126V+D142L;
A27K+Y82F+D104P+A112P+A126V+D142L;
A27K+D79L+S87P+A112P+D142L; and
D79L+S87P+D142L.

In one embodiment, the thermostable protease is a variant of the metallo protease disclosed as the mature part of SEQ ID NO: 2 disclosed in WO 2003/048353 or the mature part of SEQ ID NO: 1 in WO 2010/008841 or SEQ ID NO: 3 herein with one of the following substitutions or combinations of substitutions:

D79L+S87P+A112P+D142L;
D79L+S87P+D142L; and
A27K+ D79L+Y82F+S87G+D104P+A112P+A126V+D142L.

In one embodiment, the protease variant has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide

of SEQ ID NO: 2 disclosed in WO 2003/048353 or the mature part of SEQ ID NO: 1 in WO 2010/008841 or SEQ ID NO: 3 herein.

The thermostable protease may also be derived from any bacterium as long as the protease has the thermostability properties.

In one embodiment, the thermostable protease is derived from a strain of the bacterium *Pyrococcus*, such as a strain of *Pyrococcus furiosus* (pfu protease).

In one embodiment, the protease is one shown as SEQ ID NO: 1 in US patent No. 6,358,726-B1 (Takara Shuzo Company), or SEQ ID NO: 13 herein.

In one embodiment, the thermostable protease is SEQ ID NO: 13 herein or a protease having at least 80% identity, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 1 in US patent no. 6,358,726-B1 or SEQ ID NO: 13 herein. The *Pyroccus furiosus* protease can be purchased from Takara Bio, Japan.

The *Pyrococcus furiosus* protease is a thermostable protease. The commercial product *Pyrococcus furiosus* protease (PfuS) was found to have a thermostability of 110% (80°C/70°C) and 103% (90°C/70°C) at pH 4.5 determined as described in Example 2.

In one embodiment, a thermostable protease used in a process described herein has a thermostability value of more than 20% determined as Relative Activity at 80ºC/70ºC determined as described in Example 2.

In one embodiment, the protease has a thermostability of more than 30%, more than 40%, more than 50%, more than 60%, more than 70%, more than 80%, more than 90%, more than 100%, such as more than 105%, such as more than 110%, such as more than 115%, such as more than 120% determined as Relative Activity at 80ºC/70ºC.

In one embodiment, protease has a thermostability of between 20 and 50%, such as between 20 and 40%, such as 20 and 30% determined as Relative Activity at 80ºC/70ºC. In one embodiment, the protease has a thermostability between 50 and 115%, such as between 50 and 70%, such as between 50 and 60%, such as between 100 and 120%, such as between 105 and 115% determined as Relative Activity at 80ºC/70ºC.

In one embodiment, the protease has a thermostability value of more than 10% determined as Relative Activity at 85ºC/70ºC determined as described in Example 2.

In one embodiment, the protease has a thermostability of more than 10%, such as more than 12%, more than 14%, more than 16%, more than 18%, more than 20%, more than 30%, more than 40%, more that 50%, more than 60%, more than 70%, more than 80%, more than 90%, more than 100%, more than 110% determined as Relative Activity at 85ºC/70ºC.

In one embodiment, the protease has a thermostability of between 10% and 50%, such as between 10% and 30%, such as between 10% and 25% determined as Relative Activity at 85ºC/70ºC.

In one embodiment, the protease has more than 20%, more than 30%, more than 40%, more than 50%, more than 60%, more than 70%, more than 80%, more than 90% determined as Remaining Activity at 80ºC; and/or the protease has more than 20%, more than 30%, more than 40%, more than 50%, more than 60%, more than 70%, more than 80%, more than 90% determined as Remaining Activity at 84ºC.

Determination of "Relative Activity" and "Remaining Activity" is done as described in Example 2.

In one embodiment, the protease may have a themostability for above 90, such as above 100 at 85ºC as determined using the Zein-BCA assay as disclosed in Example 3.

In one embodiment, the protease has a themostability above 60%, such as above 90%, such as above 100%, such as above 110% at 85ºC as determined using the Zein-BCA assay.

In one embodiment, protease has a themostability between 60-120, such as between 70-120%, such as between 80-120%, such as between 90-120%, such as between 100-120%, such as 110-120% at 85ºC as determined using the Zein-BCA assay.

In one embodiment, the thermostable protease has at least 20%, such as at least 30%, such as at least 40%, such as at least 50%, such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 95%, such as at least 100% of the activity of the JTP196 protease variant or Protease Pfu determined by the AZCL-casein assay.

Glucoamylase Expressed, Present and/or Added In Liquefaction Step i)

A glucoamylase may optionally be expressed, present and/or added in liquefaction step i). In one embodiment, the glucoamylase is added together with or separately from the alpha-amylase and/or the optional protease and/or pullulanase.

In one embodiment, the glucoamylase has a Relative Activity heat stability at 85ºC of at least 20%, at least 30%, or at least 35% determined as described in Example 4 (heat stability).

In one embodiment, the glucoamylase has a relative activity pH optimum at pH 5.0 of at least 90%, e.g., at least 95%, at least 97%, or 100% determined as described in Example 4 (pH optimum).

In one embodiment, the glucoamylase has a pH stability at pH 5.0 of at least 80%, at least 85%, at least 90% determined as described in Example 4 (pH stability).

In one embodiment, the glucoamylase, such as a *Penicillium oxalicum* glucoamylase variant, used in liquefaction has a thermostability determined as DSC Td at pH 4.0 as described in Example 15 of at least 70ºC, preferably at least 75ºC, such as at least 80ºC, such as at least 81ºC, such as at least 82ºC, such as at least 83ºC, such as at least 84ºC, such as at least 85ºC, such as at least 86ºC, such as at least 87%, such as at least 88ºC, such as at least 89ºC, such as at least 90ºC. In one embodiment, the glucoamylase, such as a *Penicillium oxalicum* glucoamylase variant has a thermostability determined as DSC Td at pH 4.0 as

described in Example 15 in the range between 70ºC and 95ºC, such as between 80ºC and 90ºC.

In one embodiment, the glucoamylase, such as a *Penicillium oxalicum* glucoamylase variant, used in liquefaction has a thermostability determined as DSC Td at pH 4.8 as described in Example 15 of at least 70ºC, preferably at least 75ºC, such as at least 80ºC, such as at least 81ºC, such as at least 82ºC, such as at least 83ºC, such as at least 84ºC, such as at least 85ºC, such as at least 86ºC, such as at least 87%, such as at least 88ºC, such as at least 89ºC, such as at least 90ºC, such as at least 91ºC. In one embodiment, the glucoamylase, such as a *Penicillium oxalicum* glucoamylase variant has a thermostability determined as DSC Td at pH 4.8 as described in Example 15 in the range between 70ºC and 95ºC, such as between 80ºC and 90ºC.

In one embodiment, the glucoamylase, such as a *Penicillium oxalicum* glucoamylase variant, used in liquefaction has a residual activity determined as described in Example 16 of at least 100% such as at least 105%, such as at least 110%, such as at least 115%, such as at least 120%, such as at least 125%. In one embodiment, the glucoamylase, such as a *Penicillium oxalicum* glucoamylase variant has a thermostability determined as residual activity as described in Example 16 in the range between 100% and 130%.

In one embodiment, the glucoamylase, e.g., of fungal origin such as a filamentous fungi, from a strain of the genus *Penicillium*, e.g., a strain of *Penicillium oxalicum*, in particular the *Penicillium oxalicum* glucoamylase disclosed as SEQ ID NO: 2 in WO 2011/127802 (which is hereby incorporated by reference) and shown in SEQ ID NO: 9 or 14 herein.

In one embodiment, the glucoamylase has at least 80%, e.g., 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% identity to the mature polypeptide shown in SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NOs: 9 or 14 herein.

In one embodiment, the glucoamylase is a variant of the *Penicillium oxalicum* glucoamylase disclosed as SEQ ID NO: 2 in WO 2011/127802 and shown in SEQ ID NO: 9 and 14 herein, having a K79V substitution (using the mature sequence shown in SEQ ID NO: 14 herein for numbering). The K79V glucoamylase variant has reduced sensitivity to protease degradation relative to the parent as disclosed in WO 2013/036526 (which is hereby incorporated by reference).

In one embodiment, the glucoamylase is derived from *Penicillium oxalicum*.

In one embodiment, the glucoamylase is a variant of the *Penicillium oxalicum* glucoamylase disclosed as SEQ ID NO: 2 in WO 2011/127802 and shown in SEQ ID NO: 9 and 14 herein. In one embodiment, the *Penicillium oxalicum* glucoamylase is the one disclosed as SEQ ID NO: 2 in WO 2011/127802 and shown in SEQ ID NO: 9 and 14 herein having Val (V) in position 79 (using SEQ ID NO: 14 herein for numbering).

Contemplated *Penicillium oxalicum* glucoamylase variants are disclosed in WO 2013/053801 which is hereby incorporated by reference.

In one embodiment, these variants have reduced sensitivity to protease degradation.

In one embodiment, these variant have improved thermostability compared to the parent.

In one embodiment, the glucoamylase has a K79V substitution (using SEQ ID NO: 14 herein for numbering), corresponding to the PE001 variant, and further comprises one of the following alterations or combinations of alterations:

T65A; Q327F; E501V; Y504T; Y504*; T65A + Q327F; T65A + E501V; T65A + Y504T; T65A + Y504*; Q327F + E501V; Q327F + Y504T; Q327F + Y504*; E501V + Y504T; E501V + Y504*; T65A + Q327F + E501V; T65A + Q327F + Y504T; T65A + E501V + Y504T; Q327F + E501V + Y504T; T65A + Q327F + Y504*; T65A + E501V + Y504*; Q327F + E501V + Y504*; T65A + Q327F + E501V + Y504T; T65A + Q327F + E501V + Y504*; E501V + Y504T; T65A + K161S; T65A + Q405T; T65A + Q327W; T65A + Q327F; T65A + Q327Y; P11F + T65A + Q327F; R1K + D3W + K5Q + G7V + N8S + T10K + P11S + T65A + Q327F; P2N + P4S + P11F + T65A + Q327F; P11F + D26C + K33C + T65A + Q327F; P2N + P4S + P11F + T65A + Q327W + E501V + Y504T; R1E + D3N + P4G + G6R + G7A + N8A + T10D+ P11D + T65A + Q327F; P11F + T65A + Q327W; P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; P11F + T65A + Q327W + E501V + Y504T; T65A + Q327F + E501V + Y504T; T65A + S105P + Q327W; T65A + S105P + Q327F; T65A + Q327W + S364P; T65A + Q327F + S364P; T65A + S103N + Q327F; P2N + P4S + P11F + K34Y + T65A + Q327F; P2N + P4S + P11F + T65A + Q327F + D445N + V447S; P2N + P4S + P11F + T65A + I172V + Q327F; P2N + P4S + P11F + T65A + Q327F + N502*; P2N + P4S + P11F + T65A + Q327F + N502T + P563S + K571E; P2N + P4S + P11F + R31S + K33V + T65A + Q327F + N564D + K571S; P2N + P4S + P11F + T65A + Q327F + S377T; P2N + P4S + P11F + T65A + V325T+ Q327W; P2N + P4S + P11F + T65A + Q327F + D445N + V447S + E501V + Y504T; P2N + P4S + P11F + T65A + I172V + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + S377T + E501V + Y504T; P2N + P4S + P11F + D26N + K34Y + T65A + Q327F; P2N + P4S + P11F + T65A + Q327F + I375A + E501V + Y504T; P2N + P4S + P11F + T65A + K218A + K221D + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + S103N + Q327F + E501V + Y504T; P2N + P4S + T10D + T65A + Q327F + E501V + Y504T; P2N + P4S + F12Y + T65A + Q327F + E501V + Y504T; K5A + P11F + T65A + Q327F + E501V + Y504T; P2N + P4S + T10E + E18N + T65A + Q327F + E501V + Y504T; P2N + T10E + E18N + T65A + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T568N; P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + K524T + G526A; P2N + P4S + P11F + K34Y + T65A + Q327F + D445N + V447S + E501V + Y504T; P2N + P4S + P11F + R31S + K33V + T65A + Q327F + D445N + V447S + E501V + Y504T; P2N + P4S + P11F + D26N + K34Y + T65A + Q327F + E501V +

Y504T; P2N + P4S + P11F + T65A + F80* + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + K112S + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T516P + K524T + G526A; P2N + P4S + P11F + T65A + Q327F + E501V + N502T + Y504*; P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + S103N + Q327F + E501V + Y504T; K5A + P11F + T65A + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T516P + K524T + G526A; P2N + P4S + P11F + T65A + V79A + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + V79G + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + V79I + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + V79L + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + V79S + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + L72V + Q327F + E501V + Y504T; S255N + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + E74N + V79K + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + G220N + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + Y245N + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + Q253N + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + D279N + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + S359N + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + D370N + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + V460S + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + V460T + P468T + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + T463N + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + S465N + E501V + Y504T; and P2N + P4S + P11F + T65A + Q327F + T477N + E501V + Y504T.

In one embodiment, the *Penicillium oxalicum* glucoamylase variant has a K79V substitution (using SEQ ID NO: 14 herein for numbering), corresponding to the PE001 variant, and further comprises one of the following substitutions or combinations of substitutions:

P11F + T65A + Q327F;

P2N + P4S + P11F + T65A + Q327F;

P11F + D26C + K33C + T65A + Q327F;

P2N + P4S + P11F + T65A + Q327W + E501V + Y504T;

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; and

P11F + T65A + Q327W + E501V + Y504T.

The glucoamylase may be added in amounts from 0.1-100 micrograms EP/g, such as 0.5-50 micrograms EP/g, such as 1-25 micrograms EP/g, such as 2-12 micrograms EP/g DS.

Pullulanase Present and/or Added In Liquefaction step i)

Optionally a pullulanase may be present and/or added during liquefaction step i) together with an alpha-amylase, and/or optional protease and/or glucoamylase.

The pullulanase may be present and/or added in liquefaction step i) and/or saccharification step ii) or simultaneous saccharification and fermentation (SSF).

Pullulanases (E.C. 3.2.1.41, pullulan 6-glucano-hydrolase), are debranching enzymes characterized by their ability to hydrolyze the alpha-1,6-glycosidic bonds in, for example, amylopectin and pullulan.

Contemplated pullulanases include the pullulanases from *Bacillus amyloderamificans* disclosed in U.S. Patent No. 4,560,651 (hereby incorporated by reference), the pullulanase disclosed as SEQ ID NO: 2 in WO 01/151620 (hereby incorporated by reference), the *Bacillus deramificans* disclosed as SEQ ID NO: 4 in WO 01/151620 (hereby incorporated by reference), and the pullulanase from *Bacillus acidopullulyticus* disclosed as SEQ ID NO: 6 in WO 01/151620 (hereby incorporated by reference) and also described in FEMS Mic. Let. (1994) 115, 97-106.

Additional pullulanases contemplated include the pullulanases from *Pyrococcus woesei*, specifically from *Pyrococcus woesei* DSM No. 3773 disclosed in WO92/02614.

In one embodiment, the pullulanase is a family GH57 pullulanase. In one embodiment, the pullulanase includes an X47 domain as disclosed in US 61/289,040 published as WO 2011/087836 (which are hereby incorporated by reference). More specifically the pullulanase may be derived from a strain of the genus *Thermococcus*, including *Thermococcus litoralis* and *Thermococcus hydrothermalis*, such as the *Thermococcus hydrothermalis* pullulanase shown in SEQ ID NO: 11 truncated at site X4 right after the X47 domain (i.e., amino acids 1-782 in SEQ ID NOS: 11 and 12 herein). The pullulanase may also be a hybrid of the *Thermococcus litoralis* and *Thermococcus hydrothermalis* pullulanases or a *T. hydrothermalis/T. litoralis* hybrid enzyme with truncation site X4 disclosed in US 61/289,040 published as WO 2011/087836 (which is hereby incorporated by reference) and disclosed in SEQ ID NO: 12 herein.

In another embodiment the pullulanase is one comprising an X46 domain disclosed in WO 2011/076123 (Novozymes).

The pullulanase may be added in an effective amount which include the preferred amount of about 0.0001-10 mg enzyme protein per gram DS, preferably 0.0001-0.10 mg enzyme protein per gram DS, more preferably 0.0001-0.010 mg enzyme protein per gram DS. Pullulanase activity may be determined as NPUN. An Assay for determination of NPUN is described in the “Materials & Methods”-section below.

Suitable commercially available pullulanase products include PROMOZYME D, PROMOZYME™ D2 (Novozymes A/S, Denmark), OPTIMAX L-300 (DuPont-Danisco, USA), and AMANO 8 (Amano, Japan).

Glucoamylase Expressed,Present And/Or Added in Saccharification and/or Fermentation

Glucoamylase may be expressed, present and/or added in saccharification, fermentation or simultaneous saccharification and fermentation (SSF). The glucoamylse 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), p. 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 (Agric. Biol. Chem. (1991), 55 (4), p. 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 position A435 and S436 (Li et al. (1997), Protein Eng. 10, 1199-1204.

Other glucoamylases include *Athelia rolfsii* (previously denoted *Corticium rolfsii*) glucoamylase (see US patent no. 4,727,026 and (Nagasaka et al. (1998) "Purification and properties of the raw-starch-degrading glucoamylases from *Corticium rolfsii*, Appl Microbiol Biotechnol 50:323-330), *Talaromyces* glucoamylases, in particular derived from *Talaromyces emersonii* (WO 99/28448), *Talaromyces leycettanus* (US patent no. Re. 32,153), *Talaromyces duponti, Talaromyces thermophilus* (US patent no. 4,587,215). In one embodiment, the glucoamylase used during saccharification and/or fermentation is the *Talaromyces emersonii* glucoamylase disclosed in WO 99/28448.

Bacterial glucoamylases contemplated include glucoamylases from the genus *Clostridium*, in particular *C. thermoamylolyticum* (EP 135,138), and *C. thermohydrosulfuricum* (WO 86/01831).

Contemplated fungal glucoamylases include *Trametes cingulate* (SEQ ID NO: 20), *Pachykytospora papyracea*; and *Leucopaxillus giganteus* all disclosed in WO 2006/069289; or *Peniophora rufomarginata* disclosed in WO2007/124285; or a mixture thereof. Also hybrid glucoamylase are contemplated. Examples include the hybrid glucoamylases disclosed in WO 2005/045018. Specific examples include the hybrid glucoamylase disclosed in Table 1 and 4 of Example 1 (which hybrids are hereby incorporated by reference).

In one embodiment, the glucoamylase is derived from a strain of the genus *Pycnoporus*, in particular a strain of *Pycnoporus* as described in WO 2011/066576 (SEQ ID NOs 2, 4 or 6), including the *Pycnoporus sanguineus* glucoamylase disclosed as SEQ ID NO: 18 herein, or from a strain of the genus *Gloeophyllum*, such as a strain of *Gloeophyllum sepiarium* or *Gloeophyllum trabeum*, in particular a strain of *Gloeophyllum* as described in WO

2011/068803 (SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16). In one embodiment, the glucoamylase is SEQ ID NO: 2 in WO 2011/068803 or SEQ ID NO: 15 herein (i.e. *Gloeophyllum sepiarium* glucoamylase).

In one embodiment, the glucoamylase is SEQ ID NO: 17 herein (i.e., *Gloeophyllum trabeum* glucoamylase disclosed as SEQ ID NO: 3 in WO2014/177546). In another embodiment the glucoamylase is derived from a strain of the genus *Nigrofomes,* in particular a strain of *Nigrofomes sp.* disclosed in WO 2012/064351 (SEQ ID NO: 2) (all references hereby incorporated by reference).

Contemplated are also glucoamylases which exhibit a high identity to any of the above mentioned glucoamylases, i.e., at least 60%, such as 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 any one of the mature parts of the enzyme sequences mentioned above, such as any of SEQ ID NOs: 15, 17, 18 or 19 herein, respectively, preferably SEQ ID NO: 15 herein or SEQ ID NO: 17 herein.

Glucoamylases may be added to the saccharification and/or fermentation in an amount of 0.0001-20 AGU/g DS, preferably 0.001-10 AGU/g DS, especially between 0.01-5 AGU/g DS, such as 0.1-2 AGU/g DS.

Glucoamylases may be added to the saccharification and/or fermentation in an amount of 1-1,000 µg EP/g DS, preferably 10-500 µg/gDS, especially between 25-250 µg/g DS.

In one embodiment, the glucoamylase is added as a blend further comprising an alpha-amylase. In one embodiment, the alpha-amylase is a fungal alpha-amylase, especially an acid fungal alpha-amylase. The alpha-amylase is typically a side activity.

In one embodiment, the glucoamylase is a blend comprising *Talaromyces emersonii* glucoamylase disclosed in WO 99/28448 as SEQ ID NO: 34 and SEQ ID NO: 19 herein and *Trametes cingulata* glucoamylase disclosed as SEQ ID NO: 2 in WO 06/069289 and SEQ ID NO: 20 herein.

In one embodiment, the glucoamylase is a blend comprising *Talaromyces emersonii* glucoamylase disclosed in WO 99/28448 (SEQ ID NO: 19 herein), *Trametes cingulata* glucoamylase disclosed as SEQ ID NO: 2 in WO 06/69289 and SEQ ID NO: 20 herein, and an alpha-amylase.

In one embodiment, the glucoamylase is a blend comprising *Talaromyces emersonii* glucoamylase disclosed in WO99/28448 (SEQ ID NO: 19 herein), *Trametes cingulata* glucoamylase disclosed in WO 06/69289 (SEQ ID NO: 20 herein), and *Rhizomucor pusillus* alpha-amylase with *Aspergillus niger* glucoamylase linker and SBD disclosed as V039 in Table 5 in WO 2006/069290 or SEQ ID NO: 16 herein.

In one embodiment, the glucoamylase is a blend comprising *Gloeophyllum sepiarium* glucoamylase shown as SEQ ID NO: 2 in WO 2011/068803 (SEQ ID NO: 15 herein) and an

alpha-amylase, in particular *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), disclosed SEQ ID NO: 3 in WO 2013/006756, in particular with the following substitutions: G128D+D143N.

In one embodiment, the alpha-amylase may be derived from a strain of the genus *Rhizomucor*, preferably a strain the *Rhizomucor pusillus*, such as the one shown in SEQ ID NO: 3 in WO2013/006756, or the genus *Meripilus*, preferably a strain of *Meripilus giganteus*. In one embodiment, the alpha-amylase is derived from a *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), disclosed as V039 in Table 5 in WO 2006/069290 or SEQ ID NO: 16 herein.

In one embodiment, the *Rhizomucor pusillus* alpha-amylase or the *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) has at least one of the following substitutions or combinations of substitutions: D165M; Y141W; Y141R; K136F; K192R; P224A; P224R; S123H+Y141W; G20S + Y141W; A76G + Y141W; G128D + Y141W; G128D + D143N; P219C + Y141W; N142D + D143N; Y141W + K192R; Y141W + D143N; Y141W + N383R; Y141W + P219C + A265C; Y141W + N142D + D143N; Y141W + K192R V410A; G128D + Y141W + D143N; Y141W + D143N + P219C; Y141W + D143N + K192R; G128D + D143N + K192R; Y141W + D143N + K192R + P219C; and G128D + Y141W + D143N + K192R; or G128D + Y141W + D143N + K192R + P219C (using SEQ ID NO: 3 in WO 2013/006756 for numbering or SEQ ID NO: 16 herein).

In one embodiment, the glucoamylase blend comprises *Gloeophyllum sepiarium glucoamylase* (e.g., SEQ ID NO: 2 in WO 2011/068803 or SEQ ID NO: 15 herein) and *Rhizomucor pusillus* alpha-amylase.

In one embodiment, the glucoamylase blend comprises *Gloeophyllum sepiarium* glucoamylase shown as SEQ ID NO: 2 in WO 2011/068803 or SEQ ID NO: 15 herein and *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), disclosed SEQ ID NO: 3 in WO 2013/006756 and SEQ ID NO: 16 herein with the following substitutions: G128D+D143N.

Commercially available compositions comprising glucoamylase include AMG 200L; AMG 300 L; SAN™ SUPER, SAN™ EXTRA L, SPIRIZYME™ PLUS, SPIRIZYME™ FUEL, SPIRIZYME™ B4U, SPIRIZYME™ ULTRA, SPIRIZYME™ EXCEL, SPIRIZYME ACHIEVE™, and AMG™ E (from Novozymes A/S); OPTIDEX™ 300, GC480, GC417 (from DuPont-Danisco); AMIGASE™ and AMIGASE™ PLUS (from DSM); G-ZYME™ G900, G-ZYME™ and G990 ZR (from DuPont-Danisco).

<u>Cellulolytic Enzyme Composition present and/or added during Saccharification and/or Fermentation</u>

A cellulolytic enzyme composition comprising a beta-glucosidase, a cellobiohydrolase and an endoglucanase may be present in saccharification or fermentation or simultaneous saccharification and fermentation (SSF).

Examples of suitable cellulolytic enzyme composition can be found in WO 2008/151079 and WO 2013/028928 which are incorporated by reference.

In preferred embodiments the cellulolytic enzyme composition is derived from a strain of *Trichoderma*, *Humicola,* or *Chrysosporium*.

In one embodiment, the cellulolytic enzyme composition is derived from a strain of *Trichoderma reesei*, *Humicola insolens* and/or *Chrysosporium lucknowense*.

In one embodiment, the cellulolytic enzyme composition comprises a beta-glucosidase, e.g., one derived from a strain of the genus *Aspergillus*, such as *Aspergillus oryzae*, such as the one disclosed in WO 2002/095014 or the fusion protein having beta-glucosidase activity disclosed in WO 2008/057637, or *Aspergillus fumigatus*, such as one disclosed in WO 2005/047499 (SEQ ID NO: 29 herein) or an *Aspergillus fumigatus* beta-glucosidase variant disclosed in WO 2012/044915 (Novozymes), such as one with the following substitutions: F100D, S283G, N456E, F512Y (using SEQ ID NO: 29 herein for numbering); or a strain of the genus a strain *Penicillium*, such as a strain of the *Penicillium brasilianum* disclosed in WO 2007/019442, or a strain of the genus *Trichoderma*, such as a strain of *Trichoderma reesei*.

In one embodiment, the cellulolytic enzyme composition comprises a GH61 polypeptide having cellulolytic enhancing activity such as one derived from the genus *Thermoascus*, such as a strain of *Thermoascus aurantiacus*, such as the one described in WO 2005/074656 as SEQ ID NO: 2 and SEQ ID NO: 30 herein; or one derived from the genus *Thielavia*, such as a strain of *Thielavia terrestris*, such as the one described in WO 2005/074647 as SEQ ID NO: 7 and SEQ ID NO: 8; or one derived from a strain of *Aspergillus*, such as a strain of *Aspergillus fumigatus*, such as the one described in WO 2010/138754 as SEQ ID NO: 1 and SEQ ID NO: 2; or one derived from a strain derived from *Penicillium*, such as a strain of *Penicillium emersonii*, such as the one disclosed in WO 2011/041397 as SEQ ID NO: 2 or SEQ ID NO: 31 herein.

In one embodiment, the cellulolytic enzyme composition comprises a cellobiohydrolase I (CBH I), such as one derived from a strain of the genus *Aspergillus*, such as a strain of *Aspergillus fumigatus*, such as the Cel7a CBH I disclosed in SEQ ID NO: 2 in WO 2013/028928 or SEQ ID NO: 32 herein, or a strain of the genus *Trichoderma*, such as a strain of *Trichoderma reesei*.

In one embodiment, the cellulolytic enzyme composition comprises a cellobiohydrolase II (CBH II, such as one derived from a strain of the genus *Aspergillus*, such as a strain of *Aspergillus fumigatus*, such as the one disclosed as SEQ ID NO: 4 in WO

2013/028928 or SEQ ID NO: 33 herein); or a strain of the genus *Trichoderma*, such as *Trichoderma reesei*, or a strain of the genus *Thielavia*, such as a strain of *Thielavia terrestris*, such as cellobiohydrolase II CEL6A from *Thielavia terrestris*.

In one embodiment, the cellulolytic enzyme composition comprises a GH61 polypeptide having cellulolytic enhancing activity and a beta-glucosidase.

In one embodiment, the cellulolytic enzyme composition comprises a GH61 polypeptide having cellulolytic enhancing activity, a beta-glucosidase, and a CBH I.

In one embodiment, the cellulolytic enzyme composition comprises a GH61 polypeptide having cellulolytic enhancing activity, a beta-glucosidase, a CBH I, and a CBH II.

In one embodiment, the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition, further comprising *Thermoascus aurantiacus* GH61A polypeptide having cellulolytic enhancing activity (SEQ ID NO: 2 in WO 2005/074656 or SEQ ID NO: 30 herein), and *Aspergillus oryzae* beta-glucosidase fusion protein (WO 2008/057637).

In one embodiment, the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition, further comprising *Thermoascus aurantiacus* GH61A polypeptide having cellulolytic enhancing activity (SEQ ID NO: 2 in WO 2005/074656 or SEQ ID NO: 30 herein) and *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 29 herein).

In one embodiment, the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition further comprising *Penicillium emersonii* GH61A polypeptide having cellulolytic enhancing activity disclosed as SEQ ID NO: 2 in WO 2011/041397 or SEQ ID NO: 31 herein and *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 29 herein) or a variant thereof with the following substitutions F100D, S283G, N456E, F512Y (using SEQ ID NO; 29 for numbering).

In one embodiment, the cellulolytic enzyme composition comprising one or more of the following components:

(i) an *Aspergillus fumigatus* cellobiohydrolase I;

(ii) an *Aspergillus fumigatus* cellobiohydrolase II;

(iii) an *Aspergillus fumigatus* beta-glucosidase or variant thereof; and

(iv) a *Penicillium* sp. GH61 polypeptide having cellulolytic enhancing activity; or homologs thereof.

In one embodiment, the cellulolytic enzyme composition is derived from *Trichoderma reesei* comprising GH61A polypeptide having cellulolytic enhancing activity derived from a strain of *Penicillium emersonii* (SEQ ID NO: 2 in WO 2011/041397 or SEQ ID NO: 31 herein, *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 2 in WO 2005/047499 or SEQ ID NO: 29 herein) variant with the following substitutions: F100D, S283G, N456E, F512Y) disclosed in WO 2012/044915; *Aspergillus fumigatus* Cel7A CBH1 disclosed as SEQ ID NO: 6 in

WO2011/057140 or SEQ ID NO: 32 herein and *Aspergillus fumigatus* CBH II disclosed as SEQ ID NO: 18 in WO 2011/057140 or SEQ ID NO: 33 herein.

In one embodiment, the cellulolytic enzyme composition is dosed from 0.0001-3 mg EP/g DS, preferably, 0.0005-2 mg EP/g DS, preferably 0.001-1 mg/g DS, more preferably 0.005-0.5 mg EP/g DS, and even more preferably 0.01-0.1 mg EP/g DS.

Examples of Preferred Processes

In one embodiment, is a process for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using an alpha-amylase derived from *Bacillus stearothermophilus* (e.g., SEQ ID NO: 1 herein);

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is:

Saccharomyces cerevisiae strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, a protease is added in saccharification and/or fermentation or SSF.

In one embodiment, is processes for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using an alpha-amylase derived from *Bacillus stearothermophilus* comprising a double deletion at positions I181 + G182, and optionally a N193F substitution; (using SEQ ID NO: 1 for numbering);

ii) saccharifying using a glucoamylase, e.g., one derived from a strain of *Gloephyllum*, such as *Gloephyllum serpiarium* or *Gloephyllum trabeum*;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining

characteristics of strain *Saccharomyces* strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, is a processes for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:

an alpha-amylase derived from *Bacillus stearothermophilus* (e.g., SEQ ID NO: 1);

a protease having a thermostability value of more than 20% determined as Relative Activity at 80ºC/70ºC, preferably derived from *Pyrococcus furiosus* and/or *Thermoascus aurantiacus*; and

optionally a *Penicillium oxalicum* glucoamylase;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain *Saccharomyces* strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, is a processes for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using an alpha-amylase, preferably derived from *Bacillus stearothermophilus,* in particular one comprising a double deletion at positions I181 + G182, and optionally a N193F substitution (using SEQ ID NO: 1 for numbering) and having a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$ of at least 10;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain

MBG4985 having defining characteristics of strain *Saccharomyces* strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, is a processes for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature between 80-90°C:

an alpha-amylase, preferably derived from *Bacillus stearothermophilus,* in particular one having a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$ of at least 10;

a protease, preferably derived from *Pyrococcus furiosus* and/or *Thermoascus aurantiacus,* having a thermostability value of more than 20% determined as Relative Activity at 80ºC/70ºC;

optionally a *Penicillium oxalicum* glucoamylase

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain *Saccharomyces* strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, is processes for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:

an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion at positions I181 + G182, and optional substitution N193F; and optionally further one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S:

V59A+Q89R+E129V+K177L+R179E+Q254S+M284V;

V59A+ E129V+ K177L+R179E+Q254S+M284V; and

E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering);

ii) saccharifying using a glucoamylase, such as one from a strain of *Gloephyllum*, such as a strain of *Gloeophyllum serpiarium*;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain *Saccharomyces* strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, is a processes for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:

an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion at positions I181 + G182, and optional substitution N193F, and optionally further one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S:

V59A+Q89R+E129V+K177L+R179E+Q254S+M284V;

V59A+ E129V+ K177L+R179E+Q254S+M284V; and

E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering).

a protease having a thermostability value of more than 20% determined as Relative Activity at 80ºC/70ºC, preferably derived from *Pyrococcus furiosus* and/or *Thermoascus aurantiacus*; and

optionally a *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 14 having one of the following substitutions or combinations of substitutions:

K79V;

K79V+ P11F + T65A + Q327F;

K79V+P2N + P4S + P11F + T65A + Q327F;

K79V +P11F + D26C + K33C + T65A + Q327F;

K79V +P2N + P4S + P11F + T65A + Q327W + E501V + Y504T;

K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; and

K79V +P11F + T65A + Q327W + E501V + Y504T (using SEQ ID NO: 14 for numbering);

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain *Saccharomyces* strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, is a processes for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature between 80-90°C using:

an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion at positions I181 + G182, and optional substitution N193F, and further optionally one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

V59A+Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V;

V59A+E129V+K177L+R179E+Q254S+M284V; and

E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering),

a protease having a thermostability value of more than 20% determined as Relative Activity at 80ºC/70ºC, preferably derived from *Pyrococcus furiosus* and/or *Thermoascus aurantiacus*;

a *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 14 having one of the following substitutions or combinations of substitutions:

K79V;

K79V+ P11F + T65A + Q327F;

K79V+P2N + P4S + P11F + T65A + Q327F;

K79V +P11F + D26C + K33C + T65A + Q327F;

K79V +P2N + P4S + P11F + T65A + Q327W + E501V + Y504T;

K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; and

K79V +P11F + T65A + Q327W + E501V + Y504T (using SEQ ID NO: 14 for numbering);

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research

Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain *Saccharomyces* strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, is a processes for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:

an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion at positions I181 + G182, and optional substitution N193F;

a protease having a thermostability value of more than 20% determined as Relative Activity at 80ºC/70ºC, preferably derived from *Pyrococcus furiosus* and/or *Thermoascus aurantiacus*; and

optionally a pullulanase;

a *Penicillium oxalicum* glucoamylase having a K79V substilution (using SEQ ID NO: 14 for numbering);

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain *Saccharomyces* strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, is a processes for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:

an alpha-amylase, preferably derived from *Bacillus stearothermophilus,* having a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$ of at least 10;

between 0.5 and 10 micro grams *Pyrococcus furiosus* protease per g DS;

ii) saccharifying using a glucoamylase selected from the group of glucoamylase derived from a strain of *Aspergillus*, preferably *A. niger*, *A. awamori*, or *A. oryzae*; or a strain of *Trichoderma*, preferably *T. reesei*; or a strain of *Talaromyces*, preferably *T. emersonii*, or a strain of *Pycnoporus*, or a strain of *Gloeophyllum*, such as *G. serpiarium* or *G. trabeum,* or a strain of the *Nigrofomes;*

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain *Saccharomyces* strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, is a processes for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature between 80-90°C using;

an alpha-amylase, preferably derived from *Bacillus stearothermophilus* having a double deletion at positions I181 + G182, and optional substitution N193F and having a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$ of at least 10;

between 0.5 and 10 micro grams *Pyrococcus furiosus* protease per g DS;

optionally a pullulanase;

a *Penicillium oxalicum* glucoamylase;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain *Saccharomyces* strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, is a processes for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature a temperature between 80-90°C using;

an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion I181 + G182 and optional substitution N193F; and optionally further one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

V59A+Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V;

V59A+E129V+ K177L+ R179E+ Q254S+ M284V; and

E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering);

between 0.5 and 10 micro grams *Pyrococcus furiosus* protease per g DS; and optionally a pullulanase;

a *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 14 having one of the following substitutions or combinations of substitutions:

K79V;

K79V+ P11F + T65A + Q327F;

K79V+P2N + P4S + P11F + T65A + Q327F;

K79V +P11F + D26C + K33C + T65A + Q327F;

K79V +P2N + P4S + P11F + T65A + Q327W + E501V + Y504T;

K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; and

K79V +P11F + T65A + Q327W + E501V + Y504T (using SEQ ID NO: 14 for numbering);

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain *Saccharomyces* strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, is a processes for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature between 80-90°C using:

an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion I181 + G182 and optional substitution N193F; and further one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

V59A+Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V;

V59A+E129V+ K177L+ R179E+ Q254S+ M284V; and

E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering);

a protease having a thermostability value of more than 20% determined as Relative Activity at 80ºC/70ºC, preferably derived from *Pyrococcus furiosus* and/or *Thermoascus aurantiacus*;

optionally a pullulanase; and

a *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 14 having one of the following substitutions or combinations of substitutions:

K79V;

K79V+ P11F + T65A + Q327F;

K79V+P2N + P4S + P11F + T65A + Q327F;

K79V +P11F + D26C + K33C + T65A + Q327F;

K79V +P2N + P4S + P11F + T65A + Q327W + E501V + Y504T;

K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; and

K79V +P11F + T65A + Q327W + E501V + Y504T (using SEQ ID NO: 14 for numbering);

ii) saccharifying using a glucoamylase selected from the group of glucoamylase derived from a strain of *Aspergillus*; or a strain of *Trichoderma;* a strain of *Talaromyces*, a strain of *Pycnoporus*; a strain of *Gloeophyllum;* and a strain of the *Nigrofomes;*

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain *Saccharomyces* strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, is a processes for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature between 80-90°C at a pH between 5.0 and 6.5 using:

an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion I181 + G182 and optional substitution N193F; and optionally further one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

V59A+Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V;

V59A+E129V+ K177L+ R179E+ Q254S+ M284V; and

E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering).

a protease derived from *Pyrococcus furiosus*, preferably the one shown in SEQ ID NO: 13 herein;

a *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 14 having one of the following substitutions or combinations of substitutions:

K79V;

K79V+ P11F + T65A + Q327F;

K79V+P2N + P4S + P11F + T65A + Q327F;

K79V +P11F + D26C + K33C + T65A + Q327F;

K79V +P2N + P4S + P11F + T65A + Q327W + E501V + Y504T;

K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; and

K79V +P11F + T65A + Q327W + E501V + Y504T (using SEQ ID NO: 14 for numbering);

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain *Saccharomyces* strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, is a process comprises the steps of:

i) liquefying the starch-containing material at a temperature between 80-90°C at a pH between 5.0 and 6.5 using:

an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion I181 + G182, and optional substitution N193F; and optionally further one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

V59A+Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V;

V59A+E129V+ K177L+ R179E+ Q254S+ M284V; and

E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering);

a protease derived from *Pyrococcus furiosus*, preferably the one shown in SEQ ID NO: 13 herein present and/or added in a dosage of 1-5 micro gram protease per gram DS, such as about 1.5 or 3 micro gram protease per gram DS; and

a *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 14 having one of the following substitutions or combinations of substitutions:

K79V;

K79V+ P11F + T65A + Q327F;

K79V+P2N + P4S + P11F + T65A + Q327F;

K79V +P11F + D26C + K33C + T65A + Q327F;

K79V +P2N + P4S + P11F + T65A + Q327W + E501V + Y504T;

K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; and

K79V +P11F + T65A + Q327W + E501V + Y504T (using SEQ ID NO: 14 for numbering);

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain *Saccharomyces* strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, is a processes for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using an alpha-amylase derived from *Bacillus stearothermophilus*;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism increases ethanol yield compared to Ethanol Red™ under the same process conditions, e.g., conditions as described herein.

Raw Starch Hydrolysis Process

This aspect concerns improved raw starch hydrolysis processes for producing ethanol using a fermenting organism and yeast strains suitable for use in processes and methods thereof.

More specifically, this aspect relates to processes of producing ethanol from starch-containing material, such as granular starch, comprising:

(i) saccharifying a starch-containing material at a temperature below the initial gelatinization temperature; and

(ii) fermenting using a fermentation organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain *Saccharomyces* strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one aspect is a processes of producing ethanol from starch-containing material, such as granular starch, comprising:

(i) saccharifying a starch-containing material at a temperature below the initial gelatinization temperature; and

(ii) fermenting using a fermentation organism;

wherein

saccharification and/or fermentation is done in the presence of the following enzymes: glucoamylase and alpha-amylase, and optionally protease; and

the fermenting organism is a *Saccharomyces* yeast strain providing one or more, such as all, of the following improvements:

an ethanol yield boost compared to ETHANOL RED™ under the same fermentation conditions (e.g., conditions as described herein);

reduced acetaldehyde production compared to ETHANOL RED™ under the same process conditions (e.g., conditions as described herein);

increased temperature tolerance compared to ETHANOL RED™ under the same process conditions (e.g., conditions as described herein);

decreased glycerol production compared to ETHANOL RED™ under the same process conditions (e.g., conditions as described herein).

In one embodiment, the fermenting organism used in a process is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA). In one embodiment, the fermenting organism used in a process is a derivative of *Saccharomyces cerevisiae* strain MBG4985 that expresses a glucoamylase and/or an alpha-amylase.

A raw starch hydrolysis process using the yeast described herein results in one or more, such as all, of the following improvements compared to a corresponding process carried out under the same conditions using ETHANOL RED™ ("ER") as the fermenting organism:

an ethanol yield boost compared to ETHANOL RED™ under the same fermentation conditions (e.g., conditions as described herein);

reduced acetaldehyde production compared to ETHANOL RED™ under the same process conditions (e.g., conditions as described herein);

increased temperature tolerance compared to ETHANOL RED™ under the same process conditions (e.g., conditions as described herein);

decreased glycerol production compared to ETHANOL RED™ under the same process conditions (e.g., conditions as described herein).

Examples of suitable enzymes used, especially glucoamylases, alpha-amylases, proteases, cellulolytic enzyme compositions etc are described in the "Enzymes And Enzyme Blends Used In A Raw Starch Hydrolysis Process" section below.

In one embodiment, the following enzymes are expressed, present and/or added in saccharification and/or fermentation: *Trametes cingulata* glucoamylase, e.g., the one shown in SEQ ID NO: 20 herein and an alpha-amylase. In one embodiment, the alpha-amylase is a *Rhizomucor pusillus* alpha-amylase, preferably the *Rhizomucor pusillus* alpha-amylase with a linker and starch-binding domain (SBD), in particular the *Rhizomucor pusillus* alpha-amylase with *Aspergillus niger* glucoamylase linker and starch-binding domain shown in SEQ ID NO: 16 herein.

In one embodiment, the following enzymes are expressed, present and/or added in saccharification and/or fermentation: *Gloeophyllum trabeum* glucoamylase, e.g., the one shown in SEQ ID NO: 17 herein, especially one further having one or more of the following substitutions: S95P, A121P, especially S95P+A121P and an alpha-amylase. In one embodiment, the alpha-amylase is derived from *Rhizomucor pusillus*, e.g., *Rhizomucor pusillus* alpha-amylase with a linker and starch-binding domain (SBD), in particular the *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) disclosed as V039 in Table 5 in WO 2006/069290 or SEQ ID NO: 16 herein.

In one embodiment of the process the following enzymes are expressed, present and/or added in saccharification and/or fermentation: *Gloeophyllum trabeum* glucoamylase, preferably the one shown in SEQ ID NO: 17 herein, preferably one further having one or more of the following substitutions: S95P, A121P, especially S95P+A121P and an alpha-amylase. The alpha-amylase may be derived from *Rhizomucor pusillus*, preferably *Rhizomucor pusillus* alpha-amylase with a linker and starch-binding domain (SBD), in particular the *Rhizomucor pusillus* alpha-amylase with *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) shown in SEQ ID NO: 16 herein, preferably one further having one or more of the following substitutions: G128D, D143N, especially G128D+143N.

In one embodiment, the following enzymes are expressed, present and/or added in saccharification and/or fermentation: *Pycnoporus sanguineus* glucoamylase, preferably the one shown in SEQ ID NO: 18 herein and an alpha-amylase. In one embodiment, the alpha-amylase is derived from *Rhizomucor pusillus,* preferably with a linker and starch-binding domain (SBD), e.g., the *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) disclosed as V039 in Table 5 in WO 2006/069290 or SEQ ID NO: 16 herein, such as one further having one or more of the following substitutions: G128D, D143N (e.g., G128D+D143N).

In one embodiment, a protease is present and/or added in saccharification and/or fermentation. In one embodiment, the protease is a metallo protease or a serine protease. In one embodiment, the metallo protease is derived from a strain of the genus *Thermoascus*, such as a strain of *Thermoascus aurantiacus*, e.g., *Thermoascus aurantiacus* CGMCC No. 0670 (e.g., the metallo protease disclosed as the mature part of SEQ ID NO: 2 disclosed in WO 2003/048353 or the mature polypeptide of SEQ ID NO: 3 herein).

In one embodiment, a cellulolytic enzyme composition is present and/or added in saccharification and/or fermentation.

In one embodiment, the cellulolytic enzyme composition is derived from *Trichoderma reesei*, preferably further comprising *Thermoascus aurantiacus* GH61A polypeptide having cellulolytic enhancing activity (e.g., SEQ ID NO: 2 in WO 2005/074656 or SEQ ID NO: 30 herein) and *Aspergillus fumigatus* beta-glucosidase (e.g., SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 29 herein), or a cellulolytic enzyme composition derived from *Trichoderma reesei*, preferably further comprising *Penicillium emersonii* GH61A polypeptide, e.g., the one disclosed as SEQ ID NO: 2 in WO 2011/041397 or SEQ ID NO: 31 herein, and *Aspergillus fumigatus* beta-glucosidase, e.g., the one disclosed as SEQ ID NO: 2 in WO 2005/047499 or SEQ ID NO: 29 herein, or a variant thereof, such as a variant having one of, or all of, the following substitutions: F100D, S283G, N456E, F512Y, *Aspergillus fumigatus* CBH1, e.g., the one disclosed as SEQ ID NO: 6 in WO2011/057140 and SEQ ID NO: 32 herein, and

Aspergillus fumigatus CBH II, e.g., the one disclosed as SEQ ID NO: 18 in WO 2011/057140 and as SEQ ID NO: 33 herein.

In one embodiment, the glucoamylase to alpha-amylase ratio is between 99:1 and 1:2, such as between 98:2 and 1:1, such as between 97:3 and 2:1, such as between 96:4 and 3:1, such as 97:3, 96:4, 95:5, 94:6, 93:7, 90:10, 85:15, 83:17 or 65:35 (mg EP glucoamylase: mg EP alpha-amylase).

In one embodiment, the glucoamylase to alpha-amylase ratio is between 100:1 and 1:2, such as between 90:1 and 1:1, such as between 80:1 and 2:1, such as between 70:1 and 3:1, such as 16:1 (determined as AGU: FAU-F).

In one embodiment, the total dose of glucoamylase and alpha-amylase is from 10-1,000 µg/g DS, such as from 50-500 µg/g DS, such as 75-250 µg/g DS.

In one embodiment, the total dose of cellulolytic enzyme composition added is from 10-500 µg/g DS, such as from 20-400 µg/g DS, such as 20-300 µg/g DS.

In one embodiment, the dose of protease added is from 1-200 µg/g DS, such as from 2-100 µg/g DS, such as 3-50 µg/g DS.

In one embodiment, saccharification step (a) and fermentation step (b) are carried out simultaneously.

In one embodiment, the fermenting organism is a non-recombinant *Saccharomyces* strain, e.g., a non-recombinant *Saccharomyces cerevisiae* strain produced using the method described and concerned in US patent no. 8,257,959-BB.

Enzymes And Enzyme Blends Used In A Raw Starch Hydrolysis Process

Glucoamylase and an alpha-amylase may be present and/or added in saccharification step (i) and/or fermentation step (ii) (e.g., simultaneous saccharification and fermentation (SSF)). Optionally a protease and/or a cellulolytic enzyme composition is(are) also present and/or added. Other enzymes such as pullulanases, pectinases, and/or trehalases may also be present and/or added.

A non-exhaustive list of suitable and specifically contemplated enzymes and enzyme combinations (e.g., blends) are described below.

In one embodiment, the following enzymes are present and/or added during saccharification and/or fermentation: *Trametes* glucoamylase, preferably *Trametes cingulata* glucoamylase shown in SEQ ID NO: 20 herein and an alpha-amylase.

In one embodiment, the glucoamylase is derived from *Trametes cingulata*, such as the one shown in SEQ ID NO: 20 herein, or a glucoamylase selected from the group consisting of:

(i) a glucoamylase comprising the mature polypeptide of SEQ ID NO: 20 herein;

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, e.g., at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 20 herein.

In one embodiment, the following enzymes are present and/or added during saccharification and/or fermentation: *Gloeophyllum* glucoamylase, preferably *Gloeophyllum trabeum* glucoamylase, especially the *Gloeophyllum trabeum* glucoamylase shown in SEQ ID NO: 17 herein and an alpha-amylase.

In one embodiment, the glucoamylase is derived from *Gloeophyllum trabeum*, such as the one shown in SEQ ID NO: 17 herein, or a glucoamylase selected from the group consisting of:

(i) a glucoamylase comprising the mature polypeptide of SEQ ID NO: 17 herein;

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 17 herein.

In one embodiment, the *Gloeophyllum* glucoamylase, such as the *Gloeophyllum trabeum* glucoamylase shown in SEQ ID NO: 17, has one of the following substitutions: V59A; S95P; A121P; T119W; S95P+A121P; V59A+S95P; S95P+T119W; V59A+S95P+A121P; or S95P+T119W+A121P, especially S95P+A121P (using SEQ ID NO: 17 for numbering).

The alpha-amylase used in a process described herein is typically a fungal alpha-amylase, such as an acid fungal alpha-amylase. In one embodiment, the alpha-amylase is derived from *Rhizomucor,* such as a *Rhizomucor pusillus* alpha-amylase with a linker and starch-binding domain (SBD), e.g., the *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) disclosed as V039 in Table 5 in WO 2006/069290 or SEQ ID NO: 16 herein.

In one embodiment, the alpha-amylase is a *Rhizomucor* alpha-amylase or the *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) shown in SEQ ID NO: 16 herein, e.g., one having at least one of the following substitutions or combinations of substitutions: D165M; Y141W; Y141R; K136F; K192R; P224A; P224R; S123H+Y141W; G20S + Y141W; A76G + Y141W; G128D + Y141W; G128D + D143N; P219C + Y141W; N142D + D143N; Y141W + K192R; Y141W + D143N; Y141W + N383R; Y141W + P219C + A265C; Y141W + N142D + D143N; Y141W + K192R V410A; G128D + Y141W + D143N; Y141W + D143N + P219C; Y141W + D143N + K192R; G128D + D143N + K192R; Y141W + D143N + K192R + P219C; G128D + Y141W + D143N + K192R; or G128D + Y141W + D143N + K192R + P219C, especially G128D+D143N (using SEQ ID NO: 16 for numbering).

In one embodiment, the alpha-amylase is selected from the group consisting of:

(i) an alpha-amylase comprising the mature polypeptide of SEQ ID NO: 16 herein;

(ii) an alpha-amylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 16 herein.

In one embodiment, the following enzymes are present and/or added in saccharification and/or fermentation: the *Trametes cingulata* glucoamylase shown in SEQ ID NO: 20 herein and an alpha-amylase derived from *Rhizomucor pusillus,* preferably with a linker and starch-binding domain (SBD), in particular the *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) disclosed as V039 in Table 5 in WO 2006/069290 or SEQ ID NO: 16 herein.

In one embodiment, the following enzymes are present and/or added in saccharification and/or fermentation: *Gloeophyllum* glucoamylase, preferably the *Gloeophyllum trabeum* glucoamylase shown in SEQ ID NO: 17 herein and an alpha-amylase derived from *Rhizomucor pusillus,* preferably with a linker and starch-binding domain (SBD), in particular the *Rhizomucor pusillus alpha-amylase* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) disclosed as V039 in Table 5 in WO 2006/069290 or SEQ ID NO: 16 herein.

In one embodiment, the enzymes present and/or added comprises the *Gloeophyllum trabeum* glucoamylase shown in SEQ ID NO: 17 herein having one or more of the following substitutions: S95P, A121P, especially S95P+A121P (using SEQ ID NO: 17 herein for numbering) and the alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably one shown in SEQ ID NO: 16 herein, preferably one having one or more of the following substitutions: G128D, D143N, especially especially G128D+D143N (using SEQ ID NO: 16 for numbering).

In one embodiment, the following enzymes are present and/or added in saccharification and/or fermentation: *Pycnoporus* glucoamylase, in particular the *Pycnoporus sanguineus* glucoamylase shown in SEQ ID NO: 18 and the *Rhizomucor pusillus* alpha-amylase with a linker and starch-binding domain (SBD), in particular the *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) shown in SEQ ID NO: 16 herein.

In one embodiment, the enzymes present and/or added in saccharification and/or fermentation comprises a *Pycnoporus* glucoamylase, such as the *Pycnoporus sanguineus* glucoamylase shown in SEQ ID NO: 18 herein and the alpha-amylase, in particular an alpha-amylase derived from *Rhizomucor pusillus* with a linker and starch-binding domain (SBD), preferably the *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) shown in SEQ ID NO: 16 herein, preferably having one or more of the following substitutions: G128D, D143N, especially G128D+D143N.

The enzymes present and/or added in saccharification and/or fermentation in a process described herein include i) glucoamylase and ii) alpha-amylase; and may optionally further comprise iii) a cellulolytic enzyme composition and/or iv) a protease.

In one embodiment, the protease is a metallo protease, preferably derived from a strain of the genus *Thermoascus*, preferably a strain of *Thermoascus aurantiacus*, especially *Thermoascus aurantiacus* CGMCC No. 0670, such as the metallo protease disclosed as the mature part of SEQ ID NO: 2 disclosed in WO 2003/048353 or the mature polypeptide of SEQ ID NO: 3 herein.

In one embodiment, the protease, in particular a protease derived from *Thermoascus aurantiacus*, is selected from the group consisting of:

(i) a protease comprising the mature polypeptide of SEQ ID NO: 3 herein;

(ii) a protease comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 3 herein.

In one embodiment, the enzymes present and/or added in saccharification and/or fermentation comprises the *Trametes cingulata* glucoamylase shown in SEQ ID NO: 20 herein and the alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), such as the one shown in SEQ ID NO: 16 herein, and may have one or more of the following substitutions: G128D, D143N (e.g., G128D+D143N) and optionally further a cellulolytic enzyme composition derived from *Trichoderma reesei*, e.g., further comprising *Thermoascus aurantiacus* GH61A polypeptide having cellulolytic enhancing activity (SEQ ID NO: 2 in WO 2005/074656 or SEQ ID NO: 30 herein) and *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 29 herein); or a cellulolytic enzyme composition derived from *Trichoderma reesei*, e.g., further comprising *Penicillium emersonii* GH61A polypeptide disclosed as SEQ ID NO: 2 in WO 2011/041397 or SEQ ID NO: 31 herein and *Aspergillus fumigatus* beta-glucosidase disclosed as SEQ ID NO: 2 in WO 2005/047499 or SEQ ID NO: 29 herein, or a variant thereof, e.g., a variant having one of, preferably all of, the following substitutions: F100D, S283G, N456E, F512Y, *Aspergillus fumigatus* Cel7A CBH1 disclosed as SEQ ID NO: 6 in WO2011/057140 and SEQ ID NO: 32 herein and *Aspergillus fumigatus* CBH II disclosed as SEQ ID NO: 18 in WO 2011/057140 and as SEQ ID NO: 33 herein.

In one embodiment the enzymes present and/or added in saccharification and/or fermentation comprises the *Gloeophyllum trabeum* glucoamylase shown in SEQ ID NO: 17 herein, preferably having one or more of the following substitutions: S95P, A121P, especially S95P+A121P and the alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably the one shown in SEQ

ID NO: 16 herein, preferably having one or more of the following substitutions: G128D, D143N, especially G128D+D143N, and optionally further a cellulolytic enzyme composition derived from *Trichoderma reesei*, preferably further comprising *Thermoascus aurantiacus* GH61A polypeptide having cellulolytic enhancing activity (SEQ ID NO: 2 in WO 2005/074656 or SEQ ID NO: 30 herein) and *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 29 herein); or a cellulolytic enzyme composition derived from *Trichoderma reesei*, preferably further comprising *Penicillium emersonii* GH61A polypeptide disclosed as SEQ ID NO: 2 in WO 2011/041397 or SEQ ID NO: 31 herein and *Aspergillus fumigatus* beta-glucosidase disclosed as SEQ ID NO: 2 in WO 2005/047499 or SEQ ID NO: 29 herein, or a variant thereof, preferably a variant having one of, preferably all of, the following substitutions: F100D, S283G, N456E, F512Y, *Aspergillus fumigatus* Cel7A CBH1 disclosed as SEQ ID NO: 6 in WO2011/057140 and SEQ ID NO: 32 herein and *Aspergillus fumigatus* CBH II disclosed as SEQ ID NO: 18 in WO 2011/057140 and as SEQ ID NO: 33 herein.

In one embodiment the enzymes present and/or added in saccharification and/or fermentation comprises the *Pycnoporus sanguineus* glucoamylase shown in SEQ ID NO: 18 herein and the alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably the one shown in SEQ ID NO: 16 herein, preferably having one or more of the following substitutions: G128D, D143N, especially G128D+D143N, and optionally further a cellulolytic enzyme composition derived from *Trichoderma reesei*, preferably further comprising *Thermoascus aurantiacus* GH61A polypeptide having cellulolytic enhancing activity (SEQ ID NO: 2 in WO 2005/074656 or SEQ ID NO: herein) and *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 30 herein); or a cellulolytic enzyme composition derived from *Trichoderma reesei*, preferably further comprising *Penicillium emersonii* GH61A polypeptide disclosed as SEQ ID NO: 2 in WO 2011/041397 or SEQ ID NO: 31 herein and *Aspergillus fumigatus* beta-glucosidase disclosed as SEQ ID NO: 2 in WO 2005/047499 or SEQ ID NO: 29 herein, or a variant thereof, preferably a variant having one of, preferably all of, the following substitutions: F100D, S283G, N456E, F512Y, *Aspergillus fumigatus* Cel7A CBH I disclosed as SEQ ID NO: 6 in WO2011/057140 and SEQ ID NO: 29 herein and *Aspergillus fumigatus* CBH II disclosed as SEQ ID NO: 18 in WO 2011/057140 and as SEQ ID NO: 33 herein.

In one embodiment, a cellulolytic enzyme composition is one described below in the "Cellulolytic Enzyme Compositions"-section.

The optional cellulolytic enzyme composition, protease or other enzymes, may be added in the process described herein at the same time as the glucoamylase and the alpha-amylase. The enzymes, e.g., in the form of an enzyme composition, may be added to the saccharification and/or fermentation, preferably simultaneous saccharification and fermentation (i.e., one-step process). It should be understood that the enzymes may also be

added individually or as two, three, four or more enzyme components/compositions. In one embodiment, the glucoamylase and the alpha-amylase are added as one blend composition and the optional cellulolytic enzyme composition and/and optional protease are added separately. In another embodiment the glucoamylase, the alpha-amylase, and the cellulolytic enzyme composition are added as one enzyme composition and the optional protease is added separately. All enzymes may also in one embodiment be added as one enzyme composition comprising a glucoamylase, an alpha-amylase, a cellulolytic enzyme composition and/or a protease, and optionally other enzymes including pullulanase, trehalase and/or pectinase, such as pectin lyase or polygalacturonase.

Other enzymes may also be present. Specifically contemplated enzymes are described further below.

Glucoamylase

The glucoamylase used in a process described herein may be of any origin, such as of bacterial or fungal origin. Fungal glucoamylases are preferred.

In one embodiment, the glucoamylase may be one derived from a strain of *Trametes*, such as a strain of *Trametes cingulata* (SEQ ID NO: 20 herein); or a strain of *Pachykytospora*, such as a strain of *Pachykytospora papyracea*; or a strain of *Leucopaxillus*, such as a strain of *Leucopaxillus giganteus (*all disclosed in WO 2006/069289).

In one embodiment, the glucoamylase, in particular derived from a strain of *Trametes cingulata*, is selected from the group consisting of:

(i) a glucoamylase comprising the mature polypeptide of SEQ ID NO: 20 herein;

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 20 herein.

In one embodiment, the glucoamylase is from a strain of *Aspergillus*, preferably *Aspergillus niger*, *Aspergillus awamori*, or *Aspergillus oryzae*; or a strain of *Trichoderma*, preferably *Trichoderma reesei*; or a strain of *Talaromyces*, preferably *Talaromyces emersonii* (e.g., SEQ ID NO: 19 herein).

In one embodiment, the glucoamylase, such as one derived from a strain of *Talaromyces emersonii*, is selected from the group consisting of:

(i) a glucoamylase comprising the mature polypeptide of SEQ ID NO: 19 herein;

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 19 herein.

In another embodiment the glucoamylase is derived from a strain of *Penicillium*, such as a strain of *Penicillium oxalicum.*

In one embodiment, the glucoamylase, such as one derived from a strain of *Penicillium oxalicum*, is selected from the group consisting of:

(i) a glucoamylase comprising the mature polypeptide of SEQ ID NO: 14 herein;

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 14 herein.

In one embodiment, the glucoamylase is derived from a strain of *Gloeophyllum*, such as a strain of *Gloeophyllum sepiarium* or *Gloeophyllum trabeum*, such as one disclosed in WO 2011/068803 as any of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14 or 16. In one embodiment, the glucoamylase is SEQ ID NO: 2 in WO 2011/068803 or SEQ ID NO: 15 herein. In another embodiment the glucoamylase is SEQ ID NO: 18 in WO 2011/068803 (hereby incorporated by reference).

In one embodiment, the glucoamylase, such as one derived from a strain of *Gloeophyllum sepiarium*, is selected from the group consisting of:

(i) a glucoamylase comprising the mature polypeptide of SEQ ID NO: 15 herein;

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 15 herein.

In a further embodiment the glucoamylase is derived from a strain of the genus *Pycnoporus*, in particular a strain of *Pycnoporus sanguineus*, such as a strain described in WO 2011/066576 (SEQ ID NOs 2, 4 or 6). In one embodiment, the glucoamylase is the one shown in SEQ ID NO: 4 in WO 2011/066576 or SEQ ID NO: 18 herein.

In one embodiment, the glucoamylase, such as one derived from a strain of *Pycnoporus sanguineus*, is selected from the group consisting of:

(i) a glucoamylase comprising the mature polypeptide of SEQ ID NO: 18 herein;

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 18 herein.

Contemplated are also glucoamylases which exhibit a high identity to any of the above-mentioned glucoamylases, e.g., at least 60%, 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%, such as 100% identity to any one of the mature parts of the enzyme sequences mentioned above.

In one embodiment, the glucoamylase, such as one derived from a strain of *Gloeophyllum trabeum*, is selected from the group consisting of:

(i) a glucoamylase comprising the mature polypeptide of SEQ ID NO: 17 herein;

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 17 herein.

In one embodiment, the glucoamylase, such as the one derived from *Gloeophyllum trabeum,* shown in SEQ ID NO: 17 has one of the following substitutions: V59A; S95P; A121P; T119W; S95P+A121P; V59A+S95P; S95P+T119W; V59A+S95P+A121P; or S95P+T119W+A121P, especially S95P+A121P. In one embodiment, the *Gloeophyllum trabeum* glucoamylase shown in SEQ ID NO: 18 has one of the following substitutions: V59A; S95P; A121P; T119W; S95P+A121P; V59A+S95P; S95P+T119W; V59A+S95P+A121P; or S95P+T119W+A121P, especially S95P+A121P (using SEQ ID NO: 17 herein for numbering). All *Gloeophyllum trabeum* glucoamylase variants, especially variants in SEQ ID NO: 3, disclosed in WO 2014/177546 is hereby incorporated by reference.

A glucoamylase variant may comprise an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99%, but less than 100% sequence identity to the mature polypeptide of SEQ ID NO: 17.

Alpha-Amylase

The alpha-amylase used in a process described herein may be of any origin, such as of fungal or bacterial origin. In one embodiment, the alpha-amylase is an acid alpha-amylase, such as an acid fungal alpha-amylase, i.e., having a pH optimum below pH 7.

In one embodiment, the alpha-amylase may be derived from a strain of the genus *Rhizomucor*, preferably a strain the *Rhizomucor pusillus*, such as the one shown in SEQ ID NO: 3 in WO 2013/006756 (see e.g., Table 1 in Example 1 - hereby incorporated by reference), or the genus *Meripilus*, preferably a strain of *Meripilus giganteus*.

In one embodiment, the alpha-amylase is derived from a *Rhizomucor pusillus*, such as one with a linker and a starch-binding domain (SBD), preferably *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), disclosed as V039 in Table 5 in WO 2006/069290 (incorporated by reference) or SEQ ID NO: 16 herein.

In one embodiment, the alpha-amylase is derived from a *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), disclosed in WO 2013/006756 (incorporated by reference) or SEQ ID NO: 16 herein.

In one embodiment, the *Rhizomucor pusillus* alpha-amylase or the *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding

domain (SBD) has at least one of the following substitutions or combinations of substitutions: D165M; Y141W; Y141R; K136F; K192R; P224A; P224R; S123H+Y141W; G20S + Y141W; A76G + Y141W; G128D + Y141W; G128D + D143N; P219C + Y141W; N142D + D143N; Y141W + K192R; Y141W + D143N; Y141W + N383R; Y141W + P219C + A265C; Y141W + N142D + D143N; Y141W + K192R V410A; G128D + Y141W + D143N; Y141W + D143N + P219C; Y141W + D143N + K192R; G128D + D143N + K192R; Y141W + D143N + K192R + P219C; G128D + Y141W + D143N + K192R; or G128D + Y141W + D143N + K192R + P219C, especially G128D+D143N (using SEQ ID NO: 16 herein for numbering).

In one embodiment, the *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), is selected from the group consisting of:

(i) an alpha-amylase comprising the mature polypeptide of SEQ ID NO: 16 herein;

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 16 herein.

In one embodiment, the alpha-amylase is a variant of the *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), wherein the alpha-amylase variant comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity, but less than 100% to the mature polypeptide of SEQ ID NO: 16 herein.

In one embodiment, the alpha-amylase variant has one of the above mentioned substitutions, such as: G128D, Y141W, D143W or K192R (using SEQ ID NO: 16 for numbering).

In one embodiment, the alpha-amylase (using SEQ ID NO: 16 herein for numbering) has the following substitutions: Y141W+D143N.

In one embodiment, the alpha-amylase has the following substitutions: G128D+Y141W+D143N.

In one embodiment, the alpha-amylase has the following substitutions: G128D+Y141W+D143N+K192R;

In one embodiment, the alpha-amylase has the following substitutions: G128D+D143N (using SEQ ID NO: 16 for numbering).

A variant may comprise an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99%, but less than 100% sequence identity to the mature polypeptide of SEQ ID NO: 16.

Protease

The enzymes present and/or added to saccharification and/or fermentation may optionally further include a protease. The protease may be of any origin, such as fungal or bacterial origin.

In one embodiment, the protease is of fungal origin.

In one embodiment, the protease is a metallo protease derived from a strain of the genus *Thermoascus*, preferably a strain of *Thermoascus aurantiacus*, especially *Thermoascus aurantiacus* CGMCC No. 0670, such as the metallo protease disclosed as the mature part of SEQ ID NO: 2 disclosed in WO 2003/048353 or the mature polypeptide of SEQ ID NO: 3 herein.

In one embodiment, the protease, such as one derived from a strain of *Thermoascus aurantiacus*, is selected from the group consisting of:

(i) a protease comprising the mature polypeptide of SEQ ID NO: 3 herein;

(ii) a protease comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 3 herein.

In one embodiment, the protease is of bacterial origin.

In one embodiment, the protease is derived from a strain of *Pyrococcus*, such as a strain of *Pyrococcus furiosus,* such as the protease shown in SEQ ID NO: 1 in US 6,358,726 or SEQ ID NO: 5 herein.

In one embodiment, the protease, such as one derived from *Pyrococcus furiosus*, is selected from the group consisting of:

(i) a protease comprising the mature polypeptide of SEQ ID NO: 5 herein;

(ii) a protease comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 5 herein.

Cellulolytic Enzyme Compositions

The enzymes present and/or added to saccharification and/or fermentation may optionally further include a cellulolytic enzyme composition. The cellulolytic enzyme composition may consist of or comprise one or more cellulolytic enzymes. The cellulolytic enzyme composition may be of any origin. In one embodiment, the cellulolytic enzyme composition comprises cellulolytic enzymes of fungal origin.

In one embodiment, the cellulolytic enzyme composition is derived from a strain of *Trichoderma*, such as *Trichoderma reesei*; or a strain of *Humicola*, such as *Humicola insolens;*

or a strain of *Chrysosporium*, such as *Chrysosporium lucknowense*; or a strain of *Penicillium*, such as *Penicillium decumbens.* In one embodiment, the cellulolytic enzyme composition is derived from a strain of *Trichoderma reesei*.

The cellulolytic enzyme composition may comprise a beta-glucosidase, a cellobiohydrolase, and an endoglucanase.

In one embodiment, the cellulolytic enzyme composition comprising one or more polypeptides selected from the group consisting of:

beta-glucosidase (BG);

cellobiohydrolase I (CBHI);

cellobiohydrolase II (CBHII);

or a mixture thereof.

In one embodiment, the cellulolytic enzyme composition further comprises a GH61 polypeptide having cellulolytic enhancing activity. Cellulolytic enhancing activity is defined and determined as described in WO 2011/041397 (incorporated by reference).

The term "GH61 polypeptide having cellulolytic enhancing activity" means a GH61 polypeptide that enhances the hydrolysis of a cellulosic material by enzymes having cellulolytic activity. For purposes of the processes described herein, cellulolytic enhancing activity is determined by measuring the increase in reducing sugars or the increase of the total of cellobiose and glucose from hydrolysis of a cellulosic material by cellulolytic enzyme under the following conditions: 1-50 mg of total protein/g of cellulose in PCS (Pretreated Corn Stover), 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 50°C compared to a control hydrolysis with equal total protein loading without cellulolytic enhancing activity (1-50 mg of cellulolytic protein/g of cellulose in PCS). In a preferred aspect, a mixture of CELLUCLAST™1.5L (Novozymes A/S, Bagsværd, Denmark) in the presence of 2-3% of total protein weight *Aspergillus oryzae* beta-glucosidase (recombinantly produced in *Aspergillus oryzae* according to WO 02/095014) or 2-3% of total protein weight *Aspergillus fumigatus* beta-glucosidase (recombinantly produced in *Aspergillus oryzae* as described in WO 2002/095014) of cellulase protein loading is used as the source of the cellulolytic activity.

The cellulolytic enzyme composition comprises a beta-glucosidase, preferably one derived from a strain of the genus *Aspergillus*, such as *Aspergillus oryzae*, such as the one disclosed in WO 2002/095014 or the fusion protein having beta-glucosidase activity disclosed in WO 2008/057637 (see SEQ ID NOs: 74 or 76), or *Aspergillus fumigatus*, such as one disclosed in SEQ ID NO: 2 in WO 2005/047499 or SEQ ID NO: 8 herein; or an *Aspergillus fumigatus* beta-glucosidase variant disclosed in WO 2012/044915; or a strain of the genus a strain *Penicillium*, such as a strain of the *Penicillium brasilianum* disclosed in WO 2007/019442, or a strain of the genus *Trichoderma*, such as a strain of *Trichoderma reesei.*

In one embodiment, the beta-glucosidase is from a strain of *Aspergillus*, such as a strain of *Aspergillus fumigatus*, such as *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 29 herein), or a variant thereof, which variant comprises one or more substitutions selected from the group consisting of L89M, G91L, F100D, I140V, I186V, S283G, N456E, and F512Y; such as a variant thereof with the following substitutions:

F100D + S283G + N456E + F512Y;

L89M + G91L + I186V + I140V;

I186V + L89M + G91L + I140V + F100D + S283G + N456E + F512Y.

In one embodiment, the parent beta-glucosidase has at least 60% identity, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to the mature polypeptide of SEQ ID NO: 29 herein.

In case the beta-glucosidase is a beta-glucosidase variant it has at least 60% identity, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, but less than 100% identity to the mature polypeptide of SEQ ID NO:29 herein.

In case the cellulolytic enzyme composition comprises a GH61 polypeptide, it may be one derived from the genus *Thermoascus*, such as a strain of *Thermoascus aurantiacus*, such as the one described in WO 2005/074656 as SEQ ID NO: 2 or SEQ ID NO: 30 herein; or one derived from the genus *Thielavia*, such as a strain of *Thielavia terrestris*, such as the one described in WO 2005/074647 as SEQ ID NO: 7 and SEQ ID NO: 8 (hereby incorporated by reference); or one derived from a strain of *Aspergillus*, such as a strain of *Aspergillus fumigatus*, such as the one described in WO 2010/138754 as SEQ ID NO: 1 and SEQ ID NO: 2 (hereby incorporated by reference); or one derived from a strain from *Penicillium*, such as a strain of *Penicillium emersonii*, such as the one disclosed in WO 2011/041397 as SEQ ID NO: 2 or SEQ ID NO: 31 herein.

In one embodiment, the GH61 polypeptide, such as one derived from a strain of *Thermoascus*, is selected from the group consisting of:

(i) a GH61 polypeptide comprising the mature polypeptide of SEQ ID NO: 30 herein;

(ii) a GH61 polypeptide comprising an amino acid sequence having at least 60%, such as at least 70%, e.g., at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 30 herein.

In one embodiment, the GH61 polypeptide, such as one derived from a strain of *Penicillium* sp., is selected from the group consisting of:

(i) a GH61 polypeptide comprising the mature polypeptide of SEQ ID NO: 31 herein;

(ii) a GH61 polypeptide comprising an amino acid sequence having at least 60%, such as at least 70%, e.g., at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 31 herein.

In one embodiment, the cellulolytic enzyme composition comprises a cellobiohydrolase I (CBH I), such as one derived from a strain of the genus *Aspergillus*, such as a strain of *Aspergillus fumigatus*, such as the Cel7a CBH I disclosed as SEQ ID NO: 6 in WO 2011/057140 or SEQ ID NO: 32 herein, or a strain of the genus *Trichoderma*, such as a strain of *Trichoderma reesei*.

In one embodiment, the cellobiohydrolase I, such as one derived from a strain of *Aspergillus fumigatus,* is selected from the group consisting of:

(i) a cellobiohydrolase I comprising the mature polypeptide of SEQ ID NO: 32 herein;

(ii) a cellobiohydrolase I comprising an amino acid sequence having at least 60%, such as at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 32 herein.

In one embodiment, the cellulolytic enzyme composition, comprised in an enzyme composition described herein, comprises a cellobiohydrolase II (CBH II), such as one derived from a strain of the genus *Aspergillus*, such as a strain of *Aspergillus fumigatus*; such as the one disclosed as SEQ ID NO: 33 herein or a strain of the genus *Trichoderma*, such as *Trichoderma reesei*, or a strain of the genus *Thielavia*, such as a strain of *Thielavia terrestris*, such as cellobiohydrolase II CEL6A from *Thielavia terrestris*.

In one embodiment, cellobiohydrolase II, such as one derived from a strain of *Aspergillus fumigatus*, is selected from the group consisting of:

(i) a cellobiohydrolase II comprising the mature polypeptide of SEQ ID NO: 33 herein;

(ii) a cellobiohydrolase II comprising an amino acid sequence having at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 33 herein.

In one embodiment, the cellulolytic enzyme composition comprises a GH61 polypeptide having cellulolytic enhancing activity and a beta-glucosidase.

In one embodiment, the cellulolytic enzyme composition comprises a GH61 polypeptide having cellulolytic enhancing activity derived from a strain of *Penicillium*, such as

a strain of *Penicillium emersonii*, such as the one disclosed as SEQ ID NO: 2 in WO 2011/041397 or SEQ ID NO: 31 herein, and a beta-glucosidase.

In one embodiment, the cellulolytic enzyme composition comprises a GH61 polypeptide having cellulolytic enhancing activity, a beta-glucosidase, and a CBH I.

In one embodiment, the cellulolytic enzyme composition comprises a GH61 polypeptide having cellulolytic enhancing activity derived from a strain of *Penicillium*, such as a strain of *Penicillium emersonii*, such as the one disclosed as SEQ ID NO: 2 in WO 2011/041397 or SEQ ID NO: 31 herein, a beta-glucosidase, and a CBHII.

In one embodiment, the cellulolytic enzyme composition, comprised in an enzyme composition described herein, comprises a GH61 polypeptide having cellulolytic enhancing activity, a beta-glucosidase, a CBHI, and a CBHII.

In one embodiment, the cellulolytic enzyme composition comprises a GH61 polypeptide having cellulolytic enhancing activity derived from a strain of *Penicillium*, such as a strain of *Penicillium emersonii*, such as the one disclosed as SEQ ID NO: 2 in WO 2011/041397 or SEQ ID NO: 31 herein, a beta-glucosidase, a CBH I, and a CBH II.

In one embodiment, the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic composition further comprising *Thermoascus aurantiacus* GH61A polypeptide (SEQ ID NO: 2 in WO 2005/074656 or SEQ ID NO: 30 herein), and *Aspergillus oryzae* beta-glucosidase fusion protein (WO 2008/057637).

In one embodiment, the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition further comprising *Thermoascus aurantiacus* GH61A polypeptide having cellulolytic enhancing activity (SEQ ID NO: 2 in WO 2005/074656 or SEQ ID NO: 30 herein) and *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 29 herein).

In one embodiment, the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic composition further comprising *Penicillium emersonii* GH61A polypeptide disclosed as SEQ ID NO: 2 in WO 2011/041397 or SEQ ID NO: 31 herein, and *Aspergillus fumigatus* beta-glucosidase disclosed as SEQ ID NO: 2 in WO 2005/047499 or SEQ ID NO: 29 herein, or a variant thereof, which variant has one of, preferably all of, the following substitutions: F100D, S283G, N456E, F512Y, and optionally *Aspergillus fumigatus* CBH I, e.g., the one disclosed as SEQ ID NO: 6 in WO2011/057140 and SEQ ID NO: 32 herein and *Aspergillus fumigatus* CBH II, e.g., the one disclosed as SEQ ID NO: 18 in WO 2011/057140 and as SEQ ID NO: 33 herein.

In one embodiment, the cellulolytic enzyme composition comprises one or more of the following components

(i) an *Aspergillus fumigatus* cellobiohydrolase I;

(ii) an *Aspergillus fumigatus* cellobiohydrolase II;

(iii) an *Aspergillus fumigatus* beta-glucosidase or variant thereof.

In one embodiment, the *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 29 herein), comprises one or more substitutions selected from the group consisting of L89M, G91L, F100D, I140V, I186V, S283G, N456E, and F512Y; such as a variant thereof, with one of the following substitutions or combinations of substitutions:

F100D + S283G + N456E + F512Y;

L89M + G91L + I186V + I140V; and

I186V + L89M + G91L + I140V + F100D + S283G + N456E + F512Y (using SEQ ID NO: 29 for numbering).

In one embodiment, the cellulolytic enzyme composition further comprises the *Penicillium* sp. GH61 polypeptide shown in SEQ ID NO: 31 herein; or a GH61 polypeptide comprising an amino acid sequence having at least 60%, such as at least 70%, e.g., at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 31 herein.

Pullulanase

The enzymes present and/or added to saccharification and/or fermentation may optionally further include a pullulanase. The pullulanase may be of any origin, such as fungal or bacterial origin.

In one embodiment, the pullulanase is derived from a strain of *Bacillus* sp. such as a strain of *Bacillus deramificans*.

Trehalase

The enzymes present and/or added to saccharification and/or fermentation may optionally further include a trehalase.

The trehalase may be of any origin, such as fungal or bacterial origin.

In one embodiment, the trehalase is of fungal origin, such as derived from a strain of *Trichoderma*, such as *Trichoderma reesei.*

Pectinase

The enzymes present and/or added to saccharification and/or fermentation may optionally further include a pectinase, such as a pectin lyase (also known as pectolyase) and/or a polygalacturonase, or a combination thereof.

The pectinase may be of any origin, such as fungal or bacterial origin.

In one embodiment, the pectinase is a pectin lyase (EC 4.2.2.10).

In one embodiment, the pectin lyase is derived from a strain of *Aspergillus*, such as *Aspergillus niger*.

In one embodiment, the pectinase is a polygalacturonase (EC. 3.2.1.15).

In one embodiment, the polygalacacturonase is derived from a strain of *Aspergillus*, such as *Aspergillus aculeatus*.

In one embodiment, the pectinase is a combination of pectin lyase and polygalacturonase. In one embodiment, the pectinase is a combination of pectin lyase derived from *Aspergillus niger* and polygalacturonase derived from *Aspergillus aculeatus*.

Examples of Enzymes (e.g., Blend) Suitable for Use in a Raw Starch Hydrolysis Process

In one embodiment, enzymes (e.g., blend) for use in a process described herein comprise a glucoamylase and an alpha-amylase, and optionally a protease and/or cellulolytic enzyme composition. Other optional enzymes may also be used.

In one embodiment, the enzymes (e.g., blend) used in a process described herein comprises or consists of a glucoamylase from *Trametes cingulata* (e.g., SEQ ID NO: 20) and an alpha-amylase from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), e.g., SEQ ID NO: 16.

In one embodiment, the enzymes (e.g., blend) used in a process described herein comprises the *Gloeophyllum trabeum* glucoamylase (e.g., SEQ ID NO: 17 herein) having one or more of the following substitutions: S95P, A121P, preferably S95P+A121P and an alpha-amylase, preferably an alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), shown in SEQ ID NO: 16 herein, preferably having one or more of the following substitutions: G128D, D143N, preferably G128D+D143N.

In another embodiment, the enzymes (e.g., blend) used in a process described herein comprises the *Pycnoporus sanguineus* glucoamylase shown in SEQ ID NO: 18 herein and an alpha-amylase, preferably one derived from *Rhizomucor pusillus* with a linker and starch-binding domain (SBD), preferably *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), in particular the one shown in SEQ ID NO: 16 herein, preferably having one or more of the following substitutions: G128D, D143N, especially G128D+D143N.

In one embodiment, the enzymes (e.g., blend) used in a process described herein comprises the *Gloeophyllum sepiarium* glucoamylase shown in SEQ ID NO: 15 herein and an alpha-amylase, preferably an alpha-amylase derived from *Rhizomucor pusillus* with a linker and starch-binding domain (SBD), preferably *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD)shown in SEQ ID NO: 16 herein, preferably having one or more of the following substitutions: G128D, D143N, preferably G128D+D143N.

In one embodiment, the enzymes (e.g., blend) used in a process described herein comprises the *Trametes cingulata* glucoamylase shown in SEQ ID NO: 20 herein and an

alpha-amylase, preferably an alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), shown in SEQ ID NO: 16 herein, having one or more of the following substitutions: G128D, D143N, preferably G128D+D143N.

In one embodiment, the enzymes (e.g., blend) used in a process described herein comprises

i) fungal glucoamylase;

ii) fungal alpha-amylase;

iii) cellulolytic enzyme composition derived from a strain of *Trichoderma reesei*, further comprising a GH61 polypeptide, beta-glucosidase, CBH I and CBH II;

iv) optionally a protease.

In one embodiment, the enzymes (blend) used in a process described herein comprises

i) *Trametes cingulata* glucoamylase;

ii) *Rhizomucor pusillus* alpha-amylase, or variant thereof;

iii) cellulolytic enzyme composition derived from a strain of *Trichoderma reesei*, further comprising *Penicillium emersonii* GH61A polypeptide, *Aspergillus fumigatus* beta-glucosidase with the following substitutions: F100D, S283G, N456E, F512Y, and optionally *Aspergillus fumigatus* CBH I and *Aspergillus fumigatus* CBH II;

iv) optionally a protease from *Thermoascus aurantiacus*, or variant thereof.

In one embodiment, the enzymes (e.g., blend) used in a process described herein comprises a

i) *Trametes cingulata* glucoamylase;

ii) *Rhizomucor pusillus* alpha-amylase, or variant thereof;

iii) cellulolytic enzyme composition derived from a strain of *Trichoderma reesei*, further comprising *Penicillium emersonii* GH61A polypeptide, *Aspergillus fumigatus* beta-glucosidase with the following substitutions: F100D, S283G, N456E, F512Y, and optionally *Aspergillus fumigatus* CBH I and *Aspergillus fumigatus* CBH II;

iv) optionally a protease from *Pyropoccus furiosus*.

In one embodiment, the enzymes (e.g., blend) used in a process described herein comprises

i) glucoamylase derived from *Trametes cingulata*;

ii) alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), or a variant thereof;

iii) cellulolytic enzyme composition derived from a strain of *Trichoderma reesei*;

iv) optionally a protease from *Thermoascus aurantiacus*, or a variant thereof and/or *Pyrococcus furiosus*.

In one embodiment, the enzymes (e.g., blend) used in a process described herein comprises

i) fungal glucoamylase;

ii) fungal alpha-amylase;

iii) cellulolytic enzyme composition derived from a strain of *Trichoderma reesei*, further comprising a GH61 polypeptide, beta-glucosidase CBH I and CBH II;

iv) pectinase, preferably a pectin lyase or a polygalacturonase, or a combination thereof.

In one embodiment, the pectinase is a combination of pectin lyase derived from *Aspergillus niger* and polygalacturonase derived from *Aspergillus aculeatus*.

In one embodiment, the pectinase is a combination of pectin lyase and polygalacturonase. In one embodiment, the pectinase is a combination of pectin lyase derived from *Aspergillus niger* and polygalacturonase derived from *Aspergillus aculeatus*.

In one embodiment, the enzymes (e.g., blend) used in a process described herein comprises

i) fungal glucoamylase;

ii) fungal alpha-amylase;

iii) pectinase, preferably a pectin lyase or a polygalacturonase, or a combination thereof;

iv) cellulolytic enzyme composition derived from a strain of *Trichoderma reesei*, further comprising a GH61 polypeptide, beta-glucosidase CBH I and CBH II;

v) protease.

In one embodiment, the enzymes (e.g., blend) used in a process described herein comprises a

i) fungal glucoamylase;

ii) fungal alpha-amylase;

iii) cellulolytic enzyme composition derived from a strain of *Trichoderma reesei*, further comprising a GH61 polypeptide, beta-glucosidase, CBH I and CBH II;

iv) optionally a protease.

In one embodiment, the enzymes (e.g., blend) used in a process described herein comprises

i) *Trametes cingulata* glucoamylase;

ii) *Rhizomucor pusillus* alpha-amylase, or variant thereof;

iii) cellulolytic enzyme composition derived from a strain of *Trichoderma reesei*, further comprising *Penicillium emersonii* GH61A polypeptide, *Aspergillus fumigatus* beta-glucosidase with the following substitutions: F100D, S283G, N456E, F512Y, and optionally *Aspergillus fumigatus* CBH I and *Aspergillus fumigatus* CBH II;

iv) pectin lyase derived from *Aspergillus niger* or polygalacturonase derived from *Aspergillus aculeatus,* or a combination thereof;

v) protease from *Thermoascus aurantiacus*, or a variant thereof and/or *Pyrococcus furiosus*.

In one embodiment, the enzymes (blend) used in a process described herein comprises

i) *Gloeophyllum trabeum* glucoamylase shown in SEQ ID NO: 18 herein having one or more of the following substitutions: S95P, A121P, such as S95P+A121P;

ii) alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), shown in SEQ ID NO: 13 herein, having of the following substitutions: G128D+D143N;

iii) cellulolytic enzyme composition derived from a strain of *Trichoderma reesei*, further comprising *Penicillium emersonii* GH61A polypeptide, *Aspergillus fumigatus* beta-glucosidase with the following substitutions: F100D, S283G, N456E, F512Y, and optionally *Aspergillus fumigatus* CBH I and *Aspergillus fumigatus* CBH II;

optionally iv) protease from *Thermoascus aurantiacus*, or a variant thereof.

In one embodiment, the enzymes (e.g., blend) used in a process described herein comprises

i) *Pycnoporus sanguineus* glucoamylase shown in SEQ ID NO: 18 herein;

ii) alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), shown in SEQ ID NO: 16 herein, having of the following substitutions: G128D+D143N;

iii) cellulolytic enzyme composition derived from a strain of *Trichoderma reesei*, further comprising *Penicillium emersonii* GH61A polypeptide, *Aspergillus fumigatus* beta-glucosidase with the following substitutions: F100D, S283G, N456E, F512Y, and optionally *Aspergillus fumigatus* CBH I and *Aspergillus fumigatus* CBH II;

optionally iv) protease from *Thermoascus aurantiacus*, or a variant thereof.

In one embodiment, the enzymes (e.g., blend) used in a process described herein comprises

i) *Gloeophyllum sepiarium* glucoamylase shown in SEQ ID NO: 15 herein;

ii) alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), shown in SEQ ID NO: 16 herein, having of the following substitutions: G128D+D143N;

iii) cellulolytic enzyme composition derived from a strain of *Trichoderma reesei*, further comprising *Penicillium emersonii* GH61A polypeptide, *Aspergillus fumigatus* beta-glucosidase with the following substitutions: F100D, S283G, N456E, F512Y, and optionally *Aspergillus fumigatus* CBH I and *Aspergillus fumigatus* CBH II;

optionally iv) protease from *Thermoascus aurantiacus*, or a variant thereof.

In one embodiment, the enzymes (e.g., blend) used in a process described herein comprises

i) *Trametes cingulata* glucoamylase shown in SEQ ID NO: 20 herein;

ii) alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), shown in SEQ ID NO: 16 herein, having of the following substitutions: G128D+D143N;

iii) cellulolytic enzyme composition derived from a strain of *Trichoderma reesei*, further comprising *Penicillium emersonii* GH61A polypeptide, *Aspergillus fumigatus* beta-glucosidase with the following substitutions: F100D, S283G, N456E, F512Y, and optionally *Aspergillus fumigatus* CBH I and *Aspergillus fumigatus* CBH II;

optionally iv) protease from *Thermoascus aurantiacus*, or a variant thereof.

Examples of Raw Starch Hydrolysis Processes

In one embodiment, a process of producing ethanol from starch-containing material comprises:

(i) saccharifying starch-containing material at a temperature below the initial gelatinization temperature; and

(ii) fermenting using a fermentation organism;

wherein

saccharification and/or fermentation is done in the presence of the following enzymes: glucoamylase and alpha-amylase, and optionally protease and/or cellulolytic enzyme composition; and

the fermenting organism is a *Saccharomyces* yeast strain providing an ethanol yield boost compared to ETHANOL RED™ under the same fermentation conditions.

In one embodiment, the process provides one or more, such as all, of the following improvement:

an ethanol yield boost compared to ETHANOL RED™ under the same fermentation conditions (e.g., conditions as described herein);

reduced acetaldehyde production compared to ETHANOL RED™ under the same process conditions (e.g., conditions as described herein);

increased temperature tolerance compared to ETHANOL RED™ under the same process conditions (e.g., conditions as described herein);

decreased glycerol production compared to ETHANOL RED™ under the same process conditions (e.g., conditions as described herein). In one embodiment is a process for producing ethanol from starch-containing material comprising:

(i) saccharifying starch-containing material at a temperature below the initial gelatinization temperature; and

(ii) fermenting using a fermentation organism;

wherein

saccharification and/or fermentation is done in the presence of the following enzymes: glucoamylase and alpha-amylase, and optionally protease and/or cellulolytic enzyme composition; and

the fermenting organism is a *Saccharomyces* yeast which provides one or more, such as all of the following improvements:

boosts ethanol yield;

reduces acetaldehyde production;

increased temperature tolerance; and

descreases glycose production.

In one embodiment, the process provides one or more, such as all, of the following improvement:

boosts the ethanol yield over ETHANOL RED™ (ER) of more than 0.5%, e.g., more than 1.0%, more than 2.0%, more than 2.5%, such as about 2.9%, such as between 0.5 and 5%, such as between 1-3%, under the same process conditions (e.g., conditions as described herein);

reduces acetaldehyde production more than 10%, preferably more than 20%, more preferably more than 30%, even more preferably more than 40%, especially more than 45%, such as between 5-60%, such as 30-50%, compared to ETHANOL RED™ under the same process conditions (e.g., conditions as described herein);

increases temperature tolerance compared to ETHANOL RED™ under the same process conditions (e.g., conditions as described herein); and

decreases glycerol production by more than 3%, preferably more than 4%, more preferably more than 5%, even more preferably more than 6%, especially more than 7%, such as between 2-15%, such as 5-10%, compared to ETHANOL RED™ under the same process conditions, (e.g., conditions as described herein).

In one embodiment is a process of producing ethanol from starch-containing material comprises:

(i) saccharifying starch-containing material at a temperature below the initial gelatinization temperature; and

(ii) fermenting using a fermentation organism;

wherein saccharification and/or fermentation is done in the presence of the following enzymes: glucoamylase and alpha-amylase, and optionally protease; and

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985. In one embodiment, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase.

In one embodiment, the process of producing ethanol from starch-containing material comprises:

(a) saccharifying a starch-containing material at a temperature below the initial gelatinization temperature; and

(b) fermenting using a fermentation organism;

wherein

saccharification and/or fermentation is done in the presence of the following enzymes:

i) glucoamylase derived from *Trametes cingulata, Gloeophyllum trabeum*, *Gloeophyllum sepiarium,* or *Pycnoporus sanguineus*;

ii) alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), or a variant thereof;

iii) cellulolytic enzyme composition derived from *Trichoderma reesei*;

iv) optionally a protease from *Thermoascus aurantiacus*, or a variant thereof and/or *Pyrococcus furiosus;* and

wherein

the fermenting organism is a *Saccharomyces* yeast strain providing one or more, such as all of the following improvements:

an ethanol yield boost compared to ETHANOL RED™ under the same fermentation conditions (e.g., conditions as described herein);

reduced acetaldehyde production compared to ETHANOL RED™ under the same process conditions (e.g., conditions as described herein);

increased temperature tolerance compared to ETHANOL RED™ under the same process conditions (e.g., conditions as described herein);

decreased glycerol production compared to ETHANOL RED™ under the same process conditions (e.g., conditions as described herein).

In one embodiment, the process of producing ethanol from starch-containing material comprises:

(a) saccharifying a starch-containing material at a temperature below the initial gelatinization temperature; and

(b) fermenting using a fermentation organism;

wherein

saccharification and/or fermentation is done in the presence of the following enzymes:

i) glucoamylase derived from *Gloeophyllum trabeum* disclosed in SEQ ID NO: 17, with the following substitutions: S95P+A121P;

ii) alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), or a variant thereof, shown in SEQ ID NO: 16 herein, with the following substitutions: G128D+D143N;

iii) cellulolytic enzyme composition derived from *Trichoderma reesei*;

iv) optionally a protease from *Thermoascus aurantiacus*, or a variant thereof; and

wherein

the fermenting organism is a *Saccharomyces* yeast strain providing an ethanol yield boost compared to ETHANOL RED™ under the same fermentation conditions (e.g., provides an ethanol yield boost of at least 1.0%, at least 2.0%, at least 2.5%, such as between 0.5-5%, e.g., between 1-3% compared to ETHANOL RED™ under the conditions described herein).

In one embodiment, the process of producing ethanol from starch containing material of comprises:

(a) saccharifying a starch-containing material at a temperature below the initial gelatinization temperature; and

(b) fermenting using a fermentation organism;

wherein saccharification and/or fermentation is done in the presence of the following enzymes:

i) glucoamylase derived from *Pycnoporus sanguineus* shown in SEQ ID NO: 18;

ii) alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), or a variant thereof, shown in SEQ ID NO: 16 herein, with the following substitutions: G128D+D143N;

iii) cellulolytic enzyme composition derived from *Trichoderma reesei*;

iv) optionally a protease from *Thermoascus aurantiacus*, or a variant thereof; and

wherein the fermenting organism is a *Saccharomyces* yeast strain providing an ethanol yield boost compared to ETHANOL RED™ under the same fermentation conditions (e.g., provides an ethanol yield boost of at least 0.5%, at least 1.0%, at least 2.0%, at least 2.5%, such as between 0.5-5%, e.g., between 1-3% under the same conditions compared to ETHANOL RED™).

In one embodiment, the process of producing ethanol from starch-containing material of comprises:

(a) saccharifying a starch-containing material at a temperature below the initial gelatinization temperature; and

(b) fermenting using a fermentation organism;

wherein saccharification and/or fermentation is done in the presence of the following enzymes:

i) glucoamylase derived from *Gloeophyllum sepiarium* shown in SEQ ID NO: 15;

ii) alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), or a variant thereof, shown in SEQ ID NO: 16 herein, with the following substitutions: G128D+D143N;

iii) cellulolytic enzyme composition derived from *Trichoderma reesei*;

iv) optionally a protease from *Thermoascus aurantiacus*, or a variant thereof;

wherein the fermenting organism is a *Saccharomyces* yeast strain providing an ethanol yield boost compared to ETHANOL RED™ under the same fermentation conditions (e.g., provides an ethanol yield boost of at least 0.5%, at least 1.0%, at least 2.0%, at least 2.5%, such as between 0.5-5%, e.g., between 1-3% under the conditions defined in Example 18 compared to ETHANOL RED™).

In one embodiment, the process of producing ethanol from starch-containing material of comprises:

(a) saccharifying a starch-containing material at a temperature below the initial gelatinization temperature; and

(b) fermenting using a fermentation organism;

wherein saccharification and/or fermentation is done in the presence of the following enzymes:

i) glucoamylase derived from *Trametes cingulata* shown in SEQ ID NO: 20;

ii) alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), or a variant thereof, shown in SEQ ID NO: 16 herein, with the following substitutions: G128D+D143N;

iii) cellulolytic enzyme composition derived from *Trichoderma reesei*;

iv) optionally a protease from *Thermoascus aurantiacus*, or a variant thereof; and

wherein the fermenting organism is a *Saccharomyces* yeast strain providing an ethanol yield boost compared to ETHANOL RED™ under the same fermentation conditions (e.g., provides an ethanol yield boost of at least 0.5%, at least 1.0%, at least 2.0%, at least 2.5%, such as between 0.5-5%, e.g., between 1-3% under the conditions defined in Example 18, compared to ETHANOL RED™).

Use of strain *Saccharomyces cerevisiae* strain MBG4985, or derivative of *Saccharomyces cerevisiae* strain MBG4985 expressing a glucoamylase and/or an alpha-amylase.

Saccharomyces cerevisiae strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain

MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985, may be used for increasing the ethanol yield in fermentation.

In one embodiment, the liquefied mash, to be fermented, has been subjected to alpha-amylase and from 0.5-50 micro gram protease per gram DS, such as 1-5 micro gram protease per gram DS, such as about 1.5 or 3 micro gram protease per gram DS.

The protease may be a bacterial protease. The protease may be derived from a strain of the bacterium *Pyrococcus*, such as a strain of *Pyrococcus furiosus* (pfu protease), such as or SEQ ID NO: 13 herein. The protease may be the one disclosed in SEQ ID NO: 13 herein or a protease having at least 80% identity, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 13 herein.

The alpha-amylase used for liquefying may be of bacterial origin, such as from the genus *Bacillus*, such as a strain of *Bacillus stearothermophilus*, in particular a variant of a *Bacillus stearothermophilus* alpha-amylase, such as the one shown in SEQ ID NO: 1 herein. In one embodiment, the *Bacillus stearothermophilus* alpha-amylase variant is selected from the group with the following mutations: I181*+G182* and optionally substitution N193F, and further one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

V59A+ Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V;

V59A+ E129V+ K177L+ R179E+ Q254S+ M284V; and

E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 for numbering).

In one embodiment, the liquefied mash to be fermented, has been subjected to alpha-amylase, glucoamylase and from 0.5-50 micro gram protease per gram DS, such as 1-5 micro gram protease per gram DS such as about 1.5 or 3 micro gram protease per gram DS. The glucoamylase may be derived from a strain of the genus *Penicillium*, especially a strain of *Penicillium oxalicum* disclosed in SEQ ID NOs: 9 or 14 herein.

The glucoamylase may be a variant of the *Penicillium oxalicum* glucoamylase having a K79V substitution (using the mature sequence shown in SEQ ID NO: 14 for numbering).

In one embodiment, the *Penicillium oxalicum* glucoamylase has a K79V substitution (using SEQ ID NO: 14 for numbering) and further one of the following substitutions or combinations of substitutions:

P11F + T65A + Q327F; and

P2N + P4S + P11F + T65A + Q327F (using SEQ ID NO: 14 for numbering).

Yeast

In one embodiment is a strain of *Saccharomyces cerevisiae* deposited under the Budapest Treaty at the Agricultural Research Service Patent Culture Collection (NRRL) having deposit accession no. NRRL Y67342 (*Saccharomyces cerevisiae* strain MBG4985), or a derivative thereof expressing a glucoamylase and/or an alpha-amylase

The majority of the world's fuel ethanol is produced by industrial scale fermentation of starch-based sugars, in substrates such as corn mash. During industrial scale fermentation, the yeast encounter various physiological challenges including variable concentrations of sugars, high concentrations of yeast metabolites such as ethanol, glycerol, organic acids, osmotic stress, as well as potential competition from contaminating microbes such as wild yeasts and bacteria. As a consequence, many *Saccharomyces* strains, particularly those that are naturally occurring, are not suitable for use in industrial fermentation. A widely used commercially available industrial strain of *Saccharomyces* (i.e. for industrial scale fermentation) is the *Saccharomyces cerevisiae* strain used, for example, in the product ETHANOL RED™. This strain is well suited to industrial ethanol production, however improved strains of *Saccharomyces cerevisiae* are needed.

The Applicants have produced strain NMI V14/004037 (*See*, WO2015/143324 and WO2015/143317), which is a strain of *Saccharomyces cerevisiae* which produces higher levels of ethanol from corn mash than naturally occurring strains of *Saccharomyces cerevisiae*, and strains of *Saccharomyces cerevisiae* used in the fuel ethanol industry such as ETHANOL RED™. In particular, strain NMI V14/004037 has an ethanol yield from glucose that is higher than other industrial strains such as ETHANOL RED™ during fermentation of corn mash. This means that strain NMI V14/004037 can produce more ethanol per gram of glucose than ETHANOL RED™ during fermentation of corn mash.

The Applicants have further produced strain no. V15/004035, V15/004036, and V15/004037 (*See*, WO 2016/153924) which are capable of ethanol yields from glucose that are the same or similar to strain V14/004037 under the conditions encountered in industrial scale fermentation, such as those encountered during fermentation of corn mash, and which are higher than commercially available industrial *Saccharomyces cerevisiae* strains used in the ethanol industry and naturally occurring strains of *Saccharomyces cerevisiae*.

The Applicants have further produced stain no. NRRL Y67342 as described herein which demonstate improved properties as describen in the Examples.

The Applicants have further produced derivatives of stain no. NRRL Y67342 as described herein which express a glucoamylase and demonstate improved properties as described in the Examples.

Typically, the ethanol produced from fermentation of corn mash is produced from fermentation of sugars that are endogenous to the corn mash. Sugars that are endogenous

to the corn mash are sugars that are derived from the corn rather than sugars that are added from an exogenous source.

The ability to produce ethanol rapidly in the first 20 hours of fermentation, the ethanol yield after 50 hours of fermentation, and the ability to utilize much of the glucose present in corn mash substrate within 50 hours of fermentation, are all features which can distinguish the strains herein from naturally occurring strains, and commercially available industrial strains of *Saccharomyces cerevisiae*.

Additionally, strain *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) is capable of growth in media in which xylose is the sole carbon source. In this regard, strain *Saccharomyces cerevisiae* strain MBG4985 produces more than a 10-fold increase in biomass when grown under the conditions specified in Test T1. As a consequence, the ability of strain *Saccharomyces cerevisiae* strain MBG4985 to produce more than a 10-fold increase in biomass under the conditions specified in Test T1 is a further characteristic which distinguishes this strain from:

(a) naturally occurring strains of *Saccharomyces*;

(b) contaminating strains of *Saccharomyces* that do not utilize xylose; and

(c) other strains used in the ethanol industry that do not have the ethanol producing capabilities of strain *Saccharomyces cerevisiae* strain MBG4985; and/or do not exhibit more than a 10-fold increase in biomass in Test T1.

As current wild type and industrial strains of *Saccharomyces* are not capable of growth on xylose at the rate at which strain *Saccharomyces cerevisiae* strain MBG4985 grows on xylose, strain *Saccharomyces cerevisiae* strain MBG4985 is readily differentiated from current wild type strains of *Saccharomyces* and strains of *Saccharomyces* that are used in the ethanol industry such as Ethanol Red.

The strain may also be a derivative of *Saccharomyces cerevisiae* strain MBG4985. As used herein, a “derivative” of strain *Saccharomyces cerevisiae* strain MBG4985 is a strain derived from said strain, such as through mutagenesis, recombinant DNA technology, mating, cell fusion, or cytoduction between yeast strains. The strain derived from strain *Saccharomyces cerevisiae* strain MBG4985 may be a direct progeny (i.e. the product of a mating between strain *Saccharomyces cerevisiae* strain MBG4985 and another strain or itself), or a distant progeny resulting from an initial mating between *Saccharomyces cerevisiae* strain MBG4985 and another strain or itself, followed by a large number of subsequent matings.

In one embodiment, a derivative of strain *Saccharomyces cerevisiae* strain MBG4985 is a hybrid strain produced by culturing a first yeast strain with strain *Saccharomyces*

cerevisiae strain MBG4985 under conditions which permit combining of DNA between the first yeast strain and strain *Saccharomyces cerevisiae* strain MBG4985.

In one embodiment, a recombinant derivative of strain *Saccharomyces cerevisiae* strain MBG4985 has been prepared by genetically modifying *Saccharomyces cerevisiae* strain MBG4985 (or another derivative thereof) to express an alpha-amylase and/or glucoamylase described herein.

In one embodiment is a method of producing a recombinant derivative of strain *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) comprising:

(a) transforming *Saccharomyces cerevisiae* strain MBG4985 or a derivative of strain *Saccharomyces cerevisiae* strain MBG4985 with one or more expression vectors encoding a glucoamylase and/or an alpha-amylase; and

(b) isolating the transformed strain.

In one embodiment, a derivative of strain *Saccharomyces cerevisiae* strain MBG4985 may be prepared by:

(a) culturing a first yeast strain with a second yeast strain, wherein the second yeast strain is strain *Saccharomyces cerevisiae* strain MBG4985 or a derivative of strain *Saccharomyces cerevisiae* strain MBG4985, under conditions which permit combining of DNA between the first yeast strain and the second yeast strain; and

(b) isolating hybrid strains; and

(c) optionally repeating steps (a) and (b) using a hybrid strain isolated in step (b) as the first yeast strain and/or the derivative of strain *Saccharomyces cerevisiae* strain MBG4985.

In one embodiment, the derivative of strain *Saccharomyces cerevisiae* strain MBG4985 exhibits one or more defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985. Derivatives of *Saccharomyces* which exhibit one or more defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985 are produced using strain *Saccharomyces cerevisiae* strain MBG4985. In this regard, strain *Saccharomyces cerevisiae* strain MBG4985 forms the basis for preparing other strains having the defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985. For example, strains of *Saccharomyces* which exhibit one or more defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985 can be derived from strain *Saccharomyces cerevisiae* strain MBG4985 using methods such as classical mating, cell fusion, or cytoduction between yeast strains, mutagenesis or recombinant DNA technology.

In one embodiment, a derivative of strain *Saccharomyces cerevisiae* strain MBG4985 which exhibits one or more defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985 may be produced by:

(a) culturing a first yeast strain with a second yeast strain, wherein the second yeast strain is strain *Saccharomyces cerevisiae* strain MBG4985 or a derivative of strain *Saccharomyces cerevisiae* strain MBG4985, under conditions which permit combining of DNA between the first yeast strain and the second yeast strain;

(b) screening or selecting for a derivative of strain *Saccharomyces cerevisiae* strain MBG4985, such as screening or selecting for a derivative with increased ethanol production in corn mash compared to the first strain;

(c) optionally repeating steps (a) and (b) with the screened or selected strain as the first yeast strain and/or the second yeast strain, until a derivative of strain *Saccharomyces cerevisiae* strain MBG4985 is obtained which exhibits one or more defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985.

The first yeast strain may be any strain of yeast if the DNA of the strain can be combined with the second yeast strain using methods such as classical mating, cell fusion or cytoduction. Typically, the first yeast strain is a *Saccharomyces* strain. More typically, the first yeast strain is a *Saccharomyces cerevisiae* strain. *Saccharomyces cerevisiae* is as defined by Kurtzman (2003) FEMS Yeast Research vol 4 pp. 233-245. The first yeast strain may have desired properties which are sought to be combined with the defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985. The first yeast strain may be, for example, any *Saccharomyces cerevisiae* strain, such as for example ETHANOL RED™. It will also be appreciated that the first yeast strain may be strain *Saccharomyces cerevisiae* strain MBG4985 or a strain which exhibits one or more defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985.

The first and second yeast strains are cultured under conditions which permit combining of DNA between the yeast strains. As used herein, “combining of DNA” between yeast strains refers to combining of all or a part of the genome of the yeast strains. Combining of DNA between yeast strains may be by any method suitable for combining DNA of at least two yeast cells, and may include, for example, mating methods which comprise sporulation of the yeast strains to produce haploid cells and subsequent hybridising of compatible haploid cells; cytoduction; or cell fusion such as protoplast fusion.

In one embodiment, culturing the first yeast strain with the second yeast, under conditions which permit combining of DNA between the first yeast strain and the second yeast strain, comprises:

(i) sporulating the first yeast strain and the second yeast strain;

(ii) germinating and hybridizing spores produced by the first yeast strain with spores produced by the second yeast strain.

In one embodiment, the method of producing a derivative of strain *Saccharomyces cerevisiae* strain MBG4985 which exhibits one or more defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985, comprises:

(a) providing: (i) a first yeast strain; and (ii) a second yeast strain, wherein the second yeast strain is strain *Saccharomyces cerevisiae* strain MBG4985 or a derivative of strain *Saccharomyces cerevisiae* strain MBG4985

(b) sporulating the first yeast strain and the second yeast strain;

(c) germinating and hybridising the spores of the first yeast strain with germinated spores of the second yeast strain;

(d) screening or selecting for a derivative of strain *Saccharomyces cerevisiae* strain MBG4985, such as screening or selecting for a derivative with increased ethanol production in 20hrs of fermentation in corn mash compared to the first strain, and/or higher ethanol yield from glucose during fermentation of corn mash than the first strain;

(e) optionally repeating steps (b) to (d) with the screened or selected strain as the first and/or second yeast strain.

Methods for sporulating, germinating and hybridising yeast strains, and in particular, Saccharomyces strains, are known in the art and are described in, for example, Ausubel, F. M. et al., (1997) Current Protocols in Molecular Biology, Volume 2, pages 13.2.1 to 13.2.5 (John Willey & Sons Inc); Chapter 7, “Sporulation and Hybridisation of yeast” by R.R. Fowell, in “The Yeasts” vol 1, A.H. Rose and J.S. Harrison (Eds), 1969, Academic Press.

In one embodiment, the yeast strains may be cultured under conditions which permit cell fusion. Methods for the generation of intraspecific or interspecific hybrids using cell fusion techniques are described in, for example, Spencer et al. (1990) in, Yeast Technology, Spencer JFT and Spencer DM (Eds), Springer Verlag, New York.

In another embodiment, the yeast strains may be cultured under conditions which permit cytoduction. Methods for cytoduction are described in, for example, Inge-Vechymov et al. (1986) Genetika 22: 2625-2636; Johnston (1990) in, Yeast technology, Spencer JFT and Spencer DM (Eds), Springer Verlag, New York.

In one embodiment, screening or selecting for derivatives of strain *Saccharomyces cerevisiae* strain MBG4985 comprises screening or selecting for a derivative with increased ethanol production in the first 20 hours of fermentation of corn mash compared to the first strain, and/or screening or selecting for a hybrid which has a higher ethanol yield from glucose in corn mash compared to the first strain.

As used herein, "ethanol yield from glucose" is the yield of ethanol that would be achieved from glucose if all of the glucose in a substrate were used in the fermentation. In one embodiment, ethanol yield from glucose is calculated as follows:

(G x 0.51) + E

wherein

G = % weight/volume glucose remaining following fermentation of the glucose-containing substrate; and

E = % weight/volume of ethanol present following fermentation of the glucose-containing substrate.

The derivatives may be screened or selected for ethanol yields by screening for one or more of the following characteristics:

(a) produces a % w/v of acetate that is in the range from an amount higher than that produced by strain Ethanol Red™ to the amount produced by strain *Saccharomyces cerevisiae* strain MBG4985, under the same conditions in a corn mash fermentation;

(b) produces a ratio of % w/v glycerol to % w/v acetate that is in the range from less than the ratio of % w/v glycerol to % w/v acetate produced by Ethanol Red to the ratio of % w/v glycerol to % w/v acetate produced by strain *Saccharomyces cerevisiae* strain MBG4985, under the same conditions in a corn mash fermentation;

(c) produces a ratio of % w/v ethanol to % w/v acetate that is in the range from less than the ratio of % w/v ethanol to % w/v acetate produced by Ethanol Red to the ratio of % w/v ethanol to % w/v acetate produced by strain *Saccharomyces cerevisiae* strain MBG4985, under the same conditions in a corn mash fermentation.

Methods for determining the amount of ethanol, glycerol and acetate produced by a strain are known in the art. For example, methods for testing for determining the amount of ethanol, glycerol and acetate produced by a strain during fermentation of corn mash are described in, for example, WO 2011/035392. Once the amount of ethanol, glycerol and acetate produced are known, the ratio of ethanol/acetate and glycerol/acetate can be readily determined. Accordingly, strains can be readily screened for production levels of ethanol, acetate and/or glycerol using known methods.

In one embodiment, a derivative of strain *Saccharomyces cerevisiae* strain MBG4985 which exhibits one or more defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985 may be a mutant of strain *Saccharomyces cerevisiae* strain MBG4985. Methods for producing mutants of Saccharomyces yeast, and specifically mutants of *Saccharomyces cerevisiae*, are known in the art and described in, for example, Lawrence C.W. (1991) Methods in Enzymology, 194: 273-281.

In another embodiment, a derivative of strain *Saccharomyces cerevisiae* strain MBG4985 which exhibits one or more defining characteristics of strain *Saccharomyces*

cerevisiae strain MBG4985 may be a recombinant derivative of strain *Saccharomyces cerevisiae* strain MBG4985. A recombinant derivative of strain *Saccharomyces cerevisiae* strain MBG4985 is a strain produced by introducing into strain *Saccharomyces cerevisiae* strain MBG4985 a nucleic acid using recombinant DNA technology. Methods for the introduction of nucleic acid into *Saccharomyces* yeast cells, and in particular strains of Saccharomyces, are known in the art and are described in, for example, Ausubel, F. M. et al. (1997), Current Protocols in Molecular Biology, Volume 2, pages 13.7.1 to 13.7.7, published by John Wiley & Sons Inc.

Also described are methods for the production of ethanol using the strain described herein. In one form, strain *Saccharomyces cerevisiae* strain MBG4985 or a derivative strain which exhibits one or more defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985 is incubated with a substrate comprising fermentable sugars under conditions that allow fermentation of the fermentable sugars. The fermentable sugars may be one or more of glucose, galactose, maltose, fructose and sucrose. Typically, the fermentable sugar is glucose. While strain *Saccharomyces cerevisiae* strain MBG4985 is well suited to fermentation in corn mash, it is envisaged the strain may also be suitable for other fermentation processes. Accordingly, the source of the fermentable sugar in the substrate may be, for example, hydrolysed starch, hydrolysed cellulose, molasses, cane juice, grape juice, fruit juice, glucose, maltodextrins, raw sugar juice, galactose, sucrose, or any other forms of fermentable sugars. In one form, the source of fermentable sugar in the substrate is hydrolysed starch. Typically, the starch is obtained from a substrate such as corn mash. In preparing the substrate, the grain is typically ground and mixed with water and hydrolytic enzyme(s) under conditions which result in hydrolysis of the starch and release of fermentable sugars such as glucose. Typical enzymes for hydrolysis of the starch include α-amylase, amyloglucosidase, pullulanase, alpha-amylase, glucoamylase, or mixtures thereof. Enzymes suitable for hydrolysis are available from, for example, Novozymes or Genencor Inc. In one form, substrate is provided in the form of corn mash. Corn mash is typically produced by: (a) grinding corn to form a meal; (b) mixing the meal with water; and (c) hydrolyzing the starch in the corn meal. Methods for preparation of corn mash are known in the art and described in, for example, Thomas, K. C. et al., (2001) Journal of Applied Microbiology, volume 90, pages 819-828. Methods for the preparation of other starch-based substrates including sorghum, starch streams and combinations thereof are also known in the art and described in, for example, Kwiatkowski J.R. et al. (2003) Industrial Crops and Products 23: 288-296 and Bothast R.J. and Schlicher M.A.(2005) Applied Microbial Biotechnology 67: 19-25

The fermentation is carried out at a temperature which permits fermentation of the fermentable sugars. Typically, the temperature at which the fermentation is carried out is from 25-34ºC.

The fermentation results in an alcoholic mash comprising ethanol and residual sugars in solution, and a particulate portion comprising residual solids including yeast. Ethanol is isolated from the mash using methods know in the art such as distillation or filtration.

Methods for fermentation and distillation are known in the art and are described in, for example, Kwiatkowski J.R. et al. (2003) Industrial Crops and Products 23: 288-296 and Bothast R.J. and Schlicher M.A.(2005) Applied Microbial Biotechnology 67: 19-25

Also contemplated are methods of producing distiller's grain. Distiller's grains may be produced from the residual solids produced in the fermentation using methods known in the art and described in, for example, United States Patent 7,572,353. Because *Saccharomyces* strains *Saccharomyces cerevisiae* strain MBG4985 reduce the level of residual sugars remaining following fermentation, the distiller's grain which results from fermentation using strain *Saccharomyces cerevisiae* strain MBG4985 has a lowered glucose content and is therefore more stable and less prone to charring, caramelisation or contamination with unwanted microorganisms.

Furthermore, lower glycerol content in distiller's grains is a process advantage because less time is required for drying the distiller's grains. In addition, less glycerol in the distiller's grains results in improved flowability, and further results in distiller's grains which has a higher nutrient content (e.g. higher protein).

A further aspect provides dried or compressed yeast comprising strain *Saccharomyces cerevisiae* strain MBG4985 or a derivative of strain *Saccharomyces cerevisiae* strain MBG4985, typically having the defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985.

A further aspect provides a composition comprising a *Saccharomyces* strain *Saccharomyces cerevisiae* strain MBG4985 or a derivative of strain *Saccharomyces cerevisiae* strain MBG4985 and/or a *Saccharomyces* strain having the defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985. The composition may be, for example, cream yeast, compressed yeast, wet yeast, dry yeast, semi-dried yeast, crumble yeast, stabilized liquid yeast or frozen yeast. Methods for preparing such yeast compositions are known in the art.

Test T1

Step 1: Yeast strains are streaked onto 2% w/v D-glucose 1% bacteriological peptone and 0.5% yeast extract medium solidified with 2% agar using standard microbiological techniques.
Step 2: After incubation for 72 hours at 30 deg Celsius, yeast cells are taken from plates using a sterile microbiological loop and inoculated to an OD600 (Optical Density at 600 nm) of between 0.1 and 0.2 units (OD600 at T0) in 50 ml of broth containing xylose (5% w/v), Difco Yeast Nitrogen Base w/o amino acids (0.67%), citric acid (0.3%) and trisodium citrate (0.7%)

in distilled water in a 250 ml Erlenmeyer flask. An OD600 of 0.1 unit is equal to approximately 9 x 105 yeast cells/ mL. D-(+)-Xylose, minimum 99% can be obtained from Sigma-Aldrich.

Step 3: Cultures are incubated at 30 deg Celsius with shaking at 220 rpm (10 cm orbital diameter) for 48 hours.

Step 4: After 48 hours incubation, OD600 of culture is measured (OD600 at T48).

Step 5: The fold increase in biomass is determined by the equation:

OD600 at T48/OD600 at T0.

In some embodiments, the derivative of *Saccharomyces cerevisiae* strain MBG4985 expresses a glucoamylase and/or an alpha-amylase. The derivatives expressing glucoamylase and/or alpha-amylase have been generated in order to improve ethanol yield and to improve process economy by cutting enzyme costs since part or all of the necessary enzymes needed to hydrolyse starch will be produced by the yeast organism.

One aspect of the present invention therefore relates to yeast strains comprising one or more expression vectors encoding a glucoamylase and/or an alpha-amylase, wherein the yeast is derived from a parent strain *Saccharomyces cerevisiae* strain MBG4985; and wherein the glucoamylase is selected from glucoamylases obtainable from *Gloeophyllum, Pycnoporous, Trametes*.

Another aspect of the present invention therefore relates to yeast strain comprising one or more expression vectors encoding a glucoamylase and/or an alpha-amylase, wherein the yeast is derived from a parent strain *Saccharomyces cerevisiae* strain MBG4985; and wherein the alpha-amylase is selected from a *Rhizomucor pusillus* or *Aspergillus terreus* alpha-amylase.

In one embodiment the the glucoamylase is selected from a *Gloeophyllum trabeum, Gloeophyllum sepiarium, or Gloeophyllum abietinum* glucoamylase.

In another embodiment the glucoamylase is selected from the group consisting of:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 15 or SEQ ID NO: 17;

(ii) a glucoamylase comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 15 or SEQ ID NO: 17.

In one embodiment the the glucoamylase is the *Gloeophyllum trabeum* glucoamylase shown in SEQ ID NO: 17 having one of the following substitutions: V59A; S95P; A121P; T119W; S95P+A121P; V59A+S95P; S95P+T119W; V59A+S95P+A121P; or S95P+T119W+A121P, especially S95P+A121P; and wherein the glucoamylase has 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 17.

In one particular embodiment the glucoamylase is selected from a *Trametes cingulata* glucoamylase. More particularly the glucoamylase is selected from the group consisting of:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 20;

(ii) a glucoamylase comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 20.

In one particular embodiment the glucoamylase is selected from a *Pycnoporus sanguineus* glucoamylase. More particularly the glucoamylase is selected from the group consisting of:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 18;

(ii) a glucoamylase comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 18.

In another particular embodiment the alpha-amylase is *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) as shown in SEQ ID NO: 16, preferably one having at least one of the following substitutions or combinations of substitutions: D165M; Y141W; Y141R; K136F; K192R; P224A; P224R; S123H+Y141W; G20S + Y141W; A76G + Y141W; G128D + Y141W; G128D + D143N; P219C + Y141W; N142D + D143N; Y141W + K192R; Y141W + D143N; Y141W + N383R; Y141W + P219C + A265C; Y141W + N142D + D143N; Y141W + K192R V410A; G128D + Y141W + D143N; Y141W + D143N + P219C; Y141W + D143N + K192R; G128D + D143N + K192R; Y141W + D143N + K192R + P219C; G128D + Y141W + D143N + K192R; or G128D + Y141W + D143N + K192R + P219C, especially G128D+D143N (using SEQ ID NO: 16 for numbering), and wherein the alpha-amylase has 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 16.

In another embodiment, the alpha-amylase is an *Aspergillus terreus* alpha-amylase. More particularly the alpha-amylase is selected from the group consisting of:

(i) an alpha-amylase comprising the polypeptide of SEQ ID NO: 6 of WO2017/087330 (the content of which is incorporated by reference);

(ii) an alpha-amylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 6 of WO2017/087330.

Compositions

This aspect relates to a formulated *Saccharomyces* yeast composition comprising a yeast strain described herein and a naturally occurring and/or a nonenaturally occurring component.

As mentioned above a *Saccharomyces* yeast strain described herein may be in any viable form, including crumbled, dry, including active dry and instant, compressed, cream (liquid) form etc. In one embodiment, the *Saccharomyces cerevisiae* yeast strain is dry yeast, such as active dry yeast or instant yeast. In one embodiment, the *Saccharomyces cerevisiae* yeast strain is crumbled yeast. In one embodiment, the *Saccharomyces cerevisiae* yeast strain is compressed yeast. In one embodiment, the *Saccharomyces cerevisiae* yeast strain is cream yeast.

In one embodiment is a composition comprising a *Saccharomyces* yeast described herein, in particular *Saccharomyces cerevisiae* strain MBG4985 and one or more of the component selected from the group consisting of: surfactants, emulsifiers, gums, swelling agent, and antioxidants and other processing aids.

Surfactant

The compositions described herein may comprise a *Saccharomyces* yeast described herein, in particular *Saccharomyces cerevisiae* strain MBG4985 and any suitable surfactants. In one embodiment, the surfactant(s) is/are an anionic surfactant, cationic surfactant, and/or nonionic surfactant.

Emulsifier

The compositions described herein may comprise a *Saccharomyces* yeast described herein, in particular *Saccharomyces cerevisiae* strain MBG4985 and any suitable emulsifier. In one embodiment, the emulsifier is a fatty-acid ester of sorbitan. In one embodiment, the emulsifier is selected from the group of sorbitan monostearate (SMS), citric acid esters of monodiglycerides, polyglycerolester, fatty acid esters of propylene glycol.

In one embodiment, the composition comprises a *Saccharomyces* yeast described herein, in particular *Saccharomyces cerevisiae* strain MBG4985, and Olindronal SMS, Olindronal SK, or Olindronal SPL including composition concerned in European Patent No. 1,724,336 (hereby incorporated by reference). These products are commercially available from Bussetti, Austria, for active dry yeast.

Gum

The compositions described herein may comprise a *Saccharomyces* yeast described herein, in particular *Saccharomyces cerevisiae* strain MBG4985 and any suitable gum. In one embodiment, the gum is selected from the group of carob, guar, tragacanth, arabic, xanthan and acacia gum, in particular for cream, compressed and dry yeast.

Swelling Agents

The compositions described herein may comprise a *Saccharomyces* yeast described herein, in particular *Saccharomyces cerevisiae* strain MBG4985 and any suitable swelling agent. In one embodiment, the swelling agent is methyl cellulose or carboxymethyl cellulose.

Antioxidant

The compositions described herein may comprise a *Saccharomyces* yeast described herein, in particular *Saccharomyces cerevisiae* strain MBG4985, and any suitable anti-oxidant. In one embodiment, the antioxidant is butylated hydroxyanisol (BHA) and/or butylated hydroxytoluene (BHT), or ascorbic acid (vitamin C), particular for active dry yeast.

As used herein, the singular forms "a", "an" and "the" include plural reference unless the context clearly indicates otherwise. Thus, for example, a reference to "a cell" includes a plurality of such cells. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art.

Reference to "about" a value or parameter herein includes aspects that are directed to that value or parameter *per se*. For example, description referring to "about X" includes the aspect "X".

As used herein, except where the context requires otherwise due to express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments.

The invention may further be described in the following numbered paragraphs:

Paragraph [1]. A process for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using an alpha-amylase;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [2]. The process of any of paragraph [1], wherein the fermenting organism has at least one or more, such as all, of the following properties and defining characteristics:

increases ethanol yield compared to ETHANOL RED™ under the same process

conditions, e.g., process conditions described herein; and/or

decreased glycerol production compared to ETHANOL RED™ under the same process conditions, e.g., process conditions described herein.

Paragraph [3]. The process of paragraph [1] or [2], wherein the fermenting organism provides an ethanol yield boost over ETHANOL RED™ (ER) of more than 0.5%, e.g., more than 1.0%, more than 2.0%, more than 2.5%, such as about 2.9%, such as between 0.5 and 5%, such as between 1-3%, under the same process conditins, e.g., conditions as described herein.

Paragraph [4]. The process of any of paragraphs [1]-[3], wherein the fermenting organism reduces acetaldehyde production more than 10%, e.g., more than 20%, more than 30%, more than 40%, more than 45%, such as 5-60%, such as 30-50%, compared to ETHANOL RED™ under the same process conditions, e.g., process conditions described herein.

Paragraph [5]. The process of any of paragraphs [1]-[4], wherein the fermenting organism increases temperature tolerance compared to ETHANOL RED™ under the same process conditions, e.g., process conditions described herein;

Paragraph [6]. The process of any of paragraphs [1]-[5], wherein the fermenting organism decreases glycerol production by more than 3%, e.g., more than 4%, more than 5%, more than 6%, more than 7%, such as 2-15%, such as 5-10%, compared to ETHANOL RED™ under the same process conditions, e.g., conditions described herein.

Paragraph [7]. The process of any of paragraphs [1]-[6], wherein the fermenting organism:

(a) produces a higher titre of ethanol in the first 20 hours of fermentation than ETHANOL RED™, under the same conditions in a corn mash fermentation, e.g., conditions described herein;

(b) leaves less glucose remaining following 50 hours of fermentation than ETHANOL RED™, under the same conditions in a corn mash fermentation, e.g., conditions described herein;

(c) has a higher ethanol yield than ETHANOL RED™ following 50 hours of fermentation under the same conditions in a corn mash fermentation, e.g., conditions described herein.

Paragraph [8]. The process of any of paragraphs [1]-[7], wherein a protease is added in saccharification or fermentation or SSF.

Paragraph [9]. The process of any of paragraphs [1]-[8], further comprises, prior to the liquefaction step i), the steps of:

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

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

Paragraph [10]. The process of any of paragraphs [1]-[9], wherein at least 50%, e.g., at least 70%, at least 80%, at least 90% of the starch-containing material fits through a sieve with # 6 screen.

Paragraph [11]. The process of any of paragraphs [1]-[10], wherein the pH in liquefaction is between 4-7, such as pH 4.5-6.5, such as pH 5.0-6.5, such as pH 5.0-6.0, such as pH 5.2-6.2, such as about 5.2, such as about 5.4, such as about 5.6, such as about 5.8.

Paragraph [12]. The process of any of paragraphs [1]-[11], wherein the temperature in liquefaction is in the range of 70-100ºC, such as 75-95°C, 75-90°C, 80-90°C, or 82-88°C, such as about 85ºC.

Paragraph [13]. The process of any of paragraphs [1]-[12], wherein a jet-cooking step is carried out prior to liquefaction in step i).

Paragraph [14]. The process of paragraph [13], wherein the jet-cooking is carried out at a temperature of 110-145°C, e.g, 120-140ºC, such as 125-135°C, or about 130ºC for about 1-15 minutes, e.g., for about 3-10 minutes, or about 5 minutes.

Paragraph [15]. The process of any of paragraphs [1]-[14], wherein saccharification and fermentation is carried out sequentially or simultaneously (SSF).

Paragraph [16]. The process of any of paragraphs [1]-[15], wherein saccharification is carried out at a temperature from 20-75ºC, e.g., from 40-70ºC, such as about 60ºC, and at a pH between 4 and 5.

Paragraph [17]. The process of any of paragraphs [1]-[16], wherein fermentation or simultaneous saccharification and fermentation (SSF) is carried out 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, or about 32°C. In one embodiment, fermentation is ongoing for 6 to 120 hours, in particular 24 to 96 hours.

Paragraph [18]. The process of any of paragraphs [1]-[17], wherein the fermentation product is recovered after fermentation, such as by distillation.

Paragraph [19]. The process of any of paragraphs [1]-[18], wherein the fermentation product is an alcohol, preferably ethanol, especially fuel ethanol, potable ethanol and/or industrial ethanol.

Paragraph [20]. The process of any of paragraphs [1]-[19], wherein the starch-containing starting material is whole grains.

Paragraph [21]. The process of any of paragraphs [1]-[20], wherein the starch-containing material is derived from corn, wheat, barley, rye, milo, sago, cassava, manioc, tapioca, sorghum, oats, rice or potatoes.

Paragraph [22]. The process of any of paragraphs [1]-[21], wherein the alpha-amylase used or added in liquefaction step i) is of bacterial origin.

Paragraph [23]. The process of any of paragraphs [1]-[22], wherein the alpha-amylase is from the genus *Bacillus*, such as a strain of *Bacillus stearothermophilus,* in particular a variant of a *Bacillus stearothermophilus* alpha-amylase, such as the one shown in SEQ ID NO: 3 in WO 99/019467 or SEQ ID NO: 1 herein.

Paragraph [24]. The process of paragraph [23], wherein the *Bacillus stearothermophilus* alpha-amylase or variant thereof is truncated at the C-terminal, preferably to be from 485-495 amino acids long, such as about 491 amino acids long.

Paragraph [25]. The process of any of paragraphs [23] or [24], wherein the *Bacillus stearothermophilus* alpha-amylase has a double deletion at positions I181 + G182, and optionally substitution N193F, or deletion of R179 + G180 (using SEQ ID NO: 1 for numbering).

Paragraph [26]. The process of any of paragraphs [23]-[25], wherein the *Bacillus stearothermophilus* alpha-amylase has a substitution in position S242, e.g., S242Q substitution (using SEQ ID NO: 1 for numbering).

Paragraph [27]. The process of any of paragraphs [23]-[26], wherein the *Bacillus stearothermophilus* alpha-amylase has a substitution in position E188, e.g., E188P substitution (using SEQ ID NO: 1 for numbering).

Paragraph [28]. The process of any of paragraphs [1]-[27], wherein the alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) of at least 10, such as at least 15, such as at least 20, such as at least 25, such as at least 30, such as at least 40, such as at least 50, such as at least 60, such as 10-70, such as 15-70, such as 20-70, such as 25-70, such as 30-70, such as 40-70, such as 50-70, such as 60-70.

Paragraph [29]. The process of any of paragraphs [1]-[28], wherein the alpha-amylase present and/or added in liquefaction step i) is selected from the group of *Bacillus stearothermophilus* alpha-amylase variants with one of the following substitutions or combinations of substitutions in addition to I181*+G182*, and optionally substitution N193F:

V59A+Q89R+G112D+E129V+K177L+R179E+K220P+N224L+Q254S;
V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+D269E+D281N;
V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+I270L;
V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+H274K;
V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+Y276F;
V59A+E129V+R157Y+K177L+R179E+K220P+N224L+S242Q+Q254S;
V59A+E129V+K177L+R179E+H208Y+K220P+N224L+S242Q+Q254S;
V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+H274K;
V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F;
V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+D281N;
V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+G416V;
V59A+E129V+K177L+R179E+K220P+N224L+Q254S;
V59A+E129V+K177L+R179E+K220P+N224L+Q254S+M284T;

A91L+M96I+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
E129V+K177L+R179E;
E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F+L427M;
E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+N376*+I377*;
E129V+K177L+R179E+K220P+N224L+Q254S;
E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
E129V+K177L+R179E+S242Q;
E129V+K177L+R179V+K220P+N224L+S242Q+Q254S;
K220P+N224L+S242Q+Q254S;
M284V;
V59A+Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V; and
V59A+E129V+K177L+R179E+Q254S+ M284V;

Paragraph [30]. The process of any of paragraphs [1]-[29], wherein the alpha-amylase present and/or added in liquefaction step i) is selected from the following group of *Bacillus stearothermophilus* alpha-amylase variants comprising the following mutations: I181*+G182*, and optionally substitution N193F, and further one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;
V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S
V59A+ Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V;
V59A+ E129V+ K177L+ R179E+ Q254S+ M284V; and
E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering).

Paragraph [31]. The process of any of paragraphs [1]-[30], wherein a glucoamylase is present and/or added in saccharification and/or fermentation.

Paragraph [32]. The process of paragraph [31], wherein the glucoamylase present and/or added in saccharification, fermentation or simultaneous saccharification and fermentation (SSF) is of fungal origin, preferably from a strain of *Aspergillus*, preferably *A. niger*, *A. awamori*, or *A. oryzae*; or a strain of *Trichoderma*, preferably *Trichoderma reesei*; or a strain of *Talaromyces*, preferably *Talaroomyces emersonii*, or a strain of *Pycnoporus*, or a strain of *Gloeophyllum*, such as *Gloeophyllum serpiarium* or *Gloeophyllum trabeum,* or a strain of the *Nigrofomes.*

Paragraph [33]. The process of any of paragraphs [1]-[32], wherein the glucoamylase is derived from *Talaromyces emersonii*, such as the one shown in SEQ ID NO: 19 herein.

Paragraph [34]. The process of any of paragraphs [1]-[33], wherein the glucoamylase is selected from the group consisting of:

(i) a glucoamylase comprising the mature polypeptide of SEQ ID NO: 19 herein;

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 19 herein.

Paragraph [35]. The process of any of paragraphs 1-34, wherein the glucoamylase present and/or added in saccharification is derived from *Gloeophyllum serpiarium*, such as the one shown in SEQ ID NO: 15 herein.

Paragraph [36]. The process of any of paragraphs [1]-[35], wherein the glucoamylase present and/or added in saccharification is selected from the group consisting of:

(i) a glucoamylase comprising the mature polypeptide of SEQ ID NO: 15 herein;

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 15 herein.

Paragraph [37]. The process of any of paragraphs [1]-[36], wherein the glucoamylase present and/or added in saccharification is derived from *Gloeophyllum trabeum* such as the one shown in SEQ ID NO: 17 herein.

Paragraph [38]. The process of any of paragraphs [1]-[37], wherein the glucoamylase present and/or added in saccharification is selected from the group consisting of:

(i) a glucoamylase comprising the mature polypeptide of SEQ ID NO: 17 herein;

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 17 herein.

Paragraph [39]. The process of any of paragraphs [1]-[38], wherein the glucoamylase is present and/or added in saccharification and/or fermentation in combination with an alpha-amylase.

Paragraph [40]. The process of paragraph [39], wherein the alpha-amylase is present and/or added in saccharification and/or fermentation is of fungal or bacterial origin.

Paragraph [41]. The process of paragraph [40] or [41], wherein the alpha-amylase present and/or added in saccharification and/or fermentation is derived from a strain of the genus *Rhizomucor*, preferably a strain the *Rhizomucor pusillus,* such as the one shown in SEQ ID NO: 3 in WO 2013/006756, such as a *Rhizomucor pusillus* alpha-amylase hybrid having a

linker and a starch-binding domain, in particular having an *Aspergillus niger* linker and starch-bonding domain, such as the one shown in SEQ ID NO: 16 herein.

Paragraph [42]. The process of any of paragraphs [39]-[41], wherein the alpha-amylase present and/or added in saccharification and/or fermentation is selected from the group consisting of:

(i) an alpha-amylase comprising the mature polypeptide of SEQ ID NO: 16 herein;

(ii) an alpha-amylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 16 herein.

Paragraph [43]. The process of any of paragraphs [39]-[42], wherein the alpha-amylase is a variant of the alpha-amylase shown in SEQ ID NO: 16 having at least one of the following substitutions or combinations of substitutions: D165M; Y141W; Y141R; K136F; K192R; P224A; P224R; S123H + Y141W; G20S + Y141W; A76G + Y141W; G128D + Y141W; G128D + D143N; P219C + Y141W; N142D + D143N; Y141W + K192R; Y141W + D143N; Y141W + N383R; Y141W + P219C + A265C; Y141W + N142D + D143N; Y141W + K192R V410A; G128D + Y141W + D143N; Y141W + D143N + P219C; Y141W + D143N + K192R; G128D + D143N + K192R; Y141W + D143N + K192R + P219C; and G128D + Y141W + D143N + K192R; or G128D + Y141W + D143N + K192R + P219C (using SEQ ID NO: 16 for numbering).

Paragraph [44]. The process of any of paragraphs [39]-[43], wherein the alpha-amylase is derived from a *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably disclosed as SEQ ID NO: 16 herein, e.g., having one or more of the following substitutions: G128D, D143N, such as G128D+D143N (using SEQ ID NO: 16 for numering).

Paragraph [45]. The process of any of paragraphs [39]-[44], wherein the alpha-amylase variant has at least 75% identity preferably at least 80%, e.g., 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%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 16 herein.

Paragraph [46]. The process of any of paragraphs [1]-[42], wherein liquefaction step i) is carried out using:

an alpha-amylase;

a protease having a thermostability value of more than 20% determined as Relative Activity at 80ºC/70ºC; and

optionally a glucoamylase.

Paragraph [47]. The process of [46], wherein the protease with a thermostability value of more than 25% determined as Relative Activity at 80ºC/70ºC.

Paragraph [48]. The process of paragraphs [46]-[47], wherein the protease has a thermostability of more than 30%, e.g., more than 40%, more than 50%, more than 60%, more than 70%, more than 80%, more than 90%, more than 100%, such as more than 105%, such as more than 110%, such as more than 115%, such as more than 120% determined as Relative Activity at 80ºC/70ºC.

Paragraph [49]. The process of any of paragraphs [46]-[48], wherein the protease has a thermostability of between 20 and 50%, such as between 20 and 40%, such as 20 and 30% determined as Relative Activity at 80ºC/70ºC.

Paragraph [50]. The process of any of paragraphs [46]-[49], wherein the protease has a thermostability of between 50 and 115%, such as between 50 and 70%, such as between 50 and 60%, such as between 100 and 120%, such as between 105 and 115% determined as Relative Activity at 80ºC/70ºC.

Paragraph [51]. The process of any of paragraphs [46]-[50], wherein the protease has a thermostability of more than 10%, e.g., more than 12%, more than 14%, more than 16%, more than 18%, more than 20%, more than 30%, more than 40%, more that 50%, more than 60%, more than 70%, more than 80%, more than 90%, more than 100%, more than 110% determined as Relative Activity at 85ºC/70ºC.

Paragraph [52]. The process of any of paragraphs [46]-[51], wherein the protease has a thermostability of between 10 and 50%, such as between 10 and 30%, such as between 10 and 25% determined as Relative Activity at 85ºC/70ºC.

Paragraph [53]. The process of any of paragraphs [46]-[52], wherein the protease has a themostability above 60%, such as above 90%, such as above 100%, such as above 110% at 85ºC as determined using the Zein-BCA assay.

Paragraph [54]. The process of any of paragraphs [46]-[53], wherein the protease has a themostability between 60-120, such as between 70-120%, such as between 80-120%, such as between 90-120%, such as between 100-120%, such as 110-120% at 85ºC as determined using the Zein-BCA assay.

Paragraph [55]. The process of any of paragraphs [46]-[54], wherein the protease is of fungal origin.

Paragraph [56]. The process of any of paragraphs [46]-[55], wherein the protease is a variant of the metallo protease derived from a strain of the genus *Thermoascus*, preferably a strain of *Thermoascus aurantiacus*, especially *Thermoascus aurantiacus* CGMCC No. 0670.

Paragraph [57]. The process of any of paragraphs [46]-[56], wherein the protease is a variant of the metallo protease disclosed as the mature part of SEQ ID NO: 2 disclosed in WO2003/048353 or the mature part of SEQ ID NO: 1 in WO2010/008841 or SEQ ID NO: 3 herein, with one of the following substitutions or combinations of substitutions:

S5*+D79L+S87P+A112P+D142L;

D79L+S87P+A112P+T124V+D142L;
S5*+N26R+D79L+S87P+A112P+D142L;
N26R+T46R+D79L+S87P+A112P+D142L;
T46R+D79L+S87P+T116V+D142L;
D79L+P81R+S87P+A112P+D142L;
A27K+D79L+S87P+A112P+T124V+D142L;
D79L+Y82F+S87P+A112P+T124V+D142L;
D79L+Y82F+S87P+A112P+T124V+D142L;
D79L+S87P+A112P+T124V+A126V+D142L;
D79L+S87P+A112P+D142L;
D79L+Y82F+S87P+A112P+D142L;
S38T+D79L+S87P+A112P+A126V+D142L;
D79L+Y82F+S87P+A112P+A126V+D142L;
A27K+D79L+S87P+A112P+A126V+D142L;
D79L+S87P+N98C+A112P+G135C+D142L;
D79L+S87P+A112P+D142L+T141C+M161C;
S36P+D79L+S87P+A112P+D142L;
A37P+D79L+S87P+A112P+D142L;
S49P+D79L+S87P+A112P+D142L;
S50P+D79L+S87P+A112P+D142L;
D79L+S87P+D104P+A112P+D142L;
D79L+Y82F+S87G+A112P+D142L;
S70V+D79L+Y82F+S87G+Y97W+A112P+D142L;
D79L+Y82F+S87G+Y97W+D104P+A112P+D142L;
S70V+D79L+Y82F+S87G+A112P+D142L;
D79L+Y82F+S87G+D104P+A112P+D142L;
D79L+Y82F+S87G+A112P+A126V+D142L;
Y82F+S87G+S70V+D79L+D104P+A112P+D142L;
Y82F+S87G+D79L+D104P+A112P+A126V+D142L;
A27K+D79L+Y82F+S87G+D104P+A112P+A126V+D142L;
A27K+Y82F+S87G+D104P+A112P+A126V+D142L;
A27K+D79L+Y82F+ D104P+A112P+A126V+D142L;
A27K+Y82F+D104P+A112P+A126V+D142L;
A27K+D79L+S87P+A112P+D142L; and
D79L+S87P+D142L.

Paragraph [58]. The process of any of paragraphs [46]-[57], wherein the protease is a variant of the metallo protease disclosed as the mature part of SEQ ID NO: 2 disclosed in

WO2003/048353 or the mature part of SEQ ID NO: 1 in WO 2010/008841 or SEQ ID NO: 3 herein with one of the following substitutions or combinations of substitutions:

D79L+S87P+A112P+D142L:

D79L+S87P+D142L; and

A27K+ D79L+ Y82F+S87G+D104P+A112P+A126V+D142L.

Paragraph [59]. The process of any of paragraphs [46]-[58], wherein the protease variant has at least 75% identity, e.g., 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%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 2 disclosed in WO 2003/048353 or the mature part of SEQ ID NO: 1 in WO 2010/008841 or SEQ ID NO: 3 herein.

Paragraph [60]. The process of any of paragraphs [46]-[59], wherein the protease variant of the *Thermoascus aurantiacus* protease shown in SEQ ID NO: 3 herein comprises one of the following substitutions or combinations of substitutions:

D79L S87P D142L; D79L S87P A112P D142L; D79L Y82F S87P A112P D142L; S38T D79L S87P A112P A126V D142L; D79L Y82F S87P A112P A126V D142L; A27K D79L S87P A112P A126V D142L; S49P D79L S87P A112P D142L; S50P D79L S87P A112P D142L; D79L S87P D104P A112P D142L; D79L Y82F S87G A112P D142L; S70V D79L Y82F S87G Y97W A112P D142L; D79L Y82F S87G Y97W D104P A112P D142L; S70V D79L Y82F S87G A112P D142L; D79L Y82F S87G D104P A112P D142L; D79L Y82F S87G A112P A126V D142L; Y82F S87G S70V D79L D104P A112P D142L; Y82F S87G D79L D104P A112P A126V D142L; and A27K D79L Y82F S87G D104P A112P A126V D142L.

Paragraph [61]. The process of any of paragraphs [46]-[60], wherein the protease is of bacterial origin.

Paragraph [62]. The process of any of paragraphs [46]-[61], wherein the protease is derived from a strain of *Pyrococcus*, preferably a strain of *Pyrococcus furiosus*.

Paragraph [63]. The process of any of paragraphs [46]-[62], wherein the protease is the one shown in SEQ ID NO: 1 in US 6,358,726, or SEQ ID NO: 13 herein.

Paragraph [64]. The process of any of paragraphs [46]-[63], wherein the protease is one having at least 80%, e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity to in SEQ ID NO: 1 in US 6,358,726 or SEQ ID NO: 13 herein.

Paragraph [65]. The process of any of paragraph [46]-[64], wherein 0.5-100 micro gram *Pyrococcus furiosus* protease per gram DS, such as 1-50 micro gram *Pyrococcus furiosus* protease per gram DS, such as 1-10 micro gram *Pyrococcus furiosus* protease per gram DS, such as 1.5-5 micro gram *Pyrococcus furiosus* protease per gram DS, such as about or more

than 1.5 micro gram *Pyrococcus furiosus* protease per gram DS are present and/or added in liquefaction step i).

Paragraph [66]. The process of any of paragraphs [46]-[65], wherein 2-100 micro gram *Pyrococcus furiosus* protease per gram DS, such as 2.5-50 micro gram *Pyrococcus furiosus* protease per gram DS, such as 2.5-10 micro gram *Pyrococcus furiosus* protease per gram DS, such as 2.5-5 micro gram *Pyrococcus furiosus* protease gram DS, especially about 3 micro gram *Pyrococcus furiosus* protease per gram DS are present and/or added in liquefaction step i).

Paragraph [67]. The process of any of paragraphs [1]-[66], wherein a glucoamylase is present and/or added during liquefaction step i).

Paragraph [68]. The process of paragraph [67], wherein the glucoamylase present and/or added in liquefaction has a heat stability at 85ºC, pH 5.3, of at least 20%, such as at least 30%, or at least 35%.

Paragraph [69]. The process of paragraph [67] or [68], wherein the glucoamylase present and/or added in liquefaction has a relative activity pH optimum at pH 5.0 of at least 90%, preferably at least 95%, preferably at least 97%.

Paragraph [70]. The process of any of paragraphs [67]-[68], wherein the glucoamylase present and/or added in liquefaction has pH stability at pH 5.0 of at least at least 80%, at least 85%, at least 90%.

Paragraph [71]. The process of any of paragraphs [67]-[70], wherein the glucoamylase present and/or added in liquefaction step i) is derived from a strain of the genus *Penicillium*, especially a strain of *Penicillium oxalicum* disclosed as SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NOs: 9 or 14 herein.

Paragraph [72]. The process of any of paragraphs [67]-[71], wherein the glucoamylase has at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99% or 100% identity to the mature polypeptide shown in SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NOs: 9 or 14 herein.

Paragraph [73]. The process of any of paragraphs [67]-[72], wherein the glucoamylase is a variant of the *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 2 in WO 2011/127802 having a K79V substitution (using the mature sequence shown in SEQ ID NO: 14 herein for numbering), such as a variant disclosed in WO 2013/053801.

Paragraph [74]. The process of any of paragraph [67]-[73], wherein the *Penicillium oxalicum* glucoamylase has a K79V substitution (using SEQ ID NO: 14 for numbering) and further one of the following substitutions or combinations of substitutions:

T65A; Q327F; E501V; Y504T; Y504*; T65A + Q327F; T65A + E501V; T65A + Y504T; T65A + Y504*; Q327F + E501V; Q327F + Y504T; Q327F + Y504*; E501V + Y504T; E501V + Y504*; T65A + Q327F + E501V; T65A + Q327F + Y504T; T65A + E501V + Y504T; Q327F + E501V + Y504T; T65A + Q327F + Y504*; T65A + E501V + Y504*; Q327F + E501V + Y504*; T65A + Q327F + E501V + Y504T; T65A + Q327F + E501V + Y504*; E501V + Y504T; T65A + K161S; T65A + Q405T; T65A + Q327W; T65A + Q327F; T65A + Q327Y; P11F + T65A + Q327F; R1K + D3W + K5Q + G7V + N8S + T10K + P11S + T65A + Q327F; P2N + P4S + P11F + T65A + Q327F; P11F + D26C + K33C + T65A + Q327F; P2N + P4S + P11F + T65A + Q327W + E501V + Y504T; R1E + D3N + P4G + G6R + G7A + N8A + T10D+ P11D + T65A + Q327F; P11F + T65A + Q327W; P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; P11F + T65A + Q327W + E501V + Y504T; T65A + Q327F + E501V + Y504T; T65A + S105P + Q327W; T65A + S105P + Q327F; T65A + Q327W + S364P; T65A + Q327F + S364P; T65A + S103N + Q327F; P2N + P4S + P11F + K34Y + T65A + Q327F; P2N + P4S + P11F + T65A + Q327F + D445N + V447S; P2N + P4S + P11F + T65A + I172V + Q327F; P2N + P4S + P11F + T65A + Q327F + N502*; P2N + P4S + P11F + T65A + Q327F + N502T + P563S + K571E; P2N + P4S + P11F + R31S + K33V + T65A + Q327F + N564D + K571S; P2N + P4S + P11F + T65A + Q327F + S377T; P2N + P4S + P11F + T65A + V325T+ Q327W; P2N + P4S + P11F + T65A + Q327F + D445N + V447S + E501V + Y504T; P2N + P4S + P11F + T65A + I172V + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + S377T + E501V + Y504T; P2N + P4S + P11F + D26N + K34Y + T65A + Q327F; P2N + P4S + P11F + T65A + Q327F + I375A + E501V + Y504T; P2N + P4S + P11F + T65A + K218A + K221D + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + S103N + Q327F + E501V + Y504T; P2N + P4S + T10D + T65A + Q327F + E501V + Y504T; P2N + P4S + F12Y + T65A + Q327F + E501V + Y504T; K5A + P11F + T65A + Q327F + E501V + Y504T; P2N + P4S + T10E + E18N + T65A + Q327F + E501V + Y504T; P2N + T10E + E18N + T65A + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T568N; P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + K524T + G526A; P2N + P4S + P11F + K34Y + T65A + Q327F + D445N + V447S + E501V + Y504T; P2N + P4S + P11F + R31S + K33V + T65A + Q327F + D445N + V447S + E501V + Y504T; P2N + P4S + P11F + D26N + K34Y + T65A + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + F80* + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + K112S + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T516P + K524T + G526A; P2N + P4S + P11F + T65A + Q327F + E501V + N502T + Y504*; P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + S103N + Q327F + E501V + Y504T; K5A + P11F + T65A + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T516P + K524T + G526A; P2N + P4S + P11F + T65A + K79A + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + K79G + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + K79I + Q327F + E501V + Y504T; P2N + P4S

+ P11F + T65A + K79L + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + K79S + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + L72V + Q327F + E501V + Y504T; S255N + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + E74N + V79K + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + G220N + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + Y245N + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + Q253N + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + D279N + Q327F + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + S359N + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + D370N + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + V460S + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + V460T + P468T + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + T463N + E501V + Y504T; P2N + P4S + P11F + T65A + Q327F + S465N + E501V + Y504T; andP2N + P4S + P11F + T65A + Q327F + T477N + E501V + Y504T.

Paragraph [75]. The process of any of paragraphs [67]-[74], wherein the glucoamylase present and/or added in liquefaction is the *Penicillium oxalicum* glucoamylase has a K79V substitution (using SEQ ID NO: 14 herein for numbering) and further one of the following substitutions or combinations of substitutions:P11F + T65A + Q327F; and P2N + P4S + P11F + T65A + Q327F (using SEQ ID NO: 14 herein for numbering).

Paragraph [76]. The process of any of paragraphs [67]-[75], wherein the glucoamylase variant has at least 75% identity, e.g., 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%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 14 herein.

Paragraph [77]. The process of any of paragraphs [1]-[76], further wherein a pullulanase is present during liquefaction and/or saccharification.

Paragraph [78]. The process of any of paragraphs [1]-[77], comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using an alpha-amylase derived from *Bacillus stearothermophilus*;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [79]. The process of any of paragraphs [1]-[78], comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using an alpha-amylase derived from *Bacillus stearothermophilus* comprising a double deletion at positions I181 + G182, and optionally a N193F substitution (using SEQ ID NO: 1 herein for numbering);

ii) saccharifying using a glucoamylase derived from a strain of *Gloephyllum*, such as *Gloephyllum serpiarium* or *Gloephyllum trabeum.*

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [80]. The process of any of paragraphs [1]-[79], comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:

an alpha-amylase derived from *Bacillus stearothermophilus*;

a protease having a thermostability value of more than 20% determined as Relative Activity at 80ºC/70ºC, preferably derived from *Pyrococcus furiosus* and/or *Thermoascus aurantiacus*; and

optionally a *Penicillium oxalicum* glucoamylase;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [81]. A process of paragraphs [1]-[80], comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using an alpha-amylase, preferably derived from *Bacillus stearothermophilus,* comprising a double deletion at positions I181 + G182, and optionally a N193F substitution (using SEQ ID NO: 1 for numbering) and having a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$ of at least 10;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [82]. A process of paragraphs [1]-[81], comprising the steps of:

i) liquefying the starch-containing material at a temperature between 80-90°C using:

an alpha-amylase, preferably derived from *Bacillus stearothermophilus,* having a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$ of at least 10;

a protease, preferably derived from *Pyrococcus furiosus* and/or *Thermoascus aurantiacus,* having a thermostability value of more than 20% determined as Relative Activity at 80ºC/70ºC; and

optionally a *Penicillium oxalicum* glucoamylase;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [83]. A process of paragraphs [1]-[82], comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:

an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion at positions I181 + G182, and optional substitution N193F; and optionally further one of the following substitutions or combinations of substitutions: E129V+K177L+R179E; V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

V59A+Q89R+E129V+K177L+R179E+Q254S+M284V;

V59A+ E129V+ K177L+R179E+Q254S+M284V;

and E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering);

ii) saccharifying using a glucoamylase, such as one from a strain of *Gloephyllum*, such as a strain of *Gloephyllum serpiarium*;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [84]. A process of paragraphs [1]-[83], comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:

an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion at positions I181 + G182, and optional substitution N193F, and optionally further one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

V59A+Q89R+E129V+K177L+R179E+Q254S+M284V;

V59A+ E129V+ K177L+R179E+Q254S+M284V; and

E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering);

a protease having a thermostability value of more than 20% determined as Relative Activity at 80ºC/70ºC, preferably derived from *Pyrococcus furiosus* and/or *Thermoascus aurantiacus*; and

optionally a *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 14 having one of the following substitutions or combinations of substitutions:

K79V;

K79V+ P11F + T65A + Q327F;

K79V+P2N + P4S + P11F + T65A + Q327F;

K79V +P11F + D26C + K33C + T65A + Q327F;

K79V +P2N + P4S + P11F + T65A + Q327W + E501V + Y504T;

K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; and

K79V +P11F + T65A + Q327W + E501V + Y504T (using SEQ ID NO: 14 for numbering);

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that

of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [85]. A process of paragraphs [1]-[84], comprising the steps of:

i) liquefying the starch-containing material at a temperature between 80-90°C using:

an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion at positions I181 + G182, and optionally substitution N193F, and further optionally one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

V59A+Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V;

V59A+E129V+K177L+R179E+Q254S+M284V; and

E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering),

a protease having a thermostability value of more than 20% determined as Relative Activity at 80ºC/70ºC, preferably derived from *Pyrococcus furiosus* and/or *Thermoascus aurantiacus*;

a *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 14 having one of the following substitutions or combinations of substitutions:

K79V;

K79V+ P11F + T65A + Q327F;

K79V+P2N + P4S + P11F + T65A + Q327F;

K79V +P11F + D26C + K33C + T65A + Q327F;

K79V +P2N + P4S + P11F + T65A + Q327W + E501V + Y504T;

K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; and

K79V +P11F + T65A + Q327W + E501V + Y504T (using SEQ ID NO: 14 for numbering);

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [86]. The process of any of paragraphs [1]-[85], comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:

an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion at positions I181 + G182, and optionally substitution N193F (using SEQ ID NO: 1 herein for numbering);

a protease having a thermostability value of more than 20% determined as Relative Activity at 80ºC/70ºC, preferably derived from *Pyrococcus furiosus* and/or *Thermoascus aurantiacus*;

a *Penicillium oxalicum* glucoamylase having a K79V substilution (using SEQ ID NO: 14 herein for numbering); and

optionally a pullulanase;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [87]. A process of paragraphs [1]-[86], comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:

an alpha-amylase, preferably derived from *Bacillus stearothermophilus,* having a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$ of at least 10;

between 0.5 and 10 micro grams *Pyrococcus furiosus* protease per g DS;

ii) saccharifying using a glucoamylase selected from the group of glucoamylase derived from a strain of *Aspergillus*, preferably *A. niger*, *A. awamori*, or *A. oryzae*; or a strain of *Trichoderma*, preferably *T. reesei*; or a strain of *Talaromyces*, preferably *T. emersonii*, or a strain of *Pycnoporus*, or a strain of *Gloephyllum*, such as *G. serpiarium* or *G. trabeum,* or a strain of the *Nigrofomes;*

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [88]. A process of paragraphs [1]-[87], comprising the steps of:

i) liquefying the starch-containing material at a temperature between 80-90°C using;

an alpha-amylase, preferably derived from *Bacillus stearothermophilus* having a double deletion at positions I181 + G182, and optional substitution N193F and having a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$ of at least 10;

between 0.5 and 10 micro grams *Pyrococcus furiosus* protease per g DS;

a *Penicillium oxalicum* glucoamylase; and

optionally a pullulanase;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [89]. A process of paragraphs [1]-[88], comprising the steps of:

i) liquefying the starch-containing material at a temperature a temperature between 80-90°C using;

an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion I181 + G182 and optional substitution N193F; and optionally further one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

V59A+Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V;

V59A+E129V+ K177L+ R179E+ Q254S+ M284V; and

E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering);

between 0.5 and 10 micro grams *Pyrococcus furiosus* protease per g DS;

a *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 14 having one of the following substitutions or combinations of substitutions:

K79V;

K79V+ P11F + T65A + Q327F;

K79V+P2N + P4S + P11F + T65A + Q327F;

K79V +P11F + D26C + K33C + T65A + Q327F;

K79V +P2N + P4S + P11F + T65A + Q327W + E501V + Y504T;

K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; and

K79V +P11F + T65A + Q327W + E501V + Y504T (using SEQ ID NO: 14 for numbering); and

optionally a pullulanase;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [90]. A process of paragraphs [1]-[89], comprising the steps of:

i) liquefying the starch-containing material at a temperature between 80-90°C using:

an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion I181 + G182, and optional substitution N193F; and further one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

V59A+Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V;

V59A+E129V+ K177L+ R179E+ Q254S+ M284V; and

E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering);

a protease having a thermostability value of more than 20% determined as Relative Activity at 80ºC/70ºC, preferably derived from *Pyrococcus furiosus* and/or *Thermoascus aurantiacus*;

a *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 14 having one of the following substitutions or combinations of substitutions:

K79V;

K79V+ P11F + T65A + Q327F;

K79V+P2N + P4S + P11F + T65A + Q327F;

K79V +P11F + D26C + K33C + T65A + Q327F;

K79V +P2N + P4S + P11F + T65A + Q327W + E501V + Y504T;

K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; and

K79V +P11F + T65A + Q327W + E501V + Y504T (using SEQ ID NO: 14 for numbering); and

optionally a pullulanase;

ii) saccharifying using a glucoamylase selected from the group of glucoamylase derived from a strain of *Aspergillus*; or a strain of *Trichoderma;* a strain of *Talaromyces*, a strain of *Pycnoporus*; a strain of *Gloephyllum;* and a strain of the *Nigrofomes;*

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [91]. A process of any of paragraphs [1]-[90], comprising the steps of:

i) liquefying the starch-containing material at a temperature between 80-90°C at a pH between 5.0 and 6.5 using:

an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion I181 + G182 and optionally substitution N193F; and optionally further one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

V59A+Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V;

V59A+E129V+ K177L+ R179E+ Q254S+ M284V;

E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering);

a protease derived from *Pyrococcus furiosus*, preferably the one shown in SEQ ID NO: 13 herein;

a *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 14 having one of the following substitutions or combinations of substitutions:

K79V;

K79V+ P11F + T65A + Q327F; or

K79V+P2N + P4S + P11F + T65A + Q327F; or

K79V +P11F + D26C + K33C + T65A + Q327F; or

K79V +P2N + P4S + P11F + T65A + Q327W + E501V + Y504T; or

K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; or

K79V +P11F + T65A + Q327W + E501V + Y504T (using SEQ ID NO: 14 for numbering);

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that

of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [92]. The process of any of paragraphs [1]-[91], wherein a cellulolytic enzyme composition is present in saccharification, fermentation or simultaneous saccharification and fermentation (SSF).

Paragraph [93]. The process of any of paragraphs [1]-[92], wherein the fermenting organism has properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985, or a derivative of *Saccharomyces cerevisiae* strain MBG4985 having the defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985, as it provides one or more, such as all of, the following properties or defining characteristics

an increase in ethanol yield compared to Ethanol Red™ under the same process conditions e.g., as described herein; and/or

decreased glycerol production compared to ETHANOL RED™ under the same process conditions, e.g., as described herein.

Paragraph [94]. The process of any of claims paragraph [1]-[93], wherein the fermenting organism, provides an ethanol yield boost over ETHANOL RED™ (ER) of more than 0.5%, e.g., more than 1.0%, more than 2.0%, more than 2.5%, about 2.9%, such as between 0.5 and 5%, such as 1-3%, under the same process conditins, e.g., conditions described herein.

Paragraph [95]. The process of any of paragraphs [1]-[94], wherein the fermenting organism reduces acetaldehyde production more than 10%, e.g., more than 20%, more than 30%, more than 40%, more than 45%, such as 5-60%, such as 30-50%, compared to ETHANOL RED™ under the same process conditions, e.g., conditions described herein.

Paragraph [96]. The process of any of paragraphs [1]-[95], wherein the fermenting organism increases temperature tolerance compared to ETHANOL RED™ under the same process conditions, e.g., conditions described herein.

Paragraph [97]. The process of any of paragraphs [1]-[96], wherein the fermenting organism decreases glycerol production by more than 3%, e.g., more than 4%, more than 5%, more than 6%, more than 7%, such as 2-15%, such as 5-10%, compared to ETHANOL RED™ under the same process conditions, e.g., conditions described herein.

Paragraph [98]. The process of any of paragraphs [1]-[97], wherein the fermenting organism is a non-recombinant *Saccharomyces* strain, e.g., a non-recombinant *Saccharomyces cerevisiae* strain.

Paragraph [99]. The process of any of paragraphs [1]-[98], wherein the fermenting organism is a non-recombinant *Saccharomyces* strain preferably non-recombinant *Saccharomyces cerevisiae* strain produced using the method described and concerned in US patent no. 8,257,959-BB.

Paragraph [100]. A process for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using an alpha-amylase derived from *Bacillus stearothermophilus*;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism has one or more, such as all, of the following properties and defining characteristics:

increases ethanol yield compared to ETHANOL RED™ (ER) under the same process conditions, e.g., conditions as described herein;

reduced acetaldehyde production compared to ETHANOL RED™ under the same process conditions, e.g., conditions as described herein;

increased temperature tolerance compared to ETHANOL RED™ under the same process conditions, e.g., conditions as described herein;

decreased glycerol production compared to ETHANOL RED™ under the same process conditions, e.g., conditions as described herein.

Paragraph [101]. The process of paragraph [100], wherein the fermenting organism is a *Saccharomyces cerevisiae* yeast

Paragraph [102]. The process of paragraphs [100] or [101], wherein the fermenting organism is a non-recombinant *Saccharomyces cerevisiae* yeast.

Paragraph [103]. A process of any of paragraphs [1]-[102], comprising the steps of:

i) liquefying the starch-containing material at a temperature between 80-90°C at a pH between 5.0 and 6.5 using:

an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion I181 + G182, and optional substitution N193F; and optionally further one of the following substitutions or combinations of substitutions:

E129V+K177L+R179E;

V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

V59A+Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V;

V59A+E129V+ K177L+ R179E+ Q254S+ M284V;

E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering).

a protease derived from *Pyrococcus furiosus*, preferably the one shown in SEQ ID NO: 13 herein present and/or added in a dosage of 1-5 micro gram protease per gram DS, such as about 1.5 or 3 micro gram protease per gram DS;

optionally a *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 14 having one of the following substitutions or combinations of substitutions:

K79V;

K79V+ P11F + T65A + Q327F; or

K79V+P2N + P4S + P11F + T65A + Q327F; or

K79V +P11F + D26C + K33C + T65A + Q327F; or

K79V +P2N + P4S + P11F + T65A + Q327W + E501V + Y504T; or

K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; or

K79V +P11F + T65A + Q327W + E501V + Y504T (using SEQ ID NO: 14 for numbering);

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [104]. The process of any of paragraphs [100]-[103], wherein the fermenting organism is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* strain MBG4985 or a derivative of *Saccharomyces* strain MBG4985 having defining characteristics of strain MBG4985.

Paragraph [105]. The process of paragraphs [104], wherein a protease is present or added in saccharification and/or fermentation or simultaneous saccharification and fermentation (SSF).

Paragraph [106]. A *Saccharomyces* yeast strain deposited under the Budapest Treaty and having NRRL accession no. NRRL Y67342, or a derivative of strain NRRL Y67342.

Paragraph [107]. A method of producing a derivative of strain *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA), comprising:

a. culturing a first yeast strain with a second yeast strain, wherein the second yeast strain is strain *Saccharomyces cerevisiae* strain MBG4985 or a derivative thereof, under conditions which permit combining of DNA between the first yeast strain and the second yeast strain; and
b. isolating hybrid strains; and
c. optionally repeating steps (a) and (b) using a hybrid strain isolated in step (b)

as the first yeast strain and/or the second yeast strain.

Paragraph [108]. A method of producing a derivative of strain *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) which exhibits the defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985, comprising:

(a) providing:

(i) a first yeast strain; and

(ii) a second yeast strain, wherein the second yeast strain is strain *Saccharomyces cerevisiae* strain MBG4985 or a derivative thereof;

(b) culturing the first yeast strain and the second yeast strain under conditions which permit combining of DNA between the first and second yeast strains;

(c) screening or selecting for a derivative of strain *Saccharomyces cerevisiae* strain MBG4985.

Paragraph [109]. The method of paragraph [108], wherein step (c) comprises screening or selecting for a hybrid strain which exhibits one or more defining characteristic of strain *Saccharomyces cerevisiae* strain MBG4985.

Paragraph [110]. The method of paragraph [108], comprising the further step of:

(d) repeating steps (b) and (c) with the screened or selected strain from step (c) as the first and/or second strain, until a derivative is obtained which exhibits the defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985.

Paragraph [111]. The method of paragraph [109] or [110], wherein the culturing step (b) comprises:

(i) sporulating the first yeast strain and the second yeast strain;

(ii) hybridizing germinated spores produced by the first yeast strain with germinated spores produced by the second yeast strain.

Paragraph [112]. A *Saccharomyces* strain produced by the method of any one of paragraphs [107] to [111].

Paragraph [113]. A *Saccharomyces* strain having the defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA).

Paragraph [114]. A method of producing ethanol, comprising incubating a strain of any of paragraphs [106], [112] or [113] with a substrate comprising a fermentable sugar under conditions which permit fermentation of the fermentable sugar to produce ethanol.

Paragraph [115]. Use of a strain of any of paragraphs [106], [112] or [113] in the production of ethanol.

Paragraph [116]. A method of producing distiller's grain, comprising:

(a) incubating a *Saccharomyces* strain of any of paragraphs [106], [112] or [113] with a substrate comprising fermentable sugar under conditions which allow fermentation of the fermentable sugar to produce ethanol and distiller's grains;

(b) isolating the distiller's grains.

Paragraph [117]. Distiller's grain produced by the method of paragraph [116].

Paragraph [118]. Use of a strain of paragraph [106], [112] or [113] in the production of distiller's grains.

Paragraph [119]. Use of a strain of paragraphs [106], [112] or [113] in the production of a *Saccharomyces* strain having the defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA).

Paragraph [120]. Use of strain *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) in the production of a *Saccharomyces* strain having properties that are about the same as that of strain *Saccharomyces cerevisiae* strain MBG4985 or which exhibits one or more defining characteristics of strain *Saccharomyces cerevisiae* strain MBG4985.

Paragraph [121]. Use of strain *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a strain having properties that are about the same as that of strain *Saccharomyces cerevisiae* strain MBG4985 or a derivative thereof in a process according to any of paragraphs [1]-[120].

Paragraph [122]. A composition comprising a *Saccharomyces* yeast of any of paragraphs [106], [112] or [113] and one or more naturally occurring and/or non-naturally occurring components.

Paragraph [123]. The composition of paragraph [122], wherein the components are selected from the group consisting of: surfactants, emulsifiers, gums, swelling agents, and antioxidants.

Paragraph [124]. The composition of paragraph [122] or [123], wherein the *Saccharomyces* yeast is *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA).

Paragraph [125]. The composition of any of paragraphs [122]- [124], wherein the *Saccharomyces* yeast is in a viable form, in particular in dry, cream or compressed form.

Paragraph [126]: A process of producing ethanol from starch-containing material comprising:

(a) saccharifying the starch-containing material; and

(b) fermenting using a fermentation organism;

wherein

saccharification and/or fermentation is done in the presence of at least a glucoamylase and optionally an alpha-amylase;

the fermenting organism is *Saccharomyces cerevisiae;*

and wherein a glucoamylase and/or an alpha-amylase is expressed from the fermenting organism.

Paragraph [127]: The process according to paragraph [126], wherein the starch containing material is either gelatinized or ungelatinized starch.

Paragraph [128]: The process according to paragraph [127], wherein a liquefaction step precedes the saccharification step, and wherein the liquefaction step is performed in the presence of at least a bacterial alpha-amylase, such as an alpha-amylase from *Bacillus* sp., particularly *Bacillus stearothermophilus*.

Paragraph [129]: The process according to any of paragraphs [126]-[128], wherein the *Saccharomyces cerevisiae* is MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) or a fermenting organism having properties that are about the same as that of MBG4985 or a derivative of MBG4985 having defining characteristics of strain MBG4985.

Paragraph [130]: The process of paragraphs [126]-[129], wherein the fermenting organism is a recombinant derivative of MBG4985 that expresses the glucoamylase.

[Paragraph 131] The process of paragraphs [126]-[130], wherein the glucocamylase is a *Gloeophyllum* glucoamylase, preferably *Gloeophyllum trabeum, Gloeophyllum sepiarium, or Gloeophyllum abietinum* glucoamylase.

Paragraph [132]: The process of paragraphs [126]-[131], wherein the glucoamylase is selected from the group consisting of:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 15 or SEQ ID NO: 17;

(ii) a glucoamylase comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 15 or SEQ ID NO: 17.

Paragraph [133]: The process of paragraph [131] or [132], wherein the glucoamylase is the *Gloeophyllum trabeum* glucoamylase shown in SEQ ID NO: 17 having one of the following substitutions: V59A; S95P; A121P; T119W; S95P+A121P; V59A+S95P; S95P+T119W; V59A+S95P+A121P; or S95P+T119W+A121P, especially S95P+A121P.

Paragraph [134]: The process of any of paragraphs [126]-[130], wherein the glucoamylase is expressed from the fermenting organism and is a *Trametes* glucoamylase, preferably a

Trametes cingulata glucoamylase.

Paragraph [135]: The process of paragraph [134], wherein the glucoamylase is selected from the group consisting of:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 20;

(ii) a glucoamylase comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 20.

Paragraph [136]: The process of any of paragraphs [126]-[130], wherein the glucoamylase is expressed from the fermenting organism and is a *Pycnoporus* glucoamylase, particularly *Pycnoporus sanguineus* glucoamylase.

Paragraph [137]: The process of paragraph [136], wherein the glucoamylase is selected from the group consisting of:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 18;

(ii) a glucoamylase comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 18.

Paragraph [138]: The process of any of paragraphs [126]-[137], wherein the fermenting organism is a recombinant derivative of MBG4985 that expresses the alpha-amylase.

Paragraph [139] The process of paragraphs [126]-[138], wherein the alpha-amylase expressed from the fermenting organism and is derived from *Rhizomucor pusillus* or *Aspergillus terreus.*

Paragraph [140]: The process of paragraph [139], wherein the alpha-amylase is *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) as shown in SEQ ID NO: 16, preferably one having at least one of the following substitutions or combinations of substitutions: D165M; Y141W; Y141R; K136F; K192R; P224A; P224R; S123H+Y141W; G20S + Y141W; A76G + Y141W; G128D + Y141W; G128D + D143N; P219C + Y141W; N142D + D143N; Y141W + K192R; Y141W + D143N; Y141W + N383R; Y141W + P219C + A265C; Y141W + N142D + D143N; Y141W + K192R V410A; G128D + Y141W + D143N; Y141W + D143N + P219C; Y141W + D143N + K192R; G128D + D143N + K192R; Y141W + D143N + K192R + P219C; G128D + Y141W + D143N + K192R; or G128D + Y141W + D143N + K192R + P219C, especially G128D+D143N, and wherein the alpha-amylase has 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 16.

Paragraph [141]: The process of paragraph [139], wherein the alpha-amylase is an *Aspergillus terreus* alpha-amylase selected from the group consisting of:

(i) an alpha-amylase comprising the polypeptide of SEQ ID NO: 6 of WO2017/087330;

(ii) an alpha-amylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 6 of WO2017/087330.

Paragraph [142]: A *Saccharomyces cerevisiae* yeast strain, wherein the strain is a derivative of MBG4985 comprising one or more expression vectors encoding a glucoamylase and/or an alpha-amylase.

Paragraph [143] The yeast strain according to paragraph [142],wherein the glucoamylase is selected from glucoamylases obtainable from *Gloeophyllum, Pycnoporous,* or *Trametes*.

Paragraph [144]: The yeast strain according to paragraph [143], wherein the glucoamylase is selected from a *Gloeophyllum trabeum, Gloeophyllum sepiarium, or Gloeophyllum abietinum* glucoamylase.

Paragraph [145]: The yeast strain of paragraph [144], wherein the glucoamylase is selected from the group consisting of:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 15 or SEQ ID NO: 17;

(ii) a glucoamylase comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 15 or SEQ ID NO: 17.

Paragraph [146]: The yeast strain of any of paragraphs [143]-[145], wherein the glucoamylase is the *Gloeophyllum trabeum* glucoamylase shown in SEQ ID NO: 17 having one of the following substitutions: V59A; S95P; A121P; T119W; S95P+A121P; V59A+S95P; S95P+T119W; V59A+S95P+A121P; or S95P+T119W+A121P, especially S95P+A121P.

Paragraph [147]: The yeast strain of paragraph [143], wherein the glucoamylase is a *Trametes cingulata* glucoamylase.

Paragraph [148]: The yeast strain of paragraph [147], wherein the glucoamylase is selected from the group consisting of:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 20;

(ii) a glucoamylase comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 20.

Paragraph [149]: The yeast strain of paragraph [143], wherein the glucoamylase is s a *Pycnoporus sanguineus* glucoamylase.

Paragraph [150]: The yeast strain of paragraph [149], wherein the glucoamylase is selected from the group consisting of:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 18;

(ii) a glucoamylase comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 18.

Paragraph [151]: The yeast strain of paragraph [142] expression vectorwherein the alpha-amylase is selected from a *Rhizomucor pusillus* or *Aspergillus terreus* alpha-amylase.

Paragraph [152]: The yeast strain of paragraph [151], wherein the alpha-amylase is *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) as shown in SEQ ID NO: 16, preferably one having at least one of the following substitutions or combinations of substitutions: D165M; Y141W; Y141R; K136F; K192R; P224A; P224R; S123H+Y141W; G20S + Y141W; A76G + Y141W; G128D + Y141W; G128D + D143N; P219C + Y141W; N142D + D143N; Y141W + K192R; Y141W + D143N; Y141W + N383R; Y141W + P219C + A265C; Y141W + N142D + D143N; Y141W + K192R V410A; G128D + Y141W + D143N; Y141W + D143N + P219C; Y141W + D143N + K192R; G128D + D143N + K192R; Y141W + D143N + K192R + P219C; G128D + Y141W + D143N + K192R; or G128D + Y141W + D143N + K192R + P219C, especially G128D+D143N, and wherein the alpha-amylase has 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 16.

Paragraph [153]: The yeast strain of paragraph [151], wherein the alpha-amylase is *Aspergillus terreus* alpha-amylase selected from the group consisting of:

(i) an alpha-amylase comprising the polypeptide of SEQ ID NO: 6 of WO2017/087330;

(ii) an alpha-amylase comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 6 of WO2017/087330.

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.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

Materials & Methods

Materials:

Alpha-Amylase 369 ("AA369"): *Bacillus stearothermophilus* alpha-amylase with the mutations: I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+Q254S+M284V truncated to 491 amino acids (using SEQ ID NO: 1 herein for numbering);

Penicillium oxalicum glucoamylase variant PE498 ("PoAMG498"): *Penicillium oxalicum* glucoamylase variant having the following mutations: K79V+ P2N+ P4S+ P11F+ T65A+ Q327F (using SEQ ID NO: 14 herein for numbering):

Protease Pfu ("PFU"): Protease derived from *Pyrococcus furiosus* shown in SEQ ID NO: 13 herein.

Glucoamylase SA ("GSA") comprises a blend comprising *Talaromyces emersonii* glucoamylase disclosed in WO99/28448 (SEQ ID NO: 19 herein), *Trametes cingulata* glucoamylase disclosed as SEQ ID NO: 2 in WO 06/69289 and SEQ ID NO: 20 herein, and *Rhizomucor pusillus* alpha-amylase with *Aspergillus niger* glucoamylase linker and SBD disclosed as SEQ ID NO: 16 herein with the following substitutions: G128D+D143N (activity ratio AGU:AGU:FAU(F): approx. 30:7:1).

Yeast:

ETHANOL RED ™ ("ER"): *Saccharomyces cerevisiae* yeast available from Fermentis/Lesaffre, USA.

Deposit of Biological Material

The following biological material has been deposited under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA, and given the following accession number:

Deposit	Accession Number	Date of Deposit
Saccharomyces cerevisiae strain MBG4985	NRRL Y67342	November 23, 2016

The following biological material has been deposited under the terms of the Budapest Treaty with the National Measurement Institute, 1/153 Bertie Street, Port Melbourne, Victoria 3207, Australia and given the following accession number:

Deposit	Accession Number	Date of Deposit
MBG4851	V14/004037	February 17, 2014
MBG4911	V15/001459	January 13, 2015
MBG4913	V15/001460	January 13, 2015
MBG4914	V15/001461	January 13, 2015
MBG4931	V15/004036	February 19, 2015

The strains were deposited under conditions that assure that access to the culture will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 C.F.R. §1.14 and 35 U.S.C. §122. The deposit represents a substantially pure culture of the deposited strain. The deposit is available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Methods

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

For purposes described herein, the degree of identity between two amino acid sequences, as well as the degree of identity between two nucleotide sequences, may be determined by the program "align" which is a Needleman-Wunsch alignment (i.e. a global alignment). The program is used for alignment of polypeptide, as well as nucleotide sequences. The default scoring matrix BLOSUM50 is used for polypeptide alignments, and the default identity matrix is used for nucleotide alignments. The penalty for the first residue of a gap is -12 for polypeptides and -16 for nucleotides. The penalties for further residues of a gap are -2 for polypeptides, and -4 for nucleotides.

"Align" is part of the FASTA package version v20u6 (see W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448, and W. R. Pearson (1990) "Rapid and Sensitive Sequence Comparison with FASTP and FASTA," Methods in Enzymology 183:63- 98). FASTA protein alignments use the Smith-Waterman algorithm with no limitation on gap size (see "Smith-Waterman algorithm", T. F. Smith and M. S. Waterman (1981) J. Mol. Biol. 147:195-197).

Protease assays

AZCL-casein assay

A solution of 0.2% of the blue substrate AZCL-casein is suspended in Borax/NaH_2PO_4 buffer pH9 while stirring. The solution is distributed while stirring to microtiter plate (100 microL to each well), 30 microL enzyme sample is added and the plates are incubated in an Eppendorf Thermomixer for 30 minutes at 45°C and 600 rpm. Denatured enzyme sample (100°C boiling for 20min) is used as a blank. After incubation the reaction is stopped by transferring the microtiter plate onto ice and the coloured solution is separated from the solid by centrifugation at 3000rpm for 5 minutes at 4°C. 60 microL of supernatant is transferred to a microtiter plate and the absorbance at 595nm is measured using a BioRad Microplate Reader.

pNA-assay

50 microL protease-containing sample is added to a microtiter plate and the assay is started by adding 100 microL 1mM pNA substrate (5 mg dissolved in 100 microL DMSO and further diluted to 10 mL with Borax/NaH_2PO_4 buffer pH 9.0). The increase in OD_{405} at room temperature is monitored as a measure of the protease activity.

Glucoamylase activity (AGU) Glucoamylase activity may be measured in Glucoamylase Units (AGU).

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

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

AMG incubation
Substrate: maltose 23.2 mM
Buffer: acetate 0.1 M
pH: 4.30 ± 0.05
Incubation temperature: 37°C ± 1
Reaction time: 5 minutes
Enzyme working range: 0.5-4.0 AGU/mL

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

A folder (EB-SM-0131.02/01) describing this analytical method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Acid alpha-amylase activity (AFAU)

Acid alpha-amylase activity may be measured in AFAU (Acid Fungal Alpha-amylase Units), which are determined relative to an enzyme standard. 1 AFAU is defined as the amount of enzyme which degrades 5.260 mg starch dry matter per hour under the below mentioned standard conditions.

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

$$\text{STARCH} + \text{IODINE} \xrightarrow[40^{\circ},\ \text{pH } 2,5]{\text{ALPHA - AMYLASE}} \text{DEXTRINS} + \text{OLIGOSACCHARIDES}$$

$\lambda = 590$ nm

blue/violet t = 23 sec. decoloration

Standard conditions/reaction conditions
Substrate: Soluble starch, approx. 0.17 g/L
Buffer: Citrate, approx. 0.03 M
Iodine (I_2): 0.03 g/L
$CaCl_2$: 1.85 mM
pH: 2.50 ± 0.05
Incubation temperature: 40°C
Reaction time: 23 seconds
Wavelength: 590nm
Enzyme concentration: 0.025 AFAU/mL
Enzyme working range: 0.01-0.04 AFAU/mL

A folder EB-SM-0259.02/01 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Alpha-amylase activity (KNU)

The alpha-amylase activity may be determined using potato starch as substrate. This method is based on the break-down of modified potato starch by the enzyme, and the reaction is followed by mixing samples of the starch/enzyme solution with an iodine solution. Initially, a blackish-blue color is formed, but during the break-down of the starch the blue color gets weaker and gradually turns into a reddish-brown, which is compared to a colored glass standard.

One Kilo Novo alpha amylase Unit (KNU) is defined as the amount of enzyme which, under standard conditions (i.e., at 37°C +/- 0.05; 0.0003 M Ca^{2+}; and pH 5.6) dextrinizes 5260 mg starch dry substance Merck Amylum solubile.

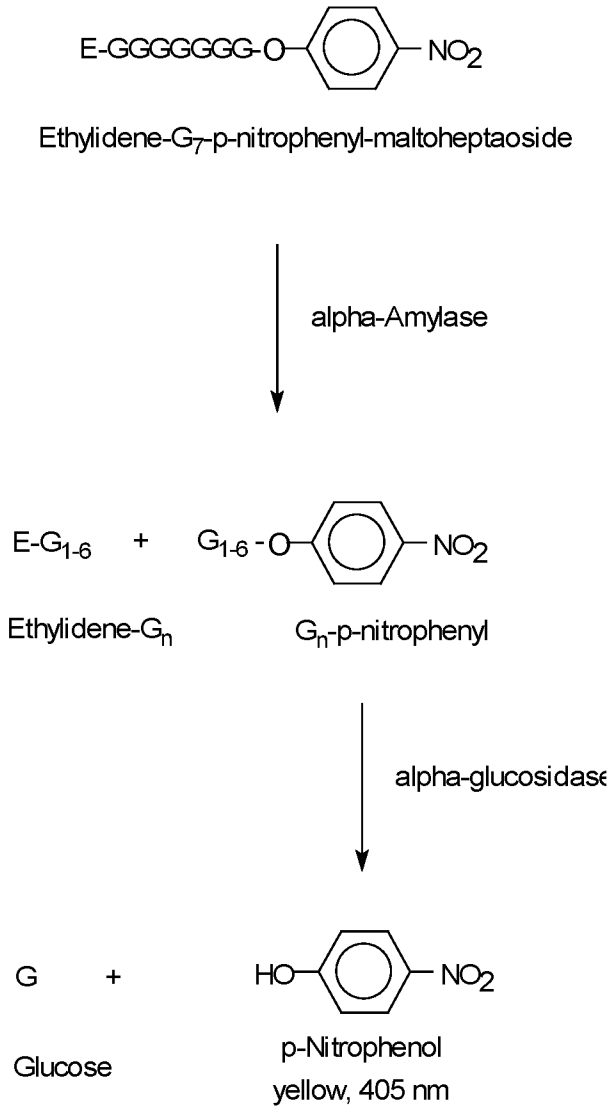
A folder EB-SM-0009.02/01 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Alpha-amylase Activity (KNU-A)

Alpha amylase activity is measured in KNU(A) Kilo Novozymes Units (A), relative to an enzyme standard of a declared strength.

Alpha amylase in samples and α-glucosidase in the reagent kit hydrolyze the substrate (4,6-ethylidene(G_7)-*p*-nitrophenyl(G_1)-α,D-maltoheptaoside (ethylidene-G_7PNP) to glucose and the yellow-colored *p*-nitrophenol.

The rate of formation of *p*-nitrophenol can be observed by Konelab 30. This is an expression of the reaction rate and thereby the enzyme activity.



The enzyme is an alpha-amylase with the enzyme classification number EC 3.2.1.1.

Parameter	Reaction conditions
Temperature	37°C
pH	7.00 (at 37°C)
Substrate conc.	Ethylidene-G_7PNP, R2: 1.86 mM
Enzyme conc. (conc. of high/low standard in reaction mixture)	1.35 -4.07 KNU(A)/L
Reaction time	2 min
Interval kinetic measuring time	7 / 18 sec.

Wave length	405nm
Conc. of reagents/chemicals critical for the analysis	α-glucosidase, R1:≥ 3.39 kU/L

A folder EB-SM-5091.02-D on determining KNU-A actitvity is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Alpha-Amylase Activity KNU(S)

BS-amylase in samples and the enzyme alpha-glucosidase in the reagent kit hydrolyze substrate (4,6-ethylidene(G7)-p-nitrophenyl(G1)-alpha-D-maltoheptaoside (ethylidene-G7PNP)) to glucose and the yellow-colored p-nitrophenol.

The rate of formation of p-nitrophenol can be observed by Konelab 30. This is an expression of the reaction rate and thereby the enzyme activity.

Reaction conditions

Reaction:
pH: 7.15
Temperature: 37 °C
Reaction Time: 180 sec
Detection:
Wavelength: 405 nm
Measuring Time: 120 sec

Unit definition

Bacillus stearothermophilus amylase (BS-amylase) activity is measured in KNU(S), Kilo Novo Units (sterarothermophilus), relative to an enzyme standard of a declared strength.

This analytical method is described in more details in EB-SM-0221.02 (incorporated by reference) available from Novozymes A/S, Denmark, on request.

Determination of FAU(F)

FAU(F) Fungal Alpha-Amylase Units (Fungamyl) is measured relative to an enzyme standard of a declared strength.

Reaction conditions
Temperature: 37°C
pH: 7.15
Wavelength: 405 nm
Reaction time: 5 min
Measuring time: 2 min

A folder (EB-SM-0216.02) describing this standard method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Determination of Pullulanase Activity (NPUN)

Endo-pullulanase activity in NPUN is measured relative to a Novozymes pullulanase standard. One pullulanase unit (NPUN) is defined as the amount of enzyme that releases 1

micro mol glucose per minute under the standard conditions (0.7% red pullulan (Megazyme), pH 5, 40ºC, 20 minutes). The activity is measured in NPUN/ml using red pullulan.

1 mL diluted sample or standard is incubated at 40ºC for 2 minutes. 0.5 mL 2% red pullulan, 0.5 M KCl, 50 mM citric acid, pH 5 are added and mixed. The tubes are incubated at 40ºC for 20 minutes and stopped by adding 2.5 ml 80% ethanol. The tubes are left standing at room temperature for 10-60 minutes followed by centrifugation 10 minutes at 4000 rpm. OD of the supernatants is then measured at 510 nm and the activity calculated using a standard curve.

The present invention is described in further detail in the following examples which are offered to illustrate the present invention, but not in any way intended to limit the scope of the invention as claimed. All references cited herein are specifically incorporated by reference for that which is described therein.

EXAMPLES

Example 1: Stability of Alpha-Amylase Variants

The stability of a reference alpha-amylase (*Bacillus stearothermophilus* alpha-amylase with the mutations I181*+G182*+N193F truncated to 491 amino acids (SEQ ID NO: 1 numbering)) and alpha-amylase variants thereof was determined by incubating the reference alpha-amylase and variants at pH 4.5 and 5.5 and temperatures of 75°C and 85°C with 0.12 mM $CaCl_2$ followed by residual activity determination using the EnzChek® substrate (EnzChek® Ultra Amylase assay kit, E33651, Molecular Probes).

Purified enzyme samples were diluted to working concentrations of 0.5 and 1 or 5 and 10 ppm (micrograms/ml) in enzyme dilution buffer (10 mM acetate, 0.01% Triton X100, 0.12 mM $CaCl_2$, pH 5.0). Twenty microliters enzyme sample was transferred to 48-well PCR MTP and 180 microliters stability buffer (150 mM acetate, 150 mM MES, 0.01% Triton X100, 0.12 mM $CaCl_2$, pH 4.5 or 5.5) was added to each well and mixed. The assay was performed using two concentrations of enzyme in duplicates. Before incubation at 75°C or 85°C, 20 microliters was withdrawn and stored on ice as control samples. Incubation was performed in a PCR machine at 75°C and 85°C. After incubation samples were diluted to 15 ng/mL in residual activity buffer (100 mM Acetate, 0.01% Triton X100, 0.12 mM $CaCl_2$, pH 5.5) and 25 microliters diluted enzyme was transferred to black 384-MTP. Residual activity was determined using the EnzChek substrate by adding 25 microliters substrate solution (100 micrograms/ml) to each well. Fluorescence was determined every minute for 15 minutes using excitation filter at 485-P nm and emission filter at 555 nm (fluorescence reader is Polarstar, BMG). The residual activity was normalized to control samples for each setup.

Assuming logarithmic decay half life time (T½ (min)) was calculated using the equation: T½ (min) = T(min)*LN(0.5)/LN(%RA/100), where T is assay incubation time in minutes, and %RA is % residual activity determined in assay.

Using this assay setup the half life time was determined for the reference alpha-amylase and variant thereof as shown in Table 1.

Table 1.

Mutations	**T½ (min) (pH 4.5, 75°C, 0.12 mM $CaCl_2$)**	**T½ (min) (pH 4.5, 85°C, 0.12 mM $CaCl_2$)**	**T½ (min) (pH 5.5, 85°C, 0.12 mM $CaCl_2$)**
Reference Alpha-Amylase A	21	4	111
Reference Alpha-Amylase A with the substitution V59A	32	6	301
Reference Alpha-Amylase A with the substitution V59E	28	5	230
Reference Alpha-Amylase A with the substitution V59I	28	5	210
Reference Alpha-Amylase A with the substitution V59Q	30	6	250
Reference Alpha-Amylase A with the substitutions V59A+Q89R+ G112D+E129V+K177L+R179E+ K220P+N224L+Q254S	149	22	ND
Reference Alpha-Amylase A with the substitutions V59A+Q89R+E129V+ K177L+R179E+H208Y+K220P+ N224L+Q254S	>180	28	ND
Reference Alpha-Amylase A with the substitutions V59A+Q89R+E129V+ K177L+R179E+K220P+N224L+ Q254S+D269E+D281N	112	16	ND
Reference Alpha-Amylase A with the substitutions V59A+Q89R+E129V+ K177L+R179E+K220P+N224L+ Q254S+I270L	168	21	ND
Reference Alpha-Amylase A with the substitutions V59A+Q89R+E129V+ K177L+R179E+K220P+N224L+ Q254S+H274K	>180	24	ND
Reference Alpha-Amylase A with the substitutions V59A+Q89R+E129V+ K177L+R179E+K220P+N224L+ Q254S+Y276F	91	15	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ R157Y+K177L+R179E+K220P+	141	41	ND

N224L+S242Q+Q254S			
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+H208Y+K220P+ N224L+S242Q+Q254S	>180	62	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S	>180	49	>480
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S+H274K	>180	53	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S+Y276F	>180	57	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S+D281N	>180	37	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S+M284T	>180	51	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S+G416V	>180	45	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ Q254S	143	21	>480
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ Q254S+M284T	>180	22	ND
Reference Alpha-Amylase A with the substitutions A91L+M96I+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S	>180	38	ND
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E	57	11	402
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+S242Q+ Q254S	174	44	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+S242Q+ Q254S+Y276F+L427M	>180	49	>480

Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+S242Q+ Q254S+M284T	>180	49	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+S242Q+ Q254S+N376*+I377*	177	36	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+Q254S	94	13	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+Q254S+ M284T	129	24	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+S242Q	148	30	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179V	78	9	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179V+K220P+N224L+S242Q+ Q254S	178	31	>480
Reference Alpha-Amylase A with the substitutions K220P+N224L+ S242Q+Q254S	66	17	>480
Reference Alpha-Amylase A with the substitutions K220P+N224L+ Q254S	30	6	159
Reference Alpha-Amylase A with the substitution M284T	35	7	278
Reference Alpha-Amylase A with the substitutions M284V	59	13	ND

ND: not determined

The results demonstrate that the alpha-amylase variants have a significantly greater half-life and stability than the reference alpha-amylase.

Example 2: Preparation of Protease Variants and Test of Thermostability

Strains and plasmids

E.coli DH12S (available from Gibco BRL) was used for yeast plasmid rescue. pJTP000 is a *S. cerevisiae* and *E.coli* shuttle vector under the control of TPI promoter, constructed from pJC039 described in WO 01/92502, in which the *Thermoascus aurantiacus* M35 protease gene (WO 03048353) has been inserted.

Saccharomyces cerevisiae YNG318 competent cells: MATa Dpep4[cir+] ura3-52, leu2-D2, his 4-539 was used for protease variants expression. It is described in J. Biol. Chem. 272 (15), pp 9720-9727, 1997.

Media and substrates

10X Basal solution: Yeast nitrogen base w/o amino acids (DIFCO) 66.8 g/l, succinate 100 g/l, NaOH 60 g/l.

SC-glucose: 20 % glucose (i.e., a final concentration of 2 % = 2 g/100 ml)) 100 ml/l, 5 % threonine 4 ml/l, 1 % tryptophan10 ml/l, 20 % casamino acids 25 ml/l, 10 X basal solution 100 ml/l. The solution is sterilized using a filter of a pore size of 0.20 micrometer. Agar (2%) and H_2O (approx. 761 ml) is autoclaved together, and the separately sterilized SC-glucose solution is added to the agar solution.

YPD: Bacto peptone 20 g/l, yeast extract 10 g/l, 20 % glucose 100 ml/l.

YPD+Zn: YPD+0.25 mM $ZnSO_4$.

PEG/LiAc solution: 40 % PEG4000 50 ml, 5 M Lithium Acetate 1 ml.

96 well Zein micro titre plate:

Each well contains 200 microL of 0.05-0.1 % of zein (Sigma), 0.25 mM $ZnSO_4$ and 1 % of agar in 20 mM sodium acetate buffer, pH 4.5.

DNA manipulations

Unless otherwise stated, DNA manipulations and transformations were performed using standard methods of molecular biology as described in Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab. Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.) "*Current protocols in Molecular Biology*", John Wiley and Sons, 1995; Harwood, C. R. and Cutting, S. M. (Eds.).

Yeast transformation

Yeast transformation was performed using the lithium acetate method. 0.5 microL of vector (digested by restriction endonucleases) and 1 microL of PCR fragments is mixed. The DNA mixture, 100 microL of YNG318 competent cells, and 10 microL of YEAST MAKER carrier DNA (Clontech) is added to a 12 ml polypropylene tube (Falcon 2059). Add 0.6 ml PEG/LiAc solution and mix gently. Incubate for 30 min at 30 ºC, and 200 rpm followed by 30 min at 42 ºC (heat shock). Transfer to an eppendorf tube and centrifuge for 5 sec. Remove the supernatant and resolve in 3 ml of YPD. Incubate the cell suspension for 45 min at 200 rpm at 30 ºC. Pour the suspension to SC-glucose plates and incubate 30 ºC for 3 days to grow colonies. Yeast total DNA are extracted by Zymoprep Yeast Plasmid Miniprep Kit (ZYMO research).

DNA sequencing

E. coli transformation for DNA sequencing was carried out by electroporation (BIO-RAD Gene Pulser). DNA Plasmids were prepared by alkaline method (Molecular Cloning, Cold

Spring Harbor) or with the Qiagen® Plasmid Kit. DNA fragments were recovered from agarose gel by the Qiagen gel extraction Kit. PCR was performed using a PTC-200 DNA Engine. The ABI PRISMTM 310 Genetic Analyzer was used for determination of all DNA sequences.

Construction of protease expression vector

The *Thermoascus* M35 protease gene was amplified with the primer pair Prot F (SEQ ID NO: 4) and Prot R (SEQ ID NO: 5). The resulting PCR fragments were introduced into *S. cerevisiae* YNG318 together with the pJC039 vector (described in WO 2001/92502) digested with restriction enzymes to remove the Humicola insolens cutinase gene.

The Plasmid in yeast clones on SC-glucose plates was recovered to confirm the internal sequence and termed as pJTP001.

Construction of yeast library and site-directed variants

Library in yeast and site-directed variants were constructed by SOE PCR method (Splicing by Overlap Extension, see "PCR: A practical approach", p. 207-209, Oxford University press, eds. McPherson, Quirke, Taylor), followed by yeast *in vivo* recombination.

General primers for amplification and sequencing

The primers AM34 (SEQ ID NO: 6) and AM35 (SEQ ID NO:7) were used to make DNA fragments containing any mutated fragments by the SOE method together with degenerated primers (AM34 + Reverse primer and AM35 + forward primer) or just to amplify a whole protease gene (AM34 + AM35).

PCR reaction system:	Conditions:	
48.5 microL H_2O	1	94 °C 2 min
2 beads puRe Taq Ready-To-Go PCR (Amersham Biosciences)	2	94 °C 30 sec
0.5 micro L X 2 100 pmole/microL of primers	3	55 °C 30 sec
0.5 microL template DNA	4	72 °C 90 sec
	2-4	25 cycles
	5	72 °C 10 min

DNA fragments were recovered from agarose gel by the Qiagen gel extraction Kit. The resulting purified fragments were mixed with the vector digest. The mixed solution was

introduced into *Saccharomyces cerevisiae* to construct libraries or site-directed variants by *in vivo* recombination.

Relative activity assay

Yeast clones on SC-glucose were inoculated to a well of a 96-well micro titre plate containing YPD+Zn medium and cultivated at 28ºC for 3 days. The culture supernatants were applied to a 96-well zein micro titer plate and incubated at at least 2 temperatures (ex. 60ºC and 65ºC, 70ºC and 75ºC, 70ºC and 80ºC) for more than 4 hours or overnight. The turbidity of zein in the plate was measured as A630 and the relative activity (higher/lower temperatures) was determined as an indicator of thermoactivity improvement. The clones with higher relative activity than the parental variant were selected and the sequence was determined.

Remaining activity assay

Yeast clones on SC-glucose were inoculated to a well of a 96-well micro titre plate and cultivated at 28ºC for 3 days. Protease activity was measured at 65ºC using azo-casein (Megazyme) after incubating the culture supernatant in 20 mM sodium acetate buffer, pH 4.5, for 10 min at a certain temperature (80ºC or 84ºC with 4ºC as a reference) to determine the remaining activity. The clones with higher remaining activity than the parental variant were selected and the sequence was determined.

Azo-casein assay

20 microL of samples were mixed with 150 microL of substrate solution (4 ml of 12.5% azo-casein in ethanol in 96 ml of 20 mM sodium acetate, pH 4.5, containing 0.01% triton-100 and 0.25 mM $ZnSO_4$) and incubated for 4 hours or longer.

After adding 20 microL/well of 100 % trichloroacetic acid (TCA) solution, the plate was centrifuge and 100 microL of supernatants were pipette out to measure A440.

Expression of protease variants in *Aspergillus oryzae*

The constructs comprising the protease variant genes were used to construct expression vectors for *Aspergillus*. The *Aspergillus* expression vectors consist of an expression cassette based on the *Aspergillus niger* neutral amylase II promoter fused to the *Aspergillus nidulans* triose phosphate isomerase non translated leader sequence (Pna2/tpi) and the *Aspergillus niger* amyloglucosidase terminator (Tamg). Also present on the plasmid was the *Aspergillus selective* marker amdS from *Aspergillus nidulans* enabling growth on acetamide as sole nitrogen source. The expression plasmids for protease variants were transformed into *Aspergillus* as described in Lassen et al. (2001), *Appl. Environ. Microbiol.* 67, 4701-4707. For each of the constructs 10-20 strains were isolated, purified and cultivated in shake flasks.

Purification of expressed variants

1. Adjust pH of the 0.22 µm filtered fermentation sample to 4.0.

2. Put the sample on an ice bath with magnetic stirring. Add (NH4)2SO4 in small aliquots (corresponding to approx. 2.0-2.2 M (NH4)2SO4 not taking the volume increase into account when adding the compound).
3. After the final addition of (NH4)2SO4, incubate the sample on the ice bath with gentle magnetic stirring for min. 45 min.
4. Centrifugation: Hitachi himac CR20G High-Speed Refrigerated Centrifuge equipped with R20A2 rotor head, 5°C, 20,000 rpm, 30 min.
5. Dissolve the formed precipitate in 200 ml 50 mM Na-acetate pH 4.0.
6. Filter the sample by vacuum suction using a 0.22 µm PES PLUS membrane (IWAKI).
7. Desalt/buffer-exchange the sample to 50 mM Na-acetate pH 4.0 using ultrafiltration (Vivacell 250 from Vivascience equipped with 5 kDa MWCO PES membrane) overnight in a cold room. Dilute the retentate sample to 200 ml using 50 mM Na-acetate pH 4.0. The conductivity of sample is preferably less than 5 mS/cm.
8. Load the sample onto a cation-exchange column equilibrated with 50 mM Na-acetate pH 4.0. Wash unbound sample out of the column using 3 column volumes of binding buffer (50 mM Na-acetate pH 4.0), and elute the sample using a linear gradient, 0-100% elution buffer (50 mM Na-acetate + 1 M NaCl pH 4.0) in 10 column volumes.
9. The collected fractions are assayed by an endo-protease assay (cf. below) followed by standard SDS-PAGE (reducing conditions) on selected fractions. Fractions are pooled based on the endo-protease assay and SDS-PAGE.

Endo-protease assay

1. Protazyme OL tablet/5 ml 250 mM Na-acetate pH 5.0 is dissolved by magnetic stirring (substrate: endo-protease Protazyme AK tablet from Megazyme – cat. # PRAK 11/08).
2. With stirring, 250 microL of substrate solution is transferred to a 1.5 ml Eppendorf tube.
3. 25 microL of sample is added to each tube (blank is sample buffer).
4. The tubes are incubated on a Thermomixer with shaking (1000 rpm) at 50°C for 15 minutes.
5. 250 microL of 1 M NaOH is added to each tube, followed by vortexing.
6. Centrifugation for 3 min. at 16,100 × G and 25°C.
7. 200 microL of the supernatant is transferred to a MTP, and the absorbance at 590 nm is recorded.

Results

Table 2. Relative activity of protease variants. Numbering of substitution(s) starts from N-terminal of the mature peptide in amino acids 1 to 177 of SEQ ID NO: 3

Variant	Substitution(s)	Relative activity 65°C/60°C
WT	none	31%

JTP004	S87P	45%
JTP005	A112P	43%
JTP008	R2P	71%
JTP009	D79K	69%
JTP010	D79L	75%
JTP011	D79M	73%
JTP012	D79L/S87P	86%
JTP013	D79L/S87P/A112P	90%
JTP014	D79L/S87P/A112P	88%
JTP016	A73C	52%
JTP019	A126V	69%
JTP021	M152R	59%

Table 3. Relative activity of protease variants. Numbering of substitution(s) starts from N-terminal of the mature peptide in amino acids 1 to 177 of SEQ ID NO: 3

Variant	Substitution(s) and/or deletion (S)	Relative activity		
		70°C/65°C	75°C/65°C	75°C/70°C
WT	none	59%	17%	
JTP036	D79L/S87P/D142L	73%	73%	
JTP040	T54R/D79L/S87P		71%	
JTP042	Q53K/D79L/S87P/I173V		108%	
JTP043	Q53R/D79L/S87P		80%	
JTP045	S41R/D79L/S87P		82%	
JTP046	D79L/S87P/Q158W		96%	
JTP047	D79L/S87P/S157K		85%	
JTP048	D79L/S87P/D104R		88%	
JTP050	D79L/S87P/A112P/D142L		88%	
JTP051	S41R/D79L/S87P/A112P/D142L			102%
JTP052	D79L/S87P/A112P/D142L/S157K			111%
JTP053	S41R/D79L/S87P/A112P/D142L/S157K			113%
JTP054	ΔS5/D79L/S87P			92%
JTP055	ΔG8/D79L/S87P			95%
JTP059	C6R/D79L/S87P			92%
JTP061	T46R/D79L/S87P			111%
JTP063	S49R/D79L/S87P			94%
JTP064	D79L/S87P/N88R			92%
JTP068	D79L/S87P/T114P			99%
JTP069	D79L/S87P/S115R			103%
JTP071	D79L/S87P/T116V			105%
JTP072	N26R/D79L/S87P		92%	
JTP077	A27K/D79L/S87P/A112P/D142L		106%	
JTP078	A27V/D79L/S87P/A112P/D142L		100%	
JTP079	A27G/D79L/S87P/A112P/D142L		104%	

Table 4. Relative activity of protease variants. Numbering of substitution(s) starts from N-terminal of the mature peptide in amino acids 1 to 177 of SEQ ID NO: 3

Variant	Substitution(s) and/or deletion(s)	Relative activity 75°C/65°C	Remaining activity	
			80°C	84°C
JTP082	ΔS5/D79L/S87P/A112P/D142L	129%		53%
JTP083	T46R/D79L/S87P/A112P/D142L	126%		
JTP088	Y43F/D79L/S87P/A112P/D142L	119%		
JTP090	D79L/S87P/A112P/T124L/D142L	141%		
JTP091	D79L/S87P/A112P/T124V/D142L	154%	43%	
JTP092	ΔS5/N26R/D79L/S87P/A112P/D142L			60%
JTP095	N26R/T46R/D79L/S87P/A112P/D142L			62%
JTP096	T46R/D79L/S87P/T116V/D142L			67%
JTP099	D79L/P81R/S87P/A112P/D142L			80%
JTP101	A27K/D79L/S87P/A112P/T124V/D142L		81%	
JTP116	D79L/Y82F/S87P/A112P/T124V/D142L		59%	
JTP117	D79L/Y82F/S87P/A112P/T124V/D142L		94%	
JTP127	D79L/S87P/A112P/T124V/A126V/D142L		53%	

Table 5. Relative activity of protease variants. Numbering of substitution(s) starts from N-terminal of the mature peptide in amino acids 1 to 177 of SEQ ID NO: 3

Variant	Substitutions	Relative activity		
		75°C/70°C	80°C/70°C	85°C/70°C
JTP050	D79L S87P A112P D142L	55%	23%	9%
JTP134	D79L Y82F S87P A112P D142L		40%	
JTP135	S38T D79L S87P A112P A126V D142L		62%	
JTP136	D79L Y82F S87P A112P A126V D142L		59%	
JTP137	A27K D79L S87P A112P A126V D142L		54%	
JTP140	D79L S87P N98C A112P G135C D142L	81%		
JTP141	D79L S87P A112P D142L T141C M161C	68%		
JTP143	S36P D79L S87P A112P D142L	69%		
JTP144	A37P D79L S87P A112P D142L	57%		
JTP145	S49P D79L S87P A112P D142L	82%	59%	
JTP146	S50P D79L S87P A112P D142L	83%	63%	
JTP148	D79L S87P D104P A112P D142L	76%	64%	
JTP161	D79L Y82F S87G A112P D142L		30%	12%
JTP180	S70V D79L Y82F S87G Y97W A112P D142L		52%	
JTP181	D79L Y82F S87G Y97W D104P A112P D142L		45%	
JTP187	S70V D79L Y82F S87G A112P D142L		45%	

JTP188	D79L Y82F S87G D104P A112P D142L		43%	
JTP189	D79L Y82F S87G A112P A126V D142L		46%	
JTP193	Y82F S87G S70V D79L D104P A112P D142L			15%
JTP194	Y82F S87G D79L D104P A112P A126V D142L			22%
JTP196	A27K D79L Y82F S87G D104P A112P A126V D142L			18%

Table 6. Relative activity of protease variants. Numbering of substitution(s) starts from N-terminal of the mature peptide in amino acids 1 to 177 of SEQ ID NO: 3

		Relative activity	
Variant	**Substitutions**	**75°C/70°C**	**80°C/70°C**
JTP196	A27K D79L Y82F S87G D104P A112P A126V D142L	102%	55%
JTP210	A27K Y82F S87G D104P A112P A126V D142L	107%	36%
JTP211	A27K D79L Y82F D104P A112P A126V D142L	94%	44%
JTP213	A27K Y82F D104P A112P A126V D142L	103%	37%

Example 3: Temperature profile of selected variants using purified enzymes

Selected variants showing good thermo-stability were purified and the purified enzymes were used in a zein-BCA assay as described below. The remaining protease activity was determined at 60°C after incubation of the enzyme at elevated temperatures as indicated for 60 min.

Zein-BCA assay:

Zein-BCA assay was performed to detect soluble protein quantification released from zein by variant proteases at various temperatures.

Protocol:

1) Mix 10ul of 10 ug/ml enzyme solutions and 100ul of 0.025% zein solution in a micro titer plate (MTP).
2) Incubate at various temperatures for 60min.
3) Add 10ul of 100% trichloroacetic acid (TCA) solution.
4) Centrifuge MTP at 3500rpm for 5min.
5) Take out 15ul to a new MTP containing 100ul of BCA assay solution (Pierce Cat#:23225, BCA Protein Assay Kit).

6) Incubate for 30min. at 60°C.

7) Measure A562.

The results are shown in Table 7. All of the tested variants showed an improved thermo-stability as compared to the wt protease.

Table 7. Zein-BCA assay

WT/Variant	Sample incubated 60 min at indicated temperatures (°C) (µg/ml Bovine serum albumin equivalent peptide released)						
	60°C	70°C	75°C	80°C	85°C	90°C	95°C
WT	94	103	107	93	58	38	
JTP050	86	101	107	107	104	63	36
JTP077	82	94	104	105	99	56	31
JTP188	71	83	86	93	100	75	53
JTP196	87	99	103	106	117	90	38

Example 4: Characterization of *Penicillium oxalicum* glucoamylase

The *Penicillium oxalicum* glucoamylase is disclosed in SEQ ID NO: 9 herein.

Substrate. Substrate: 1% soluble starch (Sigma S-9765) in deionized water

Reaction buffer: 0.1 M Acetate buffer at pH 5.3

Glucose concentration determination kit: Wako glucose assay kit (LabAssay glucose, WAKO, Cat# 298-65701).

Reaction condition. 20 microL soluble starch and 50 microL acetate buffer at pH 5.3 were mixed. 30 microL enzyme solution (50 micro g enzyme protein/ml) was added to a final volume of 100 microL followed by incubation at 37°C for 15 min.

The glucose concentration was determined by Wako kits.

All the work carried out in parallel.

Temperature optimum. To assess the temperature optimum of the *Penicillium oxalicum* glucoamylase the "Reaction condition"-assay described above was performed at 20, 30, 40, 50, 60, 70, 80, 85, 90 and 95°C. The results are shown in Table 8.

Table 8. Temperature optimum

Temperature (°C)	20	30	40	50	60	70	80	85	90	95
Relative activity (%)	63.6	71.7	86.4	99.4	94.6	100.0	92.9	92.5	82.7	82.8

From the results it can be seen that the optimal temperature for *Penicillium oxalicum* glucoamylase at the given conditions is between 50°C and 70°C and the glucoamylase maintains more than 80% activity at 95°C.

Heat stability. To assess the heat stability of the *Penicillium oxalicum* glucoamylase the Reaction condition assay was modifed in that the the enzyme solution and acetate buffer was preincubated for 15 min at 20, 30, 40, 50, 60, 70, 75, 80, 85, 90 and 95°C. Following the incubation 20 microL of starch was added to the solution and the assay was performed as described above.

The results are shown in Table 9.

Table 9. Heat stability

Temperature (°C)	20	30	40	50	60	70	80	85	90	95
Relative activity (%)	91.0	92.9	88.1	100.0	96.9	86.0	34.8	36.0	34.2	34.8

From the results it can be seen that *Penicillium oxalicum* glucoamylase is stable up to 70 °C after preincubation for 15 min in that it maintains more than 80% activity.

pH optimum. To assess the pH optimum of the *Penicillium oxalicum* glucoamylase the Reaction condition assay described above was performed at pH 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0 7.0, 8.0, 9.0, 10.0 and 11.0. Instead of using the acetate buffer described in the Reaction condition assay the following buffer was used 100mM Succinic acid, HEPES, CHES, CAPSO, 1mM $CaCl_2$, 150mM KCl, 0.01% Triton X-100, pH adjusted to 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0 7.0, 8.0, 9.0, 10.0 or 11.0 with HCl or NaOH.

The results are shown in Table 10.

Table 10. pH optimum

pH	2.0	3.0	3.5	4.0	4.5	5.0	6.0	7.0	8.0	9.0	10.0	11.0
Relative activity (%)	71.4	78.6	77.0	91.2	84.2	100.0	55.5	66.7	30.9	17.8	15.9	16.1

From the results it can be seen that *Penicillium oxalicum* glucoamylase at the given conditions has the highest activity at pH 5.0. The *Penicillium oxalicum* glucoamylase is active in a broad pH range in the it maintains more than 50% activity from pH 2 to 7.

pH stability. To assess the heat stability of the *Penicillium oxalicum* glucoamylase the Reaction condition assay was modifed in that the enzyme solution (50micro g/mL) was preincubated for 20 hours in buffers with pH 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0 7.0, 8.0, 9.0, 10.0 and 11.0 using the buffers described under pH optimum. After preincubation, 20 microL

soluble starch to a final volume of 100 microL was added to the solution and the assay was performed as described above.

The results are shown in Table 11.

Table 11. pH stability

pH	2.0	3.0	3.5	4.0	4.5	5.0	6.0	7.0	8.0	9.0	10.0	11.0
Relative activity (%)	17.4	98.0	98.0	103.2	100.0	93.4	71.2	90.7	58.7	17.4	17.0	17.2

From the results it can be seen that *Penicillium oxalicum* glucoamylase, is stable from pH 3 to pH 7 after preincubation for 20 hours and it decreases its activity at pH 8.

Example 5: Thermostability of Protease Pfu

The thermostability of the *Pyrococcus furiosus* protease (Pfu S) purchased from Takara Bio Inc, (Japan) was tested using the same methods as in Example 2. It was found that the thermostability (Relative Activity) was 110% at (80°C/70°C) and 103% (90°C/70°C) at pH 4.5.

Example 6: Cloning of *Penicillium oxalicum* strain glucoamylase gene

Preparation of *Penicillium oxalicum* strain cDNA.

The cDNA was synthesized by following the instruction of 3' Rapid Amplifiction of cDNA End System (Invitrogen Corp., Carlsbad, CA, USA).

Cloning of *Penicillium oxalicum* strain glucoamylase gene.

The *Penicillium oxalicum* glucoamylase gene was cloned using the oligonucleotide primer shown below designed to amplify the glucoamylase gene from 5' end.

Sense primer: 5'- ATGCGTCTCACTCTATTATCAGGTG-3' (SEQ ID NO: 22)

The full length gene was amplified by PCR with Sense primer and AUAP (supplied by 3' Rapid Amplifiction of cDNA End System) by using Platinum HIFI Taq DNA polymerase (Invitrogen Corp., Carlsbad, CA, USA). The amplification reaction was composed of 5 µl of 10x PCR buffer, 2 µl of 25mM $MgCl_2$, 1 µl of 10mM dNTP, 1 µl of 10uM Sense primer, 1 µl of 10 uM AUAP, 2 µl of the first strand cDNA, 0.5 µl of HIFI Taq, and 37.5 µl of deionized water. The PCR program was: 94°C, 3mins; 10 cycles of 94°C for 40secs, 60°C 40secs with 1°C decrease per cycle, 68°C for 2min; 25 cycles of 94°C for 40secs, 50°C for 40secs, 68°C for 2min; final extension at 68°C for 10 mins.

The obtained PCR fragment was cloned into pGEM-T vector (Promega Corporation, Madison, WI, USA) using a pGEM-T Vector System (Promega Corporation, Madison, WI,

USA) to generate plasmid AMG 1. The glucoamylase gene inserted in the plasmid AMG 1 was sequencing confirmed. *E. coli* strain TOP10 containing plasmid AMG 1 (designated NN059173), was deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) on November 23, 2009, and assigned accession number as DSM 23123.

Example 7: Expression of cloned *Penicillium oxalicum* glucoamylase

The *Penicillium oxalicum* glucoamylase gene was re-cloned from the plasmid AMG 1 into an *Aspergillus* expression vector by PCR using two cloning primer F and primer R shown below, which were designed based on the known sequence and added tags for direct cloning by IN-FUSION™ strategy.

Primer F: 5' ACACAACTGGGGATCCACCATGCGTCTCACTCTATTATC (SEQ ID NO: 23)

Primer R: 5' AGATCTCGAGAAGCTTAAAACTGCCACACGTCGTTGG (SEQ ID NO: 24)

A PCR reaction was performed with plasmid AMG 1 in order to amplify the full-length gene. The PCR reaction was composed of 40 µg of the plasmid AMG 1 DNA, 1 µl of each primer (100 µM); 12.5 µl of 2X Extensor Hi-Fidelity master mix (Extensor Hi-Fidelity Master Mix, ABgene, United Kingdom), and 9.5 µl of PCR-grade water. The PCR reaction was performed using a DYAD PCR machine (Bio-Rad Laboratories, Inc., Hercules, CA, USA) programmed for 2 minutes at 94°C followed by a 25 cycles of 94°C for 15 seconds, 50°C for 30 seconds, and 72°C for 1 minute; and then 10 minutes at 72°C.

The reaction products were isolated by 1.0% agarose gel electrophoresis using 1 x TAE buffer where an approximately 1.9 kb PCR product band was excised from the gel and purified using a GFX® PCR DNA and Gel Band Purification Kit (GE Healthcare, United Kingdom) according to manufacturer's instructions. DNA corresponding to the *Penicillium oxalicum* glucoamylase gene was cloned into an *Aspergillus* expression vector linearized with *Bam*HI and *Hind*III, using an IN-FUSION™ Dry-Down PCR Cloning Kit (BD Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions. The linearized vector construction is as described in WO 2005/042735 A1.

A 2 µl volume of the ligation mixture was used to transform 25 µl of Fusion Blue *E. coli* cells (included in the IN-FUSION™ Dry-Down PCR Cloning Kit). After a heat shock at 42°C for 45 sec, and chilling on ice, 250 µl of SOC medium was added, and the cells were incubated at 37°C at 225 rpm for 90 min before being plated out on LB agar plates containing 50 µg of ampicillin per ml, and cultivated overnight at 37°C. Selected colonies were inoculated in 3 ml of LB medium supplemented with 50 µg of ampicillin per ml and incubated at 37°C at 225 rpm overnight. Plasmid DNA from the selected colonies was purified using Mini JETSTAR (Genomed, Germany) according to the manufacturer's instructions. *Penicillium oxalicum* glucoamylase gene sequence was verified by Sanger sequencing before heterologous

expression. One of the plasmids was selected for further expression, and was named XYZ XYZ1471-4.

Protoplasts of *Aspergillus niger* MBin118 were prepared as described in WO 95/02043. One hundred µl of protoplast suspension were mixed with 2.5 µg of the XYZ1471-4 plasmid and 250 microliters of 60% PEG 4000 (Applichem) (polyethylene glycol, molecular weight 4,000), 10 mM $CaCl_2$, and 10 mM Tris-HCl pH 7.5 were added and gently mixed. The mixture was incubated at 37°C for 30 minutes and the protoplasts were mixed with 6% low melting agarose (Biowhittaker Molecular Applications) in COVE sucrose (Cove, 1996, Biochim. Biophys. Acta 133:51-56) (1M) plates supplemented with 10 mM acetamide and 15 mM CsCl and added as a top layer on COVE sucrose (1M) plates supplemented with 10 mM acetamide and 15 mM CsCl for transformants selection (4 ml topagar per plate). After incubation for 5 days at 37°C spores of sixteen transformants were picked up and seed on 750 µl YP-2% Maltose medium in 96 deepwell MT plates. After 5 days of stationary cultivation at 30°C, 10 µl of the culture-broth from each well was analyzed on a SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gel, Griton XT Precast gel (BioRad, CA, USA) in order to identify the best transformants based on the ability to produce large amount of glucoamylase. A selected transformant was identified on the original transformation plate and was preserved as spores in a 20% glycerol stock and stored frozen (-80°C).

Cultivation. The selected transformant was inoculated in 100ml of MLC media and cultivated at 30 °C for 2 days in 500 ml shake flasks on a rotary shaker. 3 ml of the culture broth was inoculated to 100ml of M410 medium and cultivated at 30°C for 3 days. The culture broth was centrifugated and the supernatant was filtrated using 0.2 µm membrane filters.

Alpha-cyclodextrin affinity gel. Ten grams of Epoxy-activated Sepharose 6B (GE Healthcare, Chalfont St. Giles, U.K) powder was suspended in and washed with distilled water on a sintered glass filter. The gel was suspended in coupling solution (100 ml of 12.5 mg/ml alpha-cyclodextrin, 0.5 M NaOH) and incubated at room temperature for one day with gentle shaking. The gel was washed with distilled water on a sintered glass filter, suspended in 100 ml of 1 M ethanolamine, pH 10, and incubated at 50 °C for 4 hours for blocking. The gel was then washed several times using 50 mM Tris-HCl, pH 8 and 50 mM NaOAc, pH 4.0 alternatively. The gel was finally packed in a 35-40 ml column using equilibration buffer (50 mM NaOAc, 150 mM NaCl, pH 4.5).

Purification of glucoamylase from culture broth. Culture broth from fermentation of *A. niger* MBin118 harboring the glucoamylase gene was filtrated through a 0.22 µm PES filter, and applied on a alpha-cyclodextrin affinity gel column previously equilibrated in 50 mM NaOAc, 150 mM NaCl, pH 4.5 buffer. Unbound material was washed off the column with equilibration buffer and the glucoamylase was eluted using the same buffer containing 10 mM beta-cyclodextrin over 3 column volumes.

The glucoamylase activity of the eluent was checked to see, if the glucoamylase had bound to the alpha-cyclodextrin affinity gel. The purified glucoamylase sample was then dialyzed against 20 mM NaOAc, pH 5.0. The purity was finally checked by SDS-PAGE, and only a single band was found.

Example 8: Construction and expression of a site-directed variant of *Penicillium oxalicum* glucoamylase

Two PCR reactions were performed with plasmid XYZ1471-4, described in Example 7, using primers K79V F and K79VR shown below, which were designed to substitute lysine K at position 79 from the mature sequence to valine (V) and primers F-NP003940 and R-NP003940 shown below, which were designed based on the known sequence and added tags for direct cloning by IN-FUSION™ strategy.

Primer K79V F 18mer GCAGTCTTTCCAATTGAC (SEQ ID NO: 25)

Primer K79V R 18mer AATTGGAAAGACTGCCCG (SEQ ID NO: 26)

Primer F-NP003940: 5' ACACAACTGGGGATCCACCATGCGTCTCACTCTATTATC (SEQ ID NO: 27)

Primer R-NP003940: 5' AGATCTCGAGAAGCTTAAAACTGCCACACGTCGTTGG (SEQ ID NO: 28)

The PCR was performed using a PTC-200 DNA Engine under the conditions described below.

PCR reaction system:	Conditions:		
48.5 micro L H2O	1	94°C	2 min
2 beads puRe Taq Ready-To-Go PCR Beads (Amersham Biosciences)	2	94°C	30 sec
	3	55°C	30 sec
0.5micro L X 2 100 pmole/micro L Primers (K79V F + Primer R-NP003940, K79V R + Primer F-NP003940)	4	72°C	90 sec
	2-4	25 cycles	
	5	72°C	10 min
0.5 micro L Template DNA			

DNA fragments were recovered from agarose gel by the Qiagen gel extraction Kit according to the manufacturer's instruction. The resulting purified two fragments were cloned into an *Aspergillus* expression vector linearized with *Bam*HI and *Hind*III, using an IN-FUSION™ Dry-Down PCR Cloning Kit (BD Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions. The linearized vector construction is as described in WO 2005/042735 A1.

The ligation mixture was used to transform *E. coli* DH5α cells (TOYOBO). Selected colonies were inoculated in 3 ml of LB medium supplemented with 50 µg of ampicillin per ml and incubated at 37°C at 225 rpm overnight. Plasmid DNA from the selected colonies was purified using Qiagen plasmid mini kit (Qiagen) according to the manufacturer's instructions. The sequence of *Penicillium oxalicum* glucoamylase site-directed variant gene sequence was verified before heterologous expression and one of the plasmids was selected for further expression, and was named pPoPE001.

Protoplasts of *Aspergillus niger* MBin118 were prepared as described in WO 95/02043. One hundred µl of protoplast suspension were mixed with 2.5 µg of the pPoPE001 plasmid and 250 microliters of 60% PEG 4000 (Applichem) (polyethylene glycol, molecular weight 4,000), 10 mM $CaCl_2$, and 10 mM Tris-HCl pH 7.5 were added and gently mixed. The mixture was incubated at 37°C for 30 minutes and the protoplasts were mixed with 1% agarose L (Nippon Gene) in COVE sucrose (Cove, 1996, Biochim. Biophys. Acta 133:51-56) supplemented with 10 mM acetamide and 15 mM CsCl and added as a top layer on COVE sucrose plates supplemented with 10 mM acetamide and 15 mM CsCl for transformants selection (4 ml topagar per plate). After incubation for 5 days at 37°C spores of sixteen transformants were picked up and seed on 750 µl YP-2% Maltose medium in 96 deepwell MT plates. After 5 days of stationary cultivation at 30°C, 10 µl of the culture-broth from each well was analyzed on a SDS-PAGE gel in order to identify the best transformants based on the ability to produce large amount of the glucoamylase.

Example 9: Purification of site-directed Po AMG variant PE001

The selected transformant of the variant and the strain expressing the wild type *Penicillium oxalicum* glucoamylase described in Example 6 was cultivated in 100 ml of YP-2% maltose medium and the culture was filtrated through a 0.22 µm PES filter, and applied on a alpha-cyclodextrin affinity gel column previously equilibrated in 50 mM NaOAc, 150 mM NaCl, pH 4.5 buffer. Unbound materials was washed off the column with equilibration buffer and the glucoamylase was eluted using the same buffer containing 10 mM beta-cyclodextrin over 3 column volumes.

The glucoamylase activity of the eluent was checked to see, if the glucoamylase had bound to the alpha-cyclodextrin affinity gel. The purified glucoamylase samples were then dialyzed against 20 mM NaOAc, pH 5.0.

Example 10: Characterization of PE001 Protease stability

40 µl enzyme solutions (1 mg/ml) in 50 mM sodium acetate buffer, pH 4.5, were mixed with 1/10 volume of 1mg/ml protease solutions such as aspergillopepsin I described in Biochem J. 1975 Apr; 147(1):45-53, or the commercially available product from Sigma and

aorsin described in Biochemical journal [0264-6021] Ichishima yr: 2003 vol:371 iss:Pt 2 pg:541 and incubated at 4 or 32°C overnight. As a control experiment, H_2O was added to the sample instead of proteases. The samples were loaded on SDS-PAGE to see if the glucoamylases are cleaved by proteases.

In SDS-PAGE, PE001 only showed one band corresponding to the intact molecule, while the wild type glucoamylase was degraded by proteases and showed a band at lower molecular size at 60 kCa.

Table 12. The result of SDS-PAGE after protease treatment

	Wild type glucoamylase				PE001				control
Protease	aspergillopepsin I		aorsin		aspergillopepsin I		aorsin		
Incubation temperature (°C)	4	32	4	32	4	32	4	32	4
intact glucoamylase (ca. 70 kDa)	100%	90%	40%	10%	100%	100%	100 %	100 %	100%
cleaved glucoamylase (ca. 60 kDa)	N.D.	10%	60%	90%	N.D.	N.D.	N.D.	N.D.	N.D.

N.D.: not detected.

Example 11: Less cleavage during cultivation

Aspergillus transformant of the variant and the wild type *Penicillium oxalicum* glucoamylase were cultivated in 6-well MT plates containing 4X diluted YP-2% maltose medium supplemented with 10 mM sodium acetate buffer, pH4.5, at 32°C for 1 week.

The culture supernatants were loaded on SDS-PAGE.

Table 13. The result of SDS-PAGE of the culture supernatants

	Wild type glucoamylase	PE001
intact glucoamylase(ca. 70 kDa)	90%	100%
cleaved glucoamylase (ca. 60 kDa)	10%	N.D.

N.D.: not detected.

The wild type glucoamylase was cleaved by host proteases during fermentation, while the variant yielded only intact molecule.

Example 12: Glucoamylase activity of variant compared to parent

The glucoamylase activity measures as AGU as described above was checked for the purified enzymes of the wild type *Penicillium oxalicum* and the variant glucoamylase.

The Glucoamylase Unit (AGU) was defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute under the standard conditions (37°C, pH 4.3, substrate: maltose 100 mM, buffer: acetate 0.1 M, reaction time 6 minutes).

Table 14.

Relative specific activity	AGU/mg
Penicillium oxalicum wt	100 %
Penicillium oxalicum PE001 (SEQ ID NO: 14 + K79V substitution)	102 %

Example 13: Purification of Glucoamylase variants having increased thermostability

The variants showing increased thermostability may be constructed and expressed similar to the procedure described in Example 8. All variants were derived from the PE001. After expression in YPM medium, variants comprising the T65A or Q327F substitution was micro-purified as follows:

Mycelium was removed by filtration through a 0.22 µm filter. 50 µl column material (alpha-cyclodextrin coupled to Mini-Leak divinylsulfone-activated agarose medium according to manufacturer's recommendations) was added to the wells of a filter plate (Whatman, Unifilter 800 µl, 25-30 µm MBPP). The column material was equilibrated with binding buffer (200 mM sodium acetate pH 4.5) by two times addition of 200 µl buffer, vigorous shaking for 10 min (Heidolph, Titramax 101, 1000 rpm) and removal of buffer by vacuum (Whatman, UniVac 3). Subsequently, 400 µl culture supernatant and 100 µl binding buffer was added and the plate incubated 30 min with vigorous shaking. Unbound material was removed by vacuum and the binding step was repeated. Normally 4 wells were used per variant. Three washing steps were then performed with 200 µl buffer of decreasing ionic strength added (50/10/5 mM sodium acetate, pH 4.5), shaking for 15 min and removal of buffer by vacuum. Elution of the bound AMG was achieved by two times addition of 100 µl elution buffer (250 mM sodium acetate, 0.1% alpha-cyclodextrin, pH 6.0), shaking for 15 min and collection of eluted material in a microtiter plate by vacuum. Pooled eluates were concentrated and buffer changed to 50 mM sodium acetate pH 4.5 using centrifugal filter units with 10 kDa cut-off (Millipore Microcon Ultracel YM-10). Micropurified samples were stored at -18°C until testing of thermostability.

Example 14: Protein thermal unfolding analysis (TSA, Thermal shift assay)

Protein thermal unfolding of the T65A and Q327F variants, was monitored using Sypro Orange (In-vitrogen, S-6650) and was performed using a real-time PCR instrument (Applied Biosystems; Step-One-Plus).

In a 96-well plate, 25 microliter micropurified sample in 50 mM Acetate pH4,5 at approx. 100 microgram/ml was mixed (5:1) with Sypro Orange (resulting conc. = 5X; stock solution from supplier = 5000X). The plate was sealed with an optical PCR seal. The PCR instrument was set at a scan-rate of 76°C pr. hr, starting at 25°C and finishing at 96°C.

Protein thermal unfolding of the E501V + Y504T variant, was monitored using Sypro Orange (In-vitrogen, S-6650) and was performed using a real-time PCR instrument (Applied Biosystems; Step-One-Plus).

In a 96-well plate, 15 microliter purified sample in 50 mM Acetate pH4,5 at approx. 50 microgram/ml was mixed (1:1) with Sypro Orange (resulting conc. = 5X; stock solution from supplier = 5000X) with or without 200 ppm Acarbose (Sigma A8980). The plate was sealed with an optical PCR seal. The PCR instrument was set at a scan-rate of 76 degrees C pr. hr, starting at 25°C and finishing at 96°C.

Fluorescence was monitored every 20 seconds using in-built LED blue light for excitation and ROX-filter (610 nm, emission).

Tm-values were calculated as the maximum value of the first derivative (dF/dK) (ref.: Gregory et al; *J Biomol Screen* 2009 14: 700.)

Table 15a.

Sample	Tm (Deg. Celsius) +/-0.4
PO-AMG (PE001)	80.3
Variant Q327F	82.3
Variant T65A	81.9

Table 15b.

Sample	Tm (Deg. Celsius) +/-0.4	
Acarbose:	-	+
PO-AMG (PE001)	79.5	86.9
Variant E501V Y504T	79.5	95.2

Example 15: Thermostability analysis by Differential Scanning Calorimetry (DSC)

Additional site specific variants having substitutions and /or deletions at specific positions were constructed basically as described in Example 8 and purified as described in Example 11.

The thermostability of the purified Po-AMG PE001 derived variants were determined at pH 4.0 or 4.8 (50 mM Sodium Acetate) by Differential Scanning Calorimetry (DSC) using a VP-Capillary Differential Scanning Calorimeter (MicroCal Inc., Piscataway, NJ, USA). The thermal denaturation temperature, Td (°C), was taken as the top of the denaturation peak (major endothermic peak) in thermograms (Cp vs. T) obtained after heating enzyme solutions

in selected buffers (50 mM Sodium Acetate, pH 4.0 or 4.8)at a constant programmed heating rate of 200 K/hr.

Sample- and reference-solutions (approximately 0.3 ml) were loaded into the calorimeter (reference: buffer without enzyme) from storage conditions at 10ºC and thermally pre-equilibrated for 10 minutes at 20°C prior to DSC scan from 20°C to 110°C. Denaturation temperatures were determined with an accuracy of approximately +/- 1°C.

The isolated variants and the DSC data are disclosed in Table 16 below.

Table 16.

Po-AMG name	**Mutations**	**DSC Td (°C) @ pH 4.0**	**DSC Td (°C) @ pH 4.8**
PE001 (SEQ ID NO: 14 +K79V)		82.1	83.4
GA167	E501V Y504T	82.1	
GA481	T65A K161S	84.1	86.0
GA487	T65A Q405T	83.2	
GA490	T65A Q327W	87.3	
GA491	T65A Q327F	87.7	
GA492	T65A Q327Y	87.3	
GA493	P11F T65A Q327F	87.8	88.5
GA497	R1K D3W K5Q G7V N8S T10K P11S T65A Q327F	87.8	88.0
GA498	P2N P4S P11F T65A Q327F	88.3	88.4
GA003	P11F D26C K33C T65A Q327F	83.3	84.0
GA009	P2N P4S P11F T65A Q327W E501V Y504T	88.8	
GA002	R1E D3N P4G G6R G7A N8A T10D P11D T65A Q327F	87.5	88.2
GA005	P11F T65A Q327W	87.4	88.0
GA008	P2N P4S P11F T65A Q327F E501V Y504T	89.4	90.2
GA010	P11F T65A Q327W E501V Y504T		89.7
GA507	T65A Q327F E501V Y504T		89.3
GA513	T65A S105P Q327W		87.0
GA514	T65A S105P Q327F		87.4
GA515	T65A Q327W S364P		87.8
GA516	T65A Q327F S364P		88.0
GA517	T65A S103N Q327F		88.9
GA022	P2N P4S P11F K34Y T65A Q327F		89.7
GA023	P2N P4S P11F T65A Q327F D445N V447S		89.9
GA032	P2N P4S P11F T65A I172V Q327F		88.7
GA049	P2N P4S P11F T65A Q327F N502*		88.4
GA055	P2N P4S P11F T65A Q327F N502T P563S K571E		88.0

GA057	P2N P4S P11F R31S K33V T65A Q327F N564D K571S		89.5
GA058	P2N P4S P11F T65A Q327F S377T		88.6
GA064	P2N P4S P11F T65A V325T Q327W		88.0
GA068	P2N P4S P11F T65A Q327F D445N V447S E501V Y504T		90.2
GA069	P2N P4S P11F T65A I172V Q327F E501V Y504T		90.2
GA073	P2N P4S P11F T65A Q327F S377T E501V Y504T		90.1
GA074	P2N P4S P11F D26N K34Y T65A Q327F		89.1
GA076	P2N P4S P11F T65A Q327F I375A E501V Y504T		90.2
GA079	P2N P4S P11F T65A K218A K221D Q327F E501V Y504T		90.9
GA085	P2N P4S P11F T65A S103N Q327F E501V Y504T		91.3
GA086	P2N P4S T10D T65A Q327F E501V Y504T		90.4
GA088	P2N P4S F12Y T65A Q327F E501V Y504T		90.4
GA097	K5A P11F T65A Q327F E501V Y504T		90.0
GA101	P2N P4S T10E E18N T65A Q327F E501V Y504T		89.9
GA102	P2N T10E E18N T65A Q327F E501V Y504T		89.8
GA084	P2N P4S P11F T65A Q327F E501V Y504T T568N		90.5
GA108	P2N P4S P11F T65A Q327F E501V Y504T K524T G526A		88.6
GA126	P2N P4S P11F K34Y T65A Q327F D445N V447S E501V Y504T		91.8
GA129	P2N P4S P11F R31S K33V T65A Q327F D445N V447S E501V Y504T		91.7
GA087	P2N P4S P11F D26N K34Y T65A Q327F E501V Y504T		89.8
GA091	P2N P4S P11F T65A F80* Q327F E501V Y504T		89.9
GA100	P2N P4S P11F T65A K112S Q327F E501V Y504T		89.8
GA107	P2N P4S P11F T65A Q327F E501V Y504T T516P K524T G526A		90.3
GA110	P2N P4S P11F T65A Q327F E501V N502T Y504*		90.6

Example 16: Thermostability analysis by thermo-stress test and pNPG assay

Starting from one of the identified substitution variants from Example 15, identified as GA008, additional variants were tested by a thermo-stress assay in which the supernatant

from growth cultures were assayed for glucoamylase (AMG) activity after a heat shock at 83°C for 5 min.

After the heat-shock the residual activity of the variant was measured as well as in a non-stressed sample.

Description of Po-AMG pNPG activity assay:

The *Penicillium oxalicum* glucoamylase pNPG activity assay is a spectrometric endpoint assay where the samples are split in two and measured thermo-stressed and non-thermo-stressed. The data output is therefore a measurement of residual activity in the stressed samples.

Growth:

A sterile micro titer plate (MTP) was added 200µL rich growth media (FT X-14 without Dowfax) to each well. The strains of interest were inoculated in triplicates directly from frozen stocks to the MTP. Benchmark was inoculated in 20 wells. Non-inoculated wells with media were used as assay blanks. The MTP was placed in a plastic box containing wet tissue to prevent evaporation from the wells during incubation. The plastic box was placed at 34°C for 4 days.

Assay:

50 µL supernatant was transferred to 50 µL 0.5 M NaAc pH 4.8 to obtain correct sample pH.

50 µL dilution was transferred to a PCR plate and thermo-stressed at 83°C for 5 minutes in a PCR machine. The remaining half of the dilution was kept at RT.

20 µL of both stressed and unstressed samples was transferred to a standard MTP. 20µL pNPG-substrate was added to start the reaction. The plate was incubated at RT for 1 hour.

The reaction was stopped and the colour developed by adding 50 µL 0.5M Na_2CO_3. The yellow colour was measured on a plate reader (Molecular Devices) at 405 nm.

Buffers:

0.5 M NaAc pH 4.8

0.25 M NaAc pH 4.8

Substrate, 6mM pNPG:

15mg 4-nitrophenyl D-glucopyranoside in 10 mL 0.25 NaAc pH 4.8

Stop/developing solution:

0.5 M Na_2CO_3

Data treatment:

In Excel the raw Abs405 data from both stressed and unstressed samples were blank subtracted with their respective blanks. The residual activity (% res. act. = ($Abs_{unstressed}$ –

($Abs_{unstressed}$ – $Abs_{stressed}$))/$Abs_{unstressed}$ *100%) was calculated and plotted relative to benchmark, Po-amg0008.

Table 17.

Po-AMG name	Mutations	% residual activity
GA008	P2N P4S P11F T65A Q327F E501V Y504T	100
GA085	P2N P4S P11F T65A S103N Q327F E501V Y504T	127
GA097	K5A P11F T65A Q327F E501V Y504T	106
GA107	P2N P4S P11F T65A Q327F E501V Y504T T516P K524T G526A	109
GA130	P2N P4S P11F T65A V79A Q327F E501V Y504T	111
GA131	P2N P4S P11F T65A V79G Q327F E501V Y504T	112
GA132	P2N P4S P11F T65A V79I Q327F E501V Y504T	101
GA133	P2N P4S P11F T65A V79L Q327F E501V Y504T	102
GA134	P2N P4S P11F T65A V79S Q327F E501V Y504T	104
GA150	P2N P4S P11F T65A L72V Q327F E501V Y504T	101
GA155	S255N Q327F E501V Y504T	105

Table 18.

Po-AMG name	Mutations	% residual activity
GA008	P2N P4S P11F T65A Q327F E501V Y504T	100
GA179	P2N P4S P11F T65A E74N V79K Q327F E501V Y504T	108
GA180	P2N P4S P11F T65A G220N Q327F E501V Y504T	108
GA181	P2N P4S P11F T65A Y245N Q327F E501V Y504T	102
GA184	P2N P4S P11F T65A Q253N Q327F E501V Y504T	110
GA185	P2N P4S P11F T65A D279N Q327F E501V Y504T	108
GA186	P2N P4S P11F T65A Q327F S359N E501V Y504T	108
GA187	P2N P4S P11F T65A Q327F D370N E501V Y504T	102
GA192	P2N P4S P11F T65A Q327F V460S E501V Y504T	102
GA193	P2N P4S P11F T65A Q327F V460T P468T E501V Y504T	102
GA195	P2N P4S P11F T65A Q327F T463N E501V Y504T	103

GA196	P2N P4S P11F T65A Q327F S465N E501V Y504T	106
GA198	P2N P4S P11F T65A Q327F T477N E501V Y504T	106

Example 17: Test for Glucoamylase activity of thermo-stable variants

All of the above described variants disclosed in tables 15, 16, and 17 have been verified for Glucoamylase activity on culture supernatants using the pNPG assay described in Example 16.

Example 18: Production of strain *Saccharomyces cerevisiae* strain MBG4985

Saccharomyces strains capable of utilising xylose as a sole carbon source were produced using the method described in WO2005/121337. Strains were subsequently screened for those having a low ratio of %w/v glycerol:acetate production in corn mash. Low glycerol:acetate strains were then crossed in mating with other strains having low %w/v glycerol: acetate production in corn mash, followed by screening for strains having the greatest ethanol yield. One strain identified was *Saccharomyces cerevisiae* MBG4985 (NRRL Y67342).

Example 19: Construction of yeast strains expressing alpha-amylase (AA), glucoamylase (AMG), or an alpha-amylase (AA) + a glucoamylase (AMG)

Construction of homozygous strains via two-step transformation

Expression cassettes for the desired genes were targeted to the XI-1 or XII-5 integration sites as described in Mikkelsen et al. (Metabolic Engineering v14 (2012) pp104-111). Two plasmids employing a split-marker approach were used for each integration event, each containing an expression cassette and approximately two-thirds of a dominant selection marker. The left-hand plasmid contained 5' flanking DNA homologous to the desired integration site, the *S. cerevisiae* TEF2 promoter driving expression of the gene of interest codon-optimized for expression in *S. cerevisiae*, the *S. cerevisiae* ADH3 terminator, a loxP site, and the 5' two-thirds of a dominant selection marker under control of the *Ashbya gossypii* TEF1 promoter. The right-hand plasmid contains the 3' two-thirds of the dominant selection marker with the *Ashbya gossypii* TEF1 terminator, a loxP site, an expression cassette in the reverse orientation relative to the dominant selection marker composed of the *S. cerevisiae* HXT7 promoter driving expression of the gene of interest codon-optimized for expression in *S. cerevisiae* with the *S. cerevisiae* PMA1 terminator, and 3' flanking DNA homologous to the desired integration site. A left-hand and right-hand plasmid pair was linearized with restriction enzymes and transformed into *S. cerevisiae* strain MBG4931 using lithium acetate transformation (see Gietz and Woods, 2006, Methods in Molecular Biology, v 313 pp107-120).

Since MBG4931 is a diploid yeast, the desired integration construct was first integrated using kanamycin resistance as the dominant selection marker, followed by PCR screening to confirm the desired integration event. A confirmed heterozygous transformant was then transformed again using an expression cassette pair with the nourseothricin resistance marker. PCR screening was used to confirm homozygous modification of the targeted chromosomal integration site. A diagram of the expression cassette at one chromosome of the XII-5 integration site is shown in Figure 1. Resulting strains yMHCT390, yMHCT392, yMHCT394, yMHCT396 and MEJI697 are shown in Table 19 below with corresponding expressed glucoamylase and/or alpha-amylase of interest and integration locus.

Expression cassettes for the desired genes were targeted to the XI-1 (PE096) or XII-5 (PsAMG integration sites as described in Mikkelsen et al. (Metabolic Engineering v14 (2012) pp104-111). Two plasmids employing a split-marker approach were used for each integration event, each containing an expression cassette and approximately two-thirds of a dominant selection marker. The left-hand plasmid contained 5' flanking DNA homologous to the desired integration site, the *S. cerevisiae* TEF2 promoter driving expression of the gene of interest codon-optimized for expression in *S. cerevisiae*, the *S. cerevisiae* ADH3 terminator, a loxP site, and the 5' two-thirds of a dominant selection marker under control of the *Ashbya gossypii* TEF1 promoter. The right-hand plasmid contains the 3' two-thirds of the dominant selection marker with the *Ashbya gossypii* TEF1 terminator, a loxP site, an expression cassette in the reverse orientation relative to the dominant selection marker composed of the *S. cerevisiae* HXT7 promoter driving expression of the gene of interest codon-optimized for expression in *S. cerevisiae* with the *S. cerevisiae* PMA1 terminator, and 3' flanking DNA homologous to the desired integration site. A left-hand and right-hand plasmid pair containing the AA variant PE096 expression cassettes targeting to XI-1 was linearized with restriction enzymes and transformed into *S. cerevisiae* strain MBG4911 using lithium acetate transformation (see Gietz and Woods, 2006, Methods in Molecular Biology, v 313 pp107-120). Since MBG4911 is a diploid yeast, the desired integration construct was first integrated using kanamycin resistance as the dominant selection marker, followed by PCR screening to confirm the desired integration event. A confirmed heterozygous transformant was then transformed again using an expression cassette pair with the nourseothricin resistance marker. PCR screening was used to confirm homozygous modification of the XI-1 integration site creating strain MIBa787.

The antibiotic markers present in MIBa787 are flanked by loxP sites. MIBa787 was transformed with plasmid pFYD80 that includes a gene encoding the CRE recombinase, a site specific enzyme that facilitates recombination between neighboring loxP sites (Güldener et al., 2002). Plasmid pFYD80 is maintained as a non-integrative, free replicating molecule. This approach enables the specific excision of both selective markers. MIBa787 was transformed with plasmid pFYD80, and transformants were selected on plates containing zeocin. Zeocin

resistance is encoded in pFYD80. Subsequently, screening for transformants that have lost nourseothricin and kanamycin resistance was performed. Sensitive strains were grown in YPD liquid until loss of pFYD80 plasmid was obtained. Strain MIBa795 was selected and shown to be zeocin sensitive as a result of the loss of plasmid pFYD80.

The *S. cerevisiae* strain MIBa795 expresses the AA variant PE096 at the XI-1 integration site. MIBa795 was modified to express the Ps glucoamylase at the XII-5 integration site. The Ps glucoamylase expression cassettes were introduced into MIBa795 as described above for the AA variant PE096. This resulted in strain MIBa821 which contains Ps glucoamylase expression cassettes at XII-5 and the AA variant PE096 expression cassettes at XI-1. MIBa821 still contains the antibiotic selection markers at the XII-5 integration site and therefore is resistant to kanamycin and nourseothricin. The antibiotic markers were removed from MIBa821 by transformation with pFYD80 as described above. The resulting antibiotic marker free strain is called MIBa855 and contains 4 copies of the AA variant PE096 at XI-1 and 4 copies of the Ps glucoamylase at XII-5. Resulting strains MLBA787, MLBA795, MLBA821, and MLBA855 are shown in Table 19 below with corresponding expressed glucoamylase and/or alpha-amylase of interest and integration locus.

Additional examples of homozygous strains constructed via two-step transformation are shown below in Table 19.

Construction homozygous strains via one-step transformation

To simultaneously modify both alleles of a target locus in a diploid strain in a single transformation, two left-hand and right-hand plasmid pairs were used, one pair with the kanamycin resistance marker and the other pair with the nourseothricin marker (as described *supra*). Both pairs were linearized with restriction enzymes and all four resulting linearized DNAs were simultaneously transformed into an *S. cerevisiae* host strain using electroporation (see Thompson, et al., YEAST vol. 14: 565-71, 1998). The transformation mixture was plated to a plate containing both kanamycin and nourseothricin, and colonies resistant to both selection agents were PCR screened to confirm homozygous modification of the targeted chromosomal integration site. A confirmed transformant was thus homozygous for the expression cassette at the target locus, with one chromosome containing the kanamycin marker and the other chromosome containing the nourseothricin marker. For some strains, marker removal was subsequently carried out using the plasmid pFYD80 that includes a gene encoding the CRE recombinase as described *supra*.

Examples of homozygous strains constructed via one-step transformation are shown below in Table 19.

Table 19: AA and AMG-expressing yeast strains

Strain	background	integration locus	expressed protein	copies of expressed protein	antibiotic resistance markers	construction method
yMHCT390	MBG4931	XI-1	AtAA	4	kanamycin and nourseothricin	two-step
yMHCT392	MBG4931	XII-5	AtAA	4	kanamycin and nourseothricin	two-step
yMHCT394	MBG4931	XI-1	PE096 AA	4	kanamycin and nourseothricin	two-step
yMHCT396	MBG4931	XII-5	PE096 AA	4	kanamycin and nourseothricin	two-step
MEJI697	MBG4931	XII-5	GsAMG	4	none	two-step + pFYD80
MEJI705	MBG4931	XII-5	GsAMG	4	none	two-step + pFYD80
AgJg013	MBG4911	XII-5	PsAMG	4	kanamycin and nourseothricin	two-step
MLBA787	MBG4911	XI-1	PE096 AA	4	kanamycin and nourseothricin	two-step
MLBA795	MBG4911	XI-1	PE096 AA	4	none	two-step, pFYD80
MLBA821	MBG4911	XI-1, XII-5	PE096 AA, PsAMG	4, 4	kanamycin and nourseothricin	two-step + pFYD80 at XI-1; two-step at XII-5
MLBA855	MBG4911	XI-1, XII-5	PE096 AA, PsAMG	4, 4	none	two-step + pFYD80 at XI-1; two-step at XII-5 + pFYD80
MLBA888	Ethanol Red	XI-1, XII-5	PE096 AA, PsAMG	4, 4	kanamycin and nourseothricin	two-step + pFYD80 at XII-5; one-step at XI-1 + pFYD80
MLBA889	MBG4931	XI-1, XII-5	PE096 AA, GsAMG	2, 6	none	two-step + pFYD80 at XII-5; one-step at XI-1 + pFYD80
MLBA891	MBG4931	XI-1, XII-5	PE096 AA, GsAMG	4, 4	none	two-step + pFYD80 at XII-5; one-step at XI-1 + pFYD80
PsinER1	Ethanol Red	XII-5	PsAMG	4	none	two-step + pFYD80
MHCT408	MBG4931	XII-5	GtAMG	4	none	two-step + pFYD80

Example 20: Application performance of a yeast expressing a fungal alpha amylases in a raw starch ethanol process (MBG4931 expressing AA)

The following experiments were performed using ground corn. Urea was supplemented to 500 ppm.

Experiment 1 was performed at bottle scale. 150 grams of corn mash was added to a 250 ml Wheaton bottle. All strains were dosed at 0.5 AGU/gDS using glucoamylase from *Pycnoporus sanguineus* (PsAMG/SEQ ID NO: 18). A fungal alpha-amylase variant, PE096, a *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) as shown in SEQ ID NO: 16, having substitutions G128D+D143N, was used as the exogenous alpha-amylase in this experiment. The parent strain was dosed with PE096 at 0.031, 0.016, or 0.008 FauF/ gDS (16, 32, and 64 ratios respectively). The yeast strains used as the fermenting organism expressed a fungal alpha-amylase selected as either PE096 (strains MHCT394 and MHCT396) or a wild type alpha-amylase derived from *Aspergillus terreus* (SEQ ID NO: 6 of WO2017/087330) (strains MHCT 390 and MHCT 392). Alpha-amylase expressing strains were all tested at the 64 ratio.

Experiment 2 was performed at tube scale, with approximately 5 grams of corn mash in a 15 ml conical tube. The parent strain was dosed at the 16, 32, or 64 ratio, glucoamylase (GA) only, or no exogenous enzyme added. The Alpha-amylase (AA) expressing strains were only tested under the GA only or no exogenous enzyme added conditions. When GA was added, a dose of 0.5 AGU/gDS PsAMG was used.

Results:

Experiment 1: At 55 hours the AA expressing strains at the 64 ratio outperformed the parent strain at all 3 ratios. At 73 hours of fermentation, the Alpha Amylase expressing strains, dosed at a RSH ratio of 64 have statistically identical performance to the parent strain dosed at a RSH ratio of 16. This indicates that 75% alpha amylase reduction is possible.

Experiment 2: At 72 hours, the AA expressing strains with only GA added were able to match the performance of the parent strain at a RSH16 ratio, compared to the parent which only generated ~49% of the total ethanol under these conditions. This suggests that it is possible to remove most, if not all of the AA from the enzyme mixture when using these AA expressing strains. When no exogenous enzyme is added, the strains are able to generate ~75% of the ethanol generated by the full enzyme mixture in the parent strain, whereas the parent strain generates less 2% of the total ethanol under these conditions.

Example 21: Application performance of a yeast expressing a glucoamylase (AMG) in an SSF process on liquefied starch (MBG4931 expressing AMG)

A liquefied mash prepared using a commercial bacterial alpha-amylase product, Liquozyme SCDS, was used to prepare the liquefied mash. Mash solids were read to be 33.76% using a moisture balance. Approximately 5 grams of corn mash was fermented in preweighed 15 ml flip top conical tubes with a small hole drilled for gas release. A saccharification composition, glucoamylase blend A dosed at 0.6 AGU/gDS for the parent strain (MBG4931) as the full enzyme dose. The parent strain (MBG4931), the glucoamylase producing strain producing the *Gloeophyllum sepiarium* glucoamylase (GsAMG/SEQ ID NO:

15) (MeJi697) and Transferm Yield+ were all dosed at 0.3 AGU/gDS to test a 50% enzyme replacement level. Tubes were vortexed twice daily. Twelve replicates per strain were dosed for each time point.

The MeJi697 strain was constructed by inserting the expression vectors at the location of XII-5 in the yeast genome in the MBG4931 parent strain.

At 21, 44.5, and 60 hours, 48 tubes were sampled by weighing, adding 150 µl of 40% H_2SO_4, and centrifugation for 10 minutes at 3500 rpm. Supernatant was then filtered through a 0.2 µM filter prior to being diluted in mobile phase for HPLC analysis.

Glucoamylase blend A: Blend comprising *Talaromyces emersonii* glucoamylase disclosed as SEQ ID NO: 34 in WO99/28448, *Trametes cingulata* glucoamylase disclosed as SEQ ID NO: 2 in WO 06/69289, and *Rhizomucor pusillus* alpha-amylase with *Aspergillus niger* glucoamylase linker and starch binding domain (SBD) disclosed in SEQ ID NO: 16 herein having the following substitutions G128D+D143N (activity ratio in AGU:AGU:FAU-F is about 29:8:1).

Results:

This experiment tested the performance of a GsAMG expressing strain with a 50% reduction in exogenous enzyme glucoamylase. MeJi697 dosed at 50% of the industrially recommended enzyme dose showed statistically identical performance to the parent strain at full dose at 60 hours of fermentation. The performance of this strain was also statistically identical to Transferm® Yield + (commercial yeast strain expressing a glucoamylase available from Mascoma) at this time point. At 44.5 hours, MeJi697 showed higher ethanol titers than the parent strain at full dose and Transferm Yield+ at comparable dose.

Example 22: Application performance of a yeast expressing a glucoamylase (AMG) in an SSF process (MBG4911 expressing AMG)

Mash Preparation: Ground yellow dent corn was mixed with tap water and the dry solids (DS) level was determined to be 35.4% by moisture balance. This mixture was supplemented with 3 ppm LACTROL® and 500 ppm urea. The slurry was adjusted to pH 4.5 with 40% H_2SO_4.

Yeast Strains and Preparation: The two yeast strains tested in these experiments were MBG4911 and AgJg013 (MBG4911 expressing PsAMG). AgJg013 was constructed in a similar manner to as shown above. Yeasts were propagated in filter sterilized liquid media (2% w/v D-glucose, 1% peptone, and 0.5% yeast extract). Using a sterile loop under a UV hood, cells from a lawn were transferred into 60 mL of the liquid media in 125 mL sterile vented flask and incubated at 150 rpm in a 32°C air shaker. Cells were harvested at 18 hours by spinning in 50 ml centrifuge tubes at 3000rpm for 10 minutes and decanting the supernatant. Cells were

washed once in 25 ml of water and the resulting cell pellet was resuspended in 1.5 ml tap water. Total yeast concentration was determined using the YC-100 in duplicate.

Simultaneous Saccharification and Fermentation (SSF): Approximately 5 grams of mash was transferred to test tubes having a 1/64 hole drilled in the top to allow CO_2 release. PE096 was dosed to each tube of mash at 0.028 FauF/ gDS. PsAMG was dosed at 0.45 AGU/gDS, 0.23 AGU/gDS, 0.15 AGU/gDS, or omitted entirely. Yeast was dosed at 5e6 cells/g mash. Milli-Q water was added to each tube so that a total volume of liquid added (enzyme + MQ water) to each tube would be equally proportionate to the mash weight. Fermentations took place in a 32°C incubator for 72 hours. Samples were vortexed periodically (in the morning and in the evening) throughout the fermentation. Six replicates were run per treatment.

HPLC analysis: Fermentation sampling took place after 72 hours of fermentation. Each tube was processed for HPLC analysis by deactivation with 150 µL of 40% v/v H_2SO_4, vortexing, centrifuging at 1460×*g* for 10 minutes, and filtering through a 0.2 µm SpinX column. All samples were processed at a 5X dilution. Samples were stored at 4°C prior to and during HPLC analysis.

Table 20: HPLC System and conditions

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	Waters Ion Exclusion Column 150mm x 7.8mm Part#: WAT010295 Waters Guard Cartridge SH-1011P 50mm x 6mm Part #: WAT034243
Method	0.0005M H2SO4 mobile phase Flow rate: 1 ml/min Column temperature: 75°C RI detector temperature: 40°C

Samples were analyzed for sugars (DP4+, DP3, DP2, glucose, and fructose), organic acids (lactic and acetic), glycerol, and ethanol.

Increased ethanol titers at 72 hours for AgJg013 compared to parent strain MBG4911 are shown in Table 21 below.

Table 21: Ethanol Titers at 72 Hours

Condition	MBG4911 Ethanol Titer (w/v%)	AgJg013 Ethanol Titer (w/v%)
PsAMG 0.45 AGU/gDS	14.07	14.36
PsAMG 0.23 AGU/gDS	13.51	14.05
PsAMG 0.15 AGU/gDS	12.99	13.94
PsAMG 0.00 AGU/gDS	8.28	13.43

Example 23: Application performance of a yeast expressing a glucoamylase (AMG) in an SSF process (MBG4911 expressing AMG)

Mash Preparation: Ground yellow dent corn was mixed with tap water and the dry solids (DS) level was determined to be 37.4% by moisture balance. This mixture was supplemented with 3 ppm LACTROL® and 500 ppm urea. The slurry was adjusted to pH 4.5 with 40% H_2SO_4. Water was added to bring the final dry solids to 35.0%.

Yeast Strains and Preparation: The two yeast strains tested in these experiments, MBG4911 and AgJg013 (MBG4911 expressing PsAMG), were prepared as described herein.

Simultaneous Saccharification and Fermentation (SSF): Approximately 5 grams of mash was transferred to test tubes having a 1/64 hole drilled in the top to allow CO_2 release. PE096 was dosed to each tube of mash at 0.028 FauF/ gDS. PsAMG was dosed at 0.45 AGU/gDS, or 0.23 AGU/gDS. Yeast was dosed at 5e6 cells/g mash. Milli-Q water was added to each tube so that a total volume of liquid added (enzyme + MQ water) to each tube would be equally proportionate to the mash weight. Fermentations took place in a 32°C incubator for 72 hours. Samples were vortexed periodically (in the morning and in the evening) throughout the fermentation. Seven replicates were run per treatment.

HPLC analysis: Fermentation sampling took place after 48 and 72 hours of fermentation. Each tube was processed for HPLC analysis as described herein.

Increased ethanol titers at 72 hours for AgJg013 compared to parent strain MBG4911 are shown in Table 22 below. 50% of exogenous GA is able to be removed using the PsAMG expressing yeast.

Table 22: Ethanol titers at 72 Hours

Enzyme	Yeast	72 Hours (w/v%)
0.45 AGU/gDS PsAMG	MBG4911	16.27
0.45 AGU/gDS PsAMG	AgJg013	16.46
0.23 AGU/gDS PsAMG	MBG4911	15.54
0.23 AGU/gDS PsAMG	AgJg013	16.37

Example 24: Application performance of a yeast expressing an alpha-amylase (AA) in an SSF process (MBG4911 expressing AA)

Mash Preparation: Ground yellow dent corn was mixed with tap water and the dry solids (DS) level was determined to be 35.06% by moisture balance. This mixture was supplemented with 3 ppm LACTROL® and 500 ppm urea. The slurry was adjusted to pH 4.5 with 40% H_2SO_4.

Yeast Strains and Preparation: The two yeast strains tested in these experiments were MBG4911 and MLBA795 (MBG4911 expressing PE096) were prepared as described in Example 4.

Simultaneous Saccharification and Fermentation (SSF): Approximately 5 grams of mash was transferred to test tubes having a 1/64 hole drilled in the top to allow CO_2 release. PE096 was dosed to a control set of tubes of mash for MBG4911 at 0.028 FauF/ gDS and was omitted entirely from the tubes for MLBA795. PsAMG was dosed at 0.45 AGU/gDS, or 0.23 AGU/gDS. Yeast was dosed at 10e6 cells/g mash. Milli-Q water was added to each tube so that a total volume of liquid added (enzyme + MQ water) to each tube would be equally proportionate to the mash weight. Fermentations took place in a 32°C incubator for 72 hours. Samples were vortexed periodically (in the morning and in the evening) throughout the fermentation. Ten replicates were run per treatment.

HPLC analysis: Fermentation sampling took place after 72 hours of fermentation. Each tube was processed for HPLC analysis as described herein.

Increased ethanol titers at 72 hours for strain MLBA795 compared to parent strain MBG4911 are shown in Table 23 below. With all exogenous AA removed, at 72 hours of fermentation 50% of exogenous GA is able to be removed using these AA expressing yeasts. When full enzyme dose is used, a boost in ethanol titer is observed.

Table 23: Ethanol Titers at 72 Hours

Exogenous GA (PsAMG)	Exogenous AA (PE096)	Yeast	72 Hours (w/v%)
0.45 AGU/gDS	0.028 FauF/g DS AAPE096	MBG4911	14.27
0.45 AGU/gDS	No Exogenous AA	MBG4911	6.20
0.45 AGU/gDS	No Exogenous AA	MLBA795	14.55
0.23 AGU/gDS	No Exogenous AA	MBG4911	5.33
0.23 AGU/gDS	No Exogenous AA	MLBA795	14.34

Example 25: Application performance of a yeast expressing an alpha-amylase (AA) and a glucoamylase (AMG) in an SSF process (MBG4911 expressing AA + AMG)

Mash Preparation: Ground yellow dent corn was mixed with tap water and the dry solids (DS) level was determined to be 37.4% by moisture balance. This mixture was supplemented with 3 ppm LACTROL® and 500 ppm urea. The slurry was adjusted to pH 4.5 with 40% H_2SO_4. Water was added to bring the final solids to 35.0% DS

Yeast Strains and Preparation: The two yeast strains tested in these experiments were MBG4911 and MLBA821 (MBG4911 expressing PsAMG and PE096). Yeast were propagated in filter sterilized liquid media (2% w/v D-glucose, 1% peptone, and 0.5% yeast extract). Using a sterile loop under a UV hood, cells from a lawn were transferred into 60 mL of the liquid media in 125 mL sterile vented flask and incubated at 150 rpm in a 32°C air shaker. Cells were harvested at 18 hours by spinning in 50 ml centrifuge tubes at 3000rpm for 10 minutes and decanting the supernatant. Cells were washed once in 25 ml of water and the resulting cell pellet was resuspended in 1.5 ml tap water. Total yeast concentration was determined using the YC-100 in duplicate.

Simultaneous Saccharification and Fermentation (SSF): Approximately 5 grams of mash was transferred to test tubes having a 1/64 hole drilled in the top to allow CO_2 release. PE096 was dosed to tubes of mash for MBG4911 at 0.028 FauF/ gDS and was omitted entirely from the tubes for MLBA821. PsAMG was dosed at 0.45 AGU/gDS, or 0.23 AGU/gDS for MBG4911 and 0.23 AGU/gDS, 0.15 AGU/gDS, or 0.045 AGU/gDS for MLBA821. Yeast was dosed at 10e6 cells/g mash. Milli-Q water was added to each tube so that a total volume of liquid added (enzyme + MQ water) to each tube would be equally proportionate to the mash weight. Fermentations took place in a 32°C incubator for 72 hours. Samples were vortexed periodically (in the morning and in the evening) throughout the fermentation. Ten replicates were run per treatment.

HPLC analysis: Fermentation sampling took place after 72 hours of fermentation. Each tube was processed for HPLC analysis as described herein.

Increased ethanol titers at 72 hours for strain MLBA821 compared to parent strain MBG4911 are shown in Table 24 below. The strain expressing both GA and AA is statistically identical to the host strain at full enzyme dose when dosed at only 10% of the exogenous GA with no added AA.

Table 24: Ethanol Titers at 72 Hours of Fermentation

Exogenous GA (PsAMG)	Exogenous AA (PE096)	Yeast	72 Hours (w/v%)	Tukey- Kramer Connecting Letter Report (95% confidence level)
0.45 AGU/gDS	0.028 FauF/g DS	MBG4911	16.27	A B
0.23 AGU/gDS	0.028 FauF/g DS	MBG4911	15.54	C
0.23	No Exogenous AA	MLBA821	16.43	A

AGU/gDS				
0.15 AGU/gDS	No Exogenous AA	MLBA821	16.25	A B
0.045 AGU/gDS	No Exogenous AA	MLBA821	16.10	B

Example 26: Application performance of a yeast expressing an alpha-amylase (AA) and a glucoamylase (AMG) in an SSF process (MBG4911 expressing AA + AMG)

Mash Preparation: Ground yellow dent corn was mixed with tap water and the dry solids (DS) level was determined to be 35.3% by moisture balance. This mixture was supplemented with 3 ppm LACTROL® and 500 ppm urea. The slurry was adjusted to pH 4.5 with 40% H_2SO_4. Water was added to bring the final solids to 35.0% DS

Yeast Strains and Preparation: Yeast strains ER, MBG4911, AgJg013, MLBA855, PsinER1 and MLBA888 were tested in this experiment. Yeast were propagated in filter sterilized liquid media (6% w/v D-glucose, 1% peptone, and 0.5% yeast extract). Using a sterile loop under a UV hood, cells from a lawn were transferred into 60 mL of the liquid media in 125 mL sterile vented flask and incubated at 150 rpm in a 32°C air shaker. Cells were harvested at 18 hours by spinning in 50 ml centrifuge tubes at 3000rpm for 10 minutes and decanting the supernatant. Cells were washed once in 25 ml of water and the resulting cell pellet was resuspended in 1.5 ml tap water. Total yeast concentration was determined using the YC-100 in duplicate.

Simultaneous Saccharification and Fermentation (SSF): Approximately 200 grams of mash was transferred to 250 ml Wheaton bottles with a 1/64 hole drilled in the top to allow CO_2 release. PE096 was dosed to bottles of mash for ER, MBG4911, AgJg013, and PsinER 1 at 0.025 FauF/ gDS and was omitted entirely from the bottles for MLBA855 and MLBA888. PsAMG was dosed at 0.4 AGU/gDS for ER and MBG4911 and 0.2 AGU/gDS, or 0.1 AGU/gDS for the remaining strains. Yeast was dosed at 5e6 cells/g mash. Milli-Q water was added to each tube so that a total volume of liquid added (enzyme + MQ water) to each tube would be equally proportionate to the mash weight. Fermentations took place in a 32°C incubator for 91 hours. Samples were swirled periodically (in the morning and in the evening) throughout the fermentation. Three replicates were run per treatment.

HPLC analysis: Fermentation sampling took place after 72 and 91 hours of fermentation. Each tube was processed for HPLC analysis as described herein.

Ethanol titers at 72 hours for strains AgJg013 and MLBA855 compared to parent strain MBG4911, and PsinER1 and MLBA888 compared to parent strain ER are shown in Table 25 below. The MBG4911 based strains expressing either GA or both GA and AA were able to ferment to statistically identical levels as the parent strain when dosed at 50% or 25% of the full GA dose. The combination GA and AA strains required no added AA to reach these levels.

The ER based strains were able to ferment to statistically identical levels as the parent at 50% of the full GA dose. Again the combination strain required no added AA to do so. The combination strain was also able to ferment to statistically identical levels as the parent strain at 25% of the full GA dose.

Table 25: Ethanol Titers at 72 hours

Exogenous GA (PsAMG)	Exogenous AA (PE096)	Yeast	ETOH Titer (%w/v)	Tukey Kramer Connecting Letter Report (95% Confidence)
0.2 AGU/g DS	No Exogenous AA	MLBA855	18.1	A
0.2 AGU/g DS	0.025 FauF/gDS	AgJg013	18.1	A
0.1 AGU/g DS	No Exogenous AA	MLBA855	18.1	A
0.2 AGU/g DS	No Exogenous AA	MLBA888	18.1	A
0.4 AGU/gDS	0.025 FauF/gDS	ER	18.0	A
0.4 AGU/gDS	0.025 FauF/gDS	MBG4911	18.0	AB
0.1 AGU/g DS	0.025 FauF/gDS	AgJg013	17.9	AB
0.1 AGU/g DS	No Exogenous AA	MLBA888	17.9	AB
0.2 AGU/g DS	0.025 FauF/gDS	PsinER1	17.8	AB
0.1 AGU/g DS	0.025 FauF/gDS	PsinER1	17.6	B

Ethanol titers at 91 hours for strains AgJg013 and MLBA855 compared to parent strain MBG4911, and PsinER1 and MLBA888 compared to parent strain ER are shown in Table 26 below. The MBG4911 based strains expressing either GA or both GA and AA were able to ferment to statistically identical levels as the parent strain when dosed at 50% or 25% of the full GA dose. The combination GA and AA strains required no added AA to reach these levels. The ER based strains were able to ferment to statistically identical levels as the parent at 50% of the full GA dose. Again, the combination strain required no added AA to do so. It is also noted that the MBG4911 strains all outperformed the ER based strains at this time point.

Table 26: Ethanol Titers at 91 hours

Exogenous GA (PsAMG)	Exogenous AA (PE096)	Yeast	ETOH Titer (%w/v)	Tukey Kramer Connecting Letter Report (95% Confidence)
0.1 AGU/g DS	0.025 FauF/gDS	AgJg013	19.4	A
0.1 AGU/g DS	No Exogenous AA	MLBA855	19.3	A
0.2 AGU/g DS	0.025 FauF/gDS	AgJg013	19.3	A

0.4 AGU/gDS	0.025 FauF/gDS	MBG4911	19.3	A
0.2 AGU/g DS	No Exogenous AA	MLBA855	19.2	A
0.4 AGU/gDS	0.025 FauF/gDS	ER	18.7	B
0.2 AGU/g DS	0.025 FauF/gDS	PsinER1	18.6	BC
0.2 AGU/g DS	No Exogenous AA	MLBA888	18.4	BC
0.1 AGU/g DS	0.025 FauF/gDS	PsinER1	18.3	C
0.1 AGU/g DS	No Exogenous AA	MLBA888	18.3	C

Example 27: Application performance of a yeast expressing an alpha-amylase (AA) and a glucoamylase (AMG) in an SSF process (MBG4931 expressing AA + AMG)

A liquefied mash prepared using a commercial bacterial alpha-amylase product, Avantec Amp, was obtained from a commercial plant, Valero Albert City and used for this experiment. Mash solids were read to be 33.22% using a moisture balance. Approximately 5 grams of corn mash was fermented in pre- weighed 15 ml flip top conical tubes with a small hole drilled for gas release. A saccharification composition, Glucoamylase blend A dosed at 0.6 AGU/gDS for the parent strain (MBG4931) as the full enzyme dose. A strain expressing the GsAMG (MeJi705; constructed in a similar manner to that shown herein) and two strains expressing GsAMG and PE096 (MLBA889, comprising 6X GsAMG, 2X PE096; and MLBA891, comprising 4X GsAMG, 4X Pe096; both prepared in manners similar to those described herein) were all dosed at 0.3 AGU/gDS to test a 50% enzyme replacement level or 0.15 AGU/gDS to test a 75% enzyme replacement level. Tubes were vortexed twice daily. Five replicates per strain were dosed for each condition.

At 52 hours, tubes were sampled by weighing, adding 150 µl of 40% H_2SO_4, and centrifugation for 10 minutes at 3500 rpm. Supernatant was then filtered through a 0.2 µM filter prior to being diluted in mobile phase for HPLC analysis.

This experiment tested the performance of a GsAMG and GsAMG/Pe096 expressing strains with a 50% reduction in exogenous enzyme glucoamylase. MeJi705, MLBA889, and MLBA891 dosed at 50% of the industrially recommended enzyme dose showed statistically identical performance to the parent strain at full dose at 52 hours of fermentation. The strains expressing both GsAMG and Pe096 (MLBA889 and MLBA891) dosed at 25% of the industrially recommended enzyme dose also showed statistically identical performance to the parent strain at full dose at this time point, indicating a benefit from the addition of the Pe096 expression over the GsAMG expression alone.

Example 28: Application performance of a yeast expressing a glucoamylase (AMG) in an SSF process (MBG4931 expressing AA)

A liquefied mash prepared using a commercial bacterial alpha-amylase product, Liquozyme LpH, was obtained from a commercial plant, Little Sioux and used for this experiment. Mash solids were read to be 34.5% using a moisture balance. Approximately 5 grams of corn mash was fermented in pre- weighed 15 ml flip top conical tubes with a small hole drilled for gas release. A saccharification composition, Glucoamylase blend A dosed at 0.6 AGU/gDS for the control strain (ER) as the full enzyme dose. A strain expressing GtAMG (MHCT408; prepared in a manner similar to that described herein) and parent strain MBG4931 were all dosed at 0.6, 0.45, 0.35, 0.25 and 0.15 AGU/gDS to test different enzyme replacement levels. Tubes were vortexed twice daily. Four replicates per strain were dosed for each condition.

At 54 hours, tubes were sampled by weighing, adding 50 µl of 40% H_2SO_4, and centrifuged for 10 minutes at 3500 rpm. Supernatant was then filtered through a 0.2 µM filter prior to being diluted in mobile phase for HPLC analysis.

This experiment tested the performance of a GtAMG with different reductions in exogenous enzyme glucoamylase. Results are shown in Table 27. MHCT408 dosed at 42% of the industrially recommended enzyme dose showed statistically higher performance to the control strain at full dose at 54 hours of fermentation.

Table 27. Ethanol Titers at 54 hours

	Ethanol Titers (g/L)		
Dose (AGU/gDS)	MBG4931	MHCT408	ER
0.6	139.0	139.7	138.4
0.45	138.1	139.5	
0.35	134.8	139.6	
0.25	126.7	135.3	
0.15	117.9	130.4	

Example 29: Construction of yeast strains expressing glucoamylase

Construction of homozygous strains via two-step transformation

Expression cassettes for *Gloeophyllum sepiarium* glucoamylase (GsAMG) were targeted to the XII-5 integration site as described in Mikkelsen et al. (Metabolic Engineering v14 (2012) pp104-111). Two plasmids employing a split-marker approach were used for each integration event, each containing an expression cassette and approximately two-thirds of a dominant selection marker. The left-hand plasmid contained 5' flanking DNA homologous to the desired integration site, the *S. cerevisiae* TEF2 promoter driving expression of GsAMG codon-optimized for expression in *S. cerevisiae*, the *S. cerevisiae* ADH3 terminator, a loxP

site, and the 5' two-thirds of a dominant selection marker under control of the *Ashbya gossypii* TEF1 promoter. The right-hand plasmid contains the 3' two-thirds of the dominant selection marker with the *Ashbya gossypii* TEF1 terminator, a loxP site, an expression cassette in the reverse orientation relative to the dominant selection marker composed of the *S. cerevisiae* HXT7 promoter driving expression of GsAMG codon-optimized for expression in *S. cerevisiae* with the *S. cerevisiae* PMA1 terminator, and 3' flanking DNA homologous to the XII-5 integration site. A left-hand and right-hand plasmid pair containing the GsAMG expression cassettes targeting to XII-5 was linearized with restriction enzymes and transformed into an *S. cerevisiae* strain using lithium acetate transformation (see Gietz and Woods, 2006, Methods in Molecular Biology, v 313 pp107-120). Since the ethanol yeast strains of interest are diploid yeast, the desired integration construct was first integrated using kanamycin resistance as the dominant selection marker, followed by PCR screening to confirm the desired integration event. A confirmed heterozygous transformant was then transformed again using an expression cassette pair with the nourseothricin resistance marker. PCR screening was used to confirm homozygous modification of the targeted chromosomal integration site.

The antibiotic markers present in the strains resulting from the two-step transformation method are flanked by loxP sites. These strains were transformed with plasmid pFYD80 that includes a gene encoding the CRE recombinase, a site-specific enzyme that facilitates recombination between neighboring loxP sites (Güldener et al., 2002). Plasmid pFYD80 is maintained as a non-integrative, free replicating molecule. This approach enables the specific excision of both selective markers. After a strain was transformed with plasmid pFYD80, transformants were selected on plates containing zeocin. Zeocin resistance is encoded in pFYD80. Subsequently, screening for transformants that have lost nourseothricin and kanamycin resistance was performed. Sensitive strains were grown in YPD liquid until loss of pFYD80 plasmid was obtained. Selected strains were shown to be zeocin sensitive as a result of the loss of plasmid pFYD80.

Construction of homozygous strains via one-step transformation

To simultaneously modify both alleles of the XII-5 target locus in a diploid strain in a single transformation, two left-hand and right-hand plasmid pairs were used, one pair with the kanamycin resistance marker and the other pair with the nourseothricin marker (as described *supra*). Both pairs were linearized with restriction enzymes and all four resulting linearized DNAs were simultaneously transformed into an *S. cerevisiae* host strain using electroporation (see Thompson, et al., YEAST vol. 14: 565-71, 1998). The transformation mixture was plated to a plate containing both kanamycin and nourseothricin, and colonies resistant to both selection agents were PCR screened to confirm homozygous modification of the targeted chromosomal integration site. A confirmed transformant was thus homozygous for the expression cassette at the target locus, with one chromosome containing the kanamycin

marker and the other chromosome containing the nourseothricin marker. Marker removal was subsequently carried out using the plasmid pFYD80 that includes a gene encoding the CRE recombinase as described *supra*.

Homozygous strains constructed via either the one-step or two-step transformation method express two homozygous copies of *Gloeophyllum sepiarium* glucoamylase from the XII-5 integration site on each chromosome, one copy under control of the TEF2 promoter and the other copy under control of the HXT7 promoter. A diagram of the expression cassette at one chromosome of the XII-5 integration site is shown in Figure 1.

Table 28: AMG-expressing yeast strains

Strain	background	integration locus	expressed protein	copies of expressed protein	antibiotic resistance markers	construction method
GsAMGinER1	Ethanol Red	XII-5	GsAMG	4	none	two-step + pFYD80
MLBA865	MBG4851	XII-5	GsAMG	4	none	one-step + pFYD80
yMHCT471	MBG4985	XII-5	GsAMG	4	none	one-step + pFYD80

Example 30: Fermentations of Glucoamylase-expressing yeast strains

Yeast strains were incubated overnight in YPD media (6% w/v D-glucose, 1% peptone, 0.5% yeast extract, 0.3% KH_2PO_4) at 32°C for a total of 18 hours at 150 rpm at 32°C. Cells were harvested at ~18 hours, the cultures were spun at 3500rpm for 10 minutes, and the supernatant was discarded. Cells were suspended in ~15 ml tap water, and total yeast concentration was determined in duplicate using a YC-100 Nucleocounter. Industrially obtained liquefied corn mash where liquefaction was carried out using Avantec Amp was supplemented with 3 mg/L penicillin and 350 ppm of urea. Simultaneous saccharification and fermentation (SSF) was performed via mini-scale fermentations using the condition shown in Table 29.. Approximately 5 g of liquefied corn mash was added to 15 ml conical tubes. Each vial containing a glucoamylase expressing strain was dosed with 0.3 AGU/g-DS of an exogenous glucoamylase enzyme product (Spirizyme Excel) followed by the addition of yeast strains, while each vial containing a control strain lacking glucoamylase expression was dosed with 0.6 AGU/g-DS of an exogenous glucoamylase enzyme product (Spirizyme Excel) followed by the addition of yeast strains. 10^7 yeast cells/g of corn mash were pitched. Actual Spirizyme Excel and yeast dosages were based on the exact weight of corn slurry in each vial. Vials were incubated at 32°C. Triplicates of each strain were analyzed after 54 hour fermentations. Fermentations were stopped by addition of 50 µL of 40% H_2SO_4, follow by

centrifuging, and filtration through a 0.45 micron filter. Ethanol and glycerol concentrations were determined using HPLC.

Table 29. Mini-tube fermentation reaction conditions

Substrate	Avantec Amp corn mash
Yeast pitch	10^7 cells/g corn mash
Exogenous glucoamylase product dose	0.3 AGU/g-DS
pH	5.0
Urea	350 ppm
Penicillin	3 mg/L
Incubation temperature	32°C
Reaction time	24 or 54 hours

As shown in Figs 2 and 3, strain yMHCT471 (MBG4985 background expressing glucoamylase) produced significantly more ethanol when compared to expressing glucoamylase in other background strains, and produced significantly less glycerol than (e.g., compared to expressing glucoamylase in the Ethanol Red background).

CLAIMS

1. A process of producing ethanol from starch-containing material comprising:
(a) saccharifying the starch-containing material; and
(b) fermenting using a fermentation organism;
wherein the fermenting organism is *Saccharomyces cerevisiae;*
and wherein a glucoamylase and/or an alpha-amylase is expressed from the fermenting organism.

2. The process according to claim 1, wherein the *Saccharomyces cerevisiae* is a recombinant derivative of MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) comprising one or more expression vectors encoding a glucoamylase and/or an alpha-amylase.

3. The process according to claim 2, wherein the fermenting organism comprises an expression vector encoding a glucoamylase obtainable from *Gloeophyllum, Pycnoporous,* or *Trametes*.

4. The process according to claim 2, wherein the fermenting organism comprises an expression vector encoding a glucoamylase selected from a *Gloeophyllum trabeum, Gloeophyllum sepiarium, or Gloeophyllum abietinum* glucoamylase.

5. The process according to claim 2, wherein the fermenting organism comprises an expression vector encoding a glucoamylase and wherein the encoded glucoamylase is:
(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 17; or
(ii) a glucoamylase comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 17.

6. The process according to claim 2, wherein the fermenting organism comprises an expression vector encoding a glucoamylase and wherein the encoded glucoamylase is:
(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 15; or
(ii) a glucoamylase comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 15.

7. The process according to claim 2, wherein the fermenting organism comprises an expression vector encoding a *Trametes cingulata* glucoamylase.

8. The process according to claim 2, wherein the fermenting organism comprises an expression vector encoding a glucoamylase and wherein the encoded glucoamylase is:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 20; or

(ii) a glucoamylase comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 20.

9. The process according to claim 2, wherein the fermenting organism comprises an expression vector encoding a *Pycnoporus sanguineus* glucoamylase.

10. The process according to claim 2, wherein the fermenting organism comprises an expression vector encoding a glucoamylase and wherein the encoded glucoamylase is:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 18; or

(ii) a glucoamylase comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 4.

11. The process according to any one of claims 1-10, wherein the fermenting organism comprises an expression vector encoding an alpha-amylase and wherein the encoded alpha-amylase is selected from a *Rhizomucor pusillus* or *Aspergillus terreus* alpha-amylase.

12. The process according to any one of claims 1-10, wherein the fermenting organism comprises an expression vector encoding an alpha-amylase and wherein the encoded alpha-amylase is:

(i) a *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) as shown in SEQ ID NO: 16, preferably one having at least one of the following substitutions or combinations of substitutions: D165M; Y141W; Y141R; K136F; K192R; P224A; P224R; S123H+Y141W; G20S + Y141W; A76G + Y141W; G128D + Y141W; G128D + D143N; P219C + Y141W; N142D + D143N; Y141W + K192R; Y141W + D143N; Y141W + N383R; Y141W + P219C + A265C; Y141W + N142D + D143N; Y141W + K192R V410A; G128D + Y141W + D143N; Y141W + D143N + P219C; Y141W + D143N + K192R; G128D + D143N + K192R; Y141W + D143N + K192R + P219C; G128D + Y141W + D143N + K192R; or G128D + Y141W + D143N + K192R + P219C, especially G128D+D143N (using SEQ ID NO: 5 for numbering); or

(ii) a polypeptide comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 16.

13. A recombinant *Saccharomyces cerevisiae* yeast strain, wherein the strain is a recombinant derivative of MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) comprising one or more expression vectors encoding a glucoamylase and/or an alpha-amylase.

14. The yeast strain of claim 13, wherein the fermenting organism comprises an expression vector encoding a glucoamylase and wherein the encoded glucoamylase is:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 17; or

(ii) a glucoamylase comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 17.

15. The yeast strain of claim 13, wherein the fermenting organism comprises an expression vector encoding a glucoamylase and wherein the encoded glucoamylase is:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 15; or

(ii) a glucoamylase comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 15.

16. The yeast strain any one of claims 13-15, wherein the fermenting organism comprises an expression vector encoding an alpha-amylase and wherein the encoded alpha-amylase is:

(i) a *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) as shown in SEQ ID NO: 16, preferably one having at least one of the following substitutions or combinations of substitutions: D165M; Y141W; Y141R; K136F; K192R; P224A; P224R; S123H+Y141W; G20S + Y141W; A76G + Y141W; G128D + Y141W; G128D + D143N; P219C + Y141W; N142D + D143N; Y141W + K192R; Y141W + D143N; Y141W + N383R; Y141W + P219C + A265C; Y141W + N142D + D143N; Y141W + K192R V410A; G128D + Y141W + D143N; Y141W + D143N + P219C; Y141W + D143N + K192R; G128D + D143N + K192R; Y141W + D143N + K192R + P219C; G128D + Y141W + D143N + K192R; or G128D + Y141W + D143N + K192R + P219C, especially G128D+D143N (using SEQ ID NO: 5 for numbering); or

(ii) a polypeptide comprising an amino acid sequence 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%, or at least 99% identity to the polypeptide of SEQ ID NO: 16.

17. A method of producing a recombinant derivative of strain *Saccharomyces cerevisiae* strain MBG4985 (deposited under Accession No. NRRL Y67342 at the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA) comprising:

(a) transforming *Saccharomyces cerevisiae* strain MBG4985 or a derivative of strain *Saccharomyces cerevisiae* strain MBG4985 with one or more expression vectors encoding a glucoamylase and/or an alpha-amylase; and

(b) isolating the transformed strain.

18. A recombinant *Saccharomyces cerevisiae* strain produced by the method of claim 17.

19. A process of producing ethanol from starch-containing material comprising:

(a) saccharifying the starch-containing material; and

(b) fermenting the saccharified material of step (a) with the recombinant *Saccharomyces cerevisiae* strain of claim 18.

20. Use of a recombinant yeast strain of any of claims 13-16 or 18 in the production of ethanol.

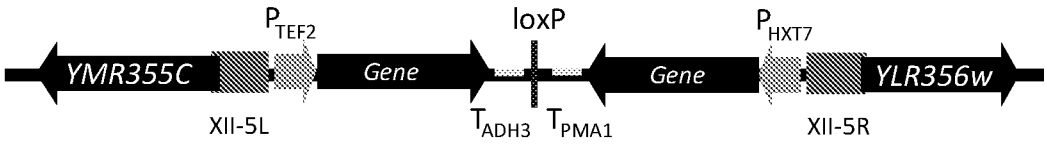

P TEF2
loxP
P HXT7
YMR355C
Gene
Gene
YLR356w
XII-5L
T ADH3
T PMA1
XII-5R

FIG. 1

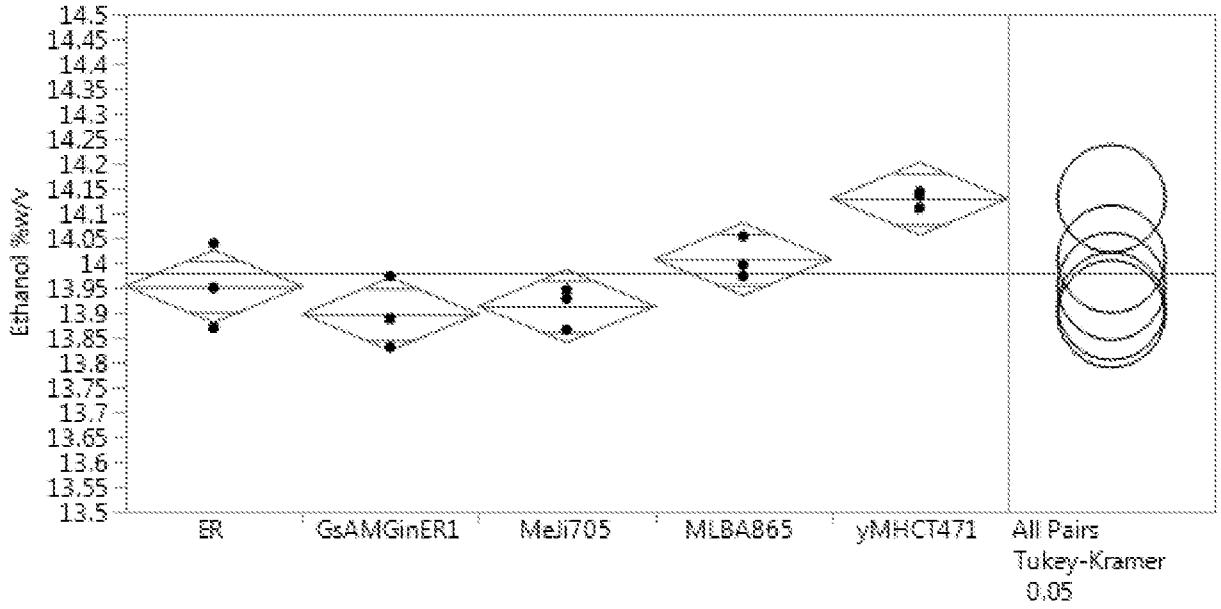

Ethanol %w/v
14.5
14.45
14.4
14.35
14.3
14.25
14.2
14.15
14.1
14.05
14
13.95
13.9
13.85
13.8
13.75
13.7
13.65
13.6
13.55
13.5
ER
GsAMGinER1
MeJi705
MLBA865
yMHCT471
All Pairs
Tukey-Kramer
0.05

FIG. 2

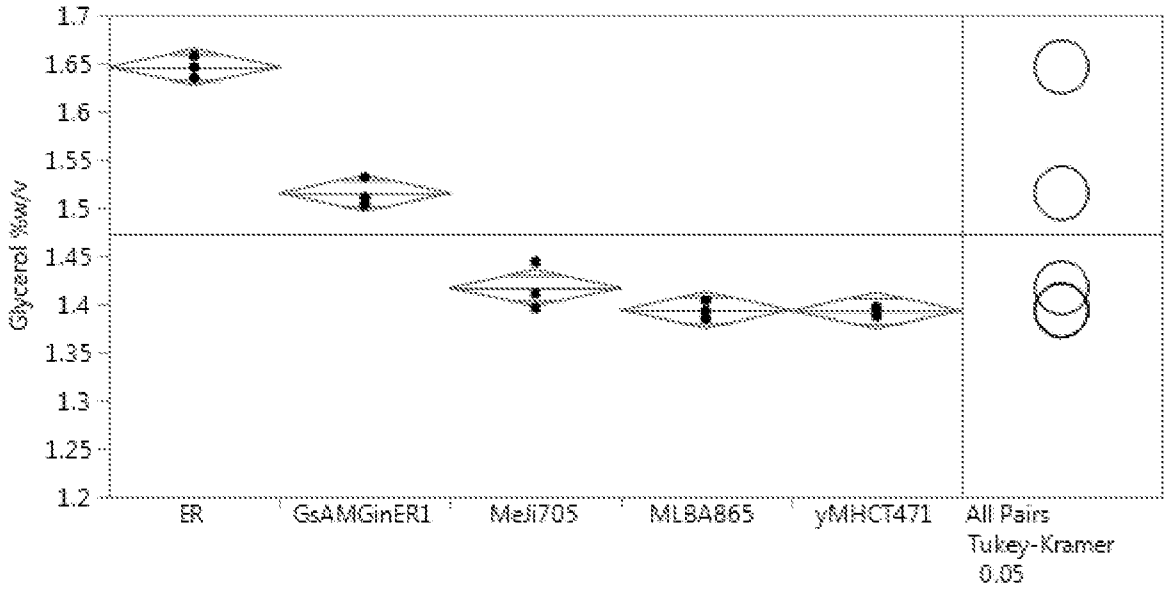

1.7
1.65
1.6
1.55
1.5
1.45
1.4
1.35
1.3
1.25
1.2
Glycerol %w/v
ER
GsAMGinER1
MeJi705
MLBA865
yMHCT471
All Pairs
Tukey-Kramer
0.05

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/063159

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/06 C12N1/19
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 C12N

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, COMPENDEX, FSTA, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FAVARO ET AL: "Consolidated bioprocessing of starchy substrates into ethanol by industrial Saccharomyces cerevisiae strains secreting fungal amylases", BIOTECHNOLOGY AND BIOENGINEERING, vol. 112, 2015, pages 1751-1760, XP02754198,	1
Y	* See page 1751 (Abstract); early online publication *	1,11,12

X	GÖRGENS ET AL: "Engineering Saccharomyces cerevisiae for direct conversion of raw, uncooked or granulated starch to ethanol", CRITICAL REVIEWS IN BIOTECHNOLOGY, vol. 35, 2015, pages 369-391, XP055318215,	1
Y	* See pages 373-375 (Table 2); early online publication *	1,11,12

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

[X] See patent family annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 January 2018	05/02/2018

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/063159

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOUZANI ET AL: "Advances in consolidated bioprocessing systems for bioethanol and butanol production from biomass: a comprehensive review", BIOFUEL RESEARCH JOURNAL, vol. 5, 2015, pages 152-195, XP02765733,	1
Y	* See section 2.3.4.3.2.1; early online publication *	1,11,12

X	DEN HAAN ET AL: "Engineering Saccharomyces cerevisaiae for next generation ethanol production", JOURNAL OF CHEMICAL TECHNOLOGY AND BIOTECHNOLOGY, vol. 88, 2013, pages 983-991, XP02765702,	1
Y	* See page 985 (left column) *	1,11,12

Y	WO 2016/138437 A1 (NOVOZYMES A/S & MICROBIOGEN PTY. LTD.) 1 September 2016 (2016-09-01) * See Sequences 4, 13, 18, 17, and 20, which correspond to the present SEQ ID NOS 15, 16, 17, 18, and 19 (only available online) *	1,11,12

X,P	WO 2017/087330 A1 (NOVOZYMES A/S) 26 May 2017 (2017-05-26)	1
Y,P	* See pages 5-6 (MBG numbers) *	1,11,12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2017/063159

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2016138437 A1	01-09-2016	AR 103811 A1	07-06-2017
		AU 2016225049 A1	24-08-2017
		WO 2016138437 A1	01-09-2016
WO 2017087330 A1	26-05-2017	NONE	