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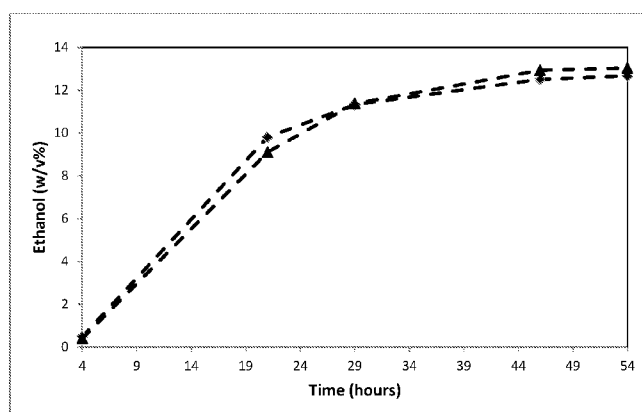


Fig. 1

(57) Abstract: The present invention relates to processes for producing ethanol from starch-containing material using a *Saccharomyces cerevisiae* yeast deposited under Accession No. V15/004035, V15/004036 or V15/004037 at National Measurement Institute, Victoria, Australia) 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* strain V15/004035, V15/004036 or V15/004037 having the defining characteristics of strain V15/004035, V15/004036 or V15/004037. The invention also relates to *Saccharomyces* yeast deposited under the Budapest Treaty and having NMI accession no. V15/004035, V15/004036 or V15/004037 or a derivative of strain V15/004035, V15/004036 or V15/004037 which exhibits one or more properties or defining characteristics of strain V15/004035, V15/004036 or V15/004037. The invention also relates to compositions comprising a *Saccharomyces* yeast of the invention and naturally occurring and/or non-naturally occurring components.





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PROCESSES FOR PRODUCING ETHANOL AND ETHANOL PRODUCING YEAST

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority benefit of U.S. provisional application serial no. 62/209,698, filed on August 25, 2015; U.S. provisional application serial no. 62/198,864, filed on July 30, 2015; and Australian patent application no. 2015901015, filed on March 20, 2015. The contents of these applications are fully incorporated herein by reference.

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 OF THE INVENTION

The present invention relates to processes, e.g., including a liquefaction step, for producing ethanol from starch-containing material using yeast for converting fermentable sugars into ethanol. The present invention also relates to a *Saccharomyces* strain having improved ability to ferment sugars to ethanol, to methods for the production of *Saccharomyces* 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 the invention relates to compositions comprising a *Saccharomyces* yeast strain of the invention and naturally occurring and/or non-naturally occurring components.

BACKGROUND OF THE INVENTION

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 (around 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 cerevisiae* yeast.

Yeast 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 have the ability to 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 also 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 OF THE INVENTION

The present invention relates to producing ethanol from starch-containing material and yeast suitable for use in such processes.

In the first aspect the invention 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;

5 wherein the fermenting organism is:

Saccharomyces cerevisiae MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

10 *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

15 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

In some embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

20 In context of the invention the terms "properties" and "defining characteristics" of *Saccharomyces* strains V15/004035, V15/004036, and V15/004037 include at least increased ethanol boost (i.e., ethanol yield) compared to Ethanol Red™ under the same process conditions (see Examples 18 and 22). Other "properties" and "defining characteristics" include reduced acetaldehyde production (see Example 23), increased temperature tolerance (see Examples 24 and 25) and decreased glycerol production (see Example 26). A fermenting organism of the invention, e.g., used in a process of the invention may have one or more the above mentioned "properties" and "defining characteristics".

30 According to the process of the invention the fermenting organism, especially *Saccharomyces cerevisiae* yeast, having properties that are about the same as that of

Saccharomyces cerevisiae MBG4930 or a derivative of *Saccharomyces* strain V15/004035 (i.e., *Saccharomyces cerevisiae* MBG4930),

Saccharomyces cerevisiae MBG4931 or a derivative of *Saccharomyces* strain V15/004036 (i.e., *Saccharomyces cerevisiae* MBG4931),

Saccharomyces cerevisiae MBG4932 or a derivative of *Saccharomyces* strain V15/004037 (i.e., *Saccharomyces cerevisiae* MBG4932),

5 having one or more, such as all of the following properties and/or defining characteristics of strain V15/004035, V15/004036, and/or V15/004037:

increased ethanol boost (i.e., ethanol yield) compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Examples 18 or 22;

10 reduced acetaldehyde production compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Example 23 below;

increased temperature tolerance compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Examples 24 or 25);

decreased glycerol production compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Example 26.

15 A fermenting organism of the invention, e.g., used in a process of the invention may have one or more, such as all, of the above mentioned “properties” or “defining characteristics”.

According to the ethanol production process of the invention 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
20 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 an embodiment the alpha-amylase is a thermostable bacterial alpha-amylase. In a preferred embodiment the alpha-amylase is from the genus *Bacillus*, such as a strain of *Bacillus stearothermophilus*, in particular a variant of a *Bacillus stearothermophilus*
25 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
30 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 around 491 amino acids long) at pH 4.5 and 5.5, 0.12 mM CaCl₂ 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 80°C/70°C. 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 a preferred embodiment the protease is the one shown in SEQ ID NO: 1 in US 6,358,726 or SEQ ID NO: 13 herein.

In a preferred 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 around 1.5 or 3 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step i).

In an embodiment of the invention 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 is preferably thermostable.

That means that the glucoamylase has a heat stability at 85°C, 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 an 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 an 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 according to the invention 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 a preferred 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 a preferred 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 an 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 70°C, preferably at least 75°C, such as at least 80°C, such as at least 81°C, such as at least 82°C, such as at least 83°C, such as at least 84°C, such as at least 85°C, such as at least 86°C, such as at least 87%, such as at least 88°C, such as at least 89°C, such as at least 90°C. In an 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 70°C and 95°C, such as between 80°C and 90°C.

In an 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 70°C, preferably at least 75°C, such as at least 80°C, such as at least 81°C, such as at least 82°C, such as at least 83°C, such as at least 84°C, such as at least 85°C, such as at least 86°C, such as at least 87%, such as at least 88°C, such as at least 89°C, such as at least 90°C, such as at least 91°C. In an 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 70°C and 95°C, such as between 80°C and 90°C.

In an 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 an 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, according to the process of the invention also a pullulanase may be present during liquefaction in combination with an alpha-amylase, a protease and/or a glucoamylase.

According to the process of the invention 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 an embodiment the glucoamylase present and/or added in saccharification and/or fermentation is of fungal origin, such as of filamentous fungus origin. In a preferred embodiment the glucoamylase is 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 *Nigrofores*.

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

In an embodiment the glucoamylase is a variant of the *Gloeophyllum trabeum* glucoamylase disclosed in WO2014/177546 (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 a preferred embodiment the glucoamylase is present and/or added in saccharification and/or fermentation in combination with an alpha-amylase and optionally a protease. The alpha-amylase may be of fungal or bacterial origin.

The alpha-amylase present and/or added in saccharification and/or fermentation 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 a preferred embodiment the alpha-amylase is derived from a strain of *Rhizomucor pusillus*, preferably with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably the one disclosed as SEQ ID NO: 16 herein, preferably having one or more of the following substitutions: G128D, D143N, preferably G128D+D143N (using SEQ ID NO: 16 for numbering).

In an embodiment a protease is present and/or added in saccharification and/or fermentation, or SSF. This results 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, in accordance with a process of the invention, to improve the oil yield.

In an embodiment of the invention 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 a preferred 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.

In a second aspect the invention 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 MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain

5 V15/004036

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain

10 V15/004037.

In some embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036

15 having defining characteristics of strain V15/004036.

More specifically the invention 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

20 (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

25 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

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

In a preferred embodiment the fermenting organism used in a process of the invention is *Saccharomyces cerevisiae* MBG4930 (deposited as V15/004035 at National Measurement Institute, Victoria, Australia), MBG4931 (deposited as V15/004036 at National Measurement

Institute, Victoria, Australia), or MBG4932 (deposited as V15/004037 at National Measurement Institute, Victoria, Australia).

In an embodiment the fermenting organism is:

a derivative of *Saccharomyces cerevisiae* MBG4930 having the defining characteristics (i.e., high ethanol yield boost, reduced acetaldehyde production, increased temperature tolerance during ethanol fermentation, and/or decreased glycerol production) of MBG4930 (strain V15/004035);

a derivative of *Saccharomyces cerevisiae* MBG4931 having the defining characteristics (i.e., high ethanol yield boost, reduced acetaldehyde production, increased temperature tolerance during ethanol fermentation, and/or decreased glycerol production) of MBG4931 (strain V15/004036); or

a derivative of *Saccharomyces cerevisiae* MBG4932 having the defining characteristics (i.e., high ethanol yield boost, reduced acetaldehyde production, increased temperature tolerance during ethanol fermentation, and/or decreased glycerol production) of MBG4932 (strain V15/004037).

In some embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

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 Of The Invention" section below.

A third aspect provides a *Saccharomyces* yeast strain deposited under the Budapest Treaty and having NMI accession no. V15/004035, or a derivative of *Saccharomyces* strain V15/004035; V15/004036, or a derivative of *Saccharomyces* strain V15/004036; or V15/004037, or a derivative of *Saccharomyces* strain V15/004037.

A fourth aspect provides a method of producing a derivative of strain V15/004035, V15/004036, or V15/004037 comprising:

(a) culturing a first yeast strain with a second yeast strain, wherein the second yeast strain is strain V15/004035 or a derivative of strain V15/004035, strain V15/004036 or a derivative of strain V15/004036, or strain V15/004037 or a derivative of strain V15/004037, 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.

5 A fifth aspect provides a method of producing a *Saccharomyces* strain having the defining characteristics of strain V15/004035, V15/004036, or V15/004037, comprising:

(a) providing: (i) a first yeast strain; and (ii) a second yeast strain, wherein the second yeast strain is strain V15/004035 or a derivative of strain V15/004035, V15/004036 or a derivative of strain V15/004036, or V15/004037 or a derivative of strain V15/004037;

10 (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 strain V15/004035, V15/004036, or V15/004037;

15 (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 V15/004035, V15/004036, or V15/004037.

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

A seventh aspect provides a *Saccharomyces* strain having the defining characteristics of strain V15/004035, V15/004036, or V15/004037.

20 An eighth 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 ninth aspect provides use of a strain of the third, sixth or seventh aspect in the production of ethanol.

25 A tenth aspect provides a method of producing distiller's grain, comprising:

(a) incubating a *Saccharomyces* strain of the third, sixth or seventh 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.

30 An eleventh aspect provides distiller's grain produced by the method of the ninth aspect.

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

A thirteenth aspect provides use of a strain of the third, sixth or seventh aspect in the production of a *Saccharomyces* strain which exhibits one or more defining characteristics of strain V15/004035, V15/004036, or V15/004037.

5 A fourteenth aspect provides a composition comprising a *Saccharomyces* strain of the third, sixth or seventh aspect.

Finally the invention also relates to compositions comprising a *Saccharomyces* yeast strain of the invention, e.g., MBG4930, MBG4931, MBG4932, or a derivative thereof, and naturally occurring and/or non-naturally occurring components.

10 BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 shows the ethanol titers during 1 L corn mash fermentations, liquefied with a blend of alpha-amylase (2.1 µg EP AA369/gDS), glucoamylase (4.5 µg EP PoAMG498/g DS) and 0.0385 µg EP Pfu/g DS (ER - -■-; MBG4931 - -▲ -- -)

15 Fig. 2 shows the ethanol titers during 500 g corn mash fermentations, liquefied with Alpha-amylase 369 ("AA369") (0.134 KNU-A/gDS).

Fig. 3 shows the acetaldehyde production during at the 12 hour time point in 500 g corn mash fermentations, liquefied with Alpha-amylase 369 ("AA369") (0.134 KNU-A/gDS).

20 Fig. 4 shows the ethanol titers during 1L corn mash fermentations, liquefied with a blend of Alpha-amylase 369 ("AA369") (2.1 µg EP AA369/gDS), PoAMG 498 (4.5 µg EP PoAMG498/g DS) and PFU (0.0385 µg EP PFU/g DS).

Fig. 5 shows the ethanol titers during 1L corn mash fermentations, liquefied with a blend of Alpha-amylase 369 ("AA369") (2.1 µg EP AA369/gDS), PoAMG 498 (4.5 µg EP PoAMG498/g DS) and PFU (0.0385 µg EP PFU/g DS).

25 Fig. 6 shows the glycerol titers during 500 g corn mash fermentations, liquefied with Alpha-amylase 369 ("AA369") (0.134 KNU-A/gDS).

Fig. 7 shows the glycerol production at the 54 hour time point in 500 g corn mash fermentations, liquefied with Alpha-amylase 369 ("AA369") (0.134 KNU-A/gDS).

Fig. 8 shows three fermentation temperature profiles (--◆--- Normal temperature profile), (---■--- Mid stress temperature profile; ---▲--- High stress temperature profile).

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DETAILED DESCRIPTION OF THE INVENTION

Processes Of The Invention

In this aspect the present invention relates to 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 the first aspect the invention 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
5 gelatinization temperature using an alpha-amylase;
- ii) saccharifying using a glucoamylase;
- iii) fermenting using a fermenting organism;

wherein the fermenting organism is:

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004035 at
10 National Measurement Institute, Victoria, Australia) or a fermenting organism strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at
15 National Measurement Institute, Victoria, Australia) or a fermenting organism strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at
20 National Measurement Institute, Victoria, Australia) or a fermenting organism strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

Steps ii) and iii) are carried out either sequentially or simultaneously (SSF). In a
25 preferred embodiment steps ii) and iii) are carried out simultaneously (SSF).

In some embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931
(deposited under Accession No. V15/004036 at National Measurement Institute, Victoria,
Australia) or a fermenting organism having properties that are about the same as that of
Saccharomyces cerevisiae MBG4931 or a derivative of *Saccharomyces* strain V15/004036
30 having defining characteristics of strain V15/004036.

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. According to the invention the nitrogen source may be organic, such as urea, DDGs, wet cake or corn mash, or inorganic, such as ammonia or ammonium hydroxide. In a preferred embodiment the nitrogen source is urea.

5 Liquefaction step i)

According to processes of the invention 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.

10 Liquefaction step i) may be carried out for 0.5-5 hours, such as 1-3 hours, such as typically around 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. In the context of this invention the initial gelatinization temperature of a given starch-containing material may be determined as the temperature at which birefringence is lost in 5% of the starch granules using the method described by Gorinstein and Lii, 1992, *Starch/Stärke* 44(12): 461-466.

20 According to the invention liquefaction is typically carried out at a temperature in the range from 70-100°C. In an embodiment the temperature in liquefaction is between 75-95°C, such as between 75-90°C, preferably between 80-90°C, such as 82-88°C, such as around 85°C.

25 According to the invention 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, preferably 120-140°C, such as 125-135°C, preferably around 130°C for about 1-15 minutes, preferably for about 3-10 minutes, especially around about 5 minutes.

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

In an embodiment, the process of the invention 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
5 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. According to the present invention dry milling is preferred.

In an embodiment the particle size is reduced to between 0.05 to 3.0 mm, preferably 0.1-
10 0.5 mm, or so that at least 30%, preferably at least 50%, more preferably at least 70%, even more preferably at least 90% of the starch-containing material fit through a sieve with a 0.05 to 3.0 mm screen, preferably 0.1-0.5 mm screen. In another embodiment at least 50%, preferably at least 70%, more preferably at least 80%, especially at least 90% of the starch-containing material fit through a sieve with # 6 screen.

15 The aqueous slurry may contain from 10-55 w/w-% dry solids (DS), preferably 25-45 w/w-% dry solids (DS), more preferably 30-40 w/w-% dry solids (DS) of 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 an embodiment only a portion of
20 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 an embodiment the alpha-amylase is a bacterial alpha-amylase. Bacterial alpha-amylases are typically thermostable. In a
25 preferred 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 an embodiment the alpha-amylase has an improved stability compared to a reference alpha-amylase (*Bacillus stearothermophilus* alpha-amylase with the mutations I181*+G182*,
30 optionally with a N193F substitution, truncated to around 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 CaCl₂ 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

5 *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;

10 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

15 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 around 491 amino acids long) at pH 4.5 and 5.5, 0.12 mM CaCl₂ can be found in WO 2011/082425 hereby incorporated by reference. (See also Example 1 below)

20 According to processes of the invention, 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 80°C/70°C determined as described in Example 2 (Relative Activity). Examples of suitable proteases are described below in the section "Protease Present and/or Added During Liquefaction".

25 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

30 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*, preferably a strain of *Pyrococcus furiosus*. In a preferred embodiment the protease is the one shown in SEQ ID NO: 1 in US 6,358,726 or SEQ ID NO: 13 herein.

In an embodiment of the invention 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 is preferably thermostable. This means that the glucoamylase has a heat stability at 85°C, 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 an 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 an 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 stability).

A suitable glucoamylase present and/or added in liquefaction step i) may according to the invention 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 a preferred 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 a preferred 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 process of the invention also 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 present and/or added in saccharification step ii) and/or fermentation step iii) or simultaneous saccharification and fermentation (SSF). The glucoamylase 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
5 step i). In a preferred embodiment the glucoamylase is 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
10 last up to from about 24 to about 72 hours. In an embodiment pre-saccharification is done. Pre-saccharification is typically done for 40-90 minutes at a temperature between 30-65°C, typically around 60°C. Pre-saccharification is in an embodiment followed by saccharification during fermentation in simultaneous saccharification and fermentation (SSF). Saccharification is typically carried out at temperatures from 20-75°C, preferably from 40-70°C, typically around
15 60°C, and typically at a pH between 4 and 5, such as around 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
20 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 according to the invention 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, preferably around about 32°C. In an embodiment fermentation is ongoing for 6 to 120 hours, in particular 24 to 96 hours. In an embodiment the pH
25 is between 4-5.

In an embodiment of the invention 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"-
30 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

According to the invention any suitable starch-containing starting material may be used. The starting material is generally selected based on the desired fermentation product, here ethanol. Examples of starch-containing starting materials, suitable for use in processes of the present invention, 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 a preferred embodiment the starch-containing starting material is corn.

In a preferred embodiment the starch-containing starting material is wheat.

In a preferred embodiment the starch-containing starting material is barley.

In a preferred embodiment the starch-containing starting material is rye.

In a preferred embodiment the starch-containing starting material is milo.

In a preferred embodiment the starch-containing starting material is sago.

In a preferred embodiment the starch-containing starting material is cassava.

In a preferred embodiment the starch-containing starting material is tapioca.

In a preferred embodiment the starch-containing starting material is sorghum.

In a preferred embodiment the starch-containing starting material is rice,

In a preferred embodiment the starch-containing starting material is peas.

In a preferred embodiment the starch-containing starting material is beans.

In a preferred embodiment the starch-containing starting material is sweet potatoes.

In a preferred 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. According to the invention 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 MBG4930, MBG4931, and MBGYYY (deposited under Accession No. V15/004035, V15/004036, and V15/004037 at National Measurement Institute,

Victoria, Australia, respectively) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930, MBG4931, MBGYYY or a derivative of *Saccharomyces* strain V15/004035, V15/004036, or V15/004037 having defining characteristics of strain V15/004035, V15/004036, or V15/004037 may be used in a process of the invention.

5 In an embodiment the fermenting organism has properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930, MBG4931, or MBGYYY as it provides an increase in ethanol yield compared to Ethanol Red™ (ER) under the same process conditions.

In an embodiment the fermenting organism strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930, MBG4931, or MBGYYY has at least the
10 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., the process conditions in Examples 18 or 22;

reduces acetaldehyde production compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Example 23 below;

15 increases temperature tolerance compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Examples 24 or 25); and

decreases glycerol production compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Example 26.

In an embodiment of the invention the fermenting organism having properties that are
20 about the same as that of *Saccharomyces cerevisiae* MBG4930, MBG4931, or MBG4932 provides an ethanol yield boost over ETHANOL RED™ (ER) of more than 1.0%, preferably more than 2.0%, such more than 2.5%, such as around 2.9% using the process set-up and conditions used in Examples 18 or 22.

In an embodiment of the invention the fermenting organism having properties that are
25 about the same as that of *Saccharomyces cerevisiae* MBG4930, MBG4931, or MBG4932 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., the process conditions in Example 23.

30 In an embodiment of the invention the fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930, MBG4931, or MBG4932 increases the temperature tolerance compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Examples 24 or 25.

Increased temperature tolerance is an advantage as the fermentation temperature may fluctuate to some degree. Fig. 8, Tables 28 and 31 in Examples 24 and 25 show a normal fermentation temperature profile (based on general targets that ethanol plants try to run).as well as two temperature profiles that stress yeast compared to a normal temperature profile. 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 around 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 an embodiment of the invention the fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930, MBG4931, or MBG4932 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., the process conditions in Example 26.

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

According to the invention an alpha-amylase is 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. Preferred the alpha-amylase is a bacterial alpha-amylases, which are typically stable at temperature, used during liquefaction.

5 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 according to the invention may, e.g., be derived from a strain of the genus *Bacillus*, which is sometimes also referred to as the genus *Geobacillus*. In an embodiment the *Bacillus* alpha-amylase is derived from a strain of *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus stearothermophilus*, or *Bacillus subtilis*, but
10 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
15 (all sequences are hereby incorporated by reference). In an embodiment the alpha-amylase may be an enzyme having a degree of identity of at least 60%, e.g., at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% to any of the sequences shown in SEQ ID NOS: 3, 4 or 5, respectively, in WO 99/19467.

In an embodiment the alpha-amylase may be an enzyme having a degree of identity of at least 60%, e.g., at least 70%, at least 80%, at least 90%, at least 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.
20

In a preferred 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 around 491 amino acids long, e.g., so that it lacks a functional
25 starch binding domain (compared to SEQ ID NO: 3 in WO 99/19467) or SEQ ID NO: 1 herein.
30

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 (all documents are 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), preferably 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). Even more preferred are *Bacillus* alpha-amylases, especially *Bacillus stearothermophilus* alpha-amylases, which 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 a preferred embodiment the variant is a S242A, E or Q variant, preferably a S242Q variant, of the *Bacillus stearothermophilus* alpha-amylase (using SEQ ID NO: 1 herein for numbering).

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

The bacterial alpha-amylase may in an embodiment be a truncated *Bacillus* alpha-amylase. Especially 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 around 491 amino acids long, such as from 480 to 495 amino acids long, or so it lack 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 a preferred 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. Also
5 preferred are variants having one or more of the following mutations (or corresponding mutations in other *Bacillus* alpha-amylases): H154Y, A181T, N190F, A209V and Q264S and/or the deletion of two residues between positions 176 and 179, preferably the deletion of E178 and G179 (using SEQ ID NO: 5 of WO 99/19467 for position numbering or SEQ ID NO: 21 herein).

In an embodiment the bacterial alpha-amylase is the mature part of the chimeric alpha-
10 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.

15 Thermostable Alpha-Amylase

According to the invention the alpha-amylase may be a thermostable alpha-amylase, such as a thermostable bacterial alpha-amylase, preferably from *Bacillus stearothermophilus*. In an embodiment the alpha-amylase used according to the invention has a T $\frac{1}{2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl₂ of at least 10 determined as described in Example 1.

20 In an embodiment the thermostable alpha-amylase has a T $\frac{1}{2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl₂, of at least 15.

In an embodiment the thermostable alpha-amylase has a T $\frac{1}{2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl₂, of as at least 20.

25 In an embodiment the thermostable alpha-amylase has a T $\frac{1}{2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl₂, of as at least 25.

In an embodiment the thermostable alpha-amylase has a T $\frac{1}{2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl₂, of as at least 30.

In an embodiment the thermostable alpha-amylase has a T $\frac{1}{2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl₂, of as at least 40.

30 In an embodiment the thermostable alpha-amylase has a T $\frac{1}{2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl₂, of at least 50.

In an embodiment the thermostable alpha-amylase has a T $\frac{1}{2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl₂, of at least 60.

In an embodiment the thermostable alpha-amylase has a $T_{1/2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl_2 , between 10-70.

In an embodiment the thermostable alpha-amylase has a $T_{1/2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl_2 , between 15-70.

5 In an embodiment the thermostable alpha-amylase has a $T_{1/2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl_2 , between 20-70.

In an embodiment the thermostable alpha-amylase has a $T_{1/2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl_2 , between 25-70.

10 In an embodiment the thermostable alpha-amylase has a $T_{1/2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl_2 , between 30-70.

In an embodiment the thermostable alpha-amylase has a $T_{1/2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl_2 , between 40-70.

In an embodiment the thermostable alpha-amylase has a $T_{1/2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl_2 , between 50-70.

15 In an embodiment the thermostable alpha-amylase has a $T_{1/2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl_2 , between 60-70.

In an embodiment of the invention the alpha-amylase is an bacterial alpha-amylase, preferably derived from the genus *Bacillus*, especially a strain of *Bacillus stearothermophilus*, in particular 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.

25 In preferred embodiments 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;

30 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;
 5 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;
 10 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;
 15 E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
 E129V+K177L+R179E+S242Q;
 E129V+K177L+R179V+K220P+N224L+S242Q+Q254S;
 K220P+N224L+S242Q+Q254S;
 M284V;
 20 V59A+Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V; and
 V59A+E129V+K177L+R179E+Q254S+ M284V;

In a preferred 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
 25 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
 30 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 around 491 amino acids long, e.g., so that it lacks a functional starch binding domain.

In a preferred 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 an 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 an 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

According to the invention a 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 a preferred embodiment the thermostable protease used according to the invention 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 an 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 of the invention 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 a preferred embodiment the thermostable protease is a variant of a metallo protease as defined above. In an embodiment the thermostable protease used in a process of the invention 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 an 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;
 5 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;
 10 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;
 15 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;
 20 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;
 25 A27K+D79L+S87P+A112P+D142L; and
 D79L+S87P+D142L.

In an preferred 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
 30 following substitutions or combinations of substitutions:

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

In an 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 defined according to the invention.

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

In an 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 another embodiment the thermostable protease is 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: 1 in US patent no. 6,358,726-B1 or SEQ ID NO: 13 herein. The *Pyrococcus furiosus* protease can be purchased from Takara Bio, Japan.

The *Pyrococcus furiosus* protease is a thermostable protease according to the invention. 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 of the invention has a thermostability value of more than 20% determined as Relative Activity at 80°C/70°C determined as described in Example 2.

In an 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 80°C/70°C.

In an 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 80°C/70°C. In an 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 80°C/70°C.

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

In an 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%,
5 more than 40%, more than 50%, more than 60%, more than 70%, more than 80%, more than 90%, more than 100%, more than 110% determined as Relative Activity at 85°C/70°C.

In an 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 85°C/70°C.

10 In an 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 80°C; and/or

In an 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
15 as Remaining Activity at 84°C.

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

In an embodiment the protease may have a thermostability for above 90, such as above 100 at 85°C as determined using the Zein-BCA assay as disclosed in Example 3.

20 In an embodiment the protease has a thermostability above 60%, such as above 90%, such as above 100%, such as above 110% at 85°C as determined using the Zein-BCA assay.

In an embodiment protease has a thermostability 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 85°C as determined using the Zein-BCA assay.

25 In an 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.

30 Glucoamylase Present and/or Added In Liquefaction Step i)

According to the invention a glucoamylase may optionally be present and/or added in liquefaction step i). In a preferred embodiment the glucoamylase is added together with or separately from the alpha-amylase and/or the optional protease and/or pullulanase.

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

5 In an embodiment the glucoamylase has a relative activity pH optimum at pH 5.0 of at least 90%, preferably at least 95%, preferably at least 97%, such as 100% determined as described in Example 4 (pH optimum).

In an 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).

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

In an 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
20 in Example 15 of at least 70°C, preferably at least 75°C, such as at least 80°C, such as at least 81°C, such as at least 82°C, such as at least 83°C, such as at least 84°C, such as at least 85°C, such as at least 86°C, such as at least 87%, such as at least 88°C, such as at least 89°C, such as at least 90°C, such as at least 91°C. In an embodiment the glucoamylase, such as a *Penicillium oxalicum* glucoamylase variant has a thermostability determined as DSC Td at pH
25 4.8 as described in Example 15 in the range between 70°C and 95°C, such as between 80°C and 90°C.

In an 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
30 least 120%, such as at least 125%. In an 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 a specific and preferred embodiment the glucoamylase, preferably of fungal origin, preferably a filamentous fungi, is from a strain of the genus *Penicillium*, especially 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 an embodiment 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.

In a preferred 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 an embodiment the glucoamylase is derived from *Penicillium oxalicum*.

In an 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 a preferred 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 an embodiment these variants have reduced sensitivity to protease degradation.

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

More specifically, in an 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 +
 5 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 +
 10 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 +
 15 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 +
 20 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 +
 25 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 +
 30 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 a preferred 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 according to the present invention include the pullulanases from *Bacillus amyloclavus* 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 according to the present invention included the pullulanases from *Pyrococcus woesei*, specifically from *Pyrococcus woesei* DSM No. 3773 disclosed in WO92/02614.

In an embodiment the pullulanase is a family GH57 pullulanase. In an 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 according to the invention 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 Present And/Or Added in Saccharification and/or Fermentation

Glucoamylase may be present and/or added in saccharification, fermentation or simultaneous saccharification and fermentation (SSF). The glucoamylase may be derived from any suitable source, e.g., derived from a microorganism or a plant. Preferred glucoamylases are of fungal or bacterial origin, selected from the group consisting of *Aspergillus* glucoamylases, in particular *Aspergillus niger* G1 or G2 glucoamylase (Boel et al. (1984), EMBO J. 3 (5), 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 a preferred 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 according to the invention. 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 an 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 a preferred embodiment the glucoamylase is SEQ ID NO: 2 in WO 2011/068803 or SEQ ID NO: 15 herein (i.e. *Gloeophyllum sepiarium* glucoamylase).

In a preferred 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 *Nigrofores*, in particular a strain of *Nigrofores* 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 in an embodiment 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 in an embodiment 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 an embodiment the glucoamylase is added as a blend further comprising an alpha-amylase. In a preferred embodiment the alpha-amylase is a fungal alpha-amylase, especially an acid fungal alpha-amylase. The alpha-amylase is typically a side activity.

In an 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 an 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 an 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 an 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 an embodiment the alpha-amylase may be derived from a strain of the genus *Rhizomucor*, preferably a strain of 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 a preferred 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 an 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 a preferred 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 a preferred 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).

Cellulolytic Enzyme Composition present and/or added during Saccharification and/or Fermentation

According to the invention a cellulolytic enzyme composition may be present in saccharification or fermentation or simultaneous saccharification and fermentation (SSF).

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

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

In an embodiment 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, 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 an 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 an 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

5 *Trichoderma reesei*.

In an 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

10 of the genus *Thielavia*, such as a strain of *Thielavia terrestris*, such as cellobiohydrolase II CEL6A from *Thielavia terrestris*.

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

In an embodiment the cellulolytic enzyme composition comprises a GH61 polypeptide

15 having cellulolytic enhancing activity, a beta-glucosidase, and a CBH I.

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

In an embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition, further comprising *Thermoascus aurantiacus* GH61A

20 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 an 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

25 NO: 30 herein) and *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 29 herein).

In an 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

30 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 a preferred 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

5 homologs thereof.

In an preferred 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 an 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 of the Invention

In a preferred embodiment the invention relates 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 herein);
- ii) saccharifying using a glucoamylase;
- iii) fermenting using a fermenting organism;

wherein the fermenting organism is:

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

5 In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

10 In an embodiment a protease is added in saccharification and/or fermentation or SSF.

In a preferred embodiment the invention relates 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* 15 *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;

20 wherein the fermenting organism is:

Saccharomyces cerevisiae MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

25 *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

30 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute,

Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

In a preferred embodiment the invention relates 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 80°C/70°C, 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 MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

In a preferred embodiment the invention relates 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 $T_{1/2}$ (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 MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

In a preferred embodiment the invention relates 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 $T_{1/2}$ (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 80°C/70°C;

optionally a *Penicillium oxalicum* glucoamylase

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is:

Saccharomyces cerevisiae MBG4930 (deposited under Accession No. V15/004035 at
5 National Measurement Institute, Victoria, Australia) or a strain having properties that are about
the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces*
strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at
National Measurement Institute, Victoria, Australia) or a strain having properties that are about
10 the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces*
strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at
National Measurement Institute, Victoria, Australia) or a strain having properties that are about
the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces*
15 strain V15/004037 having defining characteristics of strain V15/004037.

In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae*
MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute,
Victoria, Australia) or a fermenting organism having properties that are about the same as that
of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036
20 having defining characteristics of strain V15/004036.

In a preferred embodiment the invention relates 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:

25 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;

30 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 *Gloeophyllum*, such as a strain of *Gloeophyllum serpiarium*;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is:

5 *Saccharomyces cerevisiae* MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

10 *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

15 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

20 In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

In a preferred embodiment the invention relates processes for producing ethanol from starch-containing material comprising the steps of:

25 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;

30 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 80°C/70°C, 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 MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

In a preferred embodiment the invention relates 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 80°C/70°C, 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 MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about

the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about
5 the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that
10 of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

In a preferred embodiment the invention relates processes for producing ethanol from starch-containing material comprising the steps of:

i) liquefying the starch-containing material at a temperature above the initial
15 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 80°C/70°C, preferably derived from *Pyrococcus furiosus* and/or *Thermoascus*
20 *aurantiacus*; and

optionally a pullulanase;

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

ii) saccharifying using a glucoamylase;

25 iii) fermenting using a fermenting organism;

wherein the fermenting organism is:

Saccharomyces cerevisiae MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces*
30 strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

5 In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

10 In a preferred embodiment the invention relates 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 T_{1/2} (min) at pH 4.5, 85°C, 0.12 mM CaCl₂ 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 *Nigrofores*;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is:

25 *Saccharomyces cerevisiae* MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

30 *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about

the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

In a preferred embodiment the invention relates 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 T_{1/2} (min) at pH 4.5, 85°C, 0.12 mM CaCl₂ 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 MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that

of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

In a preferred embodiment the invention relates processes for producing ethanol from starch-containing material comprising the steps of:

5 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:

10 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

15 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:

20 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;

25 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;

30 wherein the fermenting organism is:

Saccharomyces cerevisiae MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

5 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

10 In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

15 In a preferred embodiment the invention relates 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:

20 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

25 herein for numbering);

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

optionally a pullulanase; and

30 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

5 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 *Nigrofores*;

iii) fermenting using a fermenting organism;

10 wherein the fermenting organism is

Saccharomyces cerevisiae MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

15 *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

20 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

25 In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

In a preferred embodiment the invention relates processes for producing ethanol from starch-containing material comprising the steps of:

30 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

5 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;

10 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;

15 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;

20 iii) fermenting using a fermenting organism;

wherein the fermenting organism is

Saccharomyces cerevisiae MBG4930 (deposited under Accession No. V15/004035 at
National Measurement Institute, Victoria, Australia) or a strain having properties that are about
the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces*
25 strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at
National Measurement Institute, Victoria, Australia) or a strain having properties that are about
the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces*
strain V15/004036 having defining characteristics of strain V15/004036; or

30 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at
National Measurement Institute, Victoria, Australia) or a strain having properties that are about
the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces*
strain V15/004037 having defining characteristics of strain V15/004037.

In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

In a preferred embodiment the process of the invention 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 around 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 MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

5 *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

10 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

15 In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

In a preferred embodiment the invention relates to processes for producing ethanol from starch-containing material comprising the steps of:

- 20 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;

25 wherein the fermenting organism increases ethanol yield compared to Ethanol Red™ under the same process conditions.

Raw Starch Hydrolysis Process Of The Invention

30 In this aspect the invention concerns improved raw starch hydrolysis processes for producing ethanol using a fermenting organism and yeast strains suitable for use in processes and methods of the invention.

More specifically in this aspect the invention 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 MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

In one aspect the invention 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

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;

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.

5 In a preferred embodiment the fermenting organism used in a process of the invention is *Saccharomyces cerevisiae* MBG4930, MBG4931, or MBG4932 (deposited as V15/004035, V15/004036, and V15/004037, respectively, at National Measurement Institute, Victoria, Australia),

10 A raw starch hydrolysis process of the invention 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;

15 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.

20 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 Of The Invention” section below.

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

30 In a preferred embodiment the following enzymes are present and/or added in saccharification and/or fermentation: *Gloeophyllum trabeum* glucoamylase, preferably 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 a preferred embodiment the alpha-amylase is 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 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.

5 In another preferred embodiment of the process of the invention the following enzymes are 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*
10 *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: 17 herein, preferably one further having one or more of the following substitutions: G128D, D143N, especially G128D+143N.

In another preferred embodiment the following enzymes are present and/or added in
15 saccharification and/or fermentation: *Pycnoporus sanguineus* glucoamylase, preferably the one shown in SEQ ID NO: 18 herein and an alpha-amylase. In a preferred embodiment the alpha-amylase is derived from *Rhizomucor pusillus*, preferably with a linker and starch-binding domain (SBD), in particular 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:
20 16 herein, preferably one further having one or more of the following substitutions: G128D, D143N, especially G128D+D143N.

In an embodiment a protease is present and/or added in saccharification and/or fermentation. In a preferred embodiment the protease is a metallo protease or a serine protease. In an embodiment the metallo protease is derived from a strain of the genus
25 *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 an embodiment a cellulolytic enzyme composition is present and/or added in saccharification and/or fermentation.

30 In a preferred 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, preferably a variant having one of, preferably 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 a preferred 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 an 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 a preferred 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 a preferred embodiment the total dose of cellulytic 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 an 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 a preferred embodiment saccharification step (a) and fermentation step (b) are carried out simultaneously.

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.

Enzymes And Enzyme Blends Used In A Raw Starch Hydrolysis Process Of The Invention

According to the invention a 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 cellulytic 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 an 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 an 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 an 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 an 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 a preferred 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 of the invention is typically a fungal alpha-amylase, such as an acid fungal alpha-amylase. In a preferred embodiment the alpha-amylase is derived from *Rhizomucor*, preferably a *Rhizomucor pusillus* alpha-amylase 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) disclosed as V039 in Table 5 in WO 2006/069290 or SEQ ID NO: 16 herein.

In an 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, especially 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 an 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 an 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 an 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 another preferred 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 an embodiment the following enzymes are present and/or added in saccharification and/or fermentation: *Pycnopus* glucoamylase, in particular the *Pycnopus 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 an especially preferred embodiment the enzymes present and/or added in saccharification and/or fermentation comprises a *Pycnopus* glucoamylase, such as the *Pycnopus 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 of the invention include i) glucoamylase and ii) alpha-amylase; and may optionally further comprise iii) a cellulolytic enzyme composition and/or iv) a protease.

In an 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 an embodiment the protease, in particular 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 an especially preferred 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), 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 an especially preferred 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 an especially preferred embodiment the enzymes present and/or added in saccharification and/or fermentation according to the invention 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 a preferred 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 of the invention at the same time as the glucoamylase and the alpha-amylase. According to the invention 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 an embodiment the glucoamylase and the alpha-amylase are added as one blend composition and the optional cellulolytic enzyme composition and/or 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 of the invention may be of any origin, such as of bacterial or fungal origin. Fungal glucoamylases are preferred.

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

In a preferred embodiment the glucoamylase, such as one derived from a strain of *Pycnopus 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 a preferred 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.

5 In a preferred 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 a preferred embodiment the *Gloeophyllum trabeum* glucoamylase shown in SEQ ID NO: 18 has one of the following substitutions: V59A;
10 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
15 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 of the invention may be of any origin, such as of
20 fungal or bacterial origin. In a preferred 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 an 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
25 the genus *Meripilus*, preferably a strain of *Meripilus giganteus*.

In a preferred 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.

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

In a preferred embodiment the alpha-amylase has the following substitutions: G128D+Y141W+D143N.

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

In a preferred 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 an embodiment the protease is of fungal origin.

In an 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 an 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 an embodiment the protease is of bacterial origin.

In an 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 an 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 a preferred embodiment the cellulolytic enzyme composition comprises cellulolytic enzymes of fungal origin.

In an 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 a preferred 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 an 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 a preferred 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 present invention, cellulolytic enhancing activity is determined by measuring the increase in reducing sugars or the increase of the total of cellobiose and glucose from 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 an 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 an 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 a preferred 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%,
 5 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 a preferred embodiment the GH61 polypeptide, such as one derived from a strain of *Penicillium* sp., is selected from the group consisting of:

- 10 (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.

15 In an 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*.

20 In a preferred 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%,
 25 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 an embodiment the cellulolytic enzyme composition, comprised in an enzyme composition of the invention, comprises a cellobiohydrolase II (CBH II), such as one derived
 30 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 a preferred 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;

5 (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.

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

In an 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.

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

In an 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.

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

In an embodiment the cellulolytic enzyme composition comprises a GH61 polypeptide 25 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 an 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).

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

Trehalase

According to the invention the enzymes present and/or added to saccharification and/or
5 fermentation may optionally further include a trehalase.

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

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

Pectinase

10 According to the invention 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 a preferred embodiment the pectinase is a pectin lyase (EC 4.2.2.10).

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

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

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

20 In an embodiment the pectinase is a combination of pectin lyase and polygalacturonase. In an 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 of the 25 Invention

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

30 In a preferred embodiment the enzymes (e.g., blend) used in a process of the invention 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 a preferred embodiment the enzymes (e.g., blend) used in a process of the invention 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
5 G128D+D143N.

In another preferred embodiment the enzymes (e.g., blend) used in a process of the invention 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*
10 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 a preferred embodiment the enzymes (e.g., blend) used in a process of the invention comprises the *Gloeophyllum sepiarium* glucoamylase shown in SEQ ID NO: 15 herein and an
15 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 a preferred embodiment the enzymes (e.g., blend) used in a process of the invention 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,
20 having one or more of the following substitutions: G128D, D143N, preferably G128D+D143N.

In an embodiment the enzymes (e.g., blend) used in a process of the invention comprises

- i) fungal glucoamylase;
- ii) fungal alpha-amylase;
- iii) cellulolytic enzyme composition derived from a strain of *Trichoderma reesei*, further
30 comprising a GH61 polypeptide, beta-glucosidase, CBH I and CBH II;
- iv) optionally a protease.

In an embodiment the enzymes (blend) used in a process of the invention 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;

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

In an embodiment the enzymes (e.g., blend) used in a process of the invention comprises a

i) *Trametes cingulata* glucoamylase;

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

10 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 an embodiment the enzymes (e.g., blend) used in a process of the invention comprises

15 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*;

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

In an embodiment the enzymes (e.g., blend) used in a process of the invention comprises

i) fungal glucoamylase;

ii) fungal alpha-amylase;

25 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 an embodiment the pectinase is a combination of pectin lyase derived from *Aspergillus niger* and polygalacturonase derived from *Aspergillus aculeatus*.

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

In an embodiment the enzymes (e.g., blend) used in a process of the invention comprises

- i) fungal glucoamylase;
- ii) fungal alpha-amylase;
- iii) pectinase, preferably a pectin lyase or a polygalacturonase, or a combination thereof;
- 5 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 an embodiment the enzymes (e.g., blend) used in a process of the invention comprises a

- i) fungal glucoamylase;
- 10 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 an embodiment the enzymes (e.g., blend) used in a process of the invention

- 15 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
 - 20 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*
 - 25 *furiosus*.

In a preferred embodiment the enzymes (blend) used in a process of the invention 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;
- 30 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.

5 In a preferred embodiment the enzymes (e.g., blend) used in a process of the invention 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;

10 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.

15 In a preferred embodiment the enzymes (e.g., blend) used in a process of the invention comprises

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

20 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;

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

In a preferred embodiment the enzymes (e.g., blend) used in a process of the invention comprises

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

30 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.

5 Examples of Raw Starch Hydrolysis Processes of the Invention

A process of the invention of producing ethanol from starch-containing material comprises:

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

10 (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

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

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

20 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;

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

A process of the invention of producing ethanol from starch-containing material comprises:

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

30 (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;

5 reduces acetaldehyde production;

increased temperature tolerance; and

decreases glycerol production.

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

10 boosts the ethanol yield over ETHANOL RED™ (ER) of more than 1.0%, preferably more than 2.0%, such more than 2.5%, such as around 2.9%, such as between 0.5 and 5%, such as between 1-3%, under the same process conditions;

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%,
15 such as between 5-60%, such as 30-50%, compared to ETHANOL RED™ under the same process conditions;

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

decreases glycerol production by more than 3%, preferably more than 4%, more
20 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.

A process of the invention of producing ethanol from starch-containing material comprises:

25 (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

30 wherein the fermenting organism is:

Saccharomyces cerevisiae MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

5 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a strain having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

10 In some of these embodiments, the fermenting organism is *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036.

15 In a preferred embodiment the process of producing ethanol from starch-containing material of the invention comprises:

- (a) saccharifying a starch-containing material at a temperature below the initial gelatinization temperature; and
- (b) fermenting using a fermentation organism;

wherein

20 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;

25 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

30 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;

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.

5 In a preferred embodiment the process of producing ethanol from starch-containing material of the invention comprises:

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

(b) fermenting using a fermentation organism;

10 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;

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; and

wherein

20 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 defined in Example 18).

25 In a preferred embodiment the process of producing ethanol from starch containing material of the invention 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:

30 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 1.0%, at least 2.0%, at least 2.5%, such as between 0.5-5%, e.g.,
5 between 1-3% under the conditions defined in Example 18 compared to ETHANOL RED™).

In a preferred embodiment the process of producing ethanol from starch-containing material of the invention comprises:

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

10 (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
15 NO: 16 herein, with the following substitutions: G128D+D143N;

iii) cellulosytic 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
20 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% under the conditions defined in Example 18 compared to ETHANOL RED™).

In a preferred embodiment the process of producing ethanol from starch-containing material of the invention comprises:

(a) saccharifying a starch-containing material at a temperature below the initial
25 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
30 NO: 16 herein, with the following substitutions: G128D+D143N;

iii) cellulosytic 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% under the conditions defined in Example 18, compared to ETHANOL RED™).

5

Use of strains V15/004035 (*Saccharomyces cerevisiae* MBG4930), V15/004036 (*Saccharomyces cerevisiae* MBG4931), and V15/004037 (*Saccharomyces cerevisiae* MBG4932), and derivatives thereof

Strain V15/004035 (*Saccharomyces cerevisiae* MBG4930) or a derivative of strain
10 V15/004035; strain V15/004036 (*Saccharomyces cerevisiae* MBG4931) or a derivative of strain V15/004036; and strain V15/004037 (*Saccharomyces cerevisiae* MBG4932) or a derivative of strain V15/004036 may according to the invention be used for increasing the ethanol yield in fermentation.

In an embodiment the liquefied mash, to be fermented, has been subjected to alpha-
15 amylase and from 0.5-50 micro gram protease per gram DS, such as 1-5 micro gram protease per gram DS, such as around 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
20 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
25 *Bacillus stearothermophilus* alpha-amylase, such as the one shown in SEQ ID NO: 1 herein. In a preferred 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;
30 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).

The liquefied mash, to be fermented, has in an embodiment 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 around 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
5 *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 a preferred embodiment the *Penicillium oxalicum* glucoamylase has a K79V substitution (using SEQ ID NO: 14 for numbering) and further one of the following substitutions
10 or combinations of substitutions:

P11F + T65A + Q327F; and

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

Yeast of the Invention

15 The invention relates in one embodiment to a strain of *Saccharomyces cerevisiae* deposited under the Budapest Treaty at the National Measurement Institute (NMI) having deposit accession no. V15/004035 (strain V15/004035), deposit accession no. V15/004036 (strain V15/004036), or deposit accession no. V15/004037 (strain V15/004037).

20 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
25 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.

30 The inventors 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 inventors have further produced strain no. V15/004035, V15/004036, and V15/004037 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*.

For example, strain no. V15/004036 produces greater amounts of ethanol during the first 20 hours of fermentation than ETHANOL RED™ and strain no. V14/004037 during fermentation of corn mash. As described in the Examples, the levels of ethanol produced by strain no. V15/004036 under the conditions encountered during fermentation of corn mash are greater than that of strain V14/004037 and the commercially available industrial yeast strain ETHANOL RED™, in the first 20 hours of fermentation of corn mash. While *Saccharomyces cerevisiae* is resistant to the inhibitory effects of ethanol, bacteria are not and therefore growth of bacteria is inhibited by ethanol production early in the fermentation (e.g. in the first 20 hours). As bacterial growth utilises the fermentable carbohydrates in the substrate, inhibition of bacterial growth allows more fermentable carbohydrate to be available for ethanol production. The production of ethanol from corn mash using strain no. V15/004036 is less likely to be adversely affected by bacterial growth due to the high levels of ethanol produced by strain V15/004036 early in the fermentation.

The strain V15/004036, for example, is a non-recombinant *Saccharomyces cerevisiae* strain which:

- (a) produces a higher titre of ethanol in the first 20 hours of fermentation than strains V14/004037 and ETHANOL RED™, under the same conditions in a corn mash fermentation;
- (b) leaves less glucose remaining following 50 hours of fermentation than ETHANOL RED™ and V14/004037, under the same conditions in a corn mash fermentation;
- (c) has a higher ethanol yield than ETHANOL RED™ following 50 hours of fermentation under the same conditions in a corn mash fermentation.

The defining characteristics of the strains (e.g., strain no. V15/004035, V15/004036, and V15/004037) are:

- (i) the amount of ethanol produced by strain (e.g., V15/004035, V15/004036, and V15/004037) in the first 20 hours of fermentation of corn mash;

(ii) its ethanol yield from glucose following 50 hours of fermentation of corn mash; and

(iii) the amount of glucose remaining (residual glucose) following 50 hours of fermentation of corn mash.

5 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.

10 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*.

15 Additionally, strain V15/004035, V15/004036, and V15/004037 are capable of growth in media in which xylose is the sole carbon source. In this regard, strains V15/004035, V15/004036, and V15/004037 produce more than a 10-fold increase in biomass when grown under the conditions specified in Test T1. As a consequence, the ability of strains V15/004035, V15/004036, and V15/004037 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:

20 (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 strains V15/004035, V15/004036, and V15/004037; and/or do not exhibit more than a 10-fold increase in biomass in Test T1.

25 As current wild type and industrial strains of *Saccharomyces* are not capable of growth on xylose at the rate at which strains V15/004035, V15/004036, and V15/004037 grow on xylose, strains V15/004035, V15/004036, and V15/004037 are readily differentiated from current wild type strains of *Saccharomyces* and strains of *Saccharomyces* that are used in the ethanol industry prior to the present invention such as Ethanol Red.

30 The invention also relates to a derivative of *Saccharomyces* strains V15/004035, V15/004036, and V15/004037. As used herein, a "derivative" of strain V15/004035, V15/004036, or V15/004037 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 V15/004035, V15/004036, or V15/004037 may be a direct progeny (i.e.

the product of a mating between strain V15/004035, V15/004036, or V15/004037 and another strain or itself), or a distant progeny resulting from an initial mating between V15/004035, V15/004036, or V15/004037 and another strain or itself, followed by a large number of subsequent matings.

5 In one embodiment, a derivative of strain V15/004035, V15/004036, or V15/004037 is a hybrid strain produced by culturing a first yeast strain with strain V15/004035, V15/004036, or V15/004037 under conditions which permit combining of DNA between the first yeast strain and strain V15/004035, V15/004036, or V15/004037.

10 In one embodiment, a derivative of strain V15/004035, V15/004036, or V15/004037 may be prepared by:

(a) culturing a first yeast strain with a second yeast strain, wherein the second yeast strain is strain V15/004035, V15/004036, or V15/004037 or a derivative of strain V15/004035, V15/004036, or V15/004037, under conditions which permit combining of DNA between the first yeast strain and the second yeast strain; and

15 (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 V15/004035, V15/004036, or V15/004037.

In one embodiment, the derivative of strain V15/004035, V15/004036, or V15/004037 exhibits one or more defining characteristics of strain V15/004035, V15/004036, or V15/004037.

20 Derivatives of *Saccharomyces* which exhibit one or more defining characteristics of strain V15/004035, V15/004036, or V15/004037 are produced using strain V15/004035, V15/004036, or V15/004037, respectively. In this regard, strain V15/004035, V15/004036, or V15/004037 forms the basis for preparing other strains having the defining characteristics of strain V15/004035, V15/004036, or V15/004037, respectively. For example, strains of

25 *Saccharomyces* which exhibit one or more defining characteristics of strain V15/004036 can be derived from strain V15/004036 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 V15/004035, V15/004036, or V15/004037 which exhibits one or more defining characteristics of strain V15/004035, V15/004036, or

30 V15/004037 may be produced by:

(a) culturing a first yeast strain with a second yeast strain, wherein the second yeast strain is strain V15/004035, V15/004036, or V15/004037 or a derivative of strain V15/004035, V15/004036, or V15/004037, 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 V15/004035, V15/004036, or V15/004037, such as screening or selecting for a derivative with increased ethanol production in corn mash compared to the first strain,;

5 (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 V15/004035, V15/004036, or V15/004037 is obtained which exhibits one or more defining characteristics of strain V15/004035, V15/004036, or V15/004037.

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 V15/004035, V15/004036, or V15/004037. The first yeast strain may be, for example, any 15 *Saccharomyces cerevisiae* strain, such as for example ETHANOL RED™, V09/024011. It will also be appreciated that the first yeast strain may be strain V15/004035, V15/004036, or V15/004037 or a strain which exhibits one or more defining characteristics of strain V15/004035, V15/004036, or V15/004037.

The first and second yeast strains are cultured under conditions which permit combining 20 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; 25 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; 30 (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 V15/004035, V15/004036, or V15/004037 which exhibits one or more defining characteristics of strain V15/004035, V15/004036, or V15/004037, comprises:

(a) providing: (i) a first yeast strain; and (ii) a second yeast strain, wherein the second yeast strain is strain V15/004035, V15/004036, or V15/004037 or a derivative of strain V15/004035, V15/004036, or V15/004037;

(b) sporulating the first yeast strain and the second yeast strain;

5 (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 V15/004035, V15/004036, or V15/004037, 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
10 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.
15 et al., (1997) *Current Protocols in Molecular Biology*, Volume 2, pages 13.2.1 to 13.2.5 (John Wiley & 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
20 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
25 Spencer DM (Eds), Springer Verlag, New York.

In one embodiment, screening or selecting for derivatives of strain V15/004035, V15/004036, or V15/004037 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
30 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:

$$(G \times 0.51) + E$$

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 V15/004035, V15/004036, or V15/004037, 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 V15/004035, V15/004036, or V15/004037, 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 V15/004035, V15/004036, or V15/004037, 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 V15/004035, V15/004036, or V15/004037 which exhibits one or more defining characteristics of strain V15/004035, V15/004036, or V15/004037 may be a mutant of strain V15/004036. 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 V15/004035, V15/004036, or V15/004037 which exhibits one or more defining characteristics of strain V15/004035, V15/004036, or V15/004037 may be a recombinant derivative of strain V15/004035, V15/004036, or

V15/004037. A recombinant derivative of strain V15/004035, V15/004036, or V15/004037 is a strain produced by introducing into strain V15/004035, V15/004036, or V15/004037a 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.

The invention also relates to methods for the production of ethanol using the strain described herein. In one form, strain V15/004035, V15/004036, or V15/004037 or a derivative strain which exhibits one or more defining characteristics of strain V15/004035, V15/004036, or V15/004037 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 V15/004035, V15/004036, or V15/004037 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 ~~amylase~~ 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 25-34°C.

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 known 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

The invention further relates to a method 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 V15/004035, V15/004036, and V15/004037 reduce the level of residual sugars remaining following fermentation, the distiller's grain which results from fermentation using strain V15/004035, V15/004036, or V15/004037 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 V15/004035, V15/004036, or V15/004037 or a derivative of strain V15/004035, V15/004036, or V15/004037, typically having the defining characteristics of strain V15/004035, V15/004036, or V15/004037.

A further aspect provides a composition comprising a *Saccharomyces* strain V15/004035, V15/004036, or V15/004037 or a derivative of strain V15/004035, V15/004036, or V15/004037 and/or a *Saccharomyces* strain having the defining characteristics of strain V15/004035, V15/004036, or V15/004037. 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.

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.

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 of the invention.

5 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
10 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 9×10^5 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
15 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:

$$\text{OD600 at T48} / \text{OD600 at T0}.$$

20 **Compositions of the Invention**

In this aspect the invention relates to a formulated *Saccharomyces* yeast composition comprising a yeast strain of the invention and a naturally occurring and/or a nonnaturally occurring component.

As mentioned above a *Saccharomyces* yeast strain, in particular *Saccharomyces*
25 *cerevisiae* yeast strain, of the invention, may according to the invention may be in any viable form, including crumbled, dry, including active dry and instant, compressed, cream (liquid) form etc. In a preferred embodiment the *Saccharomyces cerevisiae* yeast strain of the invention is dry yeast, such as active dry yeast or instant yeast. In a preferred embodiment the *Saccharomyces cerevisiae* yeast strain of the invention is crumbled yeast. In a preferred
30 embodiment the *Saccharomyces cerevisiae* yeast strain is compressed yeast. In an embodiment the *Saccharomyces cerevisiae* yeast strain of the invention is cream yeast.

In an embodiment the invention relates to a composition comprising a *Saccharomyces* yeast of the invention, in particular *Saccharmyces* MBG4931 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

5 According to the invention the composition may comprise a *Saccharomyces* yeast of the invention, in particular *Saccharmyces* MBG4931 and any suitable surfactants. In an embodiment the surfactant(s) is/are an anionic surfactant, cationic surfactant, and/or nonionic surfactant.

Emulsifier

10 According to the invention the composition may comprise a *Saccharomyces* yeast of the invention, in particular *Saccharmyces* MBG4931 and any suitable emulsifier. In an embodiment the emulsifier is a fatty-acid ester of sorbitan. In an embodiment the emulsifier is selected from the group of sorbitan monostearate (SMS), citric acid esters of monodiglycerides, polyglycerolester, fatty acid esters of propylene glycol.

15 In an embodiment the composition of the invention comprises a *Saccharomyces* yeast of the invention, in particular *Saccharmyces* MBG4931, 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

20 According to the invention the composition may comprise a *Saccharomyces* yeast of the invention, in particular *Saccharmyces* MBG4931 and any suitable gum. In an 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

25 According to the invention the composition may comprise a *Saccharomyces* yeast of the invention, in particular *Saccharmyces* MBG4931 and any suitable swelling agent. In an embodiment the swelling agent is methyl cellulose or carboxymethyl cellulose.

Antioxidant

30 According to the invention the composition may comprise a *Saccharomyces* yeast of the invention, in particular *Saccharmyces* MBG4931, and any suitable anti-oxidant. In an 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.

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 of the invention.

The invention is further described in the following numbered paragraphs:

[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 MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

[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., the process conditions in Examples 18 or 22;

reduced acetaldehyde production compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Example 23;

increased temperature tolerance compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Examples 24 or 25;

decreased glycerol production compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Example 26.

[3]. The process of paragraph [1] or [2], wherein the fermenting organism provides an ethanol yield boost over ETHANOL RED™ (ER) of more than 1.0%, preferably more than 2.0%, such
5 more than 2.5%, such as around 2.9%, such as between 0.5 and 5%, such as between 1-3%,
under the same process conditions, in particular under the process conditions in Examples 18 or 22.

[4]. The process of any of paragraphs [1]-[3], wherein the fermenting organism reduces
acetaldehyde production more than 10%, preferably more than 20%, more preferably more than
10 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.,
the process conditions in Example 23.

[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.,
15 the process conditions in Examples 24 or 25;

[6]. The process of any of paragraphs [1]-[5], wherein the fermenting organism 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., the process
20 conditions in Example 26.

[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 in particular under the process
conditions in Example 21;

25 (b) leaves less glucose remaining following 50 hours of fermentation than ETHANOL
RED™, under the same conditions in a corn mash fermentation, in particular under the process
conditions in Example 21;

(c) has a higher ethanol yield than ETHANOL RED™ following 50 hours of fermentation
under the same conditions in a corn mash fermentation in particular under the process
30 conditions in Example 21.

[8]. The process of any of paragraphs [1]-[7], wherein a protease is added in saccharification or
fermentation or SSF.

[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.

[10]. The process of any of paragraphs [1]-[9], wherein at least 50%, preferably at least 70%, more preferably at least 80%, especially at least 90% of the starch-containing material fit
5 through a sieve with # 6 screen.

[11]. The process of any of paragraphs [1]-[10], wherein the pH in liquefaction is between 4-7, such as between pH 4.5-6.5, such as between pH 5.0-6.5, such as between pH 5.0-6.0, such as between pH 5.2-6.2, such as around 5.2, such as around 5.4, such as around 5.6, such as around 5.8.

10 [12]. The process of any of paragraphs [1]-[11], wherein the temperature in liquefaction is in the range from 70-100°C, such as between 75-95°C, such as between 75-90°C, preferably between 80-90°C, such as 82-88°C, such as around 85°C.

[13]. The process of any of paragraphs [1]-[12], wherein a jet-cooking step is carried out prior to liquefaction in step i).

15 [14]. The process of paragraph [13], wherein the jet-cooking is carried out at a temperature between 110-145°C, preferably 120-140°C, such as 125-135°C, preferably around 130°C for about 1-15 minutes, preferably for about 3-10 minutes, especially around about 5 minutes.

[15]. The process of any of paragraphs [1]-[14], wherein saccharification and fermentation is carried out sequentially or simultaneously (SSF).

20 [16]. The process of any of paragraphs [1]-[15], wherein saccharification is carried out at a temperature from 20-75°C, preferably from 40-70°C, such as around 60°C, and at a pH between 4 and 5.

[17]. The process of any of paragraphs [1]-[16], wherein fermentation or simultaneous saccharification and fermentation (SSF) is carried out at a temperature from 25°C to
25 40°C, such as from 28°C to 35°C, such as from 30°C to 34°C, preferably around about 32°C. In an embodiment fermentation is ongoing for 6 to 120 hours, in particular 24 to 96 hours.

[18]. The process of any of paragraphs [1]-[17], wherein the fermentation product is recovered after fermentation, such as by distillation.

[19]. The process of any of paragraphs [1]-[18], wherein the fermentation product is an alcohol,
30 preferably ethanol, especially fuel ethanol, potable ethanol and/or industrial ethanol.

[20]. The process of any of paragraphs [1]-[19], wherein the starch-containing starting material is whole grains.

[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.

[22]. The process of any of paragraphs [1]-[21], wherein the alpha-amylase used or added in liquefaction step i) is of bacterial origin.

[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.

[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 around 491 amino acids long.

[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).

[26]. The process of any of paragraphs [23]-[25], wherein the *Bacillus stearothermophilus* alpha-amylase has a substitution in position S242, preferably S242Q substitution (using SEQ ID NO: 1 for numbering).

[27]. The process of any of paragraphs [23]-[26], wherein the *Bacillus stearothermophilus* alpha-amylase has a substitution in position E188, preferably E188P substitution (using SEQ ID NO: 1 for numbering).

[28]. The process of any of paragraphs [1]-[27], wherein the alpha-amylase has a T $\frac{1}{2}$ (min) at pH 4.5, 85°C, 0.12 mM CaCl₂) 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 between 10-70, such as between 15-70, such as between 20-70, such as between 25-70, such as between 30-70, such as between 40-70, such as between 50-70, such as between 60-70.

[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;
5 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;
10 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;
15 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*;
20 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;
25 M284V;
V59A+Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V; and
V59A+E129V+K177L+R179E+Q254S+ M284V;

[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).

5 [31]. The process of any of paragraphs [1]-[30], wherein a glucoamylase is present and/or added in saccharification and/or fermentation.

[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 *Talaromyces emersonii*, or a strain of *Pycnoporus*, or a strain of *Gloeophyllum*, such as *Gloeophyllum serpiarium* or *Gloeophyllum trabeum*, or a strain of the *Nigrofores*.

[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,

15 [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.

[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.

25 [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.

[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.

[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.

[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.

[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.

[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.

[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.

[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).

[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, preferably having one or more of the following substitutions: G128D, D143N, preferably G128D+D143N (using SEQ ID NO: 16 for numbering).

[45]. The process of any of paragraphs [39]-[44], wherein the alpha-amylase 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: 16 herein.

[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 80°C/70°C; and
optionally a glucoamylase.

[47]. The process of [46], wherein the protease with a thermostability value of more than 25% determined as Relative Activity at 80°C/70°C.

[48]. The process of paragraphs [46]-[47], wherein 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 80°C/70°C.

[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 80°C/70°C.

[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 80°C/70°C.

[51]. The process of any of paragraphs [46]-[50], wherein 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 than 50%, more than 60%, more than 70%, more than 80%, more than 90%, more than 100%, more than 110% determined as Relative Activity at 85°C/70°C.

[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 85°C/70°C.

[53]. The process of any of paragraphs [46]-[52], wherein the protease has a thermostability above 60%, such as above 90%, such as above 100%, such as above 110% at 85°C as determined using the Zein-BCA assay.

[54]. The process of any of paragraphs [46]-[53], wherein the protease has a thermostability 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 85°C as determined using the Zein-BCA assay.

[55]. The process of any of paragraphs [46]-[54], wherein the protease is of fungal origin.

[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.

[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 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:

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;
 5 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;
 10 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;
 15 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
 20 D79L+S87P+D142L.

[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 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:

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

- [59]. The process of any of paragraphs [46]-[58], wherein the protease variant has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%,
 30 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.

[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;
- 5 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;
- 10 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;
- 15 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;
- 20 Y82F S87G D79L D104P A112P A126V D142L; and
- A27K D79L Y82F S87G D104P A112P A126V D142L.

[61]. The process of any of paragraphs [46]-[60], wherein the protease is of bacterial origin.

[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*.

- 25 [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.

- [64]. The process of any of paragraphs [46]-[63], wherein the protease is one having at least 80%, 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 in SEQ ID NO: 1 in US 6,358,726 or SEQ ID NO: 13 herein.
- 30

[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 around or more than 1.5 micro

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

[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 per gram DS, especially around 3 micro gram *Pyrococcus furiosus* protease per gram DS are present and/or added in liquefaction step i).

[67]. The process of any of paragraphs [1]-[66], wherein a glucoamylase is present and/or added during liquefaction step i).

[68]. The process of paragraph [67], wherein the glucoamylase present and/or added in liquefaction has a heat stability at 85°C, pH 5.3, of at least 20%, such as at least 30%, preferably at least 35%.

[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%.

[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%.

[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.

[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.

[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.

[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:

- 5 T65A;
- Q327F;
- E501V;
- Y504T;
- Y504*;
- 10 T65A + Q327F;
- T65A + E501V;
- T65A + Y504T;
- T65A + Y504*;
- Q327F + E501V;
- Q327F + Y504T;
- 15 Q327F + Y504*;
- E501V + Y504T;
- E501V + Y504*;
- T65A + Q327F + E501V;
- T65A + Q327F + Y504T;
- 20 T65A + E501V + Y504T;
- Q327F + E501V + Y504T;
- T65A + Q327F + Y504*;
- T65A + E501V + Y504*;
- Q327F + E501V + Y504*;
- 25 T65A + Q327F + E501V + Y504T;
- T65A + Q327F + E501V + Y504*;
- E501V + Y504T;
- T65A + K161S;
- T65A + Q405T;
- 30 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;
5 P11F + T65A + Q327W;
P2N + P4S + P11F + T65A + Q327F + E501V + Y504T;
P11F + T65A + Q327W + E501V + Y504T;
T65A + Q327F + E501V + Y504T;
T65A + S105P + Q327W;
10 T65A + S105P + Q327F;
T65A + Q327W + S364P;
T65A + Q327F + S364P;
T65A + S103N + Q327F;
P2N + P4S + P11F + K34Y + T65A + Q327F;
15 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;
20 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;
25 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;
30 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
5 + 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 +
10 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;

15 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;

20 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;

25 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;

30 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.

[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).

[76]. The process of any of paragraphs [67]-[75], wherein the glucoamylase 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: 14 herein.

[77]. The process of any of paragraphs [1]-[76], further wherein a pullulanase is present during liquefaction and/or saccharification.

[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 MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

[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);

5 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:

10 *Saccharomyces cerevisiae* MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

15 *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

20 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

[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*;

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

optionally a *Penicillium oxalicum* glucoamylase;

ii) saccharifying using a glucoamylase;

30 iii) fermenting using a fermenting organism;

wherein the fermenting organism is

Saccharomyces cerevisiae MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties

that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties
5 that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties
10 that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

[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
15 N193F substitution (using SEQ ID NO: 1 for numbering) and having a T_{1/2} (min) at pH 4.5, 85°C, 0.12 mM CaCl₂ of at least 10;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is:

20 *Saccharomyces cerevisiae* MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at
25 National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties
30 that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

[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 T_{1/2} (min) at pH 4.5, 85°C, 0.12 mM CaCl₂ 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 80°C/70°C; and

optionally a *Penicillium oxalicum* glucoamylase;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is:

Saccharomyces cerevisiae MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

[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:

5 *Saccharomyces cerevisiae* MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

10 *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

15 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

[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:

20 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;

25 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);

30 a protease having a thermostability value of more than 20% determined as Relative Activity at 80°C/70°C, 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;

5 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;

10 wherein the fermenting organism is:

Saccharomyces cerevisiae MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

15 *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

20 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

[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:

25 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;

30 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 80°C/70°C, 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 MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

[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 80°C/70°C, preferably derived from *Pyrococcus furiosus* and/or *Thermoascus aurantiacus*;

a *Penicillium oxalicum* glucoamylase having a K79V substitution (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 MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

[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 T_{1/2} (min) at pH 4.5, 85°C, 0.12 mM CaCl₂ 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 *Nigrofores*;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is:

Saccharomyces cerevisiae MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

5 *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

10 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

[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;

15 an alpha-amylase, preferably derived from *Bacillus stearothermophilus* having a double deletion at positions I181 + G182, and optional substitution N193F and having a T_{1/2} (min) at pH 4.5, 85°C, 0.12 mM CaCl₂ of at least 10;
 between 0.5 and 10 micro grams *Pyrococcus furiosus* protease per g DS;
 a *Penicillium oxalicum* glucoamylase; and
 20 optionally a pullulanase;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism:

25 *Saccharomyces cerevisiae* MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

30 *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties

that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

[89]. A process of paragraphs [1]-[88], comprising the steps of:

i) liquefying the starch-containing material at a temperature a temperature between 80-
5 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;

10 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);

15 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;

20 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

25 numbering); and

optionally a pullulanase;

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is:

30 *Saccharomyces cerevisiae* MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

[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 80°C/70°C, 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 *Gloeophyllum*; and a strain of the *Nigrofomes*;

iii) fermenting using a fermenting organism;

5 wherein the fermenting organism is:

Saccharomyces cerevisiae MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

10 *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

15 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

[91]. A process of any of paragraphs [1]-[90], comprising the steps of:

20 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;

25 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);

30 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

5 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;

10 wherein the fermenting organism is:

Saccharomyces cerevisiae MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

15 *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

20 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

[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).

25 [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* MBG4931, or a derivative of *Saccharomyces* strain V15/004035, V15/004036, or V15/004037 having the defining characteristics of strain V15/004035, V15/004036, or V15/004037, respectively, 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., the process conditions in Example 18 or 22;

reduced acetaldehyde production compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Example 23;

increased temperature tolerance compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Examples 24 or 25; and

decreased glycerol production compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Example 26.

[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 1.0%, preferably more than 2.0%, such more than 2.5%, such as around 2.9%, such as between 0.5 and 5%, such as between 1-3%, under the same process conditions, in particular under the process conditions in Examples 18 or 22.

[95]. The process of any of paragraphs [1]-[94], wherein the fermenting organism 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., the process conditions in Example 23.

[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., the process conditions in Examples 24 or 25;

[97]. The process of any of paragraphs [1]-[96], wherein the fermenting organism 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., the process conditions in Example 26.

[98]. The process of any of paragraphs [1]-[97], wherein the fermenting organism is a non-recombinant *Saccharomyces* strain, preferably non-recombinant *Saccharomyces cerevisiae* strain.

[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.

[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., the process conditions in Examples 18 or 22;

5 reduced acetaldehyde production compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Example 23;

increased temperature tolerance compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Examples 24 or 25;

10 decreased glycerol production compared to ETHANOL RED™ under the same process conditions, e.g., the process conditions in Example 26.

[101]. The process of paragraph [100], wherein the fermenting organism is a *Saccharomyces cerevisiae* yeast.

[102]. The process of paragraphs [100] or [101], wherein the fermenting organism is a non-recombinant *Saccharomyces cerevisiae* yeast.

15 [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
20 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;

25 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 around 1.5 or 3 micro gram protease per gram DS;

30 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

5 numbering);

ii) saccharifying using a glucoamylase;

iii) fermenting using a fermenting organism;

wherein the fermenting organism is:

10 *Saccharomyces cerevisiae* MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

15 *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

20 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

[104]. The process of any of paragraphs [100]-[103], wherein the fermenting organism is *Saccharomyces cerevisiae* MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia), or the fermenting organism strain has properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930, or a derivative of

25 *Saccharomyces* strain V15/004035 having the defining characteristics of strain V15/004035; *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia), or the fermenting organism strain has properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931, or a derivative of *Saccharomyces* strain V15/004036 having the defining characteristics of strain V15/004036; or

30 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia), or the fermenting organism strain has properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932, or a derivative of *Saccharomyces* strain V15/004037 having the defining characteristics of strain V15/004037.

[105]. The process of paragraphs [104], wherein a protease is present or added in saccharification and/or fermentation or simultaneous saccharification and fermentation (SSF).

[106]. A *Saccharomyces* yeast strain deposited under the Budapest Treaty and having NMI accession no. V15/004035, V15/004036, or V15/004037, or a derivative of strain V15/004035, V15/004036, or V15/004037.

[107]. A method of producing a derivative of strain V15/004035, V15/004036, or V15/004037, comprising:

- a. culturing a first yeast strain with a second yeast strain, wherein the second yeast strain is strain V15/004035, V15/004036, or V15/004037 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.

[108]. A method of producing a derivative of strain V15/004035, V15/004036, or V15/004037 which exhibits the defining characteristics of strain V15/004035, V15/004036, or V15/004037, comprising:

- (a) providing:
 - (i) a first yeast strain; and
 - (ii) a second yeast strain, wherein the second yeast strain is strain V15/004035, V15/004036, or V15/004037 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 V15/004035, V15/004036, or V15/004037.

[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 V15/004035, V15/004036, or V15/004037.

[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 V15/004035, V15/004036, or V15/004037.

[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.

[112]. A *Saccharomyces* strain produced by the method of any one of paragraphs [107] to [111].

[113]. A *Saccharomyces* strain having the defining characteristics of strain V15/004035, V15/004036, or V15/004037.

5 [113]. 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.

[114]. Use of a strain of any of paragraphs [106], [112] or [113] in the production of ethanol.

[115]. A method of producing distiller's grain, comprising:

- 10 (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.

[116]. Distiller's grain produced by the method of paragraph [115].

15 [117]. Use of a strain of paragraph [106], [112] or [113] in the production of distiller's grains.

[118]. Use of a strain of paragraphs [106], [112] or [113] in the production of a *Saccharomyces* strain having the defining characteristics of strain V15/004035, V15/004036, or V15/004037.

[119]. Use of strain V15/004035 (*Saccharomyces cerevisiae* MBG4930), V15/004036 (*Saccharomyces cerevisiae* MBG4931), or V15/004037 (*Saccharomyces cerevisiae* MBG4932)

20 in the production of a *Saccharomyces* strain having properties that are about the same as that of strain V15/004035, V15/004036, or V15/004037 or which exhibits one or more defining characteristics of strain V15/004035, V15/004036, or V15/004037.

[120]. Use of strain V15/004035 (*Saccharomyces cerevisiae* MBG4930), V15/004036 (*Saccharomyces cerevisiae* MBG4931), or V15/004037 (*Saccharomyces cerevisiae* MBG4932)

25 or a strain having properties that are about the same as that of strain V15/004035, V15/004036, or V15/004037 or a derivative thereof in a process according to any of paragraphs [1]-[119].

[121]. 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.

30 [122]. The composition of paragraph [121], wherein the components are selected from the group consisting of: surfactants, emulsifiers, gums, swelling agents, and antioxidants.

[123]. The composition of paragraph [121] or [122], wherein the *Saccharomyces* yeast is *Saccharomyces* MBG4930, MBG4931, or MBG4932.

[124]. The composition of paragraph [121] or [123], wherein the *Saccharomyces* yeast is in a viable form, in particular in dry, cream or compressed form.

35 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.

MBG4930: *Saccharomyces cerevisiae* yeast (non-recombinant) deposited by Microbiogen Pty Ltd, Unit E2, Lane Cove Business Park, 16 Mars Road, Lane Cove, NSW 2066, Australia under the terms of the Budapest Treaty with the National Measurement Institute, Victoria, Australia) and given the following accession number:

Deposit	Accession Number	Date of Deposit
MBG4930	V15/004035	February 19, 2015

MBG4931: *Saccharomyces cerevisiae* yeast (non-recombinant) deposited by Microbiogen Pty Ltd, Unit E2, Lane Cove Business Park, 16 Mars Road, Lane Cove, NSW 2066, Australia under the terms of the Budapest Treaty with the National Measurement Institute, Victoria, Australia)and given the following accession number:

5	Deposit	Accession Number	Date of Deposit
	MBG4931	V15/004036	February 19, 2015

MBG4932: *Saccharomyces cerevisiae* yeast (non-recombinant) deposited by Microbiogen Pty Ltd, Unit E2, Lane Cove Business Park, 16 Mars Road, Lane Cove, NSW 2066, Australia under the terms of the Budapest Treaty with the National Measurement Institute, Victoria, Australia)and given the following accession number:

10	Deposit	Accession Number	Date of Deposit
	MBG4932	V15/004037	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".

25 For purposes of the present invention 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).

5 **Protease assays**

AZCL-casein assay

A solution of 0.2% of the blue substrate AZCL-casein is suspended in Borax/NaH₂PO₄ 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
10 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.

15 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/NaH₂PO₄ buffer pH 9.0). The increase in OD₄₀₅ at room temperature is monitored as a measure of the protease activity.

20 **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.

25 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.

30 AMG incubation

Substrate: maltose 23.2 mM

Buffer: acetate 0.1 M

pH: 4.30 ± 0.05

Incubation temperature: 37°C ± 1

35 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: $37^{\circ}\text{C} \pm 1$

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 dextrans and oligosaccharides with different chain lengths. The intensity of color formed with iodine is directly proportional to the concentration of starch. Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under the specified analytical conditions.

ALPHA - AMYLASE $\lambda = 590 \text{ nm}$

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 (I₂): 0.03 g/LCaCl₂: 1.85 mMpH: 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)

5 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
10 standard.

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

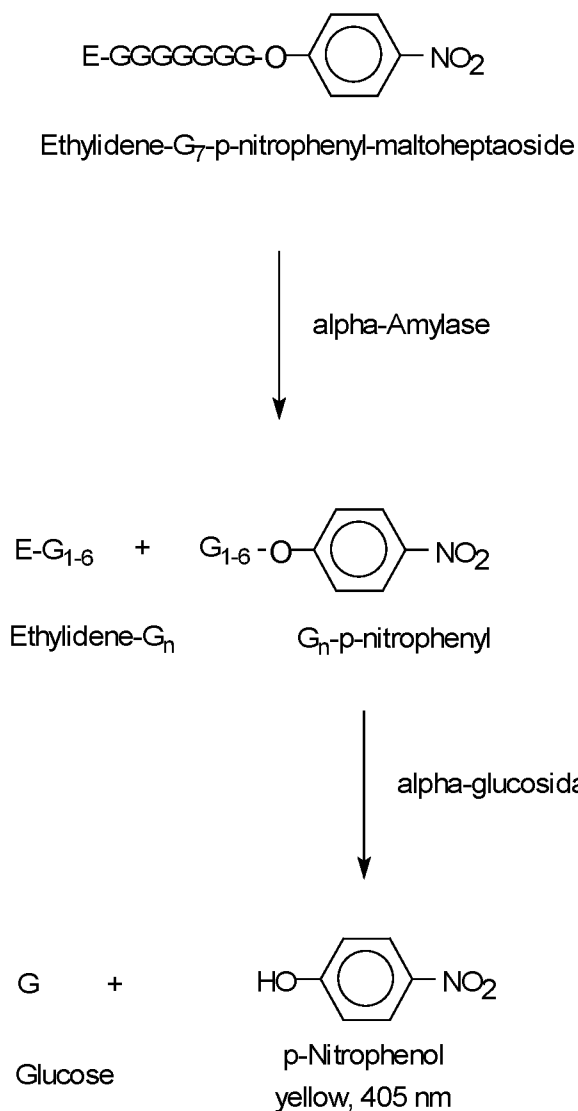
15 A folder EB-SM-0009.02/01 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

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.

20 Alpha amylase in samples and α -glucosidase in the reagent kit hydrolyze the substrate (4,6-ethylidene(G₇)-*p*-nitrophenyl(G₁)- α ,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.



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 ₇ PNP, 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 activity 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 (*stearothermophilus*), 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, 40°C, 20 minutes). The activity is measured in NPUN/ml using red pullulan.

1 mL diluted sample or standard is incubated at 40°C 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 40°C 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 CaCl₂ 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 CaCl₂, 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 CaCl₂, 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 CaCl₂, 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 ($T_{1/2}$ (min)) was calculated using the equation:
 $T_{1/2}$ (min) = $T(\text{min}) \cdot \ln(0.5) / \ln(\%RA/100)$, where T is assay incubation time in minutes, and
 5 %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	$T_{1/2}$ (min) (pH 4.5, 75°C, 0.12 mM CaCl ₂)	$T_{1/2}$ (min) (pH 4.5, 85°C, 0.12 mM CaCl ₂)	$T_{1/2}$ (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	91	15	ND

the substitutions V59A+Q89R+E129V+ K177L+R179E+K220P+N224L+ Q254S+Y276F			
Reference Alpha-Amylase A with the substitutions V59A+E129V+ R157Y+K177L+R179E+K220P+ N224L+S242Q+Q254S	141	41	ND
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	57	11	402

the substitutions E129V+K177L+R179E			
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

5 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
10 (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

15 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 % tryptophan 10 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 H₂O
20 (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 ZnSO₄.

PEG/LiAc solution: 40 % PEG4000 50 ml, 5 M Lithium Acetate 1 ml.

25 96 well Zein micro titre plate:

Each well contains 200 microL of 0.05-0.1 % of zein (Sigma), 0.25 mM ZnSO₄ 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
30 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 °C, and 200 rpm followed by 30 min at 42 °C (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 °C. Pour the suspension to SC-glucose plates and incubate 30 °C 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 ₂ O	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

5

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.

10 **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 28°C for 3 days. The culture supernatants were applied to a 96-well zein micro titer plate and incubated at at least 2 temperatures (ex. 60°C and 65°C, 70°C and 75°C, 70°C and 80°C) for more than 4 hours or overnight. The turbidity of zein
 15 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
 20 cultivated at 28°C for 3 days. Protease activity was measured at 65°C 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 (80°C or 84°C with 4°C 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.

25 **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 ZnSO₄) 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 (Tang). 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

- 5 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
10 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.

15 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%

20 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.

Variant	Substitutions	Relative activity	
		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.

5 **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).

10 **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.

15 **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

20 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.

25 **Heat stability.** To assess the heat stability of the *Penicillium oxalicum* glucoamylase the Reaction condition assay was modified 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 CaCl₂, 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 that 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 modified 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.

5

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.

10

Example 6

Cloning of *Penicillium oxalicum* strain glucoamylase gene

15 Preparation of *Penicillium oxalicum* strain cDNA.

The cDNA was synthesized by following the instruction of 3' Rapid Amplification of cDNA End System (Invitrogen Corp., Carlsbad, CA, USA).

Cloning of *Penicillium oxalicum* strain glucoamylase gene.

20 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 Amplification 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 MgCl₂, 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.

30

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),
5 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

10 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)

15 Primer R: 5' AGATCTCGAGAAGCTTAAACTGCCACACGTCGTTGG (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
20 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
25 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
30 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 CaCl₂, 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 AATTGGAAAGACTGCCCCG (SEQ ID NO: 26)

Primer F-NP003940: 5' ACACAACTGGGGATCCACCATGCGTCTCACTCTATTATC (SEQ ID NO: 27)

Primer R-NP003940: 5' AGATCTCGAGAAGCTTAAACTGCCACACGTCGTTGG (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	H ₂ O	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	4	72°C	90 sec
(K79V F + Primer R-NP003940, K79V R +	2-4	25 cycles	
Primer F-NP003940)	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 CaCl₂, 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, H₂O 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	N.D.	10%	60%	90%	N.D.	N.D.	N.D.	N.D.	N.D.

(ca. 60 kDa)									
--------------	--	--	--	--	--	--	--	--	--

N.D.: not detected.

Example 11

5 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.

10

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
15 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
20 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).

25 Table 14

Relative specific activity	AGU/mg
<i>Penicillium oxalicum</i> wt	100 %
<i>Penicillium oxalicum</i> 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
5 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
10 800 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
15 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
20 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.
30 100 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).

T_m-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	T _m (Deg. Celsius) +/-0.4
PO-AMG (PE001)	80.3
Variant Q327F	82.3
Variant T65A	81.9

Table 15b.

Sample	T _m (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, T_d (°C), was taken as the top of the denaturation peak (major endothermic peak) in thermograms (C_p 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 10°C 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 Na₂CO₃. 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 Na₂CO₃

5 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. = $(\text{Abs}_{\text{unstressed}} - (\text{Abs}_{\text{unstressed}} - \text{Abs}_{\text{stressed}}))/\text{Abs}_{\text{unstressed}} * 100\%$) was calculated and plotted relative to benchmark, Po-amg0008.

10 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	110

	Q327F E501V Y504T	
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
5 Glucoamylase activity on culture supernatants using the pNPG assay described in Example 16.

Example 18**Increased Ethanol Titters in corn mash produced industrially using a liquefaction blend.**

Ethanol production when using MBG4931 compared to ETHANOL RED™ was
10 evaluated in industrially prepared corn mash liquefied with a blend of alpha-amylase (2.1 µg EP
AA369/gDS), glucoamylase (4.5 µg EP PoAMG498/g DS) and 0.0385 µg EP Pfu/g DS. .

Corn mash

Industrially prepared corn mash was obtained from Trenton Agri Products LLC. Solids on
this mash were measured to be 31.02 % by moisture balance.

Yeast Strains and Propagation

The two yeast strains tested in this experiment were ETHANOL RED ("ER")(Fermentis)
and MBG4931.

The target solids percentage in the propagation was 20%, 322 ml of mash was added to
178 ml of water to reach a 500 ml propagation volume at 20% solids. Lactrol was added at a
20 concentration of 0.024 grams per liter. Urea nitrogen was added at a concentration of 1500
ppm, by adding 1.5 ml of a 50% urea solution. Glucoamylase dose was calculated to be 0.037 g
per 500 ml fermenter. As an inoculum, 2.75 grams of dried yeast were weighed out, added to
50 ml of water preheated to 36.5°C, and allowed to rehydrate for 30 minutes with swirling at 15

minutes. 5.99 ml of this rehydration was then added to the propagation. Propagation time was 8 hours at 33.3°C, at which time 10 ml of propagation was transferred to the fermentation vessels as an approximately 2% inoculation.

All propagations were run in 500 ml stirred glass kettle reactors.

5 *Simultaneous Saccharification and Fermentation (SSF)*

All fermentations were run in 1L Sartorius Q+ bioreactors. Lactrol was added to each fermenter at a concentration of 0.024 grams per liter.

Urea was added to 600 ppm total urea. Glucoamylase SA was dosed to each reactor at 110 µg EP GSA/gDS. To mimic enzyme addition at the plant scale, 30% of the glucoamylase and 100% of the fermentation urea were dosed at inoculation. After 8 hours of fermentation, the remaining 70% of glucoamylase was added to the fermenter.

Temperature Profiles

All fermentations started at 32°C and then started a temperature profile as described below.

Table 19: Temperature Profiles

Ferm Time	Temp Target F	Temp Target C
10	92	33.3
18	93	33.9
25	91	32.8
35	89	31.7
45	89	31.7
60	88	31.1

15 *HPLC analysis*

Fermentation sampling took place by sampling 5 grams of mash into 15 ml tubes at 4, 21, 29, 46, and 54 hours of fermentation. Each tube was processed for HPLC analysis by deactivation with 150 µL of 40% v/v H₂SO₄, vortexing, centrifuging at 1460×g for 10 minutes, and filtering through a 0.45 µm Whatman PP filter. Samples were stored at 4°C prior to and during HPLC analysis.

Table 20: HPLC System

HPLC System	Agilent's 1100/1200 series with Chem station software Degasser, Quaternary Pump, Auto-Sampler, Column Compartment /w Heater Refractive Index Detector (RI)
Column	Bio-Rad HPX- 87H Ion Exclusion Column 300mm x 7.8mm part# 125-0140 Bio-Rad guard cartridge Cation H part# 125-0129, Holder part# 125-0131
Method	0.005M H ₂ SO ₄ mobile phase Flow rate: 0.6 ml/min

Column temperature: 65°C RI detector temperature: 55°C

Results

Table 21 below shows the final ethanol titers for 1 L corn mash fermentations, liquefied with a blend of alpha-amylase (2.1 µg EP AA369/gDS), glucoamylase (4.5 µg EP PoAMG498/g DS) and 0.0385 µg EP Pfu/g DS. MBG4931 showed a 2.9% boost over ETHANOL RED™.

Table 21

Strain	Ethanol Titer at 54 hours
ETHANOL RED™ (Fermentis)	12.66%
MBG4931	13.02%

Figure 1 shows the ethanol titers during 1 L corn mash fermentations, liquefied with a blend of alpha-amylase (2.1 µg EP AA369/gDS), glucoamylase (4.5 µg EP PoAMG498/g DS) and 0.0385 µg EP Pfu/g DS. (ER - ■- -; MBG4931 - ▲ - -)

Example 19

Production of strains V15/004035 (MBG4930), V15/004036 (MBG4931), and V15/004037 (MBG4932)

Saccharomyces strains capable of utilising xylose as a sole carbon source were produced using the method described in WO 2005/121337. 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. Of those strains identified, strains with the highest ethanol yield were strains V14/004037, V15/004035, V15/004036, and V15/004037.

The strains were verified to be a *Saccharomyces cerevisiae* strains by their ability to sporulate and produce progeny when the germinated spores were mated with standard strains of *Saccharomyces cerevisiae*, including tester strains of *Saccharomyces cerevisiae*. One such haploid tester strain is W303-1A. Specifically, germinated spores of strains V14/004037 and V15/004036 were able to produce hybrid progeny when mated with tester strain W303-1A.

In more detail, haploid strain W303-1A was obtained from the Yeast Genetic Stock Center at the ATCC, USA (ATCC #208352) Strains V14/004037 and V15/004036 was cultured to form haploid *Saccharomyces* yeast as described in Ausubel, F.M. et al. (1997), Current Protocols in Molecular Biology, Volume 2, pages 13.2.1 to 13.2.5, published by John Wiley & Sons. Subsequently, the spores were germinated on a solid medium such as GYP containing

1% w/v D-glucose, 0.5% yeast extract, 1% w/v bacteriological peptone and 1.5% w/v agar and incubated at 30°C for three to five days. The isolated germinated spores from strains V14/004037 and V15/004036 were then mated together with haploid W303-1A using the method described in, for example, Ausubel, F.M. et al. (1997), Current Protocols in molecular
5 Biology, Volume 2, pages 13.2.1 to 13.2.5, published by John Wiley & Sons. Formation of hybrid zygotes could be observed under a microscope demonstrating that both strains V14/004037 and V15/004036 are *Saccharomyces cerevisiae* strains.

Strain V14/004037 was deposited on 17 February 2014 at the National Measurement Institute, 1/153 Bertie Street, Port Melbourne, Victoria 3207, Australia under the Budapest Treaty and
10 was designated accession number V14/004037.

Strain V15/004035 was deposited on 19 February 2015 at the National Measurement Institute, 1/153 Bertie Street, Port Melbourne, Victoria 3207, Australia under the Budapest Treaty and was designated accession number V15/004035.

Strain V15/004036 was deposited on 19 February 2015 at the National Measurement Institute,
15 1/153 Bertie Street, Port Melbourne, Victoria 3207, Australia under the Budapest Treaty and was designated accession number V15/004036.

Strain V15/004037 was deposited on 19 February 2015 at the National Measurement Institute, 1/153 Bertie Street, Port Melbourne, Victoria 3207, Australia under the Budapest Treaty and was designated accession number V15/004037.

20 Example 20

Growth of strain V15/004036 on xylose minimal media

Growth of strain V15/004036 on xylose as a sole carbon source was determined using Test T1. *Saccharomyces cerevisiae* strain V15/004036 was streaked onto 2% w/v D-glucose
25 1% bacteriological peptone and 0.5% yeast extract medium (GYP) solidified with 2% agar using standard microbiological techniques. After incubation for 72 hours at 30 deg Celsius, yeast cells were 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. An OD₆₀₀ of 0.1 unit is equal to approximately 9×10^5 yeast cells/ mL. The broth contained xylose (5% w/v),
30 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. Citric acid and trisodium citrate were provided as buffering agents that are not able to be used as growth substrates by *Saccharomyces*. D-(+)-Xylose 99% pure was obtained from Sigma-Aldrich (catalogue number X1500-500G). Cultures were incubated at 30 deg Celsius with shaking at 220 rpm (10 cm

orbital diameter) for 48 hours prior to measuring OD₆₀₀ (OD₆₀₀ at T_{48hrs}). The fold increase in biomass was determined by the equation: OD₆₀₀ at T_{48hrs} divided by OD₆₀₀ at T₀.

Strain V15/004036 was inoculated at an initial OD₆₀₀ of 0.149 and increased more than 10-fold in 48 hours. Under the same conditions the biomass of V14/004037 increased 7-fold, and ETHANOL RED yeast increased less than 2-fold.

Example 21

Fermentation of Corn Mash

Corn mash may be obtained from ethanol-producing companies such as described in Devantier et al., Applied Microbiology and Biotechnology 2005, 68:622-629. A method for preparing corn mash is also described in Thomas et al., Journal of Applied Microbiology 2001, 90:819-828.

Corn mash can also be prepared as follows:

Depending on the desired corn mash dry matter target, the following ingredients are placed into a glass beaker and the total weight of ingredients plus beaker is recorded.

Table 22

CORN MASH % dry matter (DM)	UREA	STILLAGE BACKSET	WATER	GROUND CORN	α-AMYLASE
30	0.6g	162g	231g	207g	1.34ml
31	0.6g	162g	224g	214g	1.34ml
32	0.6g	162g	217g	221g	1.34ml
33	0.6g	162g	210g	228g	1.34ml

Amylase may be for example, LIQUOZYME™ SC (Novozymes, Bagsvaerd, Denmark). The slurry is continuously stirred at 85°C for 3.5 hours. The mash is then cooled, and the mass of beaker is weighed and compensated with water to account for evaporation during cooking of mash based on original weight of beaker and ingredients. Mash is cooled to 32°C and adjusted to pH 5.2.

Glucoamylase is added. Glucoamylase may be for example SPIRIZYME EXCEL™ (Novozymes) and is dosed at 0.05% of dry corn solids. The mash is mixed, then dispensed in 15 g aliquots into 50 mL plastic screw capped tubes. The mash samples are placed in a static incubator at the desired temperature (typically 32°C) for 30 min prior to addition of yeast. Yeast is prepared by suspending 0.1 g active dried yeast in 5 mL water at 37°C and leaving static for

30 min. After vortex mixing to disperse the yeast evenly, 190 microlitres of the suspended yeast is inoculated per 15 g of corn mash prepared as described above.

The inoculated corn mash is incubated static for 20, 44 or 50 hours and assayed by HPLC as described in WO 2011/035392.

5 The % w/v of ethanol and residual glucose in the fermentation substrate were determined at 20 hours (Table 23), and ethanol, glycerol, acetate, glucose and maltose in the fermentation substrate were determined at 44 hrs (Table 24) and 50 hours (Table 25) of fermentation.

10 All yeasts were active dry yeasts. Ethanol Red is a commercial sample from Fermentis, BP 3029-137 rue Gabriel Péri, F-59703 Marcq-en-Baroeul, Cedex France.

V14/004037 and V15/004036 were grown and dried as described in WO 2011/035392. A representative sample of ETHANOL RED™ was deposited on 19 March 2014 under the Budapest Treaty at the National Measurement Institute, 1/153 Bertie Street, Port Melbourne, Victoria 3207 and designated accession no. V14/007039.

15 Table 23. Fermentation of corn mash after 20 hours

32%(DM)CORN MASH FERMENTATION 20 hours	Glucose %w/v	Ethanol %w/v
ETHANOL RED (V14/007039)	6.410	8.008
V14/004037	7.678	7.504
V15/004036	5.566	8.574

20 As can be seen from Table 21, after 20 hours of fermentation of corn mash, strain V15/004036 produced greater amounts of ethanol than Ethanol Red and strain V14/004037. Thus, strain V15/004036 had better fermentation performance than both strains Ethanol Red and strain V14/004037 in the first 20 hours of fermentation.

Table 24: Fermentation of corn mash after 44 hours

32%(DM)CORN MASH FERMENTATION 44 hours	Maltose %w/v	Glucose %w/v	Fructose %w/v	Glycerol %w/v	Acetate %w/v	Ethanol %w/v
ETHANOL RED (V14/007039)	0.385	0.689	0.149	1.717	0.058	13.239
V14/004037	0.322	0.598	0.116	1.515	0.154	13.612
V15/004036	0.291	0.302	0.100	1.489	0.098	13.733

25 As can be seen from Table 22, after 44 hours of fermentation of corn mash, strain V15/004036 produced greater amounts of ethanol than ETHANOL RED™ and strain

V14/004037. In addition, strain V15/004036 had lower amounts of fermentable sugars maltose, fructose and glucose remaining.

Table 25: Fermentation of corn mash for 50 hours

32%(DM)CORN MASH FERMENTATION 44 hours	Maltose %w/v	Glucose %w/v	Fructose %w/v	Glycerol %w/v	Acetate %w/v	Ethanol %w/v	Ethanol Yield
ETHANOL RED (V14/007039)	0.352	0.061	0.107	1.723	0.061	13.688	13.719
V14/004037	0.282	0.119	0.093	1.518	0.206	13.978	14.038
V15/004036	0.240	0.051	0.088	1.488	0.109	13.988	14.014

As can be seen from Table 25, after 50 hours of fermentation of corn mash, strain V15/004036 had a greater ethanol yield and produced greater amounts of ethanol than ETHANOL RED™, had a similar ethanol yield and produced similar amounts of ethanol to strain V14/004037, and had less residual glucose, fructose and maltose than ETHANOL RED™ and strain V14/004037.

These results show that the rate of ethanol production by strain V15/004036 is significantly greater than both ETHANOL RED™ and strain V14/004037 at 20 hours of fermentation of corn mash, indicating that strain V15/004036 is more efficient at ethanol production during the early stages of fermentation of corn mash. This is an important characteristic as early and rapid ethanol production can reduce or prevent the growth of contaminating microorganisms during large scale fermentation. This characteristic may also be advantageous by reducing the time necessary for fermentation.

In addition, conversion of the residual glucose to ethanol at maximum theoretical levels (0.51g ethanol/g glucose) indicates the ethanol yield of strain V15/004036 is better than ETHANOL RED™ and is similar to strain V14/004037.

Example 22

Increased Ethanol Titrers in corn mash produced industrially using a liquefaction blend

Ethanol production when using MBG4931 compared to ETHANOL RED™ was evaluated in industrially prepared corn mash liquefied with Alpha-amylase 369 ("AA369") (0.134 KNU-A/g DS).

Corn mash

Industrially prepared corn mash was obtained from GPRE Central City (Iowa). Solids on this mash were measured to be 32.35% by Mettler-Toledo HB43-S moisture balance.

Yeast Strains and Propagation

The two yeast strains tested in this experiment were ETHANOL RED™ (Fermentis) and MBG4931. The target solids percentage in the propagation was 20%, 142 g of mash was added to 87 ml of water to reach a 230 g propagation volume at 20% solids. LACTROL™ was added at a concentration of 3 ppm. Urea nitrogen was added at a concentration of 2000 ppm, by adding 0.9 ml of a 50% (w/v) urea solution. Glucoamylase SA ("GSA") dose was calculated to be 0.0075% (v/w) per 70 g mash in a sterile 125 ml baffled Erlenmeyer flask. As an inoculum, 2.00 grams of dried yeast were weighed out, added to 40 ml of water preheated to 35°C, and allowed to rehydrate for 20 minutes with swirling at 10 minutes. 2% (v/w) of this rehydration was then added to the propagation. Propagation time was 8 hours at 33.3°C, 150 rpm at which time 8 ml of propagation was transferred to round bottom fermentation vessels containing approximately 500 g liquefied mash as an approximately 1.6% (v/w) inoculation.

Simultaneous Saccharification and Fermentation (SSF)

All fermentations were run in 500mL round bottom glass vessels (Prism Research, RTP, NC).

Lactrol was added to each fermenter at a concentration of 3 ppm.

Urea was added to 600 ppm total urea. Glucoamylase SA ("GSA") was dosed to each reactor at 0.6 AGU/gDS. To mimic enzyme addition at the plant scale, 45% of the glucoamylase and 100% of the fermentation urea were dosed at inoculation. After 6 hours of fermentation, the remaining 55% of glucoamylase was added to the fermenter. All fermentations started at 32°C

HPLC analysis

Fermentation sampling took place by sampling 5 grams of mash into 15 ml tubes at 0, 6, 12, 25, 30, 36, 49 and 54 hours of fermentation. Each tube was processed for HPLC analysis by deactivation with 150 µL of 40% v/v H₂SO₄, vortexing, centrifuging at 1460×g for 10 minutes, and filtering through a 0.45 µm Whatman PP filter. Samples were stored at 4°C prior to and during HPLC analysis.

Table 26: HPLC System

HPLC System	Agilent's 1100/1200 series with Chem station software Degasser, Quaternary Pump, Auto-Sampler, Column Compartment /w Heater Refractive Index Detector (RI)
Column	Bio-Rad HPX- 87H Ion Exclusion Column 300mm x 7.8mm part# 125-0140 Bio-Rad guard cartridge Cation H part# 125-0129, Holder part# 125-0131
Method	0.005M H ₂ SO ₄ mobile phase Flow rate: 0.6 ml/min Column temperature: 65°C

RI detector temperature: 55°C

Results

Table 27 below shows the final ethanol titers for 500 g corn mash fermentations, liquefied with Alpha-amylase 369 ("AA369") (0.134 KNU-A/gDS). MBG4931 showed a 2.04% boost over

Table 27

Strain	Ethanol Titer at 54 hours
Ethanol Red (Fermentis)	12.97%
MBG4931	13.24%

Fig. 2 shows the ethanol titers during 500 g corn mash fermentations, liquefied with Alpha-amylase 369 ("AA369") (0.134 KNU-A/gDS).

Example 23

Decreased Production of acetaldehyde in corn mash produced industrially using a liquefaction blend

Ethanol production when using MBG4931 compared to ETHANOL RED™ was evaluated in industrially prepared corn mash liquefied with Alpha-amylase 369 ("AA369") (0.134 KNU-A/gDS).

Corn mash

Industrially prepared corn mash was obtained from GPRE Central City (Iowa). Solids on this mash were measured to be 32.35% by Mettler-Toledo HB43-S moisture balance.

Yeast Strains and Propagation

The two yeast strains tested in this experiment were Ethanol Red (Fermentis) and MBG4931.

The target solids percentage in the propagation was 20%, 142 g of mash was added to 87 ml of water to reach a 230 g propagation volume at 20% solids. Lactrol was added at a concentration of 3 ppm. Urea nitrogen was added at a concentration of 2000 ppm, by adding 0.9 ml of a 50% (w/v) urea solution. Glucoamylase SA ("GSA") dose was calculated to be 0.0075% (v/w) per 70 g mash in a sterile 125 ml baffled Erlenmeyer flask. As an inoculum, 2.00 grams of dried yeast were weighed out, added to 40 ml of water preheated to 35°C, and allowed to rehydrate for 20 minutes with swirling at 10 minutes. 2% (v/w) of this rehydration was then added to the propagation. Propagation time was 8 hours at 33.3°C, 150 rpm at which time 8 ml of

propagation was transferred to round bottom fermentation vessels containing approximately 500 g liquefied mash as an approximately 1.6% (v/w) inoculation.

Simultaneous Saccharification and Fermentation (SSF)

All fermentations were run in 500mL round bottom glass vessels (Prism Research, RTP, NC). Lactrol was added to each fermenter at a concentration of 3 ppm.

Urea was added to 600 ppm total urea. Glucoamylase SA ("GSA") was dosed to each reactor at 0.6 AGU/gDS. To mimic enzyme addition at the plant scale, 45% of the glucoamylase and 100% of the fermentation urea were dosed at inoculation. After 6 hours of fermentation, the remaining 55% of glucoamylase was added to the fermenter. All fermentations started at 32°C

GC analysis

Fermentation sampling took place by sampling 5 grams of mash into 15 ml tubes. Each tube was processed for GC analysis by deactivation with 150 µL of 40% v/v H₂SO₄, vortexing, centrifuging at 1460×g for 10 minutes at 4°C, and filtering through a 0.45 µm Whatman PP filter. Samples were stored at -20°C prior to and during HPLC analysis.

Table 28: GC System

GC System	Shimadzu GC-2010 with AOC-5000 Plus autosampler Degasser, Quaternary Pump, Auto-Sampler, Column Compartment /w Sample heater set to 55°C Split/Splitless Injector Flame Ionization Detector (FID)
Column	Zebron Phase ZB-624 L = 30m x ID = 0.25mm x df = 1.40 µm Helium gas carrier, 136 kPa
Method	Injector in split mode with ratio = 20, set temperature = 200°C Column held at 35°C, 2 min with ramping to 100°C (40°C/min), hold at 100°C for 3 min. Column flow rate = 2 ml/min FID = 220°C

Results

Table 29 below shows the acetaldehyde production at the 12 hour time point from 500 g corn mash fermentations, liquefied with Alpha-amylase 369 ("AA369") (0.134 KNU-AH/gDS).

MBG4931 shows a 48.71% reduction compared to Ethanol Red.

Table 29

Strain	Acetaldehyde production (ppm) at 12 hours
Ethanol Red (Fermentis)	77.06
MBG4931	39.52

Fig. 3 shows the acetaldehyde production after 12 hour fermentation in 500 g corn mash fermentations, liquefied with Alpha-amylase 369 ("AA369") (0.134 KNU-A/gDS).

Example 24**Increased temperature tolerance during ethanol production**

Ethanol production when using MBG4931 compared to Ethanol Red™ was evaluated in industrially prepared corn mash liquefied with a blend of Alpha-amylase 369 (“AA369”) (2.1 µg EP AA369/gDS), PoAMG 498 (4.5 µg EP PoAMG498/g DS) and PFU (0.0385 µg EP PFU/g DS).

Corn mash

Industrially prepared corn mash was obtained from Valero Jefferson and Valero Linden, mixed. Solids on this mash were measured to be 32.45% by Mettler-Toledo HB43-S moisture balance.

Yeast Strains and Propagation

The two yeast strains tested in this experiment were Ethanol Red (Fermentis) and MBG4931. The target solids percentage in the propagation was 20%, 100 g of mash was added to 62 ml of water to reach a 162 g propagation volume at 20% solids. LACTROL™ was added at a concentration of 3 ppm. Urea nitrogen was added at a concentration of 2000 ppm, by adding 0.65 ml of a 50% (w/v) urea solution. Glucoamylase SA (“GSA”) dose was calculated to be 0.0075% (v/w) per 70 g mash in a sterile 125 ml baffled Erlenmeyer flask. As an inoculum, 2.00 grams of dried yeast were weighed out, added to 40 ml of water preheated to 35°C, and allowed to rehydrate for 20 minutes with swirling at 10 minutes. 2% (v/w) of this rehydration was then added to the propagation. Propagation time was 8 hours at 33.3°C, 150 rpm at which time 8 ml of propagation was transferred to round bottom fermentation vessels containing approximately 500 g liquefied mash as an approximately 1.6% (v/w) inoculation.

Simultaneous Saccharification and Fermentation (SSF)

All fermentations were run in 1L Sartorius Q+ bioreactors. LACTROL was added to each fermenter at a concentration of 3 ppm.

Urea was added to 600 ppm total urea. Glucoamylase SA (“GSA”) was dosed to each reactor at 0.6 AGU/gDS. To mimic enzyme addition at the plant scale, 30% of the glucoamylase and 100% of the fermentation urea were dosed at inoculation. After 6 hours of fermentation, the remaining 70% of glucoamylase was added to the fermenter.

Temperature Profiles

All fermentations started at 32°C and then started a temperature profile as described below.

Table 30. Temperature Profiles

Ferm Time	Temp Target F	Temp Target C
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10	92	33.3
18	94	34.4
35	91	32.8
54	91	32.8

HPLC analysis

5 Fermentation sampling took place by sampling 5 grams of mash into 15 ml tubes at 0, 6, 12, 25, 30, 36, 49, and 54 hours of fermentation. Each tube was processed for HPLC analysis by deactivation with 150 μ L of 40% v/v H₂SO₄, vortexing, centrifuging at 1460 $\times$ g for 10 minutes, and filtering through a 0.45 μ m Whatman PP filter. Samples were stored at 4°C prior to and during HPLC analysis.

Table 31: HPLC System

HPLC System	Agilent's 1100/1200 series with Chem station software Degasser, Quaternary Pump, Auto-Sampler, Column Compartment /w Heater Refractive Index Detector (RI)
Column	Bio-Rad HPX- 87H Ion Exclusion Column 300mm x 7.8mm part# 125-0140 Bio-Rad guard cartridge Cation H part# 125-0129, Holder part# 125-0131
Method	0.005M H ₂ SO ₄ mobile phase Flow rate: 0.6 ml/min Column temperature: 65°C RI detector temperature: 55°C

10 Results

Table 32 below shows the final ethanol titers for 1L corn mash fermentations, liquefied with a blend of Alpha-amylase 369 ("AA369") (2.1 μ g EP AA369/gDS), PoAMG 498 (4.5 μ g EP PoAMG498/g DS) and PFU (0.0385 μ g EP PFU/g DS). MBG4931 showed a 2.16% boost over ETHANOL RED™.

15

Table 32:

Strain	Ethanol Titer at 54 hours
Ethanol Red (Fermentis)	13.71%
MBG4931	14.01%

20 Fig. 4 shows the ethanol titers during 1L corn mash fermentations, liquefied with a blend of Alpha-amylase 369 ("AA369") (2.1 μ g EP AA369/gDS), PoAMG 498 (4.5 μ g EP PoAMG498/g DS) and PFU (0.0385 μ g EP PFU/g DS).

Example 25**Increased theperature tolerance during ethanol production**

Ethanol production when using MBG4931 compared to ETHANOL RED™ was evaluated in industrially prepared corn mash liquefied with a blend of Alpha-amylase 369 (“AA369”) (2.1 µg EP AA369/gDS), PoAMG 498 (4.5 µg EP PoAMG498/g DS) and PFU (0.0385 µg EP PFU/g DS).

Corn mash

Industrially prepared corn mash was obtained from Valero Jefferson and Valero Linden, mixed. Solids on this mash were measured to be 32.45% by Mettler-Toledo HB43-S moisture balance.

Yeast Strains and Propagation

The two yeast strains tested in this experiment were ETHANOL RED™ (Fermentis) and MBG4931. The target solids percentage in the propagation was 20%, 100 g of mash was added to 62 ml of water to reach a 162 g propagation volume at 20% solids. Lactrol was added at a concentration of 3 ppm. Urea nitrogen was added at a concentration of 2000 ppm, by adding 0.65 ml of a 50% (w/v) urea solution. Glucoamylase dose was calculated to be 0.0075% (v/w) per 70 g mash in a sterile 125 ml baffled Erlenmeyer flask. As an inoculum, 2.00 grams of dried yeast were weighed out, added to 40 ml of water preheated to 35°C, and allowed to rehydrate for 20 minutes with swirling at 10 minutes. 2% (v/w) of this rehydration was then added to the propagation. Propagation time was 8 hours at 33.3°C, 150 rpm at which time 8 ml of propagation was transferred to round bottom fermentation vessels containing approximately 500 g liquefied mash as an approximately 1.6% (v/w) inoculation.

Simultaneous Saccharification and Fermentation (SSF)

All fermentations were run in 1L Sartorius Q+ bioreactors. Lactrol was added to each fermenter at a concentration of 3 ppm.

Urea was added to 600 ppm total urea. Glucoamylase SA (“GSA”) was dosed to each reactor at 0.6 AGU/gDS. To mimic enzyme addition at the plant scale, 30% of the glucoamylase and 100% of the fermentation urea were dosed at inoculation. After 6 hours of fermentation, the remaining 70% of glucoamylase was added to the fermenter.

Temperature Profiles

All fermentations started at 32°C and then started a temperature profile as described below.

Table 33: Temperature Profiles

Ferm Time	Temp Target F	Temp Target °C
10	94	34.4

18	98	36.7
25	95	35.0
35	94	34.4
45	91	32.8

HPLC analysis

Fermentation sampling took place by sampling 5 grams of mash into 15 ml tubes at 0, 6, 12, 25, 30, 36, 49, and 54 hours of fermentation. Each tube was processed for HPLC analysis by deactivation with 150 μ L of 40% v/v H₂SO₄, vortexing, centrifuging at 1460 $\times$ g for 10 minutes, and filtering through a 0.45 μ m Whatman PP filter. Samples were stored at 4°C prior to and during HPLC analysis.

Table 34: HPLC System

HPLC System	Agilent's 1100/1200 series with Chem station software Degasser, Quaternary Pump, Auto-Sampler, Column Compartment /w Heater Refractive Index Detector (RI)
Column	Bio-Rad HPX- 87H Ion Exclusion Column 300mm x 7.8mm part# 125-0140 Bio-Rad guard cartridge Cation H part# 125-0129, Holder part# 125-0131
Method	0.005M H ₂ SO ₄ mobile phase Flow rate: 0.6 ml/min Column temperature: 65°C RI detector temperature: 55°C

10 Results

Table 35 below shows the final ethanol titers for 1L corn mash fermentations, liquefied with a blend of Alpha-amylase 369 ("AA369") (2.1 μ g EP AA369/gDS), PoAMG 498 (4.5 μ g EP PoAMG498/g DS) and PFU (0.0385 μ g EP PFU/g DS). MBG4931 showed a 6.80% boost over ETHANOL RED™.

15 Table 35

Strain	Ethanol Titer at 54 hours
Ethanol Red (Fermentis)	12.89%
MBG4931	13.77%

Fig. 5 shows the ethanol titers during 1L corn mash fermentations, liquefied with a blend of Alpha-amylase 369 ("AA369") (2.1 μ g EP AA369/gDS), PoAMG 498 (4.5 μ g EP PoAMG498/g DS) and PFU (0.0385 μ g EP PFU/g DS).

Example 26**Decreased Glycerol Titer in corn mash produced industrially using a liquefaction blend**

Ethanol production when using MBG4931 compared to ETHANOL RED™ was evaluated in industrially prepared corn mash liquefied with Alpha-amylase 369 ("AA369") (0.134 KNU-A/gDS).

Corn mash

Industrially prepared corn mash was obtained from GPRE Central City (Iowa). Solids on this mash were measured to be 32.35% by Mettler-Toledo HB43-S moisture balance.

Yeast Strains and Propagation

The two yeast strains tested in this experiment were ETHANOL RED™ (Fermentis) and MBG4931. The target solids percentage in the propagation was 20%, 142 g of mash was added to 87 ml of water to reach a 230 g propagation volume at 20% solids. Lactrol was added at a concentration of 3 ppm. Urea nitrogen was added at a concentration of 2000 ppm, by adding 0.9 ml of a 50% (w/v) urea solution. Glucoamylase SA ("GSA") dose was calculated to be 0.0075% (v/w) per 70 g mash in a sterile 125 ml baffled Erlenmeyer flask. As an inoculum, 2.00 grams of dried yeast were weighed out, added to 40 ml of water preheated to 35°C, and allowed to rehydrate for 20 minutes with swirling at 10 minutes. 2% (v/w) of this rehydration was then added to the propagation. Propagation time was 8 hours at 33.3°C, 150 rpm at which time 8 ml of propagation was transferred to round bottom fermentation vessels containing approximately 500 g liquefied mash as an approximately 1.6% (v/w) inoculation.

Simultaneous Saccharification and Fermentation (SSF)

All fermentations were run in 500mL round bottom glass vessels (Prism Research, RTP, NC). Lactrol was added to each fermenter at a concentration of 3 ppm.

Urea was added to 600 ppm total urea. Glucoamylase SA ("GSA") was dosed to each reactor at 0.6 AGU/gDS. To mimic enzyme addition at the plant scale, 45% of the glucoamylase and 100% of the fermentation urea were dosed at inoculation. After 6 hours of fermentation, the remaining 55% of glucoamylase was added to the fermenter. All fermentations started at 32°C

HPLC analysis

Fermentation sampling took place by sampling 5 grams of mash into 15 ml tubes at 0, 6, 12, 25, 30, 36, 49 and 54 hours of fermentation. Each tube was processed for HPLC analysis by deactivation with 150 µL of 40% v/v H₂SO₄, vortexing, centrifuging at 1460×g for 10 minutes, and filtering through a 0.45 µm Whatman PP filter. Samples were stored at 4°C prior to and during HPLC analysis.

Table 36. HPLC System

HPLC System	Agilent's 1100/1200 series with Chem station software Degasser, Quaternary Pump, Auto-Sampler, Column Compartment /w Heater Refractive Index Detector (RI)
Column	Bio-Rad HPX- 87H Ion Exclusion Column 300mm x 7.8mm part# 125-0140 Bio-Rad guard cartridge Cation H part# 125-0129, Holder part# 125-0131
Method	0.005M H ₂ SO ₄ mobile phase Flow rate: 0.6 ml/min Column temperature: 65°C RI detector temperature: 55°C

Results

Table 37 below shows the final glycerol titers for 500 g corn mash fermentations, liquefied with Alpha-amylase 369 ("AA369") (0.134 KNU-AH/gDS). MBG4931 showed a 7.32% reduction compared to ETHANOL RED™.

Table 37.

Strain	Glycerol Titer (%w/v) at 54 hours
Ethanol Red (Fermentis)	1.62%
MBG4931	1.50%

Fig. 6 shows the glycerol titers during 500 g corn mash fermentations, liquefied with Alpha-amylase 369 ("AA369") (0.134 KNU-A/gDS).

Fig. 7 shows the glycerol production at the 54 hour time point in 500 g corn mash fermentations, liquefied with Alpha-amylase 369 ("AA369") (0.134 KNU-A/gDS).

Claims

1. A process for producing ethanol from starch-containing material comprising the steps of:

5 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

10 *Saccharomyces cerevisiae* MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

15 *Saccharomyces cerevisiae* MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

20 *Saccharomyces cerevisiae* MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

2. The process of claim 1, wherein the fermenting organism, has at least one or more, such as all, of the following properties and defining characteristics:

25 increases ethanol yield compared to ETHANOL RED™ under the same process conditions;

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

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

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

3. The process of any of claims 1 or 2, wherein the fermenting organism provides an ethanol yield boost over ETHANOL RED™ (ER) of more than 1.0%, preferably more than 2.0%, such

more than 2.5%, such as around 2.9% under the same process conditions.

4. The process of any of claims 1-3, 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;
- (b) leaves less glucose remaining following 50 hours of fermentation than ETHANOL RED™, under the same conditions in a corn mash fermentation;
- (c) has a higher ethanol yield than ETHANOL RED™ following 50 hours of fermentation under the same conditions in a corn mash fermentation.

5. A *Saccharomyces* yeast strain selected from:

Saccharomyces cerevisiae MBG4930 (deposited under Accession No. V15/004035 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4930 or a derivative of *Saccharomyces* strain V15/004035 having defining characteristics of strain V15/004035;

Saccharomyces cerevisiae MBG4931 (deposited under Accession No. V15/004036 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4931 or a derivative of *Saccharomyces* strain V15/004036 having defining characteristics of strain V15/004036; or

Saccharomyces cerevisiae MBG4932 (deposited under Accession No. V15/004037 at National Measurement Institute, Victoria, Australia) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4932 or a derivative of *Saccharomyces* strain V15/004037 having defining characteristics of strain V15/004037.

6. A method of producing a derivative of *Saccharomyces cerevisiae* strain V15/004035, V15/004036, V15/004036, comprising:

- a. culturing a first yeast strain with a second yeast strain, wherein the second yeast strain is strain V15/004035, V15/004036 or V15/004037; or a derivative of strain V15/004035, V15/004036 or V15/004037, 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.

7. A method of producing a derivative of *Saccharomyces cerevisiae* strain V15/004035,

V15/004036 or V15/004037 which exhibits the defining characteristics of strain V15/004035, V15/004036 or V15/004037, respectively, comprising:

(a) providing:

(i) a first yeast strain; and

(ii) a second yeast strain, wherein the second yeast strain is strain V15/004035, V15/004036 or V15/004037 or a derivative of strain V15/004035, V15/004036 or V15/004037;

(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.

8. The method of claim 7, wherein step (c) comprises screening or selecting for a hybrid strain which exhibits one or more defining characteristic of strain V15/004035, V15/004036 or V15/004037.

9. The method of claim 7, 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 V15/004035, V15/004036 or V15/004037.

10. The method of claim 8, or 9, 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.

11. A *Saccharomyces* strain produced by the method of any one of claims 6 to 10.

12. A *Saccharomyces* strain having the defining characteristics of strain V15/004035, V15/004036 or V15/004037.

13. A method of producing ethanol, comprising incubating a strain of claim 5, 11 or 12 with a substrate comprising a fermentable sugar under conditions which permit fermentation of the fermentable sugar to produce ethanol.

14. Use of a strain of any of claims 5, 11 or 12 in the production of ethanol.

15. A method of producing distiller's grain, comprising:

- (a) incubating a *Saccharomyces* strain of any of claims 5, 11 or 12 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.

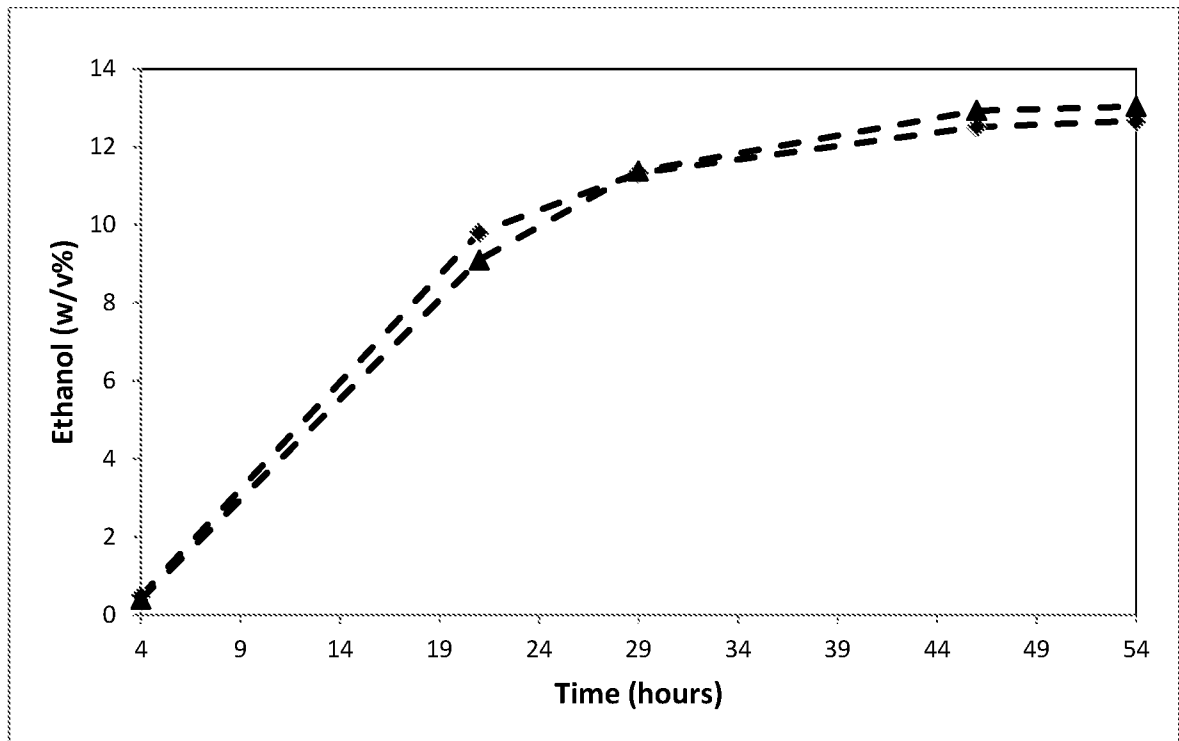
16. Distiller's grain produced by the method of claim 15.

17. Use of a strain of claim 5, 11 or 12 in the production of distiller's grains.

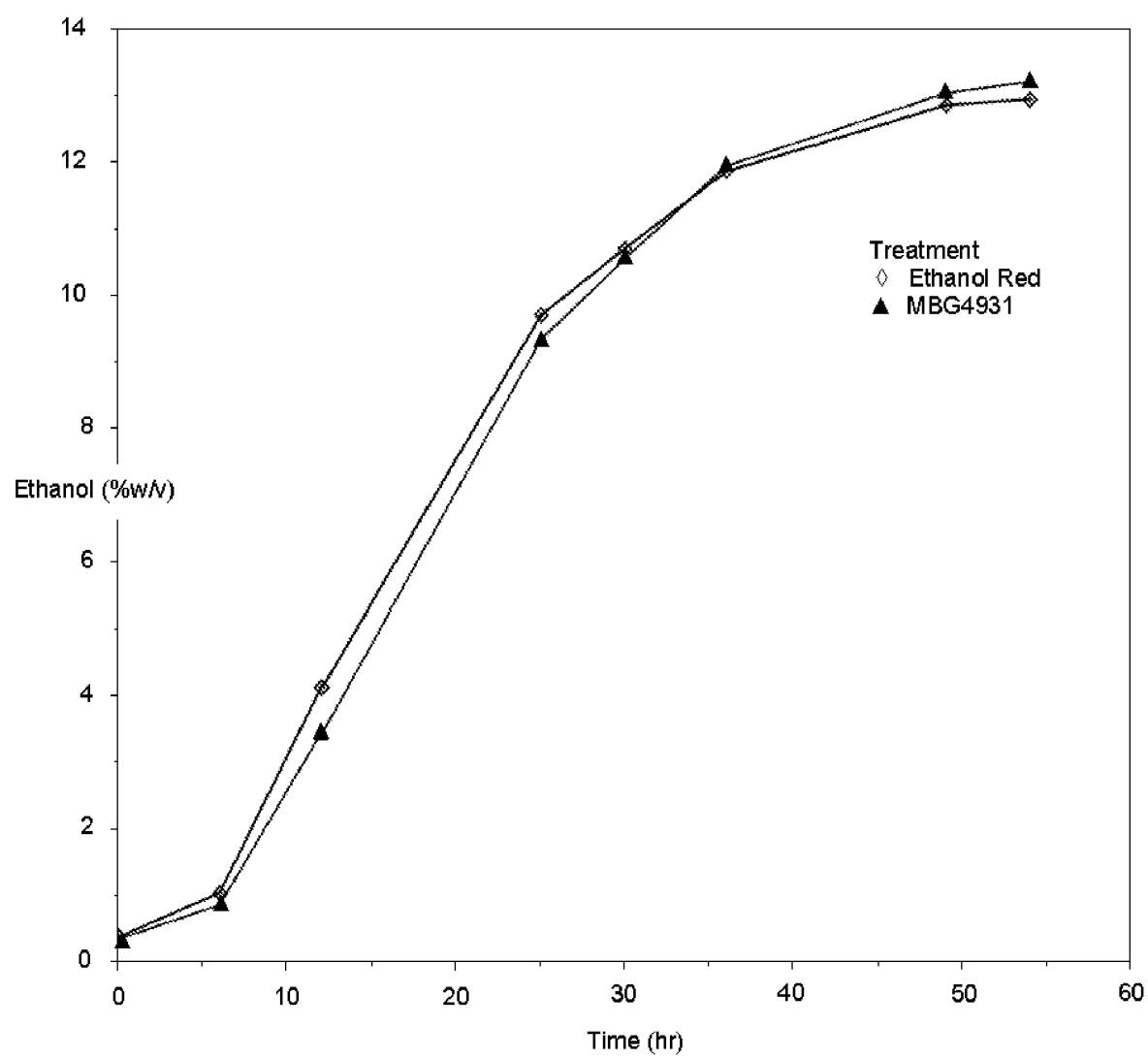
18. Use of a strain of any of claims 5, 11 or 12 in the production of a *Saccharomyces* strain having the defining characteristics of strain V15/004035, V15/004036 or V15/004037.

19. A composition comprising a *Saccharomyces* yeast strain of any of claims 5, 11 or 12 and one or more naturally occurring and/or non-naturally occurring components, such as components are selected from the group consisting of: surfactants, emulsifiers, gums, swelling agents, and antioxidants.

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**Fig. 1**

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**Fig. 2**

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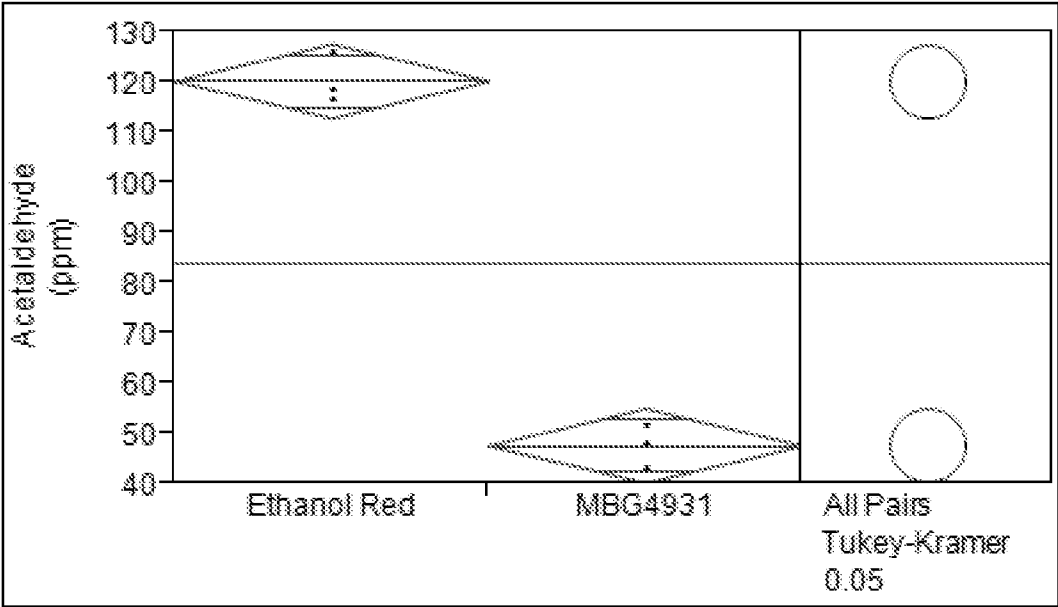
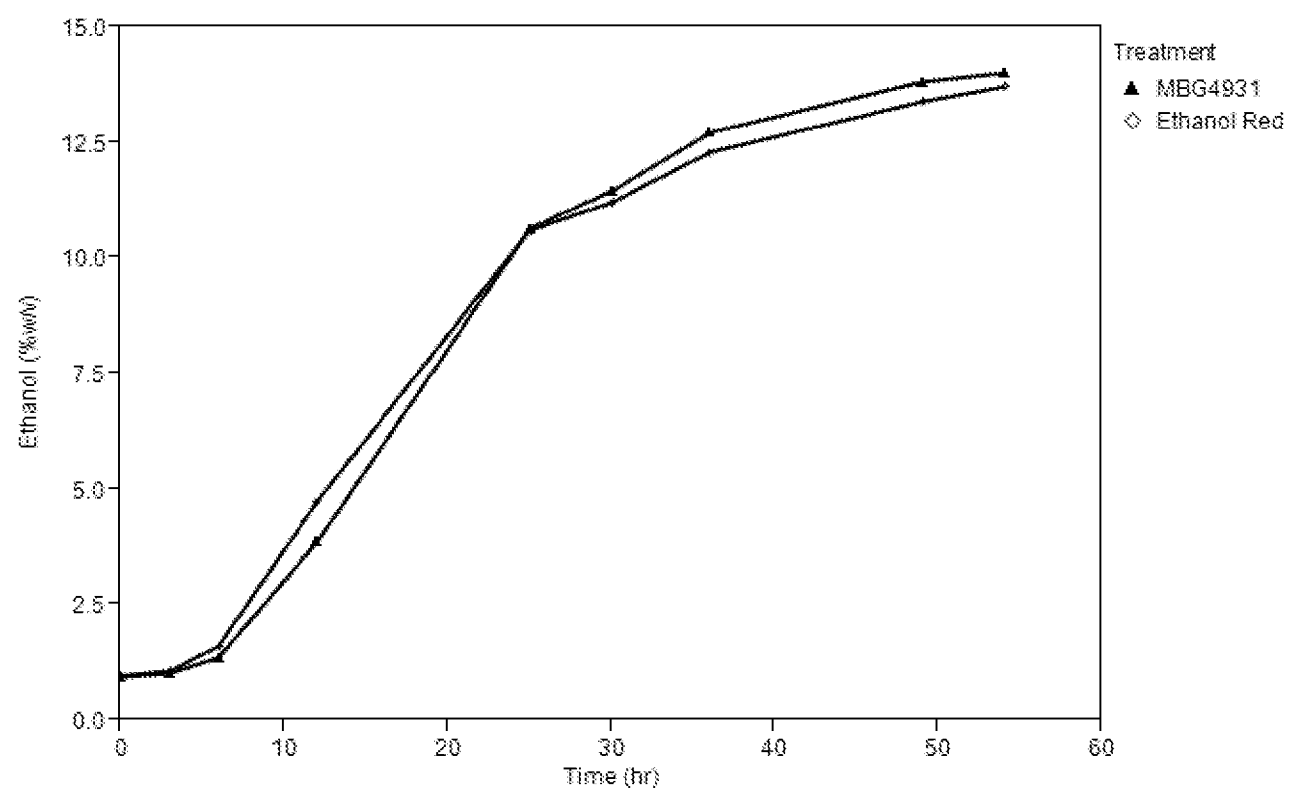
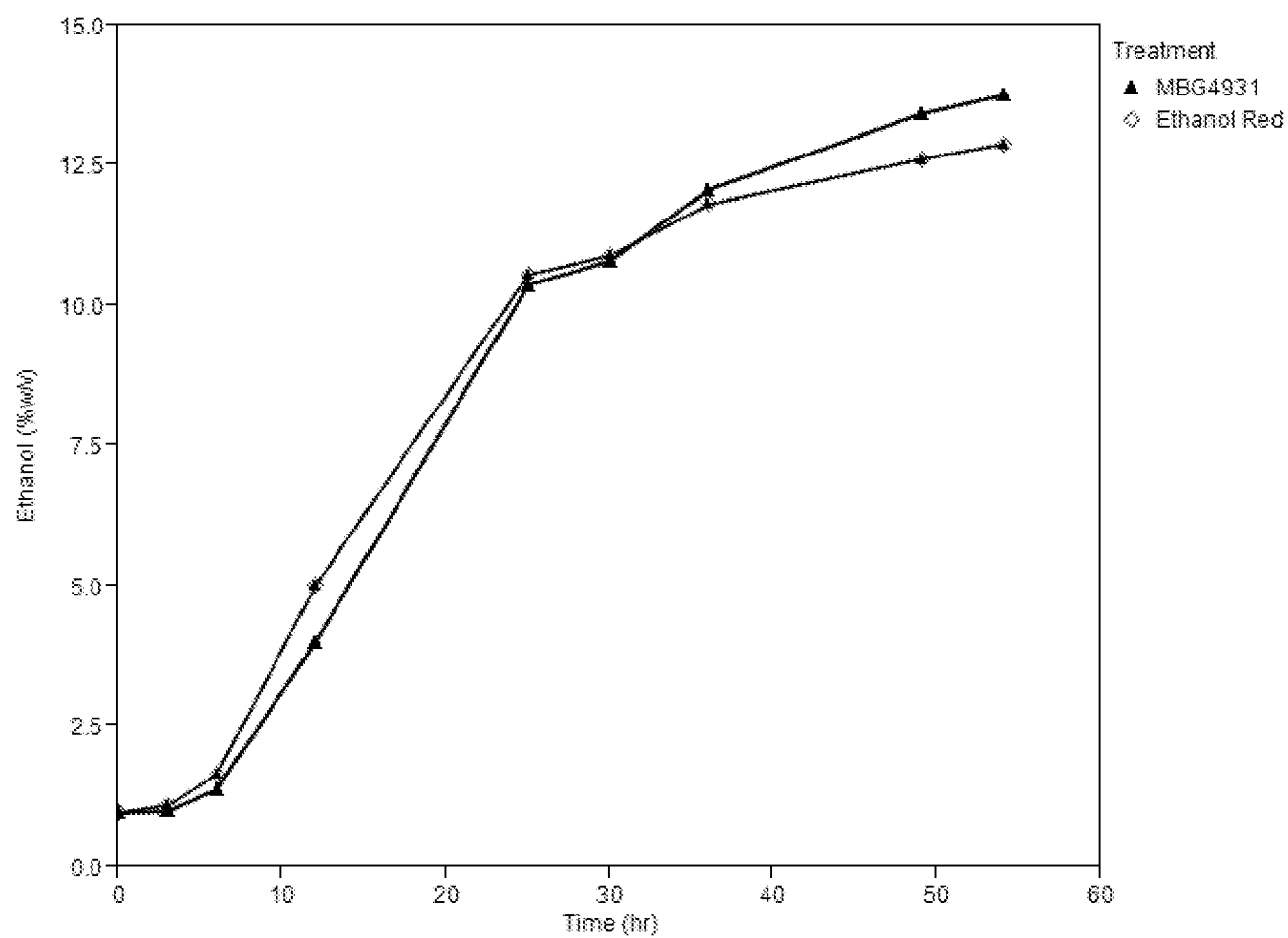


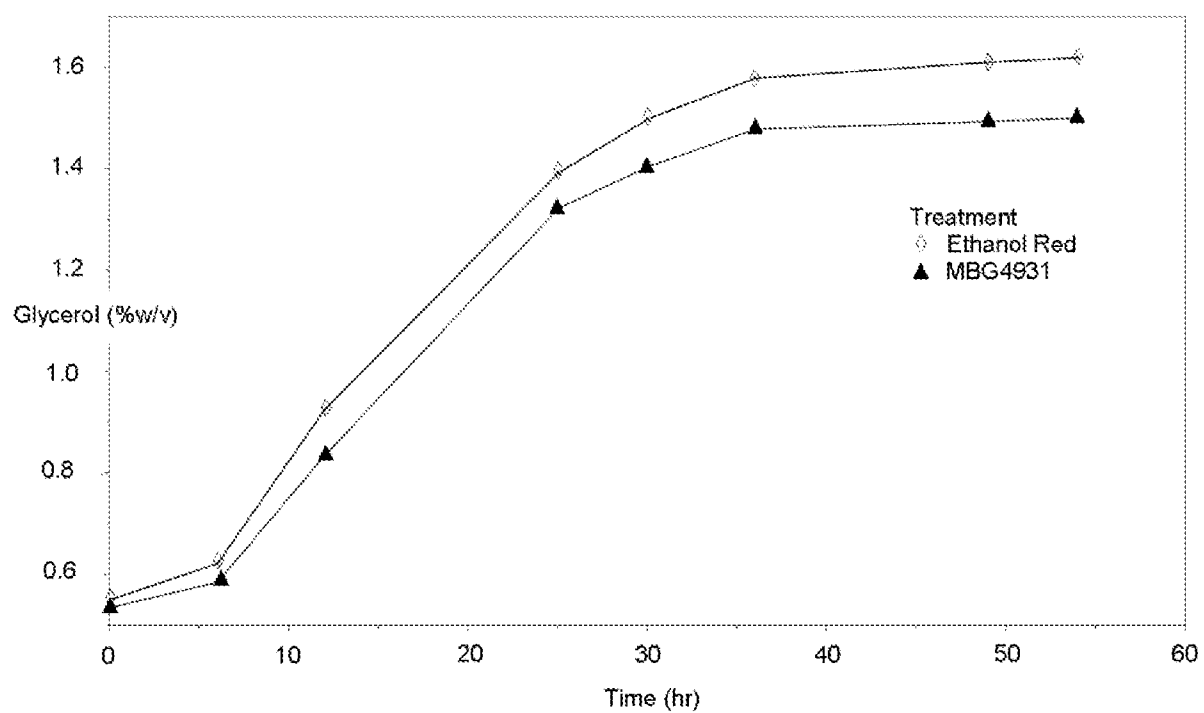
Fig. 3

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**Fig. 4**

5/8**Fig. 5**

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**Fig. 6**

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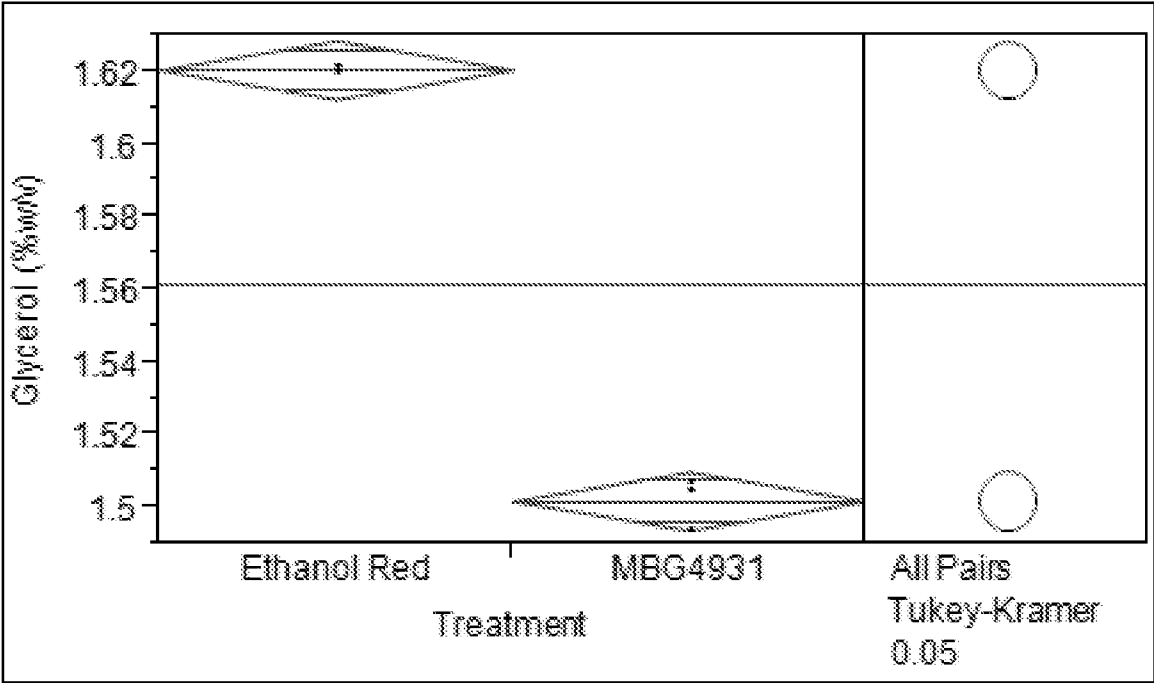
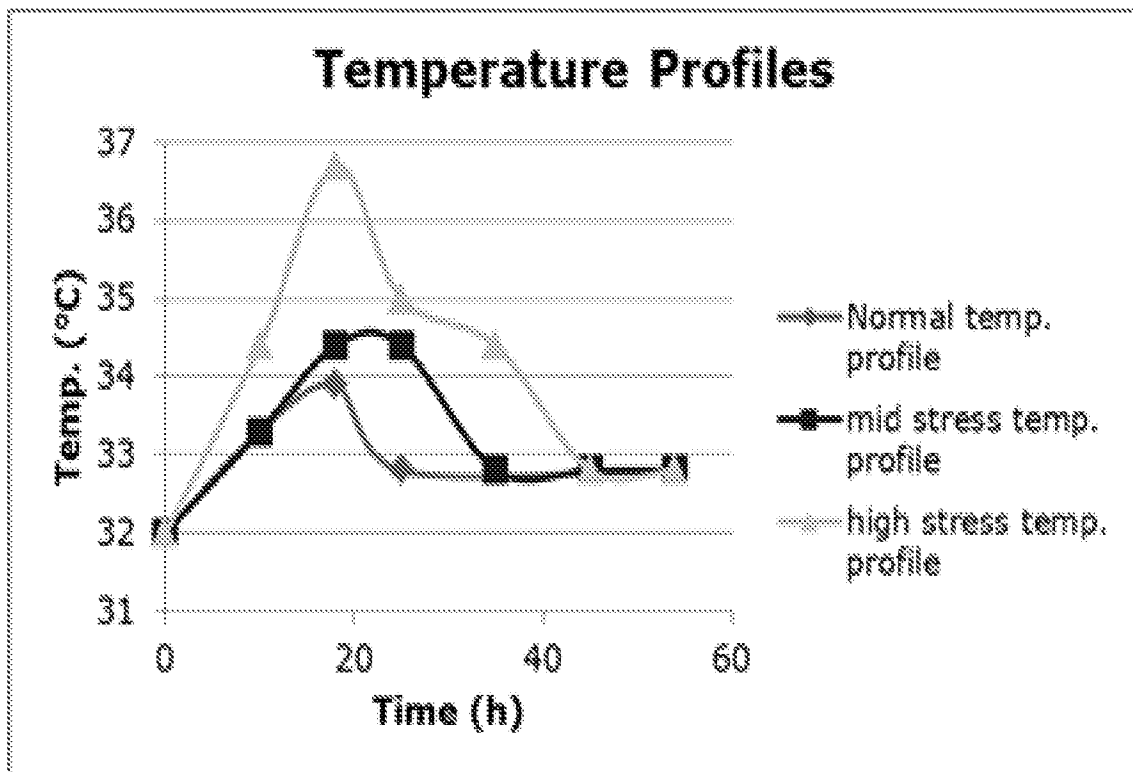


Fig. 7

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**Fig. 8**

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/022894

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/18 C12P7/06
ADD. C12R1/865

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)
C12N C12R C12P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, 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	WO 2011/035392 A1 (MICROBIOGEN PTY LTD [AU]; BELL PHILIP JOHN LIVINGSTONE [AU]; ATTFIELD) 31 March 2011 (2011-03-31) cited in the application the whole document	1-19
X	EP 2 277 989 A1 (UNIV DELFT TECH [NL]) 26 January 2011 (2011-01-26) the whole document	1-19

☐

Further documents are listed in the continuation of Box C.

☒

See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"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

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Date of the actual completion of the international search

1 June 2016

Date of mailing of the international search report

13/06/2016

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Devijver, Kristof

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2016/022894

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011035392 A1	31-03-2011	AR 078468 A1 CA 2775738 A1 US 2012309069 A1 WO 2011035392 A1	09-11-2011 31-03-2011 06-12-2012 31-03-2011
EP 2277989 A1	26-01-2011	AU 2010275085 A1 BR 112012001642 A2 CA 2768897 A1 CN 102712895 A EA 201270187 A1 EP 2277989 A1 EP 2456851 A1 JP 5836271 B2 JP 2013500006 A UA 107467 C2 US 2011275130 A1 US 2014295514 A1 WO 2011010923 A1	23-02-2012 16-06-2015 27-01-2011 03-10-2012 28-09-2012 26-01-2011 30-05-2012 24-12-2015 07-01-2013 12-01-2015 10-11-2011 02-10-2014 27-01-2011