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(54) Title: PROCESSES OF PRODUCING ETHANOL USING A FERMENTING ORGANISM

(57) Abstract: The invention relates to improved processes of producing ethanol from starch-containing material wherein saccharification and/or fermentation is done at a temperature below the initial gelatinization temperature in the presence of glucoamylase and alpha-amylase, and optionally a protease and/or a cellulolytic enzyme composition; wherein the fermenting organism is a Saccharomyces yeast strain providing a higher ethanol yield boost and lower glycerol production compared to ETHANOL RED™ under the same fermentation conditions. The invention also relates to Saccharmyces yeast strains and derivatives thereof, as well as compositions comprising such yeast strains, suitable for use in a process of the invention.

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PROCESSES OF PRODUCING ETHANOL USING A FERMENTING ORGANISM

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority benefit of U.S. provisional application Serial No. 62/121,925, filed on February 27, 2015. The content of this application is 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.

TECHNICAL FIELD

The present invention relates to improved raw starch hydrolysis processes of producing ethanol from starch-containing materials using a fermenting organism providing an ethanol yield boost and lower glycerol production compared to the current industry standard yeast ETHANOL RED™ under the same fermentation conditions. The invention also relates to *Saccharomyces* yeast strains having improved properties in raw starch hydrolysis processes and compositions comprising a *Saccharomyces* yeast strain of the invention and a naturally occurring and/or a non-naturally occurring component.

BACKGROUND ART

Processes of producing ethanol from starch-containing material are well-known in the art and used commercially today. The production of ethanol as a bio-fuel has become a major industry, with in excess of 21 billion gallons of ethanol being produced worldwide in 2012.

When producing ethanol, starch is conventionally converted into dextrins using a liquefying enzyme (e.g., *Bacillus* alpha-amylase) at temperatures above the initial gelatinization temperature of starch. The generated dextrins are hydrolyzed into sugars using a saccharifying enzyme (e.g., glucoamylase) and fermented into the desired fermentation product using a fermenting organism such as a yeast strain derived from *Saccharomyces*

cerevisiae. Typically hydrolysis and fermentation are done in a simultaneous saccharification and fermentation (SSF) step.

Another type of process is also used commercially today. Starch is converted into sugars by enzymes at temperatures below the initial gelatinization temperature of the starch in question and converted into ethanol by yeast, typically derived from *Saccharomyces cerevisiae*. This type of process is referred to as a raw starch hydrolysis (RSH) process, or alternatively a "one-step process" or "no cook" process.

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* exhibit 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 the commercially available ethanol yeast product, sold under the trade named "ETHANOL RED™" and is available from Fermentis (A Lesaffre Division).

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

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

There is therefore a need for new 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 further improved processes of producing ethanol from starch-containing material that, e.g., can provide a higher ethanol yield.

SUMMARY OF THE INVENTION

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 a first 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:
    - an ethanol yield boost compared to ETHANOL RED™;
    - lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions.

In a preferred embodiment the fermenting organism used in a process of the invention is selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia). In an embodiment the fermenting organism is a derivative of *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4914 having the defining characteristics (i.e., high ethanol yield boost and/or low glycerol production) of one or more of these *Saccharomyces cerevisiae* strains.

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:

- higher ethanol yield boost;
- lower glycerol production.

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 Process Of The Invention" section below.

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: 12 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: 13 herein.

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: 18 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: 13 herein.

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: 18 herein, preferably one further having one or more of the following substitutions: S95P, A121P, especially S95P+A121P and an alpha-amylase. The alpha-amylase may be derived from *Rhizomucor pusillus*, preferably *Rhizomucor pusillus* alpha-amylase with a linker and starch-binding domain (SBD), in particular the *Rhizomucor pusillus* alpha-amylase with *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) shown in SEQ ID NO: 13 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 saccharification and/or fermentation: *Pycnoporus sanguineus* glucoamylase, preferably the one shown in SEQ ID NO: 17 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: 13 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 *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.

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: 9 herein) and *Aspergillus fumigatus* beta-glucosidase (e.g., SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 8 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: 10 herein, and *Aspergillus fumigatus* beta-glucosidase, e.g., the one disclosed as SEQ ID NO: 2 in WO 2005/047499 or SEQ ID NO: 8 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: 6 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: 7 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 cellulolytic enzyme composition added is from 10-500 µg/g DS, such as from 20-400 µg/g DS, such as 20-300 µg/g DS.

In 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.

A second aspect provides a *Saccharomyces* yeast strain providing

- higher ethanol yield compared to ETHANOL RED™
- lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions.

In a preferred embodiment the yeast strain is selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia). In an embodiment the yeast strain is a derivative of *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4914 having the defining characteristics of one or more of these *Saccharomyces cerevisiae* strains.

A third aspect provides a method of producing a *Saccharomyces* strain having the defining characteristics of a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913 or MBG4914, comprising:

(a) providing: (i) a first yeast strain; and (ii) a second yeast strain, wherein the second yeast strain is a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913 or MBG4914, respectively, 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 yeast strain and the second yeast strain;

(c) screening or selecting for a derivative of a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913 or MBG4914;

(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 a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913 or MBG4914, respectively.

A fourth aspect provides a *Saccharomyces* yeast strain produced by the method of the third aspect.

A fifth aspect provides use of a strain of the second or fourth aspect in the production of a *Saccharomyces* strain which exhibits one or more defining characteristics of a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913 or MBG4914, respectively.

A sixth aspect provides processes of using a *Saccharomyces* yeast strain of the second or fourth aspect in a process of the first aspect.

In a final aspect the invention relates to compositions comprising a *Saccharomyces* yeast strain of the invention and a naturally occurring and/or a nonenaturally occurring component. In a preferred embodiment the naturally occurring component and/or nonenaturally occurring component is one or more of the components selected from the group consisting of: surfactants, emulsifiers, gums, swelling agents, and antioxidants.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to improved raw starch hydrolysis processes of producing ethanol from starch-containing materials using a fermenting organism providing an ethanol yield boost and lower glycerol production compared to the current industry standard yeast ETHANOL RED™ under the same fermentation conditions. A raw starch hydrolysis process is a process where starch, typically granular starch, is converted into dextrins/sugars by raw starch degrading enzymes at temperatures below the initial gelatinization temperature of the starch in question and converted into ethanol by yeast, typically Saccharomyces cerevisiae. This type of process is often alternatively referred to as a "one-step process" or "no cook" process. The invention also relates to *Saccharomyces* yeast strains having improved properties compared to ETHANOL RED™ (*Saccharomyces cerevisiae* yeast developed for the industrial ethanol industry).

Specifically, the invention relates to processes of ethanol production 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* strain providing:
    - an ethanol yield boost compared to ETHANOL RED™;
    - lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions.

In an embodiment the fermenting organism can grow on xylose as a sole carbon source, e.g., determined using the Test T1.

In an embodiment the fermenting organism shows more than two-fold increase in biomass, such as more than six-fold increase in biomass, such as more than 20-fold increase in biomass determined using the Test T1 (described in the Materials & Methods" section below.

The inventors have surprisingly found that raw starch hydrolysis (RSH) processes of the invention using MBG4911, MBG4913 or MBG4914, respectively, result in higher ethanol yield compared to corresponding processes where ETHANOL RED™ ("ER") is used under the same conditions. See for instance, Example 3, table 2; and Example 4, table 5.

Raw starch hydrolysis (RSH) processes of the invention using MBG4911, MBG4913 or MBG4914, respectively, result in lower glycerol production compared to corresponding

processes where ETHANOL RED™ ("ER") is used under the same conditions. See for instance, Example 3, table 3; and Example 4, table 6.

The process conditions may according to the invention may be as described in any of Examples 3 and 4.

As described in more details in Examples 3 and 4 the yeast strains are compared to ETHANOL RED™ by:

1) preparing a ground corn mash preparation having about 33-34% dry solids (DS), supplementing with 3 ppm LACTROL™ and 500 ppm urea and adjusting to pH 4.5 with 40% H_2SO_4.
2) rehydrating the yeast strains by weighing approximately 5 g of dried yeast into 50 ml of about 36-37°C tap water in a flask, covering the flask, incubating in a 36-37°C water bath for a total of 20 minutes, removing the flask, and enumerated the yeast.
3) Dosing enzymes at 0.5 AGU/g DS, added the yeast in question and ETHANOL RED™, respectively, at a pitch of 5 million cells per gram, adding water to a total volume added and fermenting at 32°C for 88 hours.
4) Analysing for ethanol using a HPLC.

According to the invention a yeast strain of the invention has the following defining characteristics:

- provides an ethanol yield boost of at least 0.5%, preferably at least 1.0%, more preferably at least 1.5%, more preferably at least 2.0%, more preferably at least 3.0%, more preferably at least 4.0%, even more preferably at least 5.0%, such as between 0.5-10%, e.g., between 1-6%, after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™; and/or
- provides a lower glycerol production of at least 5%, preferably at least 10%, more preferably 15%, even more preferably at least 15%, more preferably at least 20%, such as between 4 and 40%, such as between 10 and 30% after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™.

In a preferred embodiment of the invention the fermenting organism is selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia).

In another embodiment the fermenting organism used in a process of the invention is selected from the group of derivatives of *Saccharomyces cerevisiae* MBG 4911, MBG4913

or MBG4914 having the defining characteristics of one or more of these *Saccharomyces cerevisiae* strains.

According to the invention the yeast may be in any viable form, including crumbled, dry, including active dry and instant, compressed, cream form etc. In a preferred embodiment the *Saccharomyces cerevisiae* yeast strain used in a process of the invention is dry yeast, such as active dry yeast. In a preferred embodiment the *Saccharomyces cerevisiae* yeast strain used in a process of the invention is compressed yeast. In an embodiment the *Saccharomyces cerevisiae* yeast strain used in a process of the invention is cream yeast.

Raw Starch Hydrolysis Processes: In processes of the invention the starch does not gelatinize as the process is carried out at temperatures below the initial gelatinization temperature of the starch in question.

The term "initial gelatinization temperature" means the lowest temperature at which starch gelatinization commences. In general, starch heated in water begins to gelatinize between about 50°C and 75°C. The exact temperature of gelatinization depends on the specific starch and depends on the degree of cross-linking of the amylopectin. The initial gelatinization temperature can readily be determined by the skilled artisan. 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 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. S. and Lii. C., Starch/Stärke, Vol. 44 (12) pp. 461-466 (1992).

Therefore, according to the process of the invention ethanol is produced from un-gelatinized (i.e., uncooked), preferably milled grains, such as corn, or small grains such as wheat, oats, barley, rye, rice, or cereals such as sorghum. Examples of suitable starch-containing starting materials are listed in the section "Starch-Containing Materials"-section below.

In a preferred embodiment the enzymes may be added as one or more enzyme blends. According to the invention the fermentation product, i.e., ethanol, is produced without liquefying the starch-containing material. The process of the invention includes saccharifying (e.g., milled) starch-containing material, especially granular starch, below the initial gelatinization temperature, in the presence of at least a glucoamylase and an alpha-amylase and optionally a protease and/or a cellulolytic enzyme composition. The dextrins/sugars generated during saccharification can may according to the invention be simultaneously fermented into ethanol by one or more suitable fermenting organism, especially *Saccharomyces cerevisiae* MBG4911, MBG4914, and/or MBG4914 or fermenting

organism(s) having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4913, especially derivatives of *Saccharomyces* strain MBG4911, MBG4913 and/or MBG4914 having the defining characteristics of said strains. See the "Fermenting Organisms"-section below.

In a preferred embodiment step (a) and step (b) are carried out simultaneously (i.e., often referred to as "simultaneous saccharification and fermentation" or "one-step fermentation"). However, step (a) and step (b) may also be carried our sequentially.

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

In an embodiment backset, or another recycled stream, is added to the slurry before step (a), or to the saccharification (step (a)), or to the simultaneous saccharification and fermentation steps (combined step (a) and step (b)).

After being subjected to a process of the invention at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or preferably at least 99% of the dry solids in the starch-containing material are converted into a soluble starch hydrolysate.

A process of the invention is conducted at a temperature below the initial gelatinization temperature, which means that the temperature at which a separate step (a) is carried out typically lies in the range between 25-75ºC, such as between 30-70°C, or between 45-60ºC.

In a preferred embodiment the temperature during fermentation in step (b) or simultaneous saccharification and fermentation in steps (a) and (b) is between 25°C and 40°C, preferably between 28°C and 36°C, such as between 28°C and 35°C, such as between 28°C and 34°C, such as around 32°C.

In an embodiment of the invention fermentation or SSF is carried out for 30 to 150

hours, preferably 48 to 96 hours.

In an embodiment fermentation, especially SSF, is carried out so that the sugar level, such as glucose level, is kept at a low level, such as below 6 wt.-%, such as below about 3 wt.-%, such as below about 2 wt.-%, such as below about 1 wt.-%., such as below about 0.5%, or below 0.25% wt.-%, such as below about 0.1 wt.-%. Such low levels of sugar can be accomplished by simply employing adjusted quantities of enzymes and fermenting organism. A skilled person in the art can easily determine which doses/quantities of enzyme and fermenting organism, in particular MBG4911, MBG4913 and/or MBG4914, to use. The employed quantities of enzymes and fermenting organism(s) may also be selected to maintain low concentrations of maltose in the fermentation broth. For instance, the maltose level may be kept below about 0.5 wt.-%, such as below about 0.2 wt.-%.

The process of the invention may be carried out at a pH from 3 and 7, preferably from 3 to 6, or more preferably from 3.5 to 5.0.

The term “granular starch” means raw uncooked starch, i.e., starch in its natural form found in, e.g., cereal, tubers or grains. Starch is formed within plant cells as tiny granules insoluble in water. When put in cold water, the starch granules may absorb a small amount of the liquid and swell. At temperatures up to around 50°C to 75°C the swelling may be reversible. However, at higher temperatures an irreversible swelling called “gelatinization” begins. The granular starch may be a highly refined starch, preferably at least 90%, at least 95%, at least 97% or at least 99.5% pure, or it may be a more crude starch-containing materials comprising (e.g., milled) whole grains including non-starch fractions such as germ residues and fibers.

The raw material, such as whole grains, may be reduced in particle size, e.g., by milling, in order to open up the structure and allowing for further processing. Examples of suitable particle sizes are disclosed in US Patnt no. 4,514,496 (Suntory Ltd), see e.g., claim 8, and WO2004/081193 (Broin And Associates, Inc.), see, e.g., page 5, line 28 to page 6, line 2, both references hereby incorporated by reference. Two processes are preferred according to the invention: wet and dry milling. In dry milling whole kernels are milled and used. Wet milling gives a good separation of germ and meal (starch granules and protein) and is often applied at locations where the starch hydrolysate is used in production of, e.g., syrups. Both dry and wet milling is well known in the art of starch processing.

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

preferably by milling, such that at least 50% of the starch-containing material has a particle size of 0.1-0.5 mm.

According to the invention the enzymes are added so that the glucoamylase is present in an amount of 0.001 to 10 AGU/g DS, preferably from 0.01 to 5 AGU/g DS, especially 0.1 to 0.5 AGU/g DS.

According to the invention the enzymes are added so that the alpha-amylase is present or added in an amount of 0.001 to 10 AFAU/g DS, preferably from 0.01 to 5 AFAU/g DS, especially 0.3 to 2 AFAU/g DS or 0.001 to 1 FAU-F/g DS, preferably 0.01 to 1 FAU-F/g DS.

According to the invention the enzymes are added so that the cellulolytic enzyme composition is present or added in an amount 1-10,000 micro grams EP/g DS, such as 2-5,000, such as 3 and 1,000, such as 4 and 500 micro grams EP/g DS.

According to the invention the enzymes are added so that the cellulolytic enzyme composition is present or added in an amount in the range from 0.1-100 FPU per gram total solids (TS), preferably 0.5-50 FPU per gram TS, especially 1-20 FPU per gram TS.

In an embodiment of the invention the enzymes are added so that the protease is present in an amount of 0.0001-1 mg enzyme protein per g DS, preferably 0.001 to 0.1 mg enzyme protein per g DS. Alternatively, the protease is present and/or added in an amount of 0.0001 to 1 LAPU/g DS, preferably 0.001 to 0.1 LAPU/g DS and/or 0.0001 to 1 mAU-RH/g DS, preferably 0.001 to 0.1 mAU-RH/g DS.

In an embodiment of the invention the enzymes are added so that the protease is present or added in an amount in the range 1-1,000 µg EP/g DS, such as 2-500 µg EP/g DS, such as 3-250 µg EP/g DS.

In a preferred embodiment the ratio between glucoamylase and alpha-amylase 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 according to the invention 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 cellulolytic enzyme composition added is from 10-500 µg/g DS, such as from 20-400 µg/g DS, such as 20-300 µg/g DS.

In 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.

Starch-Containing Materials

According to the process of the invention any suitable starch-containing starting material, in particular granular starch (raw uncooked starch), may be used. The starting material is generally selected based on the desired fermentation product. 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, rice, peas, beans, sweet potatoes or oats, 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.

Fermenting Organisms Used In A Process Of The Invention

According to invention, the fermenting organism used in a raw starch hydrolysis process of the invention is a *Saccharomyces* strain providing:

- o an ethanol yield boost compared to ETHANOL RED™;
- o lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions.

In an embodiment the fermenting organism can grow on xylose as a sole carbon source, e.g., determined using the Test T1 (described in the Materials & Methods" section below).

In an embodiment the fermenting organism shows more than two-fold increase in

biomass, such as more than six-fold increase in biomass, such as more than 20-fold increase in biomass determined using the Test T1.

In an embodiment the fermenting organism used in a process of the invention provides an ethanol yield boost of at least 0.5%, preferably at least 1.0%, more preferably at least 1.5%, more preferably at least 2.0%, more preferably at least 3.0%, more preferably at least 4.0%, even more preferably at least 5.0%, such as between 0.5-10%, e.g., between 1-6%, after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™.

In an embodiment the fermenting organism used in a process of the invention provides a lower glycerol production of at least 5%, preferably at least 10%, more preferably 15%, even more preferably at least 15%, more preferably at least 20%, such as between 4 and 40%, such as between 10 and 30% after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™.

In a preferred embodiment of the invention the fermenting organism is selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia).

In another embodiment the fermenting organism used in a process of the invention is selected from the group of derivatives of *Saccharomyces cerevisiae* MBG 4911, MBG4913 or MBG4914, respectively, having the defining characteristics of one or more of these *Saccharomyces cerevisiae* strains.

Fermentation Medium

The term “fermentation medium” refers to the environment in which fermentation, using a fermenting organism, is carried out and which includes the fermentable substrate, that is, a carbohydrate source (e.g., glucose) that can be metabolized by the fermenting organism into a desired fermentation product, such as ethanol.

The fermentation medium may comprise nutrients and/or growth stimulator(s) for the fermenting organism. 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.

Recovery

Subsequent to fermentation, the desired fermentation product (e.g., ethanol) may be

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

Enzymes And Enzyme Blends Used In A Process Of The Invention

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

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

In 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: 12 herein and an alpha-amylase.

In an embodiment the glucoamylase is derived from *Trametes cingulata*, such as the one shown in SEQ ID NO: 12 herein, or a glucoamylase selected from the group consisting of:

(i) a glucoamylase comprising the mature polypeptide of SEQ ID NO: 12 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: 12 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: 18 herein and an alpha-amylase.

In an embodiment the glucoamylase is derived from *Gloeophyllum trabeum*, such as the one shown in SEQ ID NO: 18 herein, or a glucoamylase 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.

In a preferred embodiment the *Gloeophyllum* glucoamylase, such as the *Gloeophyllum trabeum* glucoamylase shown in SEQ ID NO: 18, has one of the following substitutions: V59A; S95P; A121P; T119W; S95P+A121P; V59A+S95P; S95P+T119W; V59A+S95P+A121P; or S95P+T119W+A121P, especially S95P+A121P (using SEQ ID NO: 18 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: 13 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: 13 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: 13 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: 13 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: 13 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: 12 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: 13 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: 18 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: 13 herein.

In another preferred embodiment the enzymes present and/or added comprises the *Gloeophyllum trabeum* glucoamylase shown in SEQ ID NO: 18 herein having one or more of the following substitutions: S95P, A121P, especially S95P+A121P (using SEQ ID NO: 13 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: 13 herein, preferably one having one or more of the following substitutions: G128D, D143N, especially especially G128D+D143N (using SEQ ID NO: 13 for numbering).

In an embodiment the following enzymes are present and/or added in saccharification and/or fermentation: *Pycnoporus* glucoamylase, in particular the *Pycnoporus sanguineus* glucoamylase shown in SEQ ID NO: 17 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: 13 herein.

In an especially preferred embodiment the enzymes present and/or added in saccharification and/or fermentation comprises a *Pycnoporus* glucoamylase, such as the *Pycnoporus sanguineus* glucoamylase shown in SEQ ID NO: 17 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: 13 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: 12 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: 13 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: 9 herein) and *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 8 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: 10 herein and *Aspergillus fumigatus* beta-glucosidase disclosed as SEQ ID NO: 2 in WO 2005/047499 or SEQ ID NO: 8 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: 6 herein and *Aspergillus fumigatus* CBH II disclosed as SEQ ID NO: 18 in WO 2011/057140 and as SEQ ID NO: 7 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: 18 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: 13 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: 9 herein) and *Aspergillus*

fumigatus beta-glucosidase (SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 8 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: 10 herein and *Aspergillus fumigatus* beta-glucosidase disclosed as SEQ ID NO: 2 in WO 2005/047499 or SEQ ID NO: 8 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: 6 herein and *Aspergillus fumigatus* CBH II disclosed as SEQ ID NO: 18 in WO 2011/057140 and as SEQ ID NO: 7 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: 17 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: 13 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: 9 herein) and *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 8 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: 10 herein and *Aspergillus fumigatus* beta-glucosidase disclosed as SEQ ID NO: 2 in WO 2005/047499 or SEQ ID NO: 8 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: 6 herein and *Aspergillus fumigatus* CBH II disclosed as SEQ ID NO: 18 in WO 2011/057140 and as SEQ ID NO: 7 herein.

In a preferred embodiment a cellulolytic enzyme composition is one described below in the “Cellulolytic Enzyme Compositions”-section.

The cellulolytic enzyme composition, protease or other enzymes, may be added in the process of the invention at the same time as the glucoamylase and alpha-amylase. According to the invention the enzymes, e.g., in the form of an enzyme composition, are 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 compositions. In an embodiment the glucoamylase and alpha-amylase are added as one blend composition and the optional

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

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

Glucoamylase

The glucoamylase used in a process 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: 12 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: 12 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: 12 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* (SEQ ID NO: 11 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: 11 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: 11 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: 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 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: 4 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: 4 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: 4 herein.

In a further embodiment the glucoamylase is derived from a strain of the genus *Pycnoporus*, in particular a strain of *Pycnoporus sanguineus*, such as a strain described in WO 2011/066576 (SEQ ID NOs 2, 4 or 6). In a preferred embodiment the glucoamylase is the one shown in SEQ ID NO: 4 in WO 2011/066576 or SEQ ID NO: 17 herein.

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

(i) a glucoamylase comprising the mature polypeptide of SEQ ID NO: 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.

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: 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.

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

A glucoamylase variant may comprise an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the mature polypeptide of SEQ ID NO: 18.

Alpha-Amylase

The alpha-amylase used in a process of the invention may be of any origin, such as of 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 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 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: 13 herein.

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: 13 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: 13 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: 13 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: 13 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: 13 herein.

In a preferred embodiment the alpha-amylase variant has one of the above mentioned substitutions, such as: G128D, Y141W, D143W or K192R.

In a preferred embodiment the alpha-amylase (using SEQ ID NO: 13 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: 13 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: 13.

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;
- cellobiohydrolase I;
- cellobiohydrolase II;

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: 8 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: 8 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: 8 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: 9 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: 10 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: 9 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: 9 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:

(i) a GH61 polypeptide comprising the mature polypeptide of SEQ ID NO: 10 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: 10 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 CBHI disclosed in SEQ ID NO: 6 in WO 2011/057140 or SEQ ID NO: 6 herein, or a strain of the genus *Trichoderma*, such as a strain of *Trichoderma reesei*.

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: 6 herein;

(ii) a cellobiohydrolase I comprising an amino acid sequence having at least 60%, such as at least 70%, *e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 6 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 from a strain of the genus *Aspergillus*, such as a strain of *Aspergillus fumigatus*; such as the one disclosed as SEQ ID NO: 7 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: 7 herein;

(ii) a cellobiohydrolase II comprising an amino acid sequence having at least 70%, *e.g.*,

at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 7 herein.

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: 10 herein, and a beta-glucosidase.

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

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: 10 herein, a beta-glucosidase, and a CBHII.

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 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: 10 herein, a beta-glucosidase, a CBHI, and a CBHII.

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: 9 herein), and *Aspergillus oryzae* beta-glucosidase fusion protein (WO 2008/057637).

In an embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic composition further comprising *Thermoascus aurantiacus* GH61A polypeptide having cellulolytic enhancing activity (SEQ ID NO: 2 in WO 2005/074656 or SEQ ID NO: 9 herein) and *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 8 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: 10 herein, and *Aspergillus fumigatus* beta-glucosidase disclosed as SEQ ID NO: 2 in WO 2005/047499 or SEQ ID NO: 8 herein, or a variant thereof, which variant has one of, preferably all of, the following

substitutions: F100D, S283G, N456E, F512Y, and optionally *Aspergillus fumigatus* CBH1, e.g., the one disclosed as SEQ ID NO: 6 in WO2011/057140 and SEQ ID NO: 6 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: 7 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: 8 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 the following substitutions:

- F100D + S283G + N456E + F512Y;
- L89M + G91L + I186V + I140V; or
- I186V + L89M + G91L + I140V + F100D + S283G + N456E + F512Y (using SEQ ID NO: 8 for numbering).

In an embodiment the cellulolytic composition further comprises the *Penicillium* sp. GH61 polypeptide shown in SEQ ID NO: 10 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: 10 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 the one shown in SEQ ID NO: 15 herein or a strain of *Bacillus deramificans*.

In an embodiment the pullulanase, such as one derived from *Bacillus* sp, is selected from the group consisting of:

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

(ii) a pullulanase 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.

Trehalase

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

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

In an embodiment the trehalase is of fungal origin, such as derived from a strain of *Trichoderma*, such as *Trichoderma reesei*, such as the one shown in SEQ ID NO: 14 herein.

In an embodiment the trehalase, such as one derived from *Trichoderma reesei*, is selected from the group consisting of:

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

(ii) a trehalase 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.

Pectinase

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).

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 polygalacacturonase is derived from a strain of *Aspergillus*, such as *Aspergillus aculeatus*.

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 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.

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: 12) and an alpha-amylase from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), e.g., SEQ ID NO: 13.

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: 18 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: 13 herein, preferably having one or more of the following substitutions: G128D, D143N, preferably 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: 17 herein and an alpha-amylase, preferably one derived from *Rhizomucor pusillus* with a linker and starch-binding domain (SBD), preferably *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), in particular the one shown in SEQ ID NO: 13 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: 4 herein and an alpha-amylase, preferably an alpha-amylase derived from *Rhizomucor pusillus* with a linker and starch-binding domain (SBD), preferably *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD)shown in SEQ ID NO: 13 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: 12 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: 13 herein, 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 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;

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;

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

i) glucoamylase derived from *Trametes cingulata*;

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

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

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

In 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 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*.

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;

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;

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 comprises

i) *Trametes cingulata* glucoamylase;

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

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

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

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

In 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;

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

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

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

In 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: 17 herein;

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

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

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

In 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: 4 herein;

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

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

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

In 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: 12 herein;

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.

Examples of 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

(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:
    - an ethanol yield boost compared to ETHANOL RED™;
    - lower glycerol production compared to ETHANOL RED™;

under the same fermentation 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

(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 which:
    - provides an ethanol yield boost of at least 0.5%, preferably at least 1.0%, more preferably at least 1.5%, more preferably at least 2.0%, more preferably at least 3.0%, more preferably at least 4.0%, even more preferably at least 5.0%, such as between 0.5-10%, e.g., between 1-6%, after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™; and/or
    - provides a lower glycerol production of at least 5%, preferably at least 10%, more preferably 15%, even more preferably at least 15%, more preferably at least 20%, such as between 4 and 40%, such as between 10 and 30% after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™.

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

(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 selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia); or
- the fermenting organism is selected from the group of derivatives of *Saccharomyces cerevisiae* MBG 4911, MBG4913 or MBG4914 having the defining characteristics of one or more of these *Saccharomyces cerevisiae* strains.

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:

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

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

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

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

wherein

- the fermenting organism is a *Saccharomyces* yeast strain providing:
  - an ethanol yield boost compared to ETHANOL RED™;
  - lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions; preferably

- provides an ethanol yield boost of at least 0.5%, preferably at least 1.0%, more preferably at least 1.5%, more preferably at least 2.0%, more preferably at least 3.0%, more preferably at least 4.0%, even more preferably at least 5.0%, such as between 0.5-10%, e.g., between 1-6%, after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™; and/or
- provides a lower glycerol production of at least 5%, preferably at least 10%, more preferably 15%, even more preferably at least 15%, more preferably at least 20%, such as between 4 and 40%, such as between 10 and 30% after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™; and/or
- the fermenting organism is selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia); or
- the fermenting organism is selected from the group of derivatives of *Saccharomyces cerevisiae* MBG 4911, MBG4913 or MBG4914 having the defining characteristics of one or more of these *Saccharomyces cerevisiae* strains.

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:

i) glucoamylase derived from *Gloeophyllum trabeum* disclosed in SEQ ID NO: 18, with the following substitutions: S95P+A121P;

ii) alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), or a variant thereof, shown in SEQ ID NO: 13 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™;
  - lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions; preferably

- provides an ethanol yield boost of at least 0.5%, preferably at least 1.0%, more preferably at least 1.5%, more preferably at least 2.0%, more preferably at least 3.0%, more preferably at least 4.0%, even more preferably at least 5.0%, such as between 0.5-10%, e.g., between 1-6%, after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™; and/or
- provides a lower glycerol production of at least 5%, preferably at least 10%, more preferably 15%, even more preferably at least 15%, more preferably at least 20%, such as between 4 and 40%, such as between 10 and 30% after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™; and/or
- the fermenting organism is selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia); or
- the fermenting organism is selected from the group of derivatives of *Saccharomyces cerevisiae* MBG 4911, MBG4913 or MBG4914 having the defining characteristics of one or more of these *Saccharomyces cerevisiae* strains.

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:

i) glucoamylase derived from *Pycnoporus sanguineus* shown in SEQ ID NO: 17;

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: 13 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™;
  - lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions; preferably

- provides an ethanol yield boost of at least 0.5%, preferably at least 1.0%, more preferably at least 1.5%, more preferably at least 2.0%, more preferably at least 3.0%, more preferably at least 4.0%, even more preferably at least 5.0%, such as between 0.5-10%, e.g., between 1-6%, after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™; and/or
- provides a lower glycerol production of at least 5%, preferably at least 10%, more preferably 15%, even more preferably at least 15%, more preferably at least 20%, such as between 4 and 40%, such as between 10 and 30% after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™; and/or
- the fermenting organism is selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia); or
- the fermenting organism is selected from the group of derivatives of *Saccharomyces cerevisiae* MBG 4911, MBG4913 or MBG4914 having the defining characteristics of one or more of these *Saccharomyces cerevisiae* strains.

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:

i) glucoamylase derived from *Gloeophyllum sepiarium* shown in SEQ ID NO: 4;

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: 13 herein, with the following substitutions: G128D+D143N;

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

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

wherein

- the fermenting organism is a *Saccharomyces* yeast strain providing:
  - an ethanol yield boost compared to ETHANOL RED™;
  - lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions; preferably

- provides an ethanol yield boost of at least 0.5%, preferably at least 1.0%, more preferably at least 1.5%, more preferably at least 2.0%, more preferably at least 3.0%, more preferably at least 4.0%, even more preferably at least 5.0%, such as between 0.5-10%, e.g., between 1-6%, after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™; and/or
- provides a lower glycerol production of at least 5%, preferably at least 10%, more preferably 15%, even more preferably at least 15%, more preferably at least 20%, such as between 4 and 40%, such as between 10 and 30% after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™; and/or
- the fermenting organism is selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia); or
- the fermenting organism is selected from the group of derivatives of *Saccharomyces cerevisiae* MBG 4911, MBG4913 or MBG4914 having the defining characteristics of one or more of these *Saccharomyces cerevisiae* strains.

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:

i) glucoamylase derived from *Trametes cingulata* shown in SEQ ID NO: 12;

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: 13 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™;
  - lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions; preferably

- provides an ethanol yield boost of at least 0.5%, preferably at least 1.0%, more preferably at least 1.5%, more preferably at least 2.0%, more preferably at least 3.0%, more preferably at least 4.0%, even more preferably at least 5.0%, such as between 0.5-10%, e.g., between 1-6%, after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™; and/or
- provides a lower glycerol production of at least 5%, preferably at least 10%, more preferably 15%, even more preferably at least 15%, more preferably at least 20%, such as between 4 and 40%, such as between 10 and 30% after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™; and/or
- the fermenting organism is selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia); or

- the fermenting organism is selected from the group of derivatives of *Saccharomyces cerevisiae* MBG 4911, MBG4913 or MBG4914, respectively, having the defining characteristics of one or more of these *Saccharomyces cerevisiae* strains.

Yeast Of The Invention

In this aspect the invention relates to *Saccharomyces* yeast strains with improved properties in ethanol production processes.

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 are not suitable for use in industrial fermentation. The most widely used commercially available industrial strain of *Saccharomyces* (i.e. for industrial scale ethanol fermentation) is the *Saccharomyces cerevisiae* strain, e.g., sold under the trade name ETHANOL RED™.

The inventors have now provided *Saccharomyces* yeast strains providing

- higher ethanol yield compared to ETHANOL RED™
- lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions.

The strains of the invention are non-recombinant *Saccharomyces* strains. The strains of the invention may be produced using the methods described in WO 2005/121337 and through matings with various strains of *Saccharomyces cerevisiae* combined with selection for characteristics including low glycerol production and high ethanol production in a raw starch ethanol production process. This is described further in Example 1 below.

As used herein, a defining characteristic of a non-recombinant *Saccharomyces cerevisiae* strain of the invention is any one or more of the following characteristics:

i) provides an ethanol yield boost of at least 0.5%, preferably at least 1.0%, more preferably at least 1.5%, more preferably at least 2.0%, more preferably at least 3.0%, more preferably at least 4.0%, even more preferably at least 5.0%, such as between 0.5-10%, e.g., between 1-6%, after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™.

ii) provides a lower glycerol production of at least 5%, preferably at least 10%, more preferably 15%, even more preferably at least 15%, more preferably at

least 20%, such as between 4 and 40%, such as between 10 and 30% after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™.

Specific embodiments of yeast strains of the invention can be selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia).

A yeast strain of the invention may also be a derivative of *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4914, respectively, having the defining characteristics of one or more of these *Saccharomyces cerevisiae* strains.

In an embodiment yeast strains of the invention grow on xylose as a sole carbon source, e.g., determined using the Test T1 described below in the "Materials & Methods" section. As current wild type and industrial strains of *Saccharomyces* are not capable of growth on xylose at the rate at which strains of the invention, in particular *Saccharomyces* MBG4911, MBG4913 and MBG4914, respectively, grow on xylose, the strains of the invention are readily differentiated from current wild type strains of *Saccharomyces* yeast and strains of *Saccharomyces* yeast that are used in the ethanol industry prior to the present invention such as ETHANOL RED™.

Further, in an embodiment a *Saccharomyces* yeast strain of the invention shows more than two-fold increase in biomass, such as more than six-fold increase in biomass, such as more than 20-fold increase in biomass determined using the Test T1 described in the "Materials & Methods" section.

According to the invention the yeast of the invention may be in any viable form, including crumbled, dry, including active dry and instant, compressed, cream form etc. In a preferred embodiment the *Saccharomyces cerevisiae* yeast strain used in a process of the invention is dry yeast, such as active dry yeast. In a preferred embodiment the *Saccharomyces cerevisiae* yeast strain used in a process of the invention is compressed yeast. In an embodiment the *Saccharomyces cerevisiae* yeast strain used in a process of the invention is cream yeast.In an embodiment a *Saccharomyces cerevisiae* strain of the invention is dry yeast.

The invention also relates to a derivative of *Saccharomyces* strain of the invention including *Saccharomyces* MBG4911, MBG4913 and MBG4914.

As used herein, a "derivative" is a yeast strain derived from a yeast strain of the invention (e.g., *Saccharomyces* MBG4911, MBG4913 and MBG4914), including through

mutagenesis, recombinant DNA technology, mating, cell fusion, or cytoduction between yeast strains. The strain may be a direct progeny (i.e. the product of a mating between a strain of the invention and another strain or itself), or a distant progeny resulting from an initial mating between a strain of the invention and another strain or itself, followed by a large number of subsequent matings.

In one embodiment, a derivative strain is a hybrid strain produced by culturing a first yeast strain with a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913 and MBG4914, under conditions which permit combining of DNA between the first yeast strain and a strain of the invention.

In an embodiment the invention relates to methods of producing a derivative of a yeast strain of the invention, in particular a derivative of *Saccharomyces* MBG 4911, MBG4913 or MBG4914, respectively, which exhibits the defining characteristics of a strain of the invention, in particular *Saccharomyces* MBG 4911, MBG4913 or MBG4914, respectively, comprising:

(a) providing:

(i) a first yeast strain; and

(ii) a second yeast strain, wherein the second yeast strain is a yeast strain of the invention, in particular strain *Saccharomyces* MBG4911, MBG4913 or MBG4914, respectively, or a derivative of yeast strain of the invention, in particular a derivative of *Saccharomyces* MBG 4911, MBG4913 or MBG4914, respectively;

(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 strain.

In an embodiment step (c) comprises screening or selecting for a hybrid strain which exhibits one or more defining characteristic of strain *Saccharomyces* MBG4911, MBG4913 or MBG4914, respectively.

In an embodiment method comprises 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 yeast strain, until a derivative is obtained which exhibits the defining characteristics of a yeast strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913, or MBG4914, respectively.

In an embodiment 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.

In an embodiment the derivative of a *Saccharomyces* yeast strain of the invention is

produced by the method described above. The method comprises incubating a yeast strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913, or MBG4914, respectively, with a substrate comprising a fermentable sugar under conditions which permit fermentation of the fermentable sugar to produce ethanol.

In an embodiment the invention relates to use of a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913, or MBG4914, respectively, in a process of producing ethanol of the invention.

In an embodiment the invention relates to the use of a strain of the invention, in particular *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4914, respectively, or a derivative thereof in a process of the invention.

In one embodiment, a derivative of a yeast strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913 and MBG4914, is a hybrid strain produced by culturing a first yeast strain with a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913 and MBG4914, under conditions which permit combining of DNA between the first yeast strain and a strain of the invention, in particular MBG4911, MBG4913 and MBG4914, respectively.

In one embodiment, a derivative yeast strain of the invention may be prepared by:

(a) culturing a first yeast strain with a second yeast strain, wherein the second yeast strain is a yeast strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913 or MBG4914, respectively, or a derivative of a strain of the invention, under conditions which permit combining of DNA between the first yeast strain and the second yeast strain; and

(b) isolating hybrid strains; and

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

In one embodiment, the derivative of a strain of the invention exhibits one or more defining characteristic of a yeast strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913 or MBG4914, respectively. Derivatives of *Saccharomyces* yeast which exhibit one or more defining characteristics are produced using a yeast strain of the invention. In this regard, a yeast strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913 or MBG4914, respectively, forms the basis for preparing other yeast strains having the defining characteristics of a yeast strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913, or MBG4914, respectively. For example, strains of *Saccharomyces* yeast which exhibit one or more defining characteristics of a yeast strain of the invention can be derived from a yeast strain of the invention using methods such as classical mating, cell fusion, or cytoduction between yeast strains, mutagenesis or

recombinant DNA technology.

In one embodiment, a derivative of a yeast strain of the invention which exhibits one or more defining characteristics of a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913 and MBG4914, respectively, may be produced by:

(a) culturing a first yeast strain with a second yeast strain, wherein the second yeast strain is a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913 or MBG4914, respectively, or a derivative thereof, under conditions which permit combining of DNA between the first yeast strain and the second yeast strain;

(b) screening or selecting for a derivative strain, such as screening or selecting for a derivative with higher ethanol yield, e.g., in corn mash, compared to the first strain, and/or screening or selecting for a hybrid which has a lower glycerol production, e.g., in corn mash, compared to the first strain;

(c) optionally repeating steps (a) and (b) with the screened or selected strain as the first yeast strain and/or the second yeast strain, until a derivative strain is obtained which exhibits one or more defining characteristics of a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913 and MBG4914, respectively.

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* yeast strain. More typically, the first yeast strain is a *Saccharomyces cerevisiae* yeast 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 a strain of the invention, in particular MBG4911, MBG4913 and MBG4914, respectively. The first yeast strain may be, for example, any *Saccharomyces cerevisiae* strain, such as for example ETHANOL RED™. It will also be appreciated that the first yeast strain may be a strain of the invention or a strain which exhibits one or more defining characteristics of a strain of the invention.

The first and second yeast strains are cultured under conditions which permit combining of DNA between the yeast strains. As used herein, “combining of DNA” between yeast strains refers to combining of all or a part of the genome of the yeast strains. Combining of DNA between yeast strains may be by any method suitable for combining DNA of at least two yeast cells, and may include, for example, mating methods which comprise sporulation of the yeast strains to produce haploid cells and subsequent hybridising of compatible haploid cells; cytoduction; or cell fusion such as protoplast fusion.

In one embodiment, culturing the first yeast strain with the second yeast strain, under conditions which permit combining of DNA between the first yeast strain and the second yeast strain, comprises:

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

(ii) germinating and hybridizing spores produced by the first yeast strain with spores produced by the second yeast strain.

In one embodiment, the method of producing a derivative of a yeast strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913, or MBG4914, respectively, which exhibits one or more defining characteristics of a yeast strain of the invention, comprises:

(a) providing: (i) a first yeast strain; and (ii) a second yeast strain, wherein the second yeast strain is a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913, or MBG4914, respectively, or a derivative thereof;

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

(c) germinating and hybridising the spores of the first yeast strain with germinated spores of the second yeast strain;

(d) screening or selecting for a derivative of a strain of the invention, such as screening or selecting for a derivative with higher ethanol yield, e.g., in corn mash, compared to the first strain, and/or screening or selecting for a hybrid with lower glycerol production, e.g., in corn mash, compared to the first strain;

(e) optionally repeating steps (b) to (d) with the screened or selected strain as the first and/or second yeast strain.

Methods for sporulating, germinating and hybridising yeast strains, and in particular, *Saccharomyces* strains, are known in the art and are described in, for example, Ausubel, F. M. et al., (1997) Current Protocols in Molecular Biology, Volume 2, pages 13.2.1 to 13.2.5 (John Willey & Sons Inc); Chapter 7, "Sporulation and Hybridisation of yeast" by R.R. Fowell, in "The Yeasts" vol 1, A.H. Rose and J.S. Harrison (Eds), 1969, Academic Press.

In one embodiment, the yeast strains may be cultured under conditions which permit cell fusion. Methods for the generation of intraspecific or interspecific hybrids using cell fusion techniques are described in, for example, Spencer et al. (1990) in, Yeast Technology, Spencer JFT and Spencer DM (Eds), Springer Verlag, New York.

In another embodiment, the yeast strains may be cultured under conditions which permit cytoduction. Methods for cytoduction are described in, for example, Inge-Vechymov et al. (1986) Genetika 22: 2625-2636; Johnston (1990) in, Yeast technology, Spencer JFT and Spencer DM (Eds), Springer Verlag, New York.

In one embodiment, screening or selecting for derivatives of a strain of the invention,

in particular *Saccharomyces* MBG4911, MBG4913, or MBG4914, respectively, comprises screening or selecting for a derivative with higher ethanol production, e.g., in corn mash, compared to the first strain, and/or screening or selecting for a hybrid which produces less glycerol, e.g., in corn mash, compared to the first strain.

Methods for determining the amount of ethanol and glycerol produced by a strain are known in the art. For example, methods for testing for determining the amount of ethanol and glycerol produced by a strain during fermentation of corn mash are described in, for example, WO 2011/035392.

Once the amount of ethanol and glycerol produced are known, the ratio of ethanol/glycerol can be readily determined. Accordingly, strains can be readily screened for production levels of ethanol and/or glycerol using known methods.

In one embodiment, a derivative of a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913, or MBG4914, respectively, which exhibits one or more defining characteristics of a strain of the invention, may be a mutant of a strain. 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 a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913, or MBG4914, which exhibits one or more defining characteristics of a strain of the invention, may be a recombinant derivative of a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913, or MBG4914.

A recombinant derivative of a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913, or MBG4914, respectively, is a strain produced by introducing into a strain of the invention, in particular *Saccharomyces* MBG4911, MBG4913, or MBG4914, a nucleic acid using recombinant DNA technology. Methods for the introduction of nucleic acid into *Saccharomyces* yeast cells, and in particular strains of *Saccharomyces*, are known in the art and are described in, for example, Ausubel, F. M. et al. (1997), Current Protocols in Molecular Biology, Volume 2, pages 13.7.1 to 13.7.7, published by John Wiley & Sons Inc.

Composition 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 nonenaturally occurring component.

As mentioned above a *Saccharomyces* yeast strain, in particular *Saccharomyces 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 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 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* MBG4911, MBG4913 or MBG4914, respectively, 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

According to the invention the composition may comprise a *Saccharomyces* yeast of the invention, in particular *Saccharmyces* MBG4911, MBG4913 or MBG4914, respectively, and any suitable surfactant. In an embodiment the surfactant(s) is/are an anionic surfactant, cationic surfactant, and/or nonionic surfactant.

Emulsifier

According to the invention the composition may comprise a *Saccharomyces* yeast of the invention, in particular *Saccharmyces* MBG4911, MBG4913 or MBG4914, respectively, 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.

In an embodiment the composition of the invention comprises a *Saccharomyces* yeast of the invention, in particular *Saccharmyces* MBG4911, MBG4913 or MBG4914, respectively, 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

According to the invention the composition may comprise a *Saccharomyces* yeast of the invention, in particular *Saccharmyces* MBG4911, MBG4913 or MBG4914, respectively, and any suitable gum. In an embodiment the gum is acacia gum, in particular for cream, compressed and dry yeast.

Swelling Agents

According to the invention the composition may comprise a *Saccharomyces* yeast of the invention, in particular *Saccharmyces* MBG4911, MBG4913 or MBG4914, respectively, and any suitable swelling agent. In an embodiment the swelling agent is methyl cellulose or carboxymethyl cellulose.

Antioxidant

According to the invention the composition may comprise a *Saccharomyces* yeast of the invention, in particular *Saccharmyces* MBG4911, MBG4913 or MBG4914, respectively, 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 may be further described by the following numbered paragraphs:

[1]. A process of producing ethanol from starch-containing material comprising:

(a) saccharifying 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: glucoamylase and alpha-amylase, and optionally protease; and
- the fermenting organism is a *Saccharomyces* yeast strain providing:
    - an ethanol yield boost compared to ETHANOL RED™;
    - lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions.

[2]. The process of paragraph [1], wherein the fermenting organism grows on xylose as a sole carbon source, e.g., determined using the Test T1.

[3]. The process of paragraph [2], wherein the fermenting organism shows more than two-fold increase in biomass, such as more than six-fold increase in biomass, such as more than 20-fold increase in biomass determined using the Test T1.

[4]. The process of any of paragraphs [1]-[3], wherein the fermenting organism provides an ethanol yield boost of at least 0.5%, preferably at least 1.0%, more preferably at least 1.5%, more preferably at least 2.0%, more preferably at least 3.0%, more preferably at least 4.0%, even more preferably at least 5.0%, such as between 0.5-10%, e.g., between 1-6%, after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™.

[5]. The process of any of paragraphs [1]-[4], wherein the fermenting organism provides a lower glycerol production of at least 5%, preferably at least 10%, more preferably 15%, even more preferably at least 15%, more preferably at least 20%, such as between 4 and 40%, such as between 10 and 30% after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™.

[6]. The process of any of paragraphs [1]-[5], wherein the fermenting organism is selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia).

[7]. The process of any of paragraphs [1]-[6], wherein the fermenting organism is selected from the group of derivatives of *Saccharomyces cerevisiae* MBG4911, MBG4913 andr MBG4914 having the defining characteristics of one or more of these *Saccharomyces cerevisiae* strains.

[8]. The process of any of paragraphs [1]-[7], wherein the *Saccharomyces cerevisiae* yeast strain is in a viable form, such as in particular dry yeast, cream yeast, or compressed yeast

[9]. The process of any of paragraphs [1]-[8], wherein the glucoamylase is a *Gloeophyllum* glucoamylase, preferably *Gloeophyllum trabeum* glucoamylase.

[10]. The process of any of paragraphs [1]-[9], wherein the glucoamylase, e.g., derived from *Gloeophyllum trabeum,* 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.

[11]. The process of any of paragraphs [1]-[10], wherein the glucoamylase is the *Gloeophyllum trabeum* glucoamylase shown in SEQ ID NO: 18 having one of the following substitutions: V59A; S95P; A121P; T119W; S95P+A121P; V59A+S95P; S95P+T119W; V59A+S95P+A121P; or S95P+T119W+A121P, especially S95P+A121P.

[12]. The process of any of paragraphs [1]-[11], wherein the glucoamylase is a *Trametes* glucoamylase, preferably *Trametes cingulata* glucoamylase.

[13]. The process of any of paragraphs [1]-[12], wherein the glucoamylase, e.g., derived from *Trametes cingulata,* is selected from the group consisting of:

(i) a glucoamylase comprising the mature polypeptide of SEQ ID NO: 12 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: 12 herein.

[14]. The process of any of paragraphs [1]-[13], wherein the alpha-amylase is, e.g., derived from *Rhizomucor pusillus*, preferably with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably the one disclosed as V039 in Table 5 in WO 2006/069290 or SEQ ID NO: 13 herein.

[15]. The process of any of paragraphs [1]-[14], wherein the alpha-amylase, e.g., derived from *Rhizomucor pusillus,* is selected from the group consisting of:

(i) an alpha-amylase comprising the mature polypeptide of SEQ ID NO: 13 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: 13 herein.

[16]. The process any of paragraphs [1]-[15], wherein the glucoamylase is the *Trametes cingulata* glucoamylase shown in SEQ ID NO: 12 and the alpha-amylase is *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD).

[17]. The process of any of paragraphs [1]-[16], wherein the alpha-amylase is *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably one having at least one of the following substitutions or combinations of substitutions: D165M; Y141W; Y141R; K136F; K192R; P224A; P224R; S123H+Y141W; G20S + Y141W; A76G + Y141W; G128D + Y141W; G128D + D143N; P219C + Y141W; N142D + D143N; Y141W + K192R; Y141W + D143N; Y141W + N383R; Y141W + P219C + A265C; Y141W + N142D + D143N; Y141W + K192R V410A; G128D + Y141W + D143N; Y141W + D143N + P219C; Y141W + D143N + K192R; G128D + D143N + K192R; Y141W + D143N + K192R + P219C; G128D + Y141W + D143N + K192R; or G128D + Y141W + D143N + K192R + P219C, especially G128D+D143N (using SEQ ID NO: 13 for numbering).

[18]. The process any of paragraphs [1]-[17], wherein the glucoamylase is the *Gloeophyllum trabeum* glucoamylase shown in SEQ ID NO: 18 having one of the following substitutions: S95P+A121P and the alpha-amylase is *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably one having the following substitutions G128D+D143N (using SEQ ID NO: 13 for numbering).

[19]. The process of any of paragraphs [1]-[18], wherein the glucoamylase is derived from *Pycnoporus*, in particular the *Pycnoporus sanguineus*, preferably the glucoamylase shown in SEQ ID NO: 17 herein.

[20]. The process of any of paragraphs [1]-[19], wherein the glucoamylase, e.g., derived from *Pycnoporus sanguineus*, 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.

[21]. The process of any of paragraphs [1]-[20], wherein the glucoamylase is the *Pycnoporus sanguineus* glucoamylase shown in SEQ ID NO: 17 herein and the alpha-amylase is the *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably the one disclosed as V039 in Table 5 in WO 2006/069290 or SEQ ID NO: 13 herein, preferably one having one or more of the following substitutions: G128D, D143N, especially G128D+D143N.

[22]. The process of any of paragraphs [1]-[21], wherein the ratio between glucoamylase and alpha-amylase 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).

[23]. The process of any of paragraphs [1]-[22], wherein the total dose of glucoamylase and alpha-amylase added is from 10-1,000 µg/g DS, such as from 50-500 µg/g DS, such as 75-250 µg/g DS.

[24]. The process of any of paragraphs 1-24, wherein the total dose of cellulolytic enzyme composition added is from 10-500 µg/g DS, such as from 20-400 µg/g DS, such as 20-300 µg/g DS.

[25]. The process of any of paragraphs [1]-[24], wherein 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.

[26]. The process of any of paragraphs [1]-[21], wherein the fermenting organism is a non-recombinant *Saccharomyces* strain, preferably non-recombinant *Saccharomyces cerevisiae* strain.

[27]. The process of any of paragraphs [1]-[26], wherein the fermenting organism strain 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.

[28]. The process of any of paragraphs [1]-[27], wherein saccharification and fermentation are done separately or simultaneously.

[29]. The process of any of paragraphs [1]-[28], wherein the ethanol (i.e., product) is recovered after fermentation.

[30]. The process of any of paragraphs [1]-[29], wherein the starch-containing material is plant material selected from the corn (maize), cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, beans, sweet potatoes, oats, or a mixture thereof, preferably corn.

[31]. The process of any of paragraphs [1]-[30], wherein the starch-containing material is granular starch.

[32]. The process of any of paragraphs [1]-[31], wherein the process is carried out at a pH in the range between 3 and 7, preferably from 3 to 6, or more preferably from 3.5 to 5.0.

[33]. The process of any of paragraphs [1]-[32], wherein the dry solid content (DS) lies in the range from 10-55 wt.-% (DS), preferably 25-45 wt.-%, more preferably 30-40% of starch-containing material.

[34]. The process of any of paragraphs [1]-[33], wherein the sugar concentration is kept at a level below about 6 wt.-%, preferably 3 wt.-%, during saccharification and fermentation, especially below 0.25 wt.-%.

[35]. The process of any of paragraphs [1]-[34], wherein a slurry comprising starch-containing material reduced in particle size and water, is prepared before step (a).

[36]. The process of any of paragraphs [1]-[35], wherein the starch-containing material is prepared by reducing the particle size of the starch-containing material, preferably by milling, such that at least 50% of the starch-containing material has a particle size of 0.1-0.5 mm.

[37]. The process of any of paragraphs [1]-[36], wherein the starch-containing plant material is reduced in particle size, such as by dry or wet milling or using particle size emulsion technology.

[38]. The process of any of paragraphs [1]-[37], wherein the fermentation is carried out for 30 to 150 hours, preferably 48 to 96 hours.

[39]. The process of any of paragraphs [1]-[34], wherein the temperature during fermentation in step (b) or simultaneous saccharification and fermentation in steps (a) and (b) is between 25°C and 40°C, preferably between 28°C and 36°C, such as between 28°C and 35°C, such as between 28°C and 34°C, such as around 32°C.

[40]. The process of any of paragraphs [1]-[39], wherein further a protease is present during saccharification and/or fermentation.

[41]. The process of any of paragraphs [1]-[40], wherein the glucoamylase is present and/or added in an amount of 0.001 to 10 AGU/g DS, preferably from 0.01 to 5 AGU/g DS, especially 0.1 to 0.5 AGU/g DS.

[42]. The process of any of paragraphs [1]-[41], wherein the glucoamylase is present and/or added in an amount of 10-1,000 micro grams Enzyme Protein/g DS

[43]. The process of any of paragraphs [1]-[42], wherein the alpha-amylase is present and/or added in an amount of 0.001 to 10 AFAU/g DS, preferably from 0.01 to 5 AFAU/g DS, especially 0.3 to 2 AFAU/g DS or 0.001 to 1 FAU-F/g DS, preferably 0.01 to 1 FAU-F/g DS.

[44]. The process of any of paragraphs [1]-[43], wherein the alpha-amylase is present and/or added in an amount of 10-1,000 micro grams Enzyme Protein/g DS.

[45]. The process of any of paragraphs [1]-[44], wherein a cellulolytic enzyme composition is present and/or added during saccharification, fermentation or simultaneous saccharification and fermentation.

[46]. The process of paragraph [45], wherein the cellulolytic enzyme composition is present and/or added in an amount 1-10,000 micrograms EP/g DS, such as 2-5,000, such as 3 and 1,000, such as 4 and 500 micrograms EP/g DS.

[47]. The process of any of paragraphs [45]-[46], wherein cellulolytic enzyme composition is present and/or added in an amount in the range from 0.1-100 FPU per gram total solids (TS), preferably 0.5-50 FPU per gram TS, especially 1-20 FPU per gram TS.

[48]. The process of any of paragraphs [1]-[47], wherein protease is present and/or added in an amount in the range 1-1,000 µg EP/g DS, such as 2-500 µg EP/g DS, such as 3-250 µg

EP/g DS.

[49]. The process of any of paragraphs [1]-[48], wherein the fermenting organism is added to fermentation, so that the count per mL of fermentation medium is in the range from 10^5 to 10^{12}, preferably from 10^7 to 10^{10}, especially about $5x10^7$.

[50]. The process of any of paragraphs [1]-[49], comprising:

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

(b) fermenting using a fermentation organism;

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

i) glucoamylase derived from *Trametes cingulata*;

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

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

- an ethanol yield boost compared to ETHANOL RED™;
- lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions; preferably

wherein the fermenting organism provides an ethanol yield boost of at least 0.5%, preferably at least 1.0%, more preferably at least 1.2%, even more preferably at least 1.5%, such as between 0.5-5%, e.g., 1.0-3.0%, after 72 hours at the conditions defined in Example 2, compared to ETHANOL RED™; and/or

wherein the fermenting organism provides a lower glycerol production of at least 5%, preferably at least 10%, more preferably at least 15%, such as between 5-25%, such as 10-20% after 72 hours, at the conditions defined in Example 3, compared to ETHANOL RED™; in particular wherein the fermenting organism is selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia); and/or

wherein the fermenting organism is selected from the group of derivatives of *Saccharomyces cerevisiae* MBG 4911, MBG4913 or MBG4914 having the defining characteristics of one or more of these *Saccharomyces cerevisiae* strains.

[51]. The process of any of paragraphs [1]-[50], comprising:

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

(b) fermenting using a fermentation organism;

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

i) glucoamylase derived from *Trametes cingulata*;

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*;

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

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

- an ethanol yield boost compared to ETHANOL RED™;
- lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions; preferably

wherein the fermenting organism provides an ethanol yield boost of at least 0.5%, preferably at least 1.0%, more preferably at least 1.2%, even more preferably at least 1.5%, such as between 0.5-5%, e.g., 1.0-3.0%, after 72 hours at the conditions defined in Example 2, compared to ETHANOL RED™; and/or

wherein the fermenting organism provides a lower glycerol production of at least 5%, preferably at least 10%, more preferably at least 15%, such as between 5-25%, such as 10-20% after 72 hours, at the conditions defined in Example 3, compared to ETHANOL RED™;

in particular wherein the fermenting organism is selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia); and/or

wherein the fermenting organism is selected from the group of derivatives of *Saccharomyces cerevisiae* MBG 4911, MBG4913 or MBG4914 having the defining characteristics of one or more of these *Saccharomyces cerevisiae* strains.

[52]. The process of any of paragraphs [1]-[51], comprising:

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

(b) fermenting using a fermentation organism;

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

i) glucoamylase derived from *Gloeophyllum trabeum* shown in SEQ ID NO: 18, preferably having at least 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: 18 for numbering);

ii) alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably one having at least one of the following substitutions or combinations of substitutions: D165M; Y141W; Y141R; K136F; K192R; P224A; P224R; S123H+Y141W; G20S + Y141W; A76G + Y141W; G128D + Y141W; G128D + D143N; P219C + Y141W; N142D + D143N; Y141W + K192R; Y141W + D143N; Y141W + N383R; Y141W + P219C + A265C; Y141W + N142D + D143N; Y141W + K192R V410A; G128D + Y141W + D143N; Y141W + D143N + P219C; Y141W + D143N + K192R; G128D + D143N + K192R; Y141W + D143N + K192R + P219C; G128D + Y141W + D143N + K192R; or G128D + Y141W + D143N + K192R + P219C, especially G128D+D143N (using SEQ ID NO: 13 for numbering);

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

- an ethanol yield boost compared to ETHANOL RED™;
- lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions; preferably

wherein the fermenting organism provides an ethanol yield boost of at least 0.5%, preferably at least 1.0%, more preferably at least 1.2%, even more preferably at least 1.5%, such as between 0.5-5%, e.g., 1.0-3.0%, after 72 hours at the conditions defined in Example 2, compared to ETHANOL RED™; and/or

wherein the fermenting organism provides a lower glycerol production of at least 5%, preferably at least 10%, more preferably at least 15%, such as between 5-25%, such as 10-20% after 72 hours, at the conditions defined in Example 3, compared to ETHANOL RED™;

in particular wherein the fermenting organism is selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia); and/or

wherein the fermenting organism is selected from the group of derivatives of *Saccharomyces cerevisiae* MBG 4911, MBG4913 or MBG4914 having the defining

characteristics of one or more of these *Saccharomyces cerevisiae* strains.

[53]. The process of any of paragraphs [1]-[52], comprising:

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

(b) fermenting using a fermentation organism;

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

i) glucoamylase derived from *Gloeophyllum trabeum* shown in SEQ ID NO: 18, preferably having at least 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: 18 for numbering);

ii) alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably one having at least one of the following substitutions or combinations of substitutions: D165M; Y141W; Y141R; K136F; K192R; P224A; P224R; S123H+Y141W; G20S + Y141W; A76G + Y141W; G128D + Y141W; G128D + D143N; P219C + Y141W; N142D + D143N; Y141W + K192R; Y141W + D143N; Y141W + N383R; Y141W + P219C + A265C; Y141W + N142D + D143N; Y141W + K192R V410A; G128D + Y141W + D143N; Y141W + D143N + P219C; Y141W + D143N + K192R; G128D + D143N + K192R; Y141W + D143N + K192R + P219C; G128D + Y141W + D143N + K192R; or G128D + Y141W + D143N + K192R + P219C, especially G128D+D143N (using SEQ ID NO: 13 for numbering);

iii) cellulolytic enzyme composition derived from a strain of *Trichoderma reesei*; preferably a cellulolytic enzyme composition derived from *Trichoderma reesei* further comprising *Penicillium emersonii* GH61A polypeptide disclosed as SEQ ID NO: 2 in WO 2011/041397 or SEQ ID NO: 10 herein, and *Aspergillus fumigatus* beta-glucosidase disclosed as SEQ ID NO: 2 in WO 2005/047499 or SEQ ID NO: 8 herein, or a variant thereof, preferably a variant having one of, preferably all of, the following substitutions: F100D, S283G, N456E, F512Y and optionally *Aspergillus fumigatus* Cel7A CBH1 disclosed as SEQ ID NO: 6 in WO2011/057140 and SEQ ID NO: 6 herein and *Aspergillus fumigatus* CBH II disclosed as SEQ ID NO: 18 in WO 2011/057140 and as SEQ ID NO: 7 herein;

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

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

- an ethanol yield boost compared to ETHANOL RED™;
- lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions; preferably

wherein the fermenting organism provides an ethanol yield boost of at least 0.5%, preferably at least 1.0%, more preferably at least 1.2%, even more preferably at least 1.5%, such as between 0.5-5%, e.g., 1.0-3.0%, after 72 hours at the conditions defined in Example 2, compared to ETHANOL RED™; and/or

wherein the fermenting organism provides a lower glycerol production of at least 5%, preferably at least 10%, more preferably at least 15%, such as between 5-25%, such as 10-20% after 72 hours, at the conditions defined in Example 3, compared to ETHANOL RED™;

in particular wherein the fermenting organism is selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia); and/or

wherein the fermenting organism is selected from the group of derivatives of *Saccharomyces cerevisiae* MBG 4911, MBG4913 or MBG4914 having the defining characteristics of one or more of these *Saccharomyces cerevisiae* strains.

[54]. The process of any of paragraphs [1]-[53], 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:

i) glucoamylase derived from *Pycnoporus sanguineus* shown in SEQ ID NO: 17 herein,

ii) alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably one having at least one of the following substitutions or combinations of substitutions: D165M; Y141W; Y141R; K136F; K192R; P224A; P224R; S123H+Y141W; G20S + Y141W; A76G + Y141W; G128D + Y141W; G128D + D143N; P219C + Y141W; N142D + D143N; Y141W + K192R; Y141W + D143N; Y141W + N383R; Y141W + P219C + A265C; Y141W + N142D + D143N; Y141W + K192R V410A; G128D + Y141W + D143N; Y141W + D143N + P219C; Y141W + D143N + K192R; G128D + D143N + K192R; Y141W + D143N + K192R + P219C; G128D + Y141W + D143N + K192R; or G128D + Y141W +

D143N + K192R + P219C, especially G128D+D143N (using SEQ ID NO: 13 for numbering).;

optionally iii) cellulolytic enzyme composition derived from a strain of *Trichoderma reesei*; preferably a cellulolytic composition derived from *Trichoderma reesei* further comprising *Penicillium emersonii* GH61A polypeptide disclosed as SEQ ID NO: 2 in WO 2011/041397 or SEQ ID NO: 10 herein, and *Aspergillus fumigatus* beta-glucosidase disclosed as SEQ ID NO: 2 in WO 2005/047499 or SEQ ID NO: 8 herein, or a variant thereof, preferably a variant having one of, preferably all of, the following substitutions: F100D, S283G, N456E, F512Y and optionally *Aspergillus fumigatus* Cel7A CBH1 disclosed as SEQ ID NO: 6 in WO2011/057140 and SEQ ID NO: 6 herein and *Aspergillus fumigatus* CBH II disclosed as SEQ ID NO: 18 in WO 2011/057140 and as SEQ ID NO: 7 herein;

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

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

- an ethanol yield boost compared to ETHANOL RED™;
- lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions; preferably

wherein the fermenting organism provides an ethanol yield boost of at least 0.5%, preferably at least 1.0%, more preferably at least 1.2%, even more preferably at least 1.5%, such as between 0.5-5%, e.g., 1.0-3.0%, after 72 hours at the conditions defined in Example 2, compared to ETHANOL RED™; and/or

wherein the fermenting organism provides a lower glycerol production of at least 5%, preferably at least 10%, more preferably at least 15%, such as between 5-25%, such as 10-20% after 72 hours, at the conditions defined in Example 3, compared to ETHANOL RED™;

in particular wherein the fermenting organism is selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia); and/or

wherein the fermenting organism is selected from the group of derivatives of *Saccharomyces cerevisiae* MBG 4911, MBG4913 or MBG4914 having the defining characteristics of one or more of these *Saccharomyces cerevisiae* strains.

[55]. The process of any of paragraphs [1]-[54], wherein the ratio between glucoamylase and alpha-amylase 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).

[56]. The process of paragraphs [1]-[55], wherein the saccharification and fermentation are carried out simultaneously.

[57]. The process of any of paragraphs [1]-[56], wherein an enzyme composition of paragraphs 1-61 is used as the enzymes in saccharification or fermentation or simultaneous saccharification and fermentation.

[58]. A *Saccharomyces* yeast strain providing:

- higher ethanol yield compared to ETHANOL RED™
- lower glycerol production compared to ETHANOL RED™;

under the same fermentation conditions.

[59]. The strain of paragraph [58], wherein the yeast strain grows on xylose as a sole carbon source, e.g., determined using the Test T1.

[60]. The strain of paragraph [59], wherein the yeast strain shows more than two-fold increase in biomass, such as more than six-fold increase in biomass, such as more than 20-fold increase in biomass determined using the Test T1.

[61]. The strain of any of paragraphs [58]-[60], wherein the yeast strain has the following defining characteristics:

- provides an ethanol yield boost of at least 0.5%, preferably at least 1.0%, more preferably at least 1.5%, more preferably at least 2.0%, more preferably at least 3.0%, more preferably at least 4.0%, even more preferably at least 5.0%, such as between 0.5-10%, e.g., between 1-6%, after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™.

[62]. The strain of any of paragraphs [58]-[61], wherein the yeast strain has the following defining characteristics:

- provides a lower glycerol production of at least 5%, preferably at least 10%, more preferably 15%, even more preferably at least 15%, more preferably at least 20%, such as between 4 and 40%, such as between 10 and 30% after 88 hours, at the conditions defined in Example 3 or Example 4, compared to ETHANOL RED™.

[63]. The strain of any of paragraphs [58]-[62], wherein the yeast strain is selected from the group consisting of *Saccharomyces cerevisiae* MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), *Saccharomyces cerevisiae* MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), and *Saccharomyces cerevisiae* MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia).

[64]. The strain of any of paragraphs [58]-[63], wherein the yeast strain is selected from the group of derivatives of *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4914 having the defining characteristics of one or more of these *Saccharomyces cerevisiae* strains.

[65]. The strain of any of paragraphs [58]-[64], wherein the *Saccharomyces cerevisiae* strain is in a viable form, such as in particular dry yeast, cream yeast or compressed yeast.

[66]. A method of producing a derivative of strain *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4914, respectively, which exhibits the defining characteristics of strains *Saccharomyces cerevisiae* MBG 4911, MBG4913 or MBG4914, respectively, comprising:

(a) providing:

(i) a first yeast strain; and

(ii) a second yeast strain, wherein the second yeast strain is a strain of the invention, in particular strain *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4914, respectively, or a derivative of strain *Saccharomyces cerevisiae* MBG 4911, MBG4913 or MBG4914, respectively;

(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 strain.

[67]. The method of paragraph [66], wherein step (c) comprises screening or selecting for a hybrid strain which exhibits one or more defining characteristic of strain *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4914 , respectively.

[68]. The method of paragraphs [66] or [67], comprising the further step of:

(d) repeating steps (b) and (c) with the screened or selected strain from step (c) as the first and/or second strain, until a derivative is obtained which exhibits the defining characteristics of strain *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4914,

respectively.

[69]. The method of paragraph [66]-[68], 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.

[70]. A *Saccharomyces* strain produced by the method of any of paragraphs [66]-[69].

[71]. A method of producing ethanol, comprising incubating a strain of any of paragraphs [58]-[70] with a substrate comprising a fermentable sugar under conditions which permit fermentation of the fermentable sugar to produce ethanol.

[72]. Use of a strain of paragraph [58]-[70] in the production of ethanol.

[73]. Use of strain *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4914, respectively, in the production of a *Saccharomyces* strain which exhibits one or more defining characteristics of strain *Saccharomyces cerevisiae* MBG 4911, MBG4913 or MBG4914, respectively.

[74]. Use of strain *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4914, respectively, or a derivative strain thereof in a process according to any of paragraphs [1]-[57].

[75]. A composition comprising a *Saccharomyces* yeast strain of any of paragraphs [58]-[65] and one or more components selected from the group consisting of: surfactants, emulsifiers, gums, swelling agents, and antioxidants.

[76]. The composition of paragraph [75], wherein the *Saccharomyces* yeast strain is *Saccharomyces* MBG4911, MBG4913 or MBG4914.

[77]. The composition of paragraphs [75] or [76], wherein the *Saccharomyces* yeast strain is in a viable form, such as in particular in dry, cream or compressed form.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as

illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. 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 be controlling.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

MATERIALS & METHODS

Materials:

PsAMG: Glucoamylase derived from *Pycnoporus sanguineus* disclosed as shown in SEQ ID NO: 4 in WO 2011/066576 and in SEQ ID NO: 17 herein.

TcAMG: Glucoamylase derived from *Trametes cingulata* shown in SEQ ID NO: 12 herein or SEQ ID NO: 2 in WO 2006/69289.

JA126: Alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) shown in SEQ ID NO: 13 herein.

AAPE096: Alpha-amylase derived from *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD) shown in SEQ ID NO: 13 herein, with the following substitutions: G128D+D143N.

Yeast:

ETHANOL RED™ ("ER"): *Saccharomyces cerevisiae* yeast available from Fermentis (A Lesaffre Division), USA.

Saccharomyces MBG4911, MBG4913 and MBG4914: Non-recombinant *Saccharomyces cerevisiae* yeast strains 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
MBG4911	V15/001459	January 13, 2015;
MBG4913	V15/001460	January 13, 2015;

MBG4914	V15/001461	January 13, 2015;
ETHANOL RED™	V14/007039	March 19, 2014.

The strains have been 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 deposits represent substantially pure cultures of the deposited strains. The deposits are 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 deposits do 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 polynucleotide sequences is described by the parameter "identity".

For purposes of the present invention, the degree of identity between two amino acid sequences is determined by the Clustal method (Higgins, 1989, *CABIOS* 5: 151-153) using the LASERGENE™ MEGALIGN™ software (DNASTAR, Inc., Madison, WI) with an identity table and the following multiple alignment parameters: Gap penalty of 10 and gap length penalty of 10. Pairwise alignment parameters are Ktuple=1, gap penalty=3, windows=5, and diagonals=5.

For purposes of the present invention, the degree of identity between two polynucleotide sequences is determined by the Wilbur-Lipman method (Wilbur and Lipman, 1983, *Proceedings of the National Academy of Science USA* 80: 726-730) using the LASERGENE™ MEGALIGN™ software (DNASTAR, Inc., Madison, WI) with an identity table and the following multiple alignment parameters: Gap penalty of 10 and gap length penalty of 10. Pairwise alignment parameters are Ktuple=3, gap penalty=3, and windows=20.

SIGMA Enzymatic Assay for Trehalase

One SIGMA unit will convert 1.0 micro mol of trehalose to 2.0 micro mol of glucose per minutes at pH 5.7 at 37°C (liberated glucose determined at pH 7.5).

Glucoamylase activity

Glucoamylase activity may be measured in Glucoamylase Units (AGU).

Glucoamylase activity (AGU)

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

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

AMG incubation:	
Substrate:	maltose 23.2 mM
Buffer:	acetate 0.1 M
pH:	4.30 ± 0.05
Incubation temperature:	37°C ± 1
Reaction time:	5 minutes
Enzyme working range:	0.5-4.0 AGU/mL

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

A folder (EB-SM-0131.02/01) describing this analytical method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Alpha-Amylase activity (KNU)

The alpha-amylase activity may be determined using potato starch as substrate. This method is based on the break-down of modified potato starch by the enzyme, and the reaction is followed by mixing samples of the starch/enzyme solution with an iodine solution.

Initially, a blackish-blue color is formed, but during the break-down of the starch the blue color gets weaker and gradually turns into a reddish-brown, which is compared to a colored glass standard.

One Kilo Novo alpha amylase Unit (KNU) is defined as the amount of enzyme which, under standard conditions (i.e., at 37°C +/- 0.05; 0.0003 M Ca^{2+}; and pH 5.6) dextrinizes 5260 mg starch dry substance Merck Amylum solubile.

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

Acid Alpha-Amylase Activity

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

Acid alpha-amylase activity (AFAU)

Acid alpha-amylase activity may be measured in AFAU (Acid Fungal Alpha-amylase Units), which are determined relative to an enzyme standard. 1 AFAU is defined as the amount of enzyme which degrades 5.260 mg starch dry matter per hour under the below mentioned standard conditions.

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

$$\text{STARCH} + \text{IODINE} \xrightarrow[40^\circ,\ \text{pH}\ 2{,}5]{\text{ALPHA - AMYLASE}} \text{DEXTRINS} + \text{OLIGOSACCHARIDES}$$

$\lambda = 590$ nm

blue/violet t = 23 sec. decoloration

Standard conditions/reaction conditions:

Substrate:	Soluble starch, approx. 0.17 g/L
Buffer:	Citrate, approx. 0.03 M
Iodine (I2):	0.03 g/L
$CaCl_2$:	1.85 mM
pH:	2.50 ± 0.05

Incubation temperature:	40°C
Reaction time:	23 seconds
Wavelength:	590nm
Enzyme concentration:	0.025 AFAU/mL
Enzyme working range:	0.01-0.04 AFAU/mL

A folder EB-SM-0259.02/01 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

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.

Measurement of Cellulase Activity Using Filter Paper Assay (FPU assay)

1. Source of Method

1.1 The method is disclosed in a document entitled "Measurement of Cellulase Activities" by Adney, B. and Baker, J. 1996. Laboratory Analytical Procedure, LAP-006, National Renewable Energy Laboratory (NREL). It is based on the IUPAC method for measuring cellulase activity (Ghose, T.K., Measurement of Cellulse Activities, Pure & Appl. Chem. 59, pp. 257-268, 1987.

2. Procedure

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

2.2 Enzyme Assay Tubes:

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

2.2.2 To the tube is added 1.0 mL of 0.05 M Na-citrate buffer (pH 4.80).

2.2.3 The tubes containing filter paper and buffer are incubated 5 min. at 50° C (± 0.1° C) in a circulating water bath.

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

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

2.2.6 After vortexing, the tubes are incubated for 60 mins. at 50° C (± 0.1° C) in a circulating water bath.

2.2.7 Immediately following the 60 min. incubation, the tubes are removed from the water bath, and 3.0 mL of DNS reagent is added to each tube to stop the reaction. The tubes are vortexed 3 seconds to mix.

2.3 *Blank and Controls*

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

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

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

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

2.4 Glucose Standards

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

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

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

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

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

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

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

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

2.5 Color Development

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

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

2.5.3 When cool, the tubes are briefly vortexed, and the pulp is allowed to settle. Then each tube is diluted by adding 50 microL from the tube to 200 microL of ddH2O in a 96-well plate. Each well is mixed, and the absorbance is read at 540 nm.

2.6 Calculations (examples are given in the NREL document)

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

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

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

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

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

Protease Assay method - AU(RH)

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

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

The AU(RH) method is described in EAL-SM-0350 and is available from Novozymes A/S Denmark on request.

Protease assay method (LAPU)

1 Leucine Amino Peptidase Unit (LAPU) is the amount of enzyme which decomposes 1 microM substrate per minute at the following conditions: 26 mM of L-leucine-p-nitroanilide as substrate, 0.1 M Tris buffer (pH 8.0), 37ºC, 10 minutes reaction time.

LAPU is described in EB-SM-0298.02/01 available from Novozymes A/S Denmark on request.

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 30ºC, yeast cells are taken from plates using a sterile microbiological loop and inoculated to an OD_{600} (Optical Density at 600 nm) of between 0.1 and 0.2 units (OD_{600} at T_0) 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 OD_{600} 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 30ºC with shaking at 220 rpm (10 cm orbital diameter) for 48 hours.

Step 4: After 48 hours incubation, OD_{600} of culture is measured (OD_{600} at T_{48}).

Step 5: The fold increase in biomass is determined by the equation:

OD_{600} at T_{48}/OD_{600} at T_0.

EXAMPLES

Example 1

Production of *Saccharomyces* MBG4911, MBG4913 and MBG4914

Novel *Saccharomyces cerevisiae* MBG4911, MBG4913 and MBG4914 were produced using the methods described in WO 2005/121337 and through matings with various strains of *Saccharomyces cerevisiae* combined with selection for characteristics including low glycerol production and high ethanol production in a raw starch ethanol production process. Strains MBG4911, MBG4913 and MBG4914 were verified to be *Saccharomyces cerevisiae* strains by their abilities to sporulate and produce progeny when the germinated spores were mated with standard strains of *Saccharomyces cerevisiae*, including haploid tester strains of *Saccharomyces cerevisiae*. One such haploid tester strain

is W303-1A. Specifically, germinated spores of strains MBG4911, MBG4913 and MBG4914 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 MBG4911, MBG4913 and MBG4914 were 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 MBG4911, MBG4913 and MBG4914 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 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 Strains MBG 4911, 4913 and 4914 are *Saccharomyces cerevisiae* strains.

Strains MBG4911, MBG4913 and MBG4914 were deposited on 19th January 2015 at the National Measurement Institute, 1/153 Bertie Street, Port Melbourne, Victoria 3207, Australia under the Budapest Treaty. Strain MBG4911 was designated accession number V15/001459, strain MBG4913 was designated accession number V15/001460, and strain MBG4914 was designated accession number V15/001461. The commercially available *Saccharomyces cerevisiae* known as ETHANOL RED™ was used for comparison in Example 2. ETHANOL RED™ yeast strain was deposited on March 19, 2014 at the National Measurement Institute, 1/153 Bertie Street, Port Melbourne, Victoria 3207, Australia, under the Budapest Treaty and was designated accession number V14/007039.

Example 2

Growth of *Saccharmyces* MBG4911, MBG4913 and MBG4914 in Xylose Minimal Media

Growth of strains MBG4911, MBG4913 and MBG4914 on xylose as a sole carbon source was determined using Test T1. *Saccharomyces cerevisiae* strains MBG4911, MBG4913 and MBG4914 were streaked onto 2% w/v D-glucose 1% bacteriological peptone and 0.5% yeast extract medium (GYP) solidified with 2% agar using standard microbiological techniques. After incubation for 72 hours at 30ºC, yeast cells were taken from plates using a sterile microbiological loop and inoculated to an OD_{600} (Optical Density at 600 nm) of between 0.1 and 0.2 units (OD_{600} at T0) in 50 ml of broth. An OD_{600} of 0.1 unit is equal to approximately 9 x 10^5 yeast cells/ mL. The broth contained xylose (5% w/v), Difco Yeast Nitrogen Base w/o amino acids (0.67%), citric acid (0.3%) and tri-sodium citrate (0.7%) in distilled water in a 250 ml Erlenmeyer flask. Citric acid and tri-sodium 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 30ºC with shaking at 220 rpm (10 cm orbital diameter) for 48 hours prior to measuring OD_{600} (OD_{600} at T_{48}hrs). The fold increase in biomass was determined by the equation: OD_{600} at T_{48}hrs divided by OD_{600} at T_0.

Strain MBG4911 showed greater than two-fold increase in biomass, whilst MBG4913 showed more than 20-fold increase in biomass, and MBG 4914 showed greater than six-fold increase in biomass in 48 hours. Under the same conditions the ETHANOL RED™ yeast (Budapest Treaty accession number V14/007039) failed to increase in biomass.

Example 3

Improved Ethanol Yield and Reduced Glycerol Production in 500 g Kettle Scale RSH Fermentations

All fermentations were done in 500 ml stirred glass kettle reactors placed in a waterbath.

Mash Preparation

Yellow dent corn (obtained from GPRE in Central City, NE in November 2014; ground in-house) was mixed with tap water and the dry solids (DS) level was determined to be 33.5% by moisture balance. This mixture was supplemented with 3 ppm Lactrol and 500 ppm urea. The slurry was adjusted to pH 4.5 with 40% H_2SO_4.

Yeast Strains and Preparation

The four yeast strains tested in this experiment were ETHANOL RED™ (Fermentis), *Saccharmyces* MBG4911, MBG4913, and MBG4914. Yeasts were rehydrated by weighing approximately 5 g of dried yeast into 50 ml of 36.5°C tap water in a 125 mL Erlenmeyer flask. The flasks were then covered with parafilm and allowed to incubate in a 36.5°C water bath. Flasks were swirled at the beginning and end of rehydration to mix, but no other agitation took place. After a total of 20 minutes, the flasks were removed from the water bath. Each yeast was enumerated using the YC-100 (Yeast Cell Counter, Chemometer).

Simultaneous Saccharification and Fermentation (SSF)

TcAMG/JA126 (ratio between AGU and FAU-F about 10:1) was dosed to each reactor at 0.51 AGU/gDS. Yeast was added at a pitch of 5 million cells per gram. Water was added to each kettle such that the total volume added to each kettle was equal. Fermentations took place at 32°C for 88 hours.

HPLC analysis

Fermentation sampling took place by sampling 5 grams of mash into 15 ml tubes at 16, 24, 40, 48, 64, 72, and 88 hours of fermentation. Each tube was processed for HPLC analysis by deactivation with 150 µL of 40% v/v H_2SO_4, vortexing, centrifuging at 1460×*g* for 10 minutes, and filtering through a 0.45 µm Whatman PP filter, followed by a 0.2 µm Whatman PP filter. Samples were stored at 4°C prior to and during HPLC analysis.

Table 1: 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_2SO_4 mobile phase Flow rate: 0.6 ml/min Column temperature: 65°C RI detector temperature: 55°C

Samples were analyzed for sugars (DP4+, DP3, DP2, glucose, and fructose), organic acids (lactic and acetic), glycerol, and ethanol.

Increased Ethanol and Faster Kinetics Results

Ethanol titers at 64, 72, and 88 hours of fermentation are shown in Table 2 below. These strains show increased kinetics, including increased speed of fermentation as evidenced by boosts exhibited at 64 and 72 hours, even when compared to the ethanol titers produced by Ethanol Red™ (ER) (Fermentis) at 88 hours of fermentation time.

Table 2: Ethanol Titers and Comparative Boosts during the later stages of fermentation

	ER	MBG4911	MBG4913	MBG4914
Time (hours)	Ethanol (w/v%)			
64	14.946	15.362	15.400	15.403
72	15.058	15.354	15.423	15.372
88	15.182	15.330	15.499	15.505

	ER	MBG4911	MBG4913	MBG4914
Time (hours)	% Boost Compared to Ethanol Red at Same Time Point			
64	0	2.78	3.03	3.05
72	0	1.97	2.42	2.08
88	0	0.97	2.08	2.13
	ER	MBG4911	MBG4913	MBG4914
Time (hours)	% Boost Compared to ER at Final Time Point			
64	-1.55	1.18	1.43	1.45
72	-0.82	1.13	1.58	1.25
88	0.00	0.97	2.08	2.13

Reduced Glycerol Results

Glycerol levels at the later stages of fermentation are shown in Table 3 below.

Table 3: Glycerol Titers and Comparisons during the later stages of fermentation.

	ER	MBG4911	MBG4913	MBG4914
Time (hours)	Glycerol (w/v%)			
64	0.922	0.755	0.872	0.809
72	0.927	0.753	0.877	0.814
88	0.935	0.755	0.889	0.819
	ER	MBG4911	MBG4913	MBG4914
Time (hours)	Comparison of Glycerol Levels to ER (%)			
64	0	-18.07	-5.34	-12.19
72	0	-18.75	-5.35	-12.18
88	0	-19.25	-5.00	-12.46

Example 4

Improved Ethanol Yield and Reduced Glycerol Production in 500 g Kettle Scale RSH Fermentations with varying RSH enzyme

All fermentations were done in 500 ml stirred glass kettle reactors placed in a waterbath.

Mash Preparation

Yellow dent corn (obtained from GPRE in Central City, NE in November 2014; ground in-house) was mixed with tap water and the dry solids (DS) level was determined to be 34.4% by moisture balance. This mixture was supplemented with 3 ppm Lactrol and 500 ppm urea.

The slurry was adjusted to pH 4.5 with 40% H_2SO_4.

Yeast Strains and Preparation

The four yeast strains tested in this experiment were ETHANOL RED™ (Fermentis), *Saccharmyces* MBG4911, MBG4913, and MBG4914. Yeasts were rehydrated by weighing approximately 5 g of dried yeast into 50 ml of 36.5°C tap water in a 125 mL Erlenmeyer flask. The flasks were then covered with parafilm and allowed to incubate in a 36.5°C water bath. Flasks were swirled at the beginning and end of rehydration to mix, but no other agitation took place. After a total of 20 minutes, the flasks were removed from the water bath. Each yeast was enumerated using the YC-100.

Simultaneous Saccharification and Fermentation (SSF)

PsAMG/ AAPE096 (ratio between AGU and FAU-F about 16:1) was dosed to each tube of mash at 0.39 AGU/gDS or Tc/JA126 (ratio about 10:1) was dosed at 0.23 AGU/ gDS. Yeast was dosed at 5x 10e6 cells/g mash. Milli-Q water was added to each tube so that a total volume of liquid added (enzyme + MQ water) to each tube would be equally proportionate to the mash weight. Fermentations took place in a 32°C water bath for 88 hours.

HPLC analysis

Fermentation sampling took place by sampling 5 grams of mash into 15 ml tubes at 24, 40, 47, 64, 70, and 88 hours of fermentation. Each tube was processed for HPLC analysis by deactivation with 150 µL of 40% v/v H_2SO_4, vortexing, centrifuging at 1460×*g* for 10 minutes, and filtering through a 0.45 µm Whatman PP filter, followed by a 0.2 µm Whatman PP filter. Samples were stored at 4°C prior to and during HPLC analysis.

Table4: 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_2SO_4 mobile phase Flow rate: 0.6 ml/min

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

Samples were analyzed for sugars (DP4+, DP3, DP2, glucose, and fructose), organic acids (lactic and acetic), glycerol, and ethanol.

Increased Ethanol Results

Ethanol titers and boosts compared to "ER" are shown in Table 5 below.

Table 5: Ethanol Titers and Boosts Compared to ER at 88 hours

TcAMG/JA126	ER	MBG4911	MBG4913	MBG4914
	Ethanol Titer (w/v%)			
	15.267	15.816	15.647	15.582
	% Boost compared to ER			
	0	3.60	2.49	2.07
PsAMG/AAPE096	ER	MBG4911	MBG4913	MBG4914
	Ethanol Titer (w/v%)			
	15.360	16.249	16.097	16.010
	% Boost compared to ER			
	0	5.79	4.80	4.23

Reduced Glycerol Results

Glycerol titers and comparisons to "ER" at 88 hours are shown in Table 6 below.

Table 3: 88 hour Glycerol Titers and Comparisons to ER

TcAMG/JA126	ER	MBG4911	MBG4913	MBG4914
	Glycerol Titer (w/v%)			
	1.000	0.760	0.880	0.847
	Comparison to ER (%)			
	0	-24.01	-12.00	-15.30
PsAMG/AAPE096	ER	MBG4911	MBG4913	MBG4914
	Glycerol Titer (w/v%)			
	0.992	0.781	0.919	0.865
	Comparison to ER (%)			
	0	-21.25	-7.34	-12.78

CLAIMS

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

(a) saccharifying 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 a glucoamylase, an alpha-amylase, and optionally protease;

wherein the fermenting organism is:

Saccharomyces cerevisiae MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), a derivative of *Saccharomyces cerevisiae* MBG4911 having defining characteristics of strain V15/001459, or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4911;

Saccharomyces cerevisiae MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), a derivative of *Saccharomyces cerevisiae* MBG4913 having defining characteristics of strain V15/001460, or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4913; or

Saccharomyces cerevisiae MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia), a derivative of *Saccharomyces cerevisiae* MBG4914 having defining characteristics of strain V15/001461, or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4914.

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:

- increases ethanol yield compared to ETHANOL RED™ under the same process conditions;
- reduced acetaldehyde production compared to ETHANOL RED™ under the same process conditions;
- increased temperature tolerance compared to ETHANOL RED™ under the same process conditions;
- decreased glycerol production compared to ETHANOL RED™ under the same process conditions.

3. The process of claim 1 or 2, wherein the fermenting organism is capable of growing on xylose as a sole carbon source (e.g., determined using the Test T1).

4. The process of any of claims 1-3, wherein the fermenting organism provides an ethanol yield boost of at least 0.5% (e.g., at least 1.0%, at least 1.5%, at least 2.0%, at least 3.0%, at least 4.0%, at least 5.0%, or between 0.5-10%, or between 1-6%), after 88 hours compared to ETHANOL RED™ under the same conditions (e.g., as defined in Example 3 or Example 4).

5. The process of any of claims 1-4, wherein the fermenting organism provides a lower glycerol production of at least 5% (e.g., at least 10%, at least 15%, at least 15%, at least 20%, or between 4 and 40%, or between 10 and 30%) after 88 hours compared to ETHANOL RED™ under the same conditions (e.g., as defined in Example 3 or Example 4).

6. A *Saccharomyces* yeast strain selected from:

Saccharomyces cerevisiae MBG4911 (deposited as V15/001459 at National Measurement Institute, Victoria, Australia), a derivative of *Saccharomyces cerevisiae* MBG4911 having defining characteristics of strain V15/001459, or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4911;

Saccharomyces cerevisiae MBG4913 (deposited as V15/001460 at National Measurement Institute, Victoria, Australia), a derivative of *Saccharomyces cerevisiae* MBG4913 having defining characteristics of strain V15/001460, or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4913; and

Saccharomyces cerevisiae MBG4914 (deposited as V15/001461 at National Measurement Institute, Victoria, Australia), a derivative of *Saccharomyces cerevisiae* MBG4914 having defining characteristics of strain V15/001461, or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* MBG4914.

7. The yeast strain of claim 6, having 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;
- 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.

8. The yeast strain of claim 6 or 7, wherein the strain is capable of growing on xylose as a sole carbon source (e.g., determined using the Test T1).

9. The yeast strain of any of claims 6-8, wherein the strain is capable of providing an ethanol yield boost of at least 0.5% (e.g., at least 1.0%, at least 1.5%, at least 2.0%, at least 3.0%, at least 4.0%, at least 5.0%, or between 0.5-10%, or between 1-6%), after 88 hours compared to ETHANOL RED™ under the same conditions (e.g., as defined in Example 3 or Example 4).

10. The yeast strain of any of claims 6-8, wherein the strain is capable of providing a lower glycerol production of at least 5% (e.g., at least 10%, at least 15%, at least 15%, at least 20%, or between 4 and 40%, or between 10 and 30%) after 88 hours compared to ETHANOL RED™ under the same conditions (e.g., as defined in Example 3 or Example 4).

11. A method of producing a derivative of strain *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4914, which exhibits the defining characteristics of strains *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4914, respectively, comprising:

(a) providing:

(i) a first yeast strain; and

(ii) a second yeast strain, wherein the second yeast strain is strain *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4914, or a derivative of strain *Saccharomyces cerevisiae* MBG 4911, MBG4913 or MBG4914;

(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 strain.

12. The method of claim 11, wherein step (c) comprises screening or selecting for a hybrid strain which exhibits one or more defining characteristic of strain *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4914.

13. The method of claim 11 or 12, comprising the further step of:

(d) repeating steps (b) and (c) with the screened or selected strain from step (c) as the first and/or second strain, until a derivative is obtained which exhibits the defining characteristics of strain *Saccharomyces cerevisiae* MBG4911, MBG4913 or MBG4914.

14. The method of any of claims 11-13, 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.

15. A *Saccharomyces* strain produced by the method of any of claims 11-14.

16. A method of producing ethanol, comprising incubating the *Saccharomyces* yeast strain of any of claims 6-10 or 15 with a substrate comprising a fermentable sugar under conditions which permit fermentation of the fermentable sugar to produce ethanol.

17. Use of the *Saccharomyces* yeast strain of any of claims 6-10 or 15 in the production of ethanol.

18. A composition comprising a *Saccharomyces* yeast strain of any of claims 6-10 or 15 and one or more components selected from the group consisting of: surfactants, emulsifiers, gums, swelling agents, and antioxidants.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/019874

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12R1/865 C12N1/18 C12N1/36 C12N15/01 C12P7/06
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P C12N C12R

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, WPI Data, BIOSIS, COMPENDEX, EMBASE, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2010/008841 A2 (NOVOZYMES AS [DK]; SOONG CHEE-LEONG [US]; OSTERGAARD PETER RAHBEK [DK]) 21 January 2010 (2010-01-21) abstract; examples 1-3; tables 1-6 -----	1-10, 16-18
Y	WO 2011/035392 A1 (MICROBIOGEN PTY LTD [AU]; BELL PHILIP JOHN LIVINGSTONE [AU]; ATTFIELD) 31 March 2011 (2011-03-31) cited in the application abstract; examples 3-5; tables 1-3 -----	1-10, 16-18
A	WO 2005/121337 A1 (MICROBIOGEN PTY LTD [AU]; BELL PHILIP JOHN LIVINGSTON [AU]; ATTFIELD P) 22 December 2005 (2005-12-22) cited in the application abstract; claims 1-75 page 84, line 12 - page 85, line 10 ----- -/--	1-10, 16-18

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

[X] 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

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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

"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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 April 2016	15/07/2016

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

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/019874

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-10, 16-18(all partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WEI ZHANG ET AL: "Improved ethanol production by a xylose-fermenting recombinant yeast strain constructed through a modified genome shuffling method", BIOTECHNOLOGY FOR BIOFUELS, BIOMED CENTRAL LTD, GB, vol. 5, no. 1, 18 July 2012 (2012-07-18), page 46, XP021108102, ISSN: 1754-6834, DOI: 10.1186/1754-6834-5-46 abstract Discussion; pages 5-9 -----	1-10, 16-18

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2016/019874

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010008841 A2	21-01-2010	CA 2726688 A1	21-01-2010
		CN 102083991 A	01-06-2011
		EP 2364363 A2	14-09-2011
		US 2011097779 A1	28-04-2011
		WO 2010008841 A2	21-01-2010
WO 2011035392 A1	31-03-2011	AR 078468 A1	09-11-2011
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		US 2012309069 A1	06-12-2012
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WO 2005121337 A1	22-12-2005	AT 527352 T	15-10-2011
		AU 2005252266 A1	22-12-2005
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		EP 1766007 A1	28-03-2007
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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-10, 16-18(all partially)

S. cerevisiae strain MBG4911 or derivatives thereof, processes for producing ethanol using said strain, use of said strain for producing ethanol, and a composition comprising said strain

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2. claims: 1-10, 16-18(all partially)

S. cerevisiae strain MBG4913 or derivatives thereof, processes for producing ethanol using said strain, use of said strain for producing ethanol, and a composition comprising said strain

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3. claims: 1-10, 16-18(all partially)

S. cerevisiae strain MBG4914 or derivatives thereof, processes for producing ethanol using said strain, use of said strain for producing ethanol, and a composition comprising said strain

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4-7. claims: 1-10, 16-18(all partially)

S. cerevisiae strains other than MBG4911, MBG4913, MBG4914 or derivatives thereof, the strains having properties that are about the same as that of said strains, wherein the properties are selected from: (1) increased ethanol yield, (2) reduced acetaldehyde production, (3) increased temperature tolerance or (4) decreased glycerol production, wherein increase or reduction is relative to the performance to strain Ethanol Red under the same process conditions; inventions 4-7 concerning strains and their uses having properties of (1)-(4), respectively.

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8. claims: 11-15(completely); 16-18(partially)

Method of producing a derivative of strain S. cerevisiae MBG4911, MBG4913 or MBG4914, and strains obtained by this method

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