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(71) Applicant: **NOVOZYMES A/S** [DK/DK]; Krogshoejvej 36, DK-2880 Bagsvaerd (DK).

(72) Inventor; and
(71) Applicant *(for BW only)*: **MATTHEWS, John** [US/US]; 402 M C Wilder Rd., Louisburg, North Carolina 27549 (US).

(72) Inventors: **CLARK, Suzanne**; 175 Bradford Ridge, Youngsville, North Carolina 27596 (US). **JUMP, Joseph**; 404 Dartmouth Rd., Raleigh, North Carolina 27609 (US). **KREEL, Nathanial**; 20 Elizabeth Ct., Louisburg, North Carolina 27549 (US).

(74) Agents: **KRENICKY, Michael, W.** et al.; Novozymes North America, Inc., 60 E. 42nd St., Suite 700, New York, New York 10165 (US).

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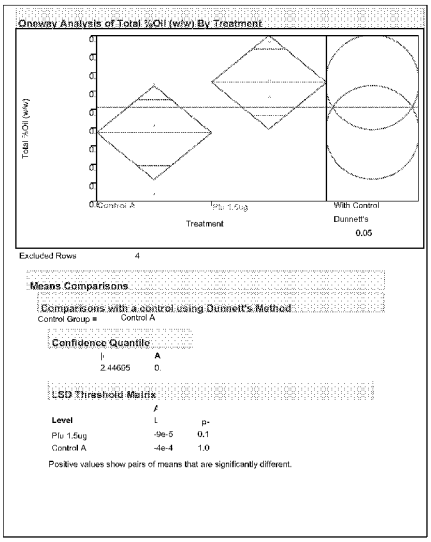
(54) Title: PROCESSES FOR RECOVERING OIL FROM FERMENTATION PRODUCT PROCESSES AND PROCESSES FOR PRODUCING FERMENTATION PRODUCTS

Fig. 1



(57) Abstract: The present invention relates to processes for recovering/extracting oil from fermentation product production processes based on starch-containing material, wherein an alpha-amylase, a high dosage of protease, and optionally a glucoamylase, are present and/or added in liquefaction. The invention also relates to processes for producing fermentation products and to enzyme compositions suitable for use in processes of the invention.

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Processes for Recovering Oil from Fermentation Product Processes and Processes for Producing Fermentation Products

REFERENCE TO A SEQUENCE LISTING

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

FIELD OF THE INVENTION

The present invention relates to processes of recovering oil from a fermentation product production process and well as processes for producing fermentation products from starch-containing material. The invention also relates to compositions suitable for use in a process of the invention.

BACKGROUND OF THE INVENTION

Fermentation products, such as ethanol, are typically produced by first grinding starch-containing material in a dry-grind or wet-milling process, then degrading the material into fermentable sugars using enzymes and finally converting the sugars directly or indirectly into the desired fermentation product using a fermenting organism. Liquid fermentation products are recovered from the fermented mash (often referred to as “beer mash”), e.g., by distillation, which separate the desired fermentation product from other liquids and/or solids. The remaining faction is referred to as “whole stillage”. The whole stillage is dewatered and separated into a solid and a liquid phase, e.g., by centrifugation. The solid phase is referred to as “wet cake” (or “wet grains”) and the liquid phase (supernatant) is referred to as “thin stillage”. Wet cake and thin stillage contain about 35 and 7% solids, respectively. Dewatered wet cake is dried to provide “Distillers Dried Grains” (DDG) used as nutrient in animal feed. Thin stillage is typically evaporated to provide condensate and syrup or may alternatively be recycled directly to the slurry tank as “backset”. Condensate may either be forwarded to a methanator before being discharged or may be recycled to the slurry tank. The syrup may be blended into DDG or added to the wet cake before drying to produce DDGS (Distillers Dried Grain with Solubles).

WO 2012/088303 (Novozymes) discloses processes for producing fermentation products by liquefying starch-containing material at a pH in the range from 4.5-5.0 at a temperature in the range from 80-90°C using a combination of alpha-amylase having a T½ (min) at pH 4.5, 85°C, 0.12 mM CaCl2) of at least 10 and a protease having a thermostability value of more than 20%

determined as Relative Activity at 80°C/70°C; followed by saccharification and fermentation.

WO 2013/082486 (Novozymes) discloses processes for producing fermentation products by liquefying starch-containing material at a pH in the range between from above 5.0-7.0 at a temperature above the initial gelatinization temperature using an alpha-amylase; a protease having a thermostability value of more than 20% determined as Relative Activity at 80°C/70°C; and optionally a carbohydrate-source generating enzyme followed by saccharification and fermentation.

An increasing number of ethanol plants extract oil from the thin stillage and/or syrup as a by-product for use in biodiesel production or other biorenewable products. Much of the work in oil recovery/extraction from fermentation product production processes has focused on improving the extractability of the oil from the thin stillage. Effective removal of oil is often accomplished by hexane extraction. However, the utilization of hexane extraction has not seen widespread application due to the high capital investment required. Therefore, other processes that improve oil extraction from fermentation product production processes have been explored.

WO 2011/126897 (Novozymes) discloses processes of recovering oil by converting starch-containing materials into dextrins with alpha-amylase; saccharifying with a carbohydrate source generating enzyme to form sugars; fermenting the sugars using fermenting organism; wherein the fermentation medium comprises a hemicellulase; distilling the fermentation prodct to form whole stillage; separating the whole stillage into thin stillage and wet cake; and recovering oil from the thin stillage. The fermentation medium may further comprise a protease.

It is an object of the present invention to provide improved processes for increasing the amount of recoverable oil from fermentation product production processes and to provide processes for producing fermentation products, such as ethanol, from starch-containing material that can provide a higher fermentation product yield, or other advantages, compared to a conventional process.

SUMMARY OF THE INVENTION

The present invention relates to processes of recovering/extracting oil from fermentation product production processes. The invention also related to producing fermentation products, such as ethanol, from starch-containing material in a process including liquefying starch-containing

material, saccharifying and fermenting the liquefied material. The invention also relates to compositions suitable for use in a process of the invention.

In the first aspect the invention relates to processes of recovering/extracting oil from a fermentation product production process comprising the steps of:

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

- an alpha-amylase;

- more than 0.5 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS);

b) saccharifying using a glucoamylase;

c) fermenting using a fermenting organism.

d) recovering the fermentation product to form whole stillage;

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

f) optionally concentrating the thin stillage into syrup;

wherein oil is recovered from the:

- liquefied starch-containing material during and/or after step a); and/or

- downstream from fermentation step c).

In an embodiment between 0.5-100 micro gram *Pyrococcus furiosus* protease per gram DS (dry solids) DS is present and/or added in liquefaction step a). In an embodiment between 0.5-10 micro gram *Pyrococcus furiosus* protease per gram DS (dry solids) is present and/or added in liquefaction step a). In an embodiment between 1-50 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a). In an embodiment between 1-10 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a). In an embodiment between 1.5-5 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a). In an embodiment around or more than 1.5 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a). In an embodiment around or more than 2 micro gram *Pyrococcus furiosus* protease per

gram DS is present and/or added in liquefaction step a). In an embodiment around or more than 3 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a).

In a preferred embodiment the *Pyrococcus furiosus* protease is the mature sequence shown in SEQ ID NO: 13 herein or one having at least 90% or 95% identity thereof.

Examples of alpha-amylase can be found below in the "Alpha-Amylases Present and/or Added In Liquefaction"-section below.

Preferred alpha-amylases are *Bacillus* sp. alpha-amylases or variants thereof, especially derived from *Bacillus stearothermophilus* or *Bacillus licheniformis*.

In a preferred embodiment the alpha-amylase is a *Bacillus stearothermophilus* alpha-amylase variant comprising a double deletion in positions I181*+G182* (using SEQ ID NO: 1 for numbering).

Preferred alpha-amylases include *Bacillus stearothermophilus* alpha-amylase variants, such as one shown in SEQ ID NO: 1 herein with the following mutations:

- I181*+G182*+N193F+E129V+K177L+R179E;

- I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S

- I181*+G182*+N193F+V59A Q89R+ E129V+ K177L+ R179E+ Q254S+M284V; and

- I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 for numbering).

In an embodiment a glucoamylase is present and/or added in liquefaction. Examples of suitable glucoamylase can be found in the "Glucoamylase Present And/Or Added In Liquefaction" section below.

In an embodiment the glucoamylase has a thermostability of at least 80°C, preferably at least 82°C, such as at least 84°C, such as at least 86°C, such as at least 88°C at pH 4.0 determined as Differential Scanning Calorimitry (DSC) as described in Example 3 below.

In an embodiment the glucoamylase has a thermostability of at least 80°C, preferably at least 82°C, such as at least 84°C, such as at least 86°C, such as at least 88°C, such as at least 90°C at pH 4.8 determined as Differential Scanning Calorimitry (DSC) as described in Example 3 below.

Examples of specifically contemplated glucoamylases can be found in Example 3 (Table 6) below.

Preferred glucoamylases include *Penicillium oxalicum* glucoamylases, such as one shown in SEQ ID NO: 14 herein having a K79V substitution and preferably further one of the following:

- P11F + T65A + Q327F;

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

In a preferred embodiment liquefaction is carried out at a temperature between 80-90°C, such as around 85°C. In a preferred embodiment liquefaction is carried out at a pH in the range pH above 5.0 to 6.0.

A glucoamylase is present and/or added in saccharification and/or fermentation. Examples of suitable glucoamylases can be found in the "Glucoamylase Present And/Or Added In Saccharification And/Or Fermentation" section below.

In a second aspect the invention relates to processes for producing fermentation products from starch-containing material comprising the steps of:

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

- an alpha-amylase;

- more than 2 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS);

b) saccharifying using a glucoamylase;

c) fermenting using a fermenting organism.

In a preferred embodiment between 2-100 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a). In a preferred embodiment between 2-10 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a). In a preferred embodiment between 2.5-50 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a). In a preferred embodiment between 2.5-10 micro gram per gram DS is present and/or added in liquefaction step a). In a preferred embodiment between 2.5-5 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a). In a preferred embodiment around or more than 3 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a).

In a preferred embodiment the *Pyrococcus furiosus* protease is the mature one shown in SEQ ID NO: 13 herein or one having at least 90% or at least 95% identity thereof.

Examples of alpha-amylase can be found below in the “Alpha-Amylases Present and/or Added In Liquefaction”-section below.

Preferred alpha-amylases are *Bacillus* sp. alpha-amylases or variants thereof, especially derived from *Bacillus stearothermophilus* or *Bacillus licheniformis*.

In a preferred embodiment the alpha-amylase is a *Bacillus stearothermophilus* alpha-amylase variant comprising a double deletion in I181*+G182* (using SEQ ID NO: 1 for numbering).

Preferred alpha-amylases include *Bacillus stearothermophilus* alpha-amylase variants, such as one shown in SEQ ID NO: 1 herein with the following mutations:

- I181*+G182*+N193F+E129V+K177L+R179E;

- I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S

- I181*+G182*+N193F +V59A Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V; and

- I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 for numbering).

In an embodiment glucoamylase is present and/or added in liquefaction. Examples of suitable glucoamylase can be found in the “Glucoamylase Present And/Or Added In Liquefaction” section below.

In an embodiment the glucoamylase has a thermostability of at least 80°C, preferably at least 82°C, such as at least 84°C, such as at least 86°C, such as at least 88°C at pH 4.0 determined as Differential Scanning Calorimitry (DSC) as described in Example 3 below.

In an embodiment the glucoamylase has a thermostability of at least 80°C, preferably at least 82°C, such as at least 84°C, such as at least 86°C, such as at least 88°C, such as at least 90°C at pH 4.8 determined as Differential Scanning Calorimitry (DSC) as described in Example 3 below.

Examples of specifically contemplated glucoamylases can be found in Example 3 (Table 6) below.

Preferred glucoamylases include *Penicillium oxalicum* glucoamylases, such as one shown in SEQ ID NO: 14 herein having a K79V substitution and preferably further one of the following:

- P11F + T65A + Q327F;

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

A glucoamylase is present and/or added in saccharification and/or fermentation. Examples of suitable glucoamylases can be found in the "Glucoamylase Present And/Or Added In Saccharification And/Or Fermentation" section below.

In a third aspect the invention relates to an enzyme composition comprising:

(i) *Bacillus* sp. alpha-amylase, or a variant thereof;

(ii) *Pyrococcus furiosus* protease;

wherein the ratio between alpha-amylase and protease is in the range from 1:1 and 1:25 (micro gram alpha-amylase : micro gram protease).

Examples of alpha-amylase can be found below in the "Alpha-Amylases Present And/Or Added In Liquefaction" section below.

Preferred alpha-amylases are *Bacillus* sp. alpha-amylases or variants thereof, especially derived from *Bacillus stearothermophilus* or *Bacillus licheniformis*.

In a preferred embodiment the alpha-amylase is a *Bacillus stearothermophilus* alpha-amylase variant comprising a double deletion in I181*+G182* (using SEQ ID NO: 1 for numbering).

Preferred alpha-amylases include *Bacillus stearothermophilus* alpha-amylase variants, such as one show in SEQ ID NO: 1 herein with the following mutations:

- I181*+G182*+N193F+E129V+K177L+R179E;

- I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S

- I181*+G182*+N193F+V59A Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V; and

- I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 for numbering).

In an embodiment a glucoamylase is present and/or added in liquefaction. Examples of suitable glucoamylase can be found in the "Glucoamylase Present And/Or Added In Liquefaction" section below.

In an embodiment the glucoamylase has a thermostability of at least 80°C, preferably at least 82°C, such as at least 84°C, such as at least 86°C, such as at least 88°C at pH 4.0 determined as Differential Scanning Calorimitry (DSC) as described in Example 3 below.

In an embodiment the glucoamylase has a thermostability of at least 80°C, preferably at least 82°C, such as at least 84°C, such as at least 86°C, such as at least 88°C, such as at least 90°C at pH 4.8 determined as Differential Scanning Calorimitry (DSC) as described in Example 3 below.

Examples of specifically contemplated glucoamylases can be found in Example 3 (Table 6) below.

Preferred glucoamylases include *Penicillium oxalicum* glucoamylases, such as one shown in SEQ ID NO: 14 herein having a K79V substitution and further one of the following:

- P11F + T65A + Q327F;

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

In a preferred embodiment the ratio between alpha-amylase and protease is in the range between 1:1.2 and 1:10, such as around 1:1.4 (micro gram alpha-amylase : micro gram protease).

In another embodiment the enzyme composition of the invention comprises a glucoamylase and the ratio between alpha-amylase and glucoamylase in liquefaction is between 1:1 and 1:10, such as around 1:2 (micro gram alpha-amylase : micro gram glucoamylase).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows an oil extraction comparison between Protease Pfu (1.5 µg/gDS) and Protease X (no statistical difference).

Fig 2 shows an oil extraction comparison between Protease Pfu (3 µg/gDS) and Protease X (statistical difference).

Fig. 3 shows an oil extraction comparison between Protease Pfu (5 µg/gDS) and Protease X (statistical difference).

Fig 4 shows the ethanol concentrations (%w/v) for no-urea fermentations.

Fig 5 shows an Oneway Analysis of the ethanol concentration (%w/v) comparison for 0, 1.5, 3 and 5 µg/gDS Protease Pfu comparison urea-free fermentations.

Fig. 6 shows the ethanol concentrations (%w/v) for fermentations operating with 200 ppm urea for Protease X added in SSF and Protease Pfu (1.5, 3 and 5 µg/gDS.

Fig. 7 shows an Oneway Analysis of the ethanol concentration (%w/v) comparison for 0, 1.5, 3 and 5 µg/gDS Protease Pfu for 200 ppm urea based fermentations.

Fig. 8 shows the 54 hour glycerol concentrations (%w/v). The highest dose of Protease Pfu (5 µg/gDS) was approximately 10% lower than the control of Protease X.

Fig. 9 shows the ethanol concentrations (% w/v) after 54 hours when from 0 (control) to 50 µg/gDS Protease Pfu was added in liquefaction.

Fig. 10 shows the glycerol concentrations (% w/v) after 54 hours when from 0 (control) to 50 µg/gDS Protease Pfu was added in liquefaction.

Fig. 11 shows the glucose concentrations (% w/v) after 54 hours when from 0 (control) to 50 µg/gDS Protease Pfu was added in liquefaction.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to processes of recovering oil from a fermentation product production process and well as processes for producing fermentation products from starch-containing material. The invention also relates to compositions suitable for use in a process of the invention.

The inventors have found that an increased amount of oil can be recovered in liquefaction or downstream from fermentation when combining an alpha-amylase, a high amount of *Pyrococcus furiosus* protease and optionally a glucoamylase compared to when adding an alpha-amylase in liquefaction and a protease during fermentation (SSF).

The inventors also found that an increased ethanol yield is obtained when combining an alpha-amylase, more than 2 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS) and a glucoamylase compared to when using alpha-amylase, less than 2 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS) and glucoamylase during liquefaction.

The inventors also found that the glycerol concentration is lower with Protease Pfu (5 µg/gDS) compared to adding protease in SSF.

It was also found that an ethanol process of the invention can be run efficiently with reduced or without adding a nitrogen source, such as urea, in SSF.

Processes Of Recovering/Extracting Oil Of The Invention

In the first aspect the invention relates to processes of recovering oil from a fermentation product production process comprising the steps of:

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

- an alpha-amylase;

- more than 0.5 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS);

b) saccharifying using a glucoamylase;

c) fermenting using a fermenting organism.

d) recovering the fermentation product to form whole stillage;

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

f) optionally concentrating the thin stillage into syrup;

wherein oil is recovered from the:

- liquefied starch-containing material after step a); and/or

- downstream from fermentation step c).

In an embodiment the oil is recovered/extracted during and/or after liquefying the starch-containing material. In an embodiment the oil is recovered from the whole stillage. In an embodiment the oil is recovered from the thin stillage. In an embodiment the oil is recovered from the syrup.

In an embodiment between 0.5-100 micro gram *Pyrococcus furiosus* protease per gram DS (dry solids) DS is present and/or added in liquefaction step a). In an embodiment between 0.5-10 micro gram *Pyrococcus furiosus* protease per gram DS (dry solids) DS is present and/or added in liquefaction step a). In an embodiment between 1-50 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a). In an embodiment between 1-10 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a). In an embodiment between 1.5-5 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a). In an embodiment around or more than 1 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a). In an embodiment around or more than 1.5 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a). In an embodiment around or more than 2 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a).

In an embodiment between 2-100 micro gram *Pyrococcus furiosus* protease per gram DS is added and/or present during liquefaction. In an embodiment between 2.5-50 micro gram *Pyrococcus furiosus* protease per gram DS is added and/or present during liquefaction. In an embodiment between 2.5-10 micro gram *Pyrococcus furiosus* protease per gram DS is added and/or present during liquefaction. In an embodiment between 2.5-5 micro gram *Pyrococcus furiosus* protease per gram DS is added and/or present during liquefaction. In an embodiment between 2.75-50 micro gram *Pyrococcus furiosus* protease per gram DS is added and/or present during liquefaction. In an embodiment between 2.75-10 micro gram *Pyrococcus furiosus* protease per gram DS is added and/or present during liquefaction. In an embodiment between 2.75-5 micro gram *Pyrococcus furiosus* protease per gram DS is added and/or present during liquefaction. In a preferred embodiment around or more than 3 micro gram *Pyrococcus furiosus* protease per gram DS are present and/or added in liquefaction step a).

In a preferred embodiment the *Pyrococcus furiosus* protease is the mature sequence shown in SEQ ID NO: 13 herein. In an embodiment the *Pyrococcus furiosus* protease is one having at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 13 herein.

In an embodiment no nitrogen-compound, such as urea, is present and/or added in steps a)-c), such as during saccharification step b), fermentation step c), or simultaneous saccharification and fermentation (SSF).

In an embodiment 10-1,000 ppm, such as 50-800 ppm, such as 100-600 ppm, such as 200-500 ppm nitrogen-compound, preferably urea, is present and/or added in steps a)-c), such as in saccharification step b) or fermentation step c) or in simultaneous saccharification and fermentation (SSF).

Process Of Producing A Fermentation Product Of The Invention

In the second aspect the invention relates to processes for producing fermentation products from starch-containing material comprising the steps of:

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

- an alpha-amylase;

- more than 2 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS);

b) saccharifying using a glucoamylase;

c) fermenting using a fermenting organism.

In an embodiment the fermentation product is recovered after fermentation. In a preferred embodiment the fermentation product is recovered after fermentation, such as by distillation. In an embodiment the fermentation product is an alcohol, preferably ethanol, especially fuel ethanol, potable ethanol and/or industrial ethanol.

In an embodiment from 2-100 micro gram *Pyrococcus furiosus* protease per gram DS is added and/or present during liquefaction. In an embodiment 2.5-50 micro gram *Pyrococcus furiosus* protease per gram DS is added and/or present during liquefaction. In an embodiment 2.5-10 micro gram *Pyrococcus furiosus* protease per gram DS is added and/or present during liquefaction. In an embodiment 2.5-5 micro gram *Pyrococcus furiosus* protease per gram DS is added and/or present during liquefaction. In an embodiment 2.75-50 micro gram *Pyrococcus furiosus* protease per gram DS is added and/or present during liquefaction. In an embodiment 2.75-10 micro gram *Pyrococcus furiosus* protease per gram DS is added and/or present during liquefaction. In an embodiment 2.75-5 micro gram *Pyrococcus furiosus* protease per gram DS is added and/or present during liquefaction. In a preferred embodiment around 3 micro gram *Pyrococcus furiosus* protease per gram DS is present and/or added in liquefaction step a).

In a preferred embodiment the *Pyrococcus furiosus* protease is the one shown in SEQ ID NO: 13 herein. In an embodiment the *Pyrococcus furiosus* protease is one having at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 13 herein.

In a preferred embodiment no nitrogen-compound is present and/or added in steps a)-c), such as during saccharification step b) or fermentation step c) or simultaneous saccharification and fermentation (SSF).

In an embodiment 10-1,000 ppm, such as 50-800 ppm, such as 100-600 ppm, such as 200-500 ppm nitrogen-compound, preferably urea, is present and/or added in steps a)-c), such as during saccharification step b) or fermentation step c) or simultaneous saccharification and fermentation (SSF).

Alpha-Amylases Present And/Or Added In Liquefaction

The alpha-amylase added during liquefaction step a) in a process of the invention (i.e., oil recovery process and fermentation product production process) may be any alpha-amylase.

Preferred are bacterial alpha-amylases, which typically are stable at a temperature used in liquefaction.

In an embodiment the alpha-amylase is from a strain of the genus *Bacillus*.

In a preferred embodiment the alpha-amylase is from a strain of *Bacillus stearothermophilus*, such as the sequence shown in SEQ ID NO: 1. In an embodiment the alpha-amylase is the *Bacillus stearothermophilus* alpha-amylase shown in SEQ ID NO: 1 herein, such as one having at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 1 herein.

In an embodiment the *Bacillus stearothermophilus* alpha-amylase or variant thereof is truncated, preferably at the C-terminal, preferably truncated to have around 491 amino acids, such as from 480-495 amino acids.

In an embodiment the *Bacillus stearothermophilus* alpha-amylase has a double deletion at positions I181 + G182, and optionally a N193F substitution (using SEQ ID NO: 1 for numbering).

In another embodiment the *Bacillus stearothermophilus* alpha-amylase has a double deletion at positions R179 + G180 and optionally a N193F substitution (using SEQ ID NO: 1 for numbering).

In an embodiment the *Bacillus stearothermophilus* alpha-amylase has a substitution at position S242, preferably S242Q substitution.

In an embodiment the *Bacillus stearothermophilus* alpha-amylase has a substitution at position E188, preferably E188P substitution.

In an embodiment the alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) of at least 10.

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

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

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

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

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

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

In embodiment the alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) of at least 60. In embodiment the alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) between 10-70.

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

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

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

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

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

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

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

In an embodiment the alpha-amylase is selected from the group of *Bacillus stearothermophilus* alpha-amylase variants with the following mutations in addition to I181*+G182*, and optionally N193F:

- V59A+Q89R+G112D+E129V+K177L+R179E+K220P+N224L+Q254S;
- V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+D269E+D281N;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+I270L;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+H274K;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+Y276F;
- V59A+E129V+R157Y+K177L+R179E+K220P+N224L+S242Q+Q254S;
- V59A+E129V+K177L+R179E+H208Y+K220P+N224L+S242Q+Q254S;
- 59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+H274K;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+D281N;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+G416V;
- V59A+E129V+K177L+R179E+K220P+N224L+Q254S;

- V59A+E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
- A91L+M96I+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- E129V+K177L+R179E;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F+L427M;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+N376*+I377*;
- E129V+K177L+R179E+K220P+N224L+Q254S;
- E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
- E129V+K177L+R179E+S242Q;
- E129V+K177L+R179V+K220P+N224L+S242Q+Q254S;
- K220P+N224L+S242Q+Q254S;
- M284V;
- V59A Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V.

In a preferred embodiment the alpha-amylase is selected from the group of *Bacillus stearothermophilus* alpha-amylase variants:

- I181*+G182*+N193F+E129V+K177L+R179E;

- I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S

- I181*+G182*+N193F +V59A Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V; and

- I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 for numbering).

According to the invention the alpha-amylase variant has at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 1 herein.

In another embodiment the alpha-amylase is a *Bacillus licheniformis* alpha-amylase, or a variant thereof. In an embodiment the *Bacillus licheniformis* alpha-amylase is the one shown in SEQ ID NO: 21 herein. According to the invention the alpha-amylase has at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99% identity to the mature part of the polypeptide of SEQ ID NO: 21 herein.

The alpha-amylase may according to the invention be present and/or added in a concentration of 0.1-100 micro gram per gram DS, such as 0.5-50 micro gram per gram DS, such as 1-25 micro gram per gram DS, such as 1-10 micro gram per gram DS, such as 2-5 micro gram per gram DS.

In an embodiment from 1-10 micro gram *Pyrococcus furiosus* protease and 1-10 micro gram *Bacillus stearothermophilus* alpha-amylase are present and/or added in liquefaction.

Glucoamylase Present And/Or Added In Liquefaction

In an embodiment a glucoamylase is present and/or added in liquefaction step a) in a process of the invention (i.e., oil recovery process and fermentation product production process).

In a preferred embodiment the glucoamylase present and/or added in liquefaction has a heat stability at 85°C, pH 5.3, of at least 20%, such as at least 30%, preferably at least 35% determined as disclosed in Example 2 herein or Example 8 in WO 2011/127802

In an embodiment the glucoamylase has a relative activity pH optimum at pH 5.0 of at least 90%, preferably at least 95%, preferably at least 97% determined as disclosed in Example 2 herein or Example 8 in WO 2011/127802.

In an embodiment the glucoamylase has a pH stability at pH 5.0 of at least at least 80%, at least 85%, at least 90% determined as disclosed in Example 2 herein or Example 8 in WO 2011/127802.

In a preferred embodiment the glucoamylase present and/or added in liquefaction step a) is derived from a strain of the genus *Penicillium*, especially a strain of *Penicillium oxalicum* disclosed as SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NOs: 9 or 14 herein.

In an embodiment the glucoamylase has at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99% or 100% identity to the mature polypeptide shown in SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NOs: 9 or 14 herein.

In an embodiment the glucoamylase has a thermostability of at least 80°C, preferably at least 82°C, such as at least 84°C, such as at least 86°C, such as at least 88°C at pH 4.0 determined as Differential Scanning Calorimitry (DSC) as described in Example 3 below.

In an embodiment the glucoamylase has a thermostability of at least 80°C, preferably at least 82°C, such as at least 84°C, such as at least 86°C, such as at least 88°C, such as at least 90°C at pH 4.8 determined as Differential Scanning Calorimitry (DSC) as described in Example 3 below.

Examples of specifically contemplated glucoamylases can be found in Example 3 (Table 6) below.

In a preferred embodiment the glucoamylase is a variant of the *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NO: 14 herein having a K79V substitution (using the mature sequence shown in SEQ ID NO: 14 for numbering), such as a variant disclosed in WO 2013/053801 (hereby incorporated by reference).

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

T65A; or

Q327F; or

E501V; or

Y504T; or

Y504*; or

T65A + Q327F; or

T65A + E501V; or

T65A + Y504T; or

T65A + Y504*; or

Q327F + E501V; or

Q327F + Y504T; or

Q327F + Y504*; or

E501V + Y504T; or

E501V + Y504*; or

T65A + Q327F + E501V; or

T65A + Q327F + Y504T; or

T65A + E501V + Y504T; or

Q327F + E501V + Y504T; or

T65A + Q327F + Y504*; or

T65A + E501V + Y504*; or

Q327F + E501V + Y504*; or

T65A + Q327F + E501V + Y504T; or

T65A + Q327F + E501V + Y504*;

E501V + Y504T; or

T65A + K161S; or

T65A + Q405T; or

T65A + Q327W; or

T65A + Q327F; or

T65A + Q327Y; or

P11F + T65A + Q327F; or

R1K + D3W + K5Q + G7V + N8S + T10K + P11S + T65A + Q327F; or

P2N + P4S + P11F + T65A + Q327F; or

P11F + D26C + K33C + T65A + Q327F; or

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

R1E + D3N + P4G + G6R + G7A + N8A + T10D+ P11D + T65A + Q327F; or

P11F + T65A + Q327W; or

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

P11F + T65A + Q327W + E501V + Y504T; or

T65A + Q327F + E501V + Y504T; or

T65A + S105P + Q327W; or

T65A + S105P + Q327F; or

T65A + Q327W + S364P; or

T65A + Q327F + S364P; or

T65A + S103N + Q327F; or

P2N + P4S + P11F + K34Y + T65A + Q327F; or

P2N + P4S + P11F + T65A + Q327F + D445N + V447S; or

P2N + P4S + P11F + T65A + I172V + Q327F; or

P2N + P4S + P11F + T65A + Q327F + N502*; or

P2N + P4S + P11F + T65A + Q327F + N502T + P563S + K571E; or

P2N + P4S + P11F + R31S + K33V + T65A + Q327F + N564D + K571S; or

P2N + P4S + P11F + T65A + Q327F + S377T; or

P2N + P4S + P11F + T65A + V325T+ Q327W; or

P2N + P4S + P11F + T65A + Q327F + D445N + V447S + E501V + Y504T; or

P2N + P4S + P11F + T65A + I172V + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + S377T + E501V + Y504T; or

P2N + P4S + P11F + D26N + K34Y + T65A + Q327F; or

P2N + P4S + P11F + T65A + Q327F + I375A + E501V + Y504T; or

P2N + P4S + P11F + T65A + K218A + K221D + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + S103N + Q327F + E501V + Y504T; or

P2N + P4S + T10D + T65A + Q327F + E501V + Y504T; or

P2N + P4S + F12Y + T65A + Q327F + E501V + Y504T; or

K5A + P11F + T65A + Q327F + E501V + Y504T; or

P2N + P4S + T10E + E18N + T65A + Q327F + E501V + Y504T; or

P2N + T10E + E18N + T65A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T568N; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + K524T + G526A; or

P2N + P4S + P11F + K34Y + T65A + Q327F + D445N + V447S + E501V + Y504T; or

P2N + P4S + P11F + R31S + K33V + T65A + Q327F + D445N + V447S + E501V + Y504T; or

P2N + P4S + P11F + D26N + K34Y + T65A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + F80* + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K112S + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T516P + K524T + G526A; or

P2N + P4S + P11F + T65A + Q327F + E501V + N502T + Y504*; or

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

P2N + P4S + P11F + T65A + S103N + Q327F + E501V + Y504T; or

K5A + P11F + T65A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T516P + K524T + G526A; or

P2N + P4S + P11F + T65A + K79A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79G + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79I + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79L + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79S + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + L72V + Q327F + E501V + Y504T; or

S255N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + E74N + V79K + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + G220N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Y245N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q253N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + D279N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + S359N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + D370N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + V460S + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + V460T + P468T + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + T463N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + S465N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + T477N + E501V + Y504T.

In a preferred embodiment the glucoamylase present and/or added in liquefaction is the *Penicillium oxalicum* glucoamylase having a K79V substitution and preferably further one of the following substitutions:

- P11F + T65A + Q327F;

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

In an embodiment the glucoamylase variant has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 14 herein.

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

Glucoamylase Present And/Or Added In Saccharification And/Or Fermentation

A glucoamylase is present and/or added in saccharification and/or fermentation, preferably simultaneous saccharification and fermentation (SSF), in a process of the invention (i.e., oil recovery process and fermentation product production process).

In an embodiment the glucoamylase present and/or added in saccharification and/or fermentation is of fungal origin, preferably from a stain of *Aspergillus*, preferably *A. niger*, *A. awamori*, or *A. oryzae*; or a strain of *Trichoderma*, preferably *T. reesei*; or a strain of *Talaromyces*, preferably *T. emersonii.*

In an embodiment the glucoamylase is derived from *Talaromyces*, such as a strain of *Talaromyces emersonii*, such as the one shown in SEQ ID NO: 19 herein,

In an embodiment the glucoamylase is selected from the group consisting of:

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

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 19 herein.

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

In an embodiment the glucoamylase is derived from a strain of the genus *Gloeophyllum*, such as a strain of *Gloeophyllum sepiarium* or *Gloeophyllum trabeum*, in particular a strain of *Gloeophyllum* as described in WO 2011/068803 (SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16). In a preferred embodiment the glucoamylase is the *Gloeophyllum sepiarium* shown in SEQ ID NO: 2 in WO 2011/068803 or SEQ ID NO: 15 herein.

In a preferred embodiment the glucoamylase is derived from *Gloephyllum serpiarium*, such as the one shown in SEQ ID NO: 15 herein. In an embodiment the glucoamylase is selected from the group consisting of:

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

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 15 herein.

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

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

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 17 herein.

In an embodiment the glucoamylase is derived from a strain of the genus *Nigrofomes,* in particular a strain of *Nigrofomes sp.* disclosed in WO 2012/064351 (SEQ ID NO: 2) (all references hereby incorporated by reference).

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

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

According to a preferred embodiment of the invention the glucoamylase is present and/or added in saccharification and/or fermentation in combination with an alpha-amylase. Examples of suitable alpha-amylase are described below.

Alpha-Amylase Present and/or Added In Saccharification And/Or Fermentation

In an embodiment an alpha-amylase is present and/or added in saccharification and/or fermentation in a process of the invention. In a preferred embodiment the alpha-amylase is of fungal or bacterial origin. In a preferred embodiment the alpha-amylase is a fungal acid stable alpha-amylase. A fungal acid stable alpha-amylase is an alpha-amylase that has activity in the pH range of 3.0 to 7.0 and preferably in the pH range from 3.5 to 6.5, including activity at a pH of about 4.0, 4.5, 5.0, 5.5, and 6.0.

In a preferred embodiment the alpha-amylase present and/or added in saccharification and/or fermentation is derived from a strain of the genus *Rhizomucor*, preferably a strain the *Rhizomucor pusillus,* such as one shown in SEQ ID NO: 3 in WO 2013/006756, such as a *Rhizomucor pusillus* alpha-amylase hybrid having an *Aspergillus niger* linker and starch-bonding domain, such as the one shown in SEQ ID NO: 16 herein, or a variant thereof.

In an embodiment the alpha-amylase present and/or added in saccharification and/or fermentation is selected from the group consisting of:

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

(ii) an alpha-amylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 16 herein.

In a preferred embodiment the alpha-amylase is a variant of the alpha-amylase shown in SEQ ID NO: 13 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 (using SEQ ID NO: 16 for numbering).

In an embodiment the alpha-amylase is derived from a *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably disclosed as SEQ ID

NO: 13 herein, preferably having one or more of the following substitutions: G128D, D143N, preferably G128D+D143N (using SEQ ID NO: 13 for numbering).

In an embodiment the alpha-amylase variant present and/or added in saccharification and/or fermentation has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 16 herein.

In an embodiment the alpha-amylase is derived from a strain of *Aspergillus*, such as *Aspergillus niger,* such as the one shown as SEQ ID NO: 9 in US patent No. 8,048,657; or *Aspergillus kawachi*, such as the one shown as SEQ ID NO: 5 in US patent No. 8,048,657.

In an embodiment the alpha-amylase is derived from a strain of *Trichoderma reesei*, such as the one shown in SEQ ID NO: 13 in US patent No. 8,048,657.

In a preferred embodiment the ratio between glucoamylase and alpha-amylase present and/or added during saccharification and/or fermentation may preferably be in the range from 500:1 to 1:1, such as from 250:1 to 1:1, such as from 100:1 to 1: 1, such as from 100: 2 to 100:50, such as from 100:3 to 100:70.

Pullulanase Present And/Or Added In Liquefaction And/Or Saccharification And/Or Fermentation.

A pullulanase may be present and/or added during liquefaction step a) and/or saccharification step b) or fermentation step c) or simultaneous saccharification and fermentation.

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

Contemplated pullulanases according to the present invention include the pullulanases from *Bacillus amyloderamificans* disclosed in U.S. Patent No. 4,560,651 (hereby incorporated by reference), the pullulanase disclosed as SEQ ID NO: 2 in WO 01/151620 (hereby incorporated by reference), the *Bacillus deramificans* disclosed as SEQ ID NO: 4 in WO 01/151620 (hereby

incorporated by reference), and the pullulanase from *Bacillus acidopullulyticus* disclosed as SEQ ID NO: 6 in WO 01/151620 (hereby incorporated by reference) and also described in FEMS Mic. Let. (1994) 115, 97-106.

Additional pullulanases contemplated according to the present invention included the pullulanases from *Pyrococcus woesei*, specifically from *Pyrococcus woesei* DSM No. 3773 disclosed in WO92/02614.

In an embodiment the pullulanase is a family GH57 pullulanase, wherein the pullulanase preferably includes an X47 domain as disclosed in WO 2011/087836. More specifically the the pullulanase may be derived from a strain from the genus *Thermococcus*, including *Thermococcus litoralis* and *Thermococcus hydrothermalis* or a hybrid thereof. In an embodiment the pullulanase is the truncated *Thermococcus hydrothermalis* pullulanase at site X4 or a *T. hydrothermalis/T. litoralis* hybrid enzyme with truncation site X4 disclosed in WO 2011/087836 or shown in SEQ ID NO: 12 herein.

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

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

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

Further Aspects Of Processes Of The Invention

Prior to liquefaction step a), processes of the invention, including processes of extracting/recovering oil and processes for producing fermentation products, may comprise the steps of:

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

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

In an embodiment at least 50%, preferably at least 70%, more preferably at least 80%, especially at least 90% of the starch-containing material fit through a sieve with # 6 screen.

In an embodiment the pH during liquefaction is between above 4.5-6.5, such as 4.5-5.0, such as around 4.8, or a pH between 5.0-6.2, such as 5.0-6.0, such as between 5.0-5.5, such as around 5.2, such as around 5.4, such as around 5.6, such as around 5.8.

In an embodiment the temperature during liquefaction is above the initial gelatinization temperature, preferably in the range from 70-100°C, such as between 75-95°C, such as between 75-90°C, preferably between 80-90°C, especially around 85°C.

In an embodiment a jet-cooking step is carried out before liquefaction in step a). In an embodiment the jet-cooking is carried out at a temperature between 110-145°C, preferably 120-140°C, such as 125-135°C, preferably around 130°C for about 1-15 minutes, preferably for about 3-10 minutes, especially around about 5 minutes.

In a preferred embodiment saccharification and fermentation is carried out sequentially or simultaneously.

In an embodiment saccharification is carried out at a temperature from 20-75°C, preferably from 40-70°C, such as around 60°C, and at a pH between 4 and 5.

In an embodiment fermentation or simultaneous saccharification and fermentation (SSF) is carried out carried out at a temperature from 25°C to 40°C, such as from 28°C to 35°C, such as from 30°C to 34°C, preferably around about 32°C. In an embodiment fermentation is ongoing for 6 to 120 hours, in particular 24 to 96 hours.

In a preferred embodiment the fermentation product is recovered after fermentation, such as by distillation.

In an embodiment the fermentation product is an alcohol, preferably ethanol, especially fuel ethanol, potable ethanol and/or industrial ethanol.

In an embodiment the starch-containing starting material is whole grains. In an embodiment the starch-containing material is selected from the group of corn, wheat, barley, rye, milo, sago, cassava, manioc, tapioca, sorghum, rice, and potatoes.

In an embodiment the fermenting organism is yeast, preferably a strain of *Saccharomyces*, especially a strain of *Saccharomyces cerevisae*.

In an embodiment the alpha-amylase is a bacterial or fungal alpha-amylase.

In an embodiment saccharification step b) and fermentation step c) are carried out simultaneously or sequentially.

In an embodiment the temperature in step (a) is above the initial gelatinization temperature, such as at a temperature between 80-90°C, such as around 85°C.

In an embodiment a process of the invention further comprises a pre-saccharification step, before saccharification step b), carried out for 40-90 minutes at a temperature between 30-65°C. In an embodiment saccharification is carried out at a temperature from 20-75°C, preferably from 40-70°C, such as around 60°C, and at a pH between 4 and 5. In an embodiment fermentation step c) or simultaneous saccharification and fermentation (SSF) (i.e., steps b) and c)) are carried out carried out at a temperature from 25°C to 40°C, such as from 28°C to 35°C, such as from 30°C to 34°C, preferably around about 32°C. In an embodiment the fermentation step c) or simultaneous saccharification and fermentation (SSF) (i.e., steps b) and c)) are ongoing for 6 to 120 hours, in particular 24 to 96 hours.

In an embodiment separation in step e) is carried out by centrifugation, preferably a decanter centrifuge, filtration, preferably using a filter press, a screw press, a plate-and-frame press, a gravity thickener or decker.

In an embodiment the fermentation product is recovered by distillation.

Examples of Specific Process Embodiments Of The Invention

Oil recovery:

In a preferred embodiment the invention concerns processes of recovering oil comprising the steps of:

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

- *Bacillus stearothermophilus* alpha-amylase comprising a double deletion at positions I181 + G182 using SEQ ID NO: 1 for numbering;

- more than 0.5 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS);

- *Penicillium oxalicum* shown in SEQ ID NO: 14 comprising a K79V substitution;

b) saccharifying using a glucoamylase;

c) fermenting using a fermenting organism.

d) recovering the fermentation product to form whole stillage;

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

f) optionally concentrating the thin stillage into syrup;

wherein oil is recovered from the:

- liquefied starch-containing material after step a); and/or

- downstream from fermentation step c).

In a preferred embodiment the invention concerns processes of recovering oil comprising the steps of:

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

- *Bacillus stearothermophilus* alpha-amylase comprising a double deletion at positions:

I181 + G182 and the following substitutions N193F+V59A+Q89R+E129V+K177L+R179E+Q254S+M284V truncated to 491 amino acids (using SEQ ID NO: 1 for numbering).

- more than 0.5 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS);

- *Penicillium oxalicum* glucoamylase having the following mutations: K79V+P2N+P4S+P11F+T65A+Q327F (using SEQ ID NO: 14 for numbering);

b) saccharifying using a glucoamylase;

c) fermenting using a fermenting organism.

d) recovering the fermentation product to form whole stillage;

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

f) optionally concentrating the thin stillage into syrup;

wherein oil is recovered from the:

- liquefied starch-containing material after step a); and/or

- downstream from fermentation step c).

In a preferred embodiment the ratio between alpha-amylase and glucoamylase in liquefaction is between 1:1 and 1:10, such as around 1:2 (micro gram alpha-amylase per g DS : micro gram glucoamylase per gram DS).

In a preferred embodiment the ratio between alpha-amylase and protease in liquefaction is in the range between 1:1 and 1:25, such between 1:1.2 and as 1:10, such as around 1:1.4 (micro gram alpha-amylase per gram DS : micro gram protease per gram DS).

Producing Fermentation Products:

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

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

- an alpha-amylase derived from *Bacillus stearothermophilus*;

- more than 2 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS); and

- optionally a *Penicillium oxalicum* glucoamylase;

b) saccharifying using a glucoamylase enzyme;

c) fermenting using a fermenting organism.

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

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

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

- more than 2 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS); and

- optionally a glucoamylase;

b) saccharifying using a glucoamylase enzyme;

c) fermenting using a fermenting organism.

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

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

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

- more than 2 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS); and

- a *Penicillium oxalicum* glucoamylasea;

b) saccharifying using a glucoamylase enzyme;

c) fermenting using a fermenting organism.

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

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

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

  - E129V+K177L+R179E;

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

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

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

- more than 2 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS);

- a *Penicillium oxalicum* glucoamylase in SEQ ID NO: 14 having substitutions selected from the group of:

- K79V;

- K79V+ P11F + T65A + Q327F; or

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

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

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

- K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; or

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

b) saccharifying using a glucoamylase enzyme;

c) fermenting using a fermenting organism.

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

a) liquefying the starch-containing material at a pH in the range between from above 4.5-6.5 at a temperature between 80-90°C using:

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

- E129V+K177L+R179E;

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

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

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

- more than 2 micro gram, such as between 2-5 micro gram, preferably around or more than 3 micro gram *Pyrococcus furiosus* protease per gram DS dry solids (DS);

- a *Penicillium oxalicum* glucoamylase in SEQ ID NO: 14 having substitutions selected from the group of:

- K79V;

- K79V+ P11F + T65A + Q327F; or

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

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

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

- K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; or

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

b) saccharifying using a glucoamylase enzyme;

c) fermenting using a fermenting organism.

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

a) liquefying the starch-containing material at a pH in the range between from above 4.5-6.5 at a temperature between 80-90°C using:

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

  - E129V+K177L+R179E;

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

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

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

- more than 2 micro gram, such as between 2-5 micro gram, preferably around or more than 3 micro gram *Pyrococcus furiosus* protease per gram DS dry solids (DS)

- a *Penicillium oxalicum* glucoamylase in SEQ ID NO: 14 having substitutions selected from the group of:

- K79V;

- K79V+ P11F + T65A + Q327F; or

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

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

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

- K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; or

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

b) saccharifying using a *Rhizomucor pusillus* glucoamylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably disclosed as SEQ ID NO: 13 herein, preferably having one or more of the following substitutions: G128D, D143N, preferably G128D+D143N (using SEQ ID NO: 13 for numbering);

c) fermenting using a fermenting organism.

In an embodiment the ratio between alpha-amylase and glucoamylase in liquefaction is between 1:1 and 1:10, such as around 1:2 (micro gram alpha-amylase per g DS : micro gram glucoamylase per gram DS).

In an embodiment the ratio between alpha-amylase and protease in liquefaction is in the range between 1:1 and 1:25, such between 1:1.2 and as 1:10, such as around 1:1.4 (micro gram alpha-amylase per g DS : micro gram protease per gram DS).

Fermentation Medium

The environment in which fermentation is carried out is often referred to as the "fermentation media" or "fermentation medium". The fermentation medium includes the fermentation substrate, that is, the carbohydrate source that is metabolized by the fermenting organism. According to the invention the fermentation medium may comprise nutrients and growth stimulator(s) for the fermenting organism(s). Nutrient and growth stimulators are widely used in the art of fermentation and include nitrogen sources, such as ammonia; urea, vitamins and minerals, or combinations thereof.

Fermenting Organisms

The term "fermenting organism" refers to any organism, including bacterial and fungal organisms, especially yeast, suitable for use in a fermentation process and capable of producing the desired fermentation product. Especially suitable fermenting organisms are able to ferment, i.e., convert, sugars, such as glucose or maltose, directly or indirectly into the desired fermentation product, such as ethanol. Examples of fermenting organisms include fungal organisms, such as yeast. Preferred yeast includes strains of *Saccharomyces* spp., in

particular, *Saccharomyces cerevisiae*.

Suitable concentrations of the viable fermenting organism during fermentation, such as SSF, are well known in the art or can easily be determined by the skilled person in the art. In one embodiment the fermenting organism, such as ethanol fermenting yeast, (e.g., *Saccharomyces cerevisiae*) is added to the fermentation medium so that the viable fermenting organism, such as yeast, count per mL of fermentation medium is in the range from 10^5 to 10^{12}, preferably from 10^7 to 10^{10}, especially about $5x10^7$.

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

Starch-Containing Materials

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

Fermentation Products

The term "fermentation product" means a product produced by a process including a fermentation step using a fermenting organism. Fermentation products contemplated according to the invention include alcohols (e.g., ethanol, methanol, butanol; polyols such as glycerol, sorbitol and inositol); organic acids (e.g., citric acid, acetic acid, itaconic acid, lactic acid, succinic acid, gluconic acid); ketones (e.g., acetone); amino acids (e.g., glutamic acid); gases (e.g., H_2 and CO_2); antibiotics (e.g., penicillin and tetracycline); enzymes; vitamins (e.g., riboflavin, B_{12}, beta-carotene); and hormones. In a preferred embodiment the fermentation product is ethanol, e.g., fuel ethanol; drinking ethanol, i.e., potable neutral spirits; or industrial ethanol or products used in the consumable alcohol industry (e.g., beer and wine), dairy

industry (e.g., fermented dairy products), leather industry and tobacco industry. Preferred beer types comprise ales, stouts, porters, lagers, bitters, malt liquors, happoushu, high-alcohol beer, low-alcohol beer, low-calorie beer or light beer. Preferably processes of the invention are used for producing an alcohol, such as ethanol. The fermentation product, such as ethanol, obtained according to the invention, may be used as fuel, which is typically blended with gasoline. However, in the case of ethanol it may also be used as potable ethanol.

Recovery of Fermentation Products

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

Recovery of Oil

According to the invention oil is recovered during and/or after liquefying, from the whole stillage, from the thin stillage or from the syrup. Oil may be recovered by extraction. In one embodiment oil is recovered by hexane extraction. Other oil recovery technologies well-known in the art may also be used.

An Enzyme Composition Of The Invention

An enzyme composition of the invention comprises an alpha-amylase and a *Pyrococcus furiosus* protease suitable for use in a liquefaction step in a process of the invention.

An enzyme composition of the invention comprises:

i) *Bacillus* sp. alpha-amylase, or a variant thereof;

ii) *Pyrococcus furiosus* protease;

wherein the ratio between alpha-amylase and protease is in the range from 1:1 and 1:25 (micro gram alpha-amylase : micro gram protease).

In a preferred embodiment the ratio between alpha-amylase and protease is in the range between 1:1.2 and 1:10, such as around 1:1.4 (micro gram alpha-amylase : micro gram protease).

In a preferred embodiment the enzyme composition of the invention comprises a glucoamylase and the ratio between alpha-amylase and glucoamylase in liquefaction is between 1:1 and 1:10, such as around 1:2 (micro gram alpha-amylase : micro gram glucoamylase).

In an embodiment the alpha-amylase in the enzyme composition of the invention is a bacterial or fungal alpha-amylase.

In an embodiment the alpha-amylase is from the genus *Bacillus*, such as a strain of *Bacillus stearothermophilus,* in particular a variant of a *Bacillus stearothermophilus* alpha-amylase, such as the one shown in SEQ ID NO: 3 in WO 99/019467 or SEQ ID NO: 1 herein.

In an embodiment the *Bacillus stearothermophilus* alpha-amylase or variant thereof is truncated, preferably to have around 491 amino acids, such as from 480-495 amino acids.

In an embodiment the *Bacillus stearothermophilus* alpha-amylase has a double deletion, preferably at positions I181 + G182 and optionally a N193F substitution, or double deletion of R179 and G180 (using SEQ ID NO: 1 for numbering).

In an embodiment the *Bacillus stearothermophilus* alpha-amylase has a substitution at position S242, preferably S242Q substitution.

In an embodiment the *Bacillus stearothermophilus* alpha-amylase has a substitution at position E188, preferably E188P substitution.

In an embodiment the alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) of at least 10, such as at least 15, such as at least 20, such as at least 25, such as at least 30, such as at least 40, such as at least 50, such as at least 60, such as between 10-70, such as between 15-70, such as between 20-70, such as between 25-70, such as between 30-70, such as between 40-70, such as between 50-70, such as between 60-70.

In an embodiment the alpha-amylase is selected from the group of *Bacillus stearothermphilus* alpha-amylase variants with the following mutations:

- I181*+G182*+N193F+E129V+K177L+R179E;

- I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

- I181*+G182*+N193F +V59A Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V; and

- I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering).

In an embodiment the alpha-amylase variant has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 1 herein.

In another embodiment the alpha-amylase is a *Bacillus licheniformis* alpha-amylase, or a variant thereof.

In an embodiment the *Bacillus licheniformis* alpha-amylase is the one shown in SEQ ID NO: 21 herein.

In an embodiment the alpha-amylase has at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99% identity to the mature part of the polypeptide of SEQ ID NO: 21 herein.

In an embodiment the enzyme composition comprises a *Bacillus licheniformis* alpha-amylase and a *Pyrococcus furiosus* protease.

In an embodiment the enzyme composition further comprises a glucoamylase.

In an embodiment the *Pyrococcus furiosus* is the one shown in SEQ ID NO: 13 herein.

In an embodiment the *Pyrococcus furiosus* protease is one having at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 13 herein.

In an embodiment the enzyme composition further comprises a glucoamylase shown in SEQ ID NO: 14, or a variant thereof.

In an embodiment the glucoamylase has a heat stability at 85°C, pH 5.3, of at least 20%, such as at least 30%, preferably at least 35% determined as disclosed in Example 2 herein or Example 8 in WO 2011/127802.

In an embodiment the glucoamylase has a relative activity pH optimum at pH 5.0 of at least 90%, preferably at least 95%, preferably at least 97% determined as disclosed in Example 2 herein or Example 8 in WO 2011/127802.

In an embodiment the glucoamylase has a pH stability at pH 5.0 of at least at least 80%, at least 85%, at least 90% determined as disclosed in Example 2 herein or Example 8 in WO 2011/127802.

In an embodiment the glucoamylase is derived from a strain of the genus *Penicillium*, especially a strain of *Penicillium oxalicum* disclosed as SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NOs: 9 or 14 herein.

In an embodiment the glucoamylase has at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99% or 100% identity to the mature polypeptide shown in SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NOs: 9 or 14 herein.

In an embodiment the glucoamylase has a thermostability of at least 80°C, preferably at least 82°C, such as at least 84°C, such as at least 86°C, such as at least 88°C at pH 4.0 determined as Differential Scanning Calorimitry (DSC) as described in Example 3 below.

In an embodiment the glucoamylase has a thermostability of at least 80°C, preferably at least 82°C, such as at least 84°C, such as at least 86°C, such as at least 88°C, such as at least 90°C at pH 4.8 determined as Differential Scanning Calorimitry (DSC) as described in Example 3 below.

Examples of specifically contemplated glucoamylases can be found in Example 3 (Table 6) below.

In an embodiment the glucoamylase is a variant of the *Penicillium oxalicum* glucoamylase disclosed as SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NO: 14 herein having a K79V substitution (using the mature sequence shown in SEQ ID NO: 14 for numbering) such as a variant disclosed in WO 2013/053801.

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

T65A; or

Q327F; or

E501V; or

Y504T; or

Y504*; or

T65A + Q327F; or

T65A + E501V; or

T65A + Y504T; or

T65A + Y504*; or

Q327F + E501V; or

Q327F + Y504T; or

Q327F + Y504*; or

E501V + Y504T; or

E501V + Y504*; or

T65A + Q327F + E501V; or

T65A + Q327F + Y504T; or

T65A + E501V + Y504T; or

Q327F + E501V + Y504T; or

T65A + Q327F + Y504*; or

T65A + E501V + Y504*; or

Q327F + E501V + Y504*; or

T65A + Q327F + E501V + Y504T; or

T65A + Q327F + E501V + Y504*;

E501V + Y504T; or

T65A + K161S; or

T65A + Q405T; or

T65A + Q327W; or

T65A + Q327F; or

T65A + Q327Y; or

P11F + T65A + Q327F; or

R1K + D3W + K5Q + G7V + N8S + T10K + P11S + T65A + Q327F; or

P2N + P4S + P11F + T65A + Q327F; or

P11F + D26C + K33C + T65A + Q327F; or

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

R1E + D3N + P4G + G6R + G7A + N8A + T10D+ P11D + T65A + Q327F; or

P11F + T65A + Q327W; or

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

P11F + T65A + Q327W + E501V + Y504T; or

T65A + Q327F + E501V + Y504T; or

T65A + S105P + Q327W; or

T65A + S105P + Q327F; or

T65A + Q327W + S364P; or

T65A + Q327F + S364P; or

T65A + S103N + Q327F; or

P2N + P4S + P11F + K34Y + T65A + Q327F; or

P2N + P4S + P11F + T65A + Q327F + D445N + V447S; or

P2N + P4S + P11F + T65A + I172V + Q327F; or

P2N + P4S + P11F + T65A + Q327F + N502*; or

P2N + P4S + P11F + T65A + Q327F + N502T + P563S + K571E; or

P2N + P4S + P11F + R31S + K33V + T65A + Q327F + N564D + K571S; or

P2N + P4S + P11F + T65A + Q327F + S377T; or

P2N + P4S + P11F + T65A + V325T+ Q327W; or

P2N + P4S + P11F + T65A + Q327F + D445N + V447S + E501V + Y504T; or

P2N + P4S + P11F + T65A + I172V + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + S377T + E501V + Y504T; or

P2N + P4S + P11F + D26N + K34Y + T65A + Q327F; or

P2N + P4S + P11F + T65A + Q327F + I375A + E501V + Y504T; or

P2N + P4S + P11F + T65A + K218A + K221D + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + S103N + Q327F + E501V + Y504T; or

P2N + P4S + T10D + T65A + Q327F + E501V + Y504T; or

P2N + P4S + F12Y + T65A + Q327F + E501V + Y504T; or

K5A + P11F + T65A + Q327F + E501V + Y504T; or

P2N + P4S + T10E + E18N + T65A + Q327F + E501V + Y504T; or

P2N + T10E + E18N + T65A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T568N; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + K524T + G526A; or

P2N + P4S + P11F + K34Y + T65A + Q327F + D445N + V447S + E501V + Y504T; or

P2N + P4S + P11F + R31S + K33V + T65A + Q327F + D445N + V447S + E501V + Y504T; or

P2N + P4S + P11F + D26N + K34Y + T65A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + F80* + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K112S + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T516P + K524T + G526A; or

P2N + P4S + P11F + T65A + Q327F + E501V + N502T + Y504*; or

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

P2N + P4S + P11F + T65A + S103N + Q327F + E501V + Y504T; or

K5A + P11F + T65A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T516P + K524T + G526A; or

P2N + P4S + P11F + T65A + K79A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79G + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79I + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79L + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79S + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + L72V + Q327F + E501V + Y504T; or

S255N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + E74N + V79K + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + G220N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Y245N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q253N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + D279N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + S359N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + D370N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + V460S + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + V460T + P468T + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + T463N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + S465N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + T477N + E501V + Y504T.

In an embodiment the glucoamylase is the *Penicillium oxalicum* glucoamylase having a K79V substitution (using SEQ ID NO: 14 for numbering) and further one of the following substitutions:

- P11F + T65A + Q327F

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

In an embodiment the glucoamylase variant has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more

preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 14 herein.

In an embodiment the composition further comprising a pullulanase.

In an embodiment the pullulanase is a family GH57 pullulanase, wherein the pullulanase preferably includes an X47 domain as disclosed in WO 2011/087836.

In an embodiment the pullulanase is derived from a strain from the genus *Thermococcus*, including *Thermococcus litoralis* and *Thermococcus hydrothermalis* or a hybrid thereof.

In an embodiment the pullulanase is the truncated *Thermococcus hydrothermalis* pullulanase at site X4 or a *T. hydrothermalis/T. litoralis* hybrid enzyme with truncation site X4 disclosed in WO 2011/087836 or shown in SEQ ID NO: 12 herein.

In an embodiment the enzyme composition comprises

- *Bacillus stearothermophilus* alpha-amylase, or a variant thereof;

- *Pyrococcus furiosus* protease; and

- *Penicillium oxalicum* glucoamylase,

wherein the ratio between alpha-amylase and protease is in the range from 1:1 and 1:25 (micro gram alpha-amylase : micro gram protease).

In an embodiment the enzyme composition of the invention comprises:

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

- *Pyrococcus furiosus* protease; and

- *Penicillium oxalicum* glucoamylase,

wherein the ratio between alpha-amylase and protease is in the range from 1:1 and 1:25 (micro gram alpha-amylase : micro gram protease).

In an embodiment the enzyme composition comprises:

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

- E129V+K177L+R179E;

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

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

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

- *Pyrococcus furiosus* protease; and

- *Penicillium oxalicum* glucoamylase in SEQ ID NO: 14 having substitutions selected from the group of:

- K79V;
- K79V+ P11F + T65A + Q327F; or
- K79V+P2N + P4S + P11F + T65A + Q327F; or
- K79V +P11F + D26C + K33C + T65A + Q327F; or
- K79V +P2N + P4S + P11F + T65A + Q327W + E501V+Y504T; or
- K79V +P2N + P4S + P11F + T65A + Q327F+E501V+Y504T; or
- K79V+P11F+T65A+Q327W+E501V+Y504T (using SEQ ID NO: 14 for numbering),

wherein the ratio between alpha-amylase and protease is in the range from 1:1 and 1:25 (micro gram alpha-amylase : micro gram protease).

In an embodiment the ratio between alpha-amylase and protease is in the range between 1:1.2 and 1:10, such as around 1:1.4 (micro gram alpha-amylase : micro gram protease).

In an embodiment the ratio between alpha-amylase and glucoamylase is between 1:1 and 1:10, such as around 1:2 (micro gram alpha-amylase : micro gram glucoamylase).

The invention described and claimed herein is not to be limited in scope by the specific aspects herein disclosed, since these aspects are intended as illustrations of several aspects of the invention. Any equivalent aspects are intended to be within the scope of this invention. Indeed,

various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

Materials & Methods

Materials:

Alpha-Amylase A (AAA): *Bacillus stearothermophilus* alpha-amylase with the mutations I181*+G182*+N193F truncated to 491 amino acids (SEQ ID NO: 1)

Alpha-Amylase 1407 (AA1407): *Bacillus stearothermophilus* alpha-amylase with the mutations I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S truncated to 491 amino acids (SEQ ID NO: 1)

Alpha-Amylase 369 (AA369): *Bacillus stearothermophilus* alpha-amylase with the mutations: I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+Q254S+M284V truncated to 491 amino acids (SEQ ID NO: 1).

Protease Pfu: Protease derived from *Pyrococcus furiosus* shown in SEQ ID NO: 13 herein.

Glucoamylase Po: Mature part of the *Penicillium oxalicum* glucoamylase disclosed as SEQ ID NO: 2 in WO 2011/127802 and shown in SEQ ID NO: 14 herein.

Glucoamylase Po PE001: Variant of *Penicillium oxalicum* glucoamylase having the following mutation: K79V (using SEQ ID NO: 14 for numbering).

Glucoamylase Po 498 (GA498): Variant of *Penicillium oxalicum* glucoamylase having the following mutations: K79V+ P2N+ P4S+ P11F+ T65A+ Q327F (using SEQ ID NO: 14 for numbering).

Glucoamylase A: Blend comprising *Talaromyces emersonii* glucoamylase disclosed as SEQ ID NO: 34 in WO99/28448, *Trametes cingulata* glucoamylase disclosed as SEQ ID NO: 2 in WO 06/69289, and *Rhizomucor pusillus* alpha-amylase with *Aspergillus niger* glucoamylase linker and starch binding domain (SBD) disclosed in SEQ ID NO: 16 herein having the following substitutions G128D+D143N (activity ratio in AGU:AGU:FAU-F is about 20:5:1).

Glucoamylase U: Blend comprising *Talaromyces emersonii* glucoamylase disclosed as SEQ ID NO: 34 in WO99/28448, *Trametes cingulata* glucoamylase disclosed as SEQ ID NO: 2 in WO 06/69289and *Rhizomucor pusillus* alpha-amylase with *Aspergillus niger* glucoamylase linker and starch binding domain (SBD) disclosed disclosed in SEQ ID NO: 16 herein (activity ratio in AGU: AGU:FAU-F is about 65:15:1).

Protease X: Metallo protease derived from *Thermoascus aurantiacus* CGMCC No. 0670 disclosed as amino acids 1-177 in SEQ ID NO: 3 herein and amino acids 1-177 in SEQ ID NO: 2 in WO 2003/048353

Yeast:
ETHANOL RED™ from Fermentis, USA

Methods

Identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "identity".

For purposes of the present invention the degree of identity between two amino acid sequences, as well as the degree of identity between two nucleotide sequences, may be determined by the program "align" which is a Needleman-Wunsch alignment (i.e. a global alignment). The program is used for alignment of polypeptide, as well as nucleotide sequences. The default scoring matrix BLOSUM50 is used for polypeptide alignments, and the default identity matrix is used for nucleotide alignments. The penalty for the first residue of a gap is -12 for polypeptides and -16 for nucleotides. The penalties for further residues of a gap are -2 for polypeptides, and -4 for nucleotides.

"Align" is part of the FASTA package version v20u6 (see W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448, and W. R. Pearson (1990) "Rapid and Sensitive Sequence Comparison with FASTP and FASTA," Methods in Enzymology 183:63- 98). FASTA protein alignments use the Smith-Waterman algorithm with no limitation on gap size (see "Smith-Waterman algorithm", T. F. Smith and M. S. Waterman (1981) J. Mol. Biol. 147:195-197).

Protease assays

AZCL-casein assay

A solution of 0.2% of the blue substrate AZCL-casein is suspended in Borax/NaH_2PO_4 buffer pH9 while stirring. The solution is distributed while stirring to microtiter plate (100 microL to each well), 30 microL enzyme sample is added and the plates are incubated in an Eppendorf Thermomixer for 30 minutes at 45°C and 600 rpm. Denatured enzyme sample (100°C boiling for 20min) is used as a blank. After incubation the reaction is stopped by transferring the microtiter plate onto ice and the colored solution is separated from the solid by centrifugation at 3000rpm for 5 minutes at 4°C. 60 microL of supernatant is transferred to a microtiter plate and the absorbance at 595nm is measured using a BioRad Microplate Reader.

pNA-assay

50 microL protease-containing sample is added to a microtiter plate and the assay is started by adding 100 microL 1mM pNA substrate (5 mg dissolved in 100 microL DMSO and further diluted to 10 mL with Borax/NaH_2PO_4 buffer pH 9.0). The increase in OD_{405} at room temperature is monitored as a measure of the protease activity.

Glucoamylase activity (AGU)

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

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

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

AMG incubation:	
Substrate:	maltose 23.2 mM

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

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

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

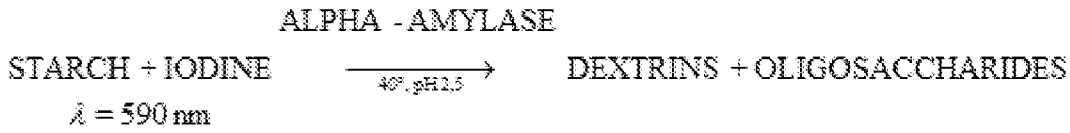
Acid Alpha-Amylase Activity

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.



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:	590 nm
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.

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.

Determination of Pullulanase Activity (NPUN)

Endo-pullulanase activity in NPUN is measured relative to a Novozymes pullulanase standard. One pullulanase unit (NPUN) is defined as the amount of enzyme that releases 1 micro mol glucose per minute under the standard conditions (0.7% red pullulan (Megazyme), pH 5, 40°C, 20 minutes). The activity is measured in NPUN/ml using red pullulan.

1 mL diluted sample or standard is incubated at 40°C for 2 minutes. 0.5 mL 2% red pullulan, 0.5 M KCl, 50 mM citric acid, pH 5 are added and mixed. The tubes are incubated at 40°C for 20

minutes and stopped by adding 2.5 ml 80% ethanol. The tubes are left standing at room temperature for 10-60 minutes followed by centrifugation 10 minutes at 4000 rpm. OD of the supernatants is then measured at 510 nm and the activity calculated using a standard curve.

The present invention is described in further detail in the following examples which are offered to illustrate the present invention, but not in any way intended to limit the scope of the invention as claimed. All references cited herein are specifically incorporated by reference for that which is described therein.

EXAMPLES

Example 1

Stability of Alpha-Amylase Variants

The stability of a reference alpha-amylase (*Bacillus stearothermophilus* alpha-amylase with the mutations I181*+G182*+N193F truncated to 491 amino acids (SEQ ID NO: 1 numbering)) and alpha-amylase variants thereof was determined by incubating the reference alpha-amylase and variants at pH 4.5 and 5.5 and temperatures of 75°C and 85°C with 0.12 mM $CaCl_2$ followed by residual activity determination using the EnzChek® substrate (EnzChek® Ultra Amylase assay kit, E33651, Molecular Probes).

Purified enzyme samples were diluted to working concentrations of 0.5 and 1 or 5 and 10 ppm (micrograms/ml) in enzyme dilution buffer (10 mM acetate, 0.01% Triton X100, 0.12 mM $CaCl_2$, pH 5.0). Twenty microliters enzyme sample was transferred to 48-well PCR MTP and 180 microliters stability buffer (150 mM acetate, 150 mM MES, 0.01% Triton X100, 0.12 mM $CaCl_2$, pH 4.5 or 5.5) was added to each well and mixed. The assay was performed using two concentrations of enzyme in duplicates. Before incubation at 75°C or 85°C, 20 microliters was withdrawn and stored on ice as control samples. Incubation was performed in a PCR machine at 75°C and 85°C. After incubation samples were diluted to 15 ng/mL in residual activity buffer (100 mM Acetate, 0.01% Triton X100, 0.12 mM $CaCl_2$, pH 5.5) and 25 microliters diluted enzyme was transferred to black 384-MTP. Residual activity was determined using the EnzChek substrate by adding 25 microliters substrate solution (100 micrograms/ml) to each well. Fluorescence was determined every minute for 15 minutes using excitation filter at 485-P nm and emission filter at 555 nm (fluorescence reader is Polarstar, BMG). The residual activity was normalized to control samples for each setup.

Assuming logarithmic decay half life time (T½ (min)) was calculated using the equation: T½ (min) = T(min)*LN(0.5)/LN(%RA/100), where T is assay incubation time in minutes, and %RA is % residual activity determined in assay.

Using this assay setup the half life time was determined for the reference alpha-amylase and variant thereof as shown in Table 1.

Table 1

Mutations	**T½ (min) (pH 4.5, 75°C, 0.12 mM $CaCl_2$)**	**T½ (min) (pH 4.5, 85°C, 0.12 mM $CaCl_2$)**	**T½ (min) (pH 5.5, 85°C, 0.12 mM $CaCl_2$)**
Reference Alpha-Amylase A	21	4	111
Reference Alpha-Amylase A with the substitution V59A	32	6	301
Reference Alpha-Amylase A with the substitution V59E	28	5	230
Reference Alpha-Amylase A with the substitution V59I	28	5	210
Reference Alpha-Amylase A with the substitution V59Q	30	6	250
Reference Alpha-Amylase A with the substitutions V59A+Q89R+ G112D+E129V+K177L+R179E+ K220P+N224L+Q254S	149	22	ND
Reference Alpha-Amylase A with the substitutions V59A+Q89R+E129V+ K177L+R179E+H208Y+K220P+	>180	28	ND

N224L+Q254S			
Reference Alpha-Amylase A with the substitutions V59A+Q89R+E129V+ K177L+R179E+K220P+N224L+ Q254S+D269E+D281N	112	16	ND
Reference Alpha-Amylase A with the substitutions V59A+Q89R+E129V+ K177L+R179E+K220P+N224L+ Q254S+I270L	168	21	ND
Reference Alpha-Amylase A with the substitutions V59A+Q89R+E129V+ K177L+R179E+K220P+N224L+ Q254S+H274K	>180	24	ND
Reference Alpha-Amylase A with the substitutions V59A+Q89R+E129V+ K177L+R179E+K220P+N224L+ Q254S+Y276F	91	15	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ R157Y+K177L+R179E+K220P+	141	41	ND

N224L+S242Q+Q254S			
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+H208Y+K220P+ N224L+S242Q+Q254S	>180	62	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S	>180	49	>480
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S+H274K	>180	53	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S+Y276F	>180	57	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S+D281N	>180	37	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+	>180	51	ND

K177L+R179E+K220P+N224L+ S242Q+Q254S+M284T			
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S+G416V	>180	45	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ Q254S	143	21	>480
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ Q254S+M284T	>180	22	ND
Reference Alpha-Amylase A with the substitutions A91L+M96I+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S	>180	38	ND
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E	57	11	402
Reference Alpha-Amylase A with	174	44	>480

the substitutions E129V+K177L+ R179E+K220P+N224L+S242Q+ Q254S			
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+S242Q+ Q254S+Y276F+L427M	>180	49	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+S242Q+ Q254S+M284T	>180	49	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+S242Q+ Q254S+N376*+I377*	177	36	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+Q254S	94	13	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+Q254S+ M284T	129	24	>480

Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+S242Q	148	30	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179V	78	9	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179V+K220P+N224L+S242Q+ Q254S	178	31	>480
Reference Alpha-Amylase A with the substitutions K220P+N224L+ S242Q+Q254S	66	17	>480
Reference Alpha-Amylase A with the substitutions K220P+N224L+ Q254S	30	6	159
Reference Alpha-Amylase A with the substitution M284T	35	7	278
Reference Alpha-Amylase A with the substitutions M284V	59	13	ND

ND not determined

The results demonstrate that the alpha-amylase variants have a significantly greater half-life and stability than the reference alpha-amylase.

Example 2

Characterization of *Penicillium oxalicum* glucoamylase

The *Penicillium oxalicum* glucoamylase is disclosed in SEQ ID NO: 9 herein.

Substrate. Substrate: 1% soluble starch (Sigma S-9765) in deionized water

Reaction buffer: 0.1M Acetate buffer at pH 5.3

Glucose concentration determination kit: Wako glucose assay kit (LabAssay glucose, WAKO, Cat# 298-65701).

Reaction condition. 20 microL soluble starch and 50 microL acetate buffer at pH 5.3 were mixed. 30 microL enzyme solution (50 micro g enzyme protein/ml) was added to a final volume of 100 microL followed by incubation at 37°C for 15 min.

The glucose concentration was determined by Wako kits.

All the work carried out in parallel.

Temperature optimum. To assess the temperature optimum of the *Penicillium oxalicum* glucoamylase the "Reaction condition"-assay described above was performed at 20, 30, 40, 50, 60, 70, 80, 85, 90 and 95°C. The results are shown in Table 2.

Table 2 Temperature optimum

Temperature (°C)	20	30	40	50	60	70	80	85	90	95
Relative activity (%)	63.6	71.7	86.4	99.4	94.6	100.0	92.9	92.5	82.7	82.8

From the results it can be seen that the optimal temperature for *Penicillium oxalicum* glucoamylase at the given conditions is between 50°C and 70°C and the glucoamylase maintains more than 80% activity at 95°C.

Heat stability. To assess the heat stability of the *Penicillium oxalicum* glucoamylase the Reaction condition assay was modifed in that the the enzyme solution and acetate buffer was

preincubated for 15 min at 20, 30, 40, 50, 60, 70, 75, 80, 85, 90 and 95°C. Following the incubation 20 microL of starch was added to the solution and the assay was performed as described above.

The results are shown in Table 3.

Table 3 Heat stability

Temperature (°C)	20	30	40	50	60	70	80	85	90	95
Relative activity (%)	91.0	92.9	88.1	100.0	96.9	86.0	34.8	36.0	34.2	34.8

From the results it can be seen that *Penicillium oxalicum* glucoamylase is stable up to 70 °C after preincubation for 15 min in that it maintains more than 80% activity.

pH optimum. To assess the pH optimum of the *Penicillium oxalicum* glucoamylase the Reaction condition assay described above was performed at pH 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0 7.0, 8.0, 9.0, 10.0 and 11.0. Instead of using the acetate buffer described in the Reaction condition assay the following buffer was used 100mM Succinic acid, HEPES, CHES, CAPSO, 1mM $CaCl_2$, 150mM KCl, 0.01% Triton X-100, pH adjusted to 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0 7.0, 8.0, 9.0, 10.0 or 11.0 with HCl or NaOH.

The results are shown in Table 4.

Table 4 pH optimum

pH	2.0	3.0	3.5	4.0	4.5	5.0	6.0	7.0	8.0	9.0	10.0	11.0
Relative activity (%)	71.4	78.6	77.0	91.2	84.2	100.0	55.5	66.7	30.9	17.8	15.9	16.1

From the results it can be seen that *Penicillium oxalicum* glucoamylase at the given conditions has the highest activity at pH 5.0. The *Penicillium oxalicum* glucoamylase is active in a broad pH range in the it maintains more than 50% activity from pH 2 to 7.

pH stability. To assess the heat stability of the *Penicillium oxalicum* glucoamylase the Reaction condition assay was modifed in that the enzyme solution (50micro g/mL) was preincubated for 20 hours in buffers with pH 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0 7.0, 8.0, 9.0, 10.0 and 11.0 using the buffers described under pH optimum. After preincubation, 20 microL soluble starch to a final volume of 100 microL was added to the solution and the assay was performed as described above.

The results are shown in Table 5.

Table 5 pH stability

pH	2.0	3.0	3.5	4.0	4.5	5.0	6.0	7.0	8.0	9.0	10.0	11.0
Relative activity (%)	17.4	98.0	98.0	103.2	100.0	93.4	71.2	90.7	58.7	17.4	17.0	17.2

From the results it can be seen that *Penicillium oxalicum* glucoamylase, is stable from pH 3 to pH 7 after preincubation for 20 hours and it decreases its activity at pH 8.

Example 3

Penicillium oxalicum Glucoamylase Variants (PoAMG) - Thermostability Analysis by Differential Scanning Calorimitry (DSC)

Site specific *Penicillium oxalicum* glucoamylase (PoAMG) variants having substitutions and/or deletions at specific positions were constructed basically as described in Example 3 and purified as described in Example 4 in WO2013/053801 (hereby incorporated by reference).

The thermostability of the purified Glucoamylase Po PE001 (SEQ ID NO: 14 with K79V) derived variants were determined at pH 4.0 or 4.8 (50 mM Sodium Acetate) by Differential Scanning Calorimetry (DSC) using a VP-Capillary Differential Scanning Calorimeter (MicroCal Inc., Piscataway, NJ, USA). The thermal denaturation temperature, Td (°C), was taken as the top of the denaturation peak (major endothermic peak) in thermograms (Cp vs. T) obtained after heating enzyme solutions in selected buffers (50 mM Sodium Acetate, pH 4.0 or 4.8)at a constant programmed heating rate of 200 K/hr.

Sample- and reference-solutions (approximately 0.3 ml) were loaded into the calorimeter (reference: buffer without enzyme) from storage conditions at 10°C and thermally pre-equilibrated for 10 minutes at 20°C prior to DSC scan from 20°C to 110°C. Denaturation temperatures were determined with an accuracy of approximately +/- 1°C.

The isolated variants and the DSC data are disclosed in Table 6 below.

Table 6.

Po-AMG name	**Mutations** (+ K79V)	**DSC Td (°C) @ pH 4.0**	**DSC Td (°C) @ pH 4.8**
Glucoamylase Po PE001 SEQ ID NO: 14 with K79V		82.1	83.4
PE167	E501V Y504T	82.1	
PE481	T65A K161S	84.1	86.0
PE487	T65A Q405T	83.2	
PE490	T65A Q327W	87.3	
PE491	T65A Q327F	87.7	
PE492	T65A Q327Y	87.3	
PE493	P11F T65A Q327F	87.8	88.5
PE497	R1K D3W K5Q G7V N8S T10K P11S T65A Q327F	87.8	88.0
PE498	P2N P4S P11F T65A Q327F	88.3	88.4
PE003	P11F D26C K33C T65A Q327F	83.3	84.0

PE009	P2N P4S P11F T65A Q327W E501V Y504T	88.8	
PE002	R1E D3N P4G G6R G7A N8A T10D P11D T65A Q327F	87.5	88.2
PE005	P11F T65A Q327W	87.4	88.0
PE008	P2N P4S P11F T65A Q327F E501V Y504T	89.4	90.2
PE010	P11F T65A Q327W E501V Y504T		89.7
PE507	T65A Q327F E501V Y504T		89.3
PE513	T65A S105P Q327W		87.0
PE514	T65A S105P Q327F		87.4
PE515	T65A Q327W S364P		87.8
PE516	T65A Q327F S364P		88.0
PE517	T65A S103N Q327F		88.9
PE022	P2N P4S P11F K34Y T65A Q327F		89.7
PE023	P2N P4S P11F T65A Q327F D445N V447S		89.9
PE032	P2N P4S P11F T65A I172V Q327F		88.7
PE049	P2N P4S P11F T65A Q327F N502*		88.4
PE055	P2N P4S P11F T65A Q327F N502T P563S K571E		88.0
PE057	P2N P4S P11F R31S K33V T65A		89.5

	Q327F N564D K571S		
PE058	P2N P4S P11F T65A Q327F S377T		88.6
PE064	P2N P4S P11F T65A V325T Q327W		88.0
PE068	P2N P4S P11F T65A Q327F D445N V447S E501V Y504T		90.2
PE069	P2N P4S P11F T65A I172V Q327F E501V Y504T		90.2
PE073	P2N P4S P11F T65A Q327F S377T E501V Y504T		90.1
PE074	P2N P4S P11F D26N K34Y T65A Q327F		89.1
PE076	P2N P4S P11F T65A Q327F I375A E501V Y504T		90.2
PE079	P2N P4S P11F T65A K218A K221D Q327F E501V Y504T		90.9
PE085	P2N P4S P11F T65A S103N Q327F E501V Y504T		91.3
PE086	P2N P4S T10D T65A Q327F E501V Y504T		90.4
PE088	P2N P4S F12Y T65A Q327F E501V Y504T		90.4
PE097	K5A P11F T65A Q327F E501V Y504T		90.0
PE101	P2N P4S T10E E18N T65A Q327F		89.9

	E501V Y504T		
PE102	P2N T10E E18N T65A Q327F E501V Y504T		89.8
PE084	P2N P4S P11F T65A Q327F E501V Y504T T568N		90.5
PE108	P2N P4S P11F T65A Q327F E501V Y504T K524T G526A		88.6
PE126	P2N P4S P11F K34Y T65A Q327F D445N V447S E501V Y504T		91.8
PE129	P2N P4S P11F R31S K33V T65A Q327F D445N V447S E501V Y504T		91.7
PE087	P2N P4S P11F D26N K34Y T65A Q327F E501V Y504T		89.8
PE091	P2N P4S P11F T65A F80* Q327F E501V Y504T		89.9
PE100	P2N P4S P11F T65A K112S Q327F E501V Y504T		89.8
PE107	P2N P4S P11F T65A Q327F E501V Y504T T516P K524T G526A		90.3
PE110	P2N P4S P11F T65A Q327F E501V N502T Y504*		90.6

Example 4

Use of High Dosage of Protease Pfu for Oil Extraction and Ethanol

Liquefaction: Nine slurries of whole ground corn, backset and tap water were prepared to a total weight of 150 g targeting 32.50% Dry Solids (DS); backset was blended at 30% weight of

backset per weight of slurry. Slurry pH was 5.0 and no further adjustments were made before applying the following treatments:

- 3 mashes were controls, meaning that they only received Alpha-Amylase 369 (AA369) during liquefaction and will be the baseline. AA369 was applied at a fixed dose of 2.1 µg/gDS in all cases when applied.
- 2 mashes were treated with AA369 and 1.5 µg/gDS Protease Pfu.
- 2 mashes were treated with AA369 and 3 µg/gDS Protease Pfu.
- 2 mashes were treated with AA369 and 5 µg/gDS Protease Pfu.

Water and enzymes were added to each canister, and then each canister was sealed and mixed well prior to loading into the Labomat. All samples were incubated in the Labomat set to the following conditions: 5°C/min. Ramp, 15 minute Ramp to 80°C, hold for 1 min, Ramp to 85°C at 1°C/min and holding for 103 min., 40 rpm for 30 seconds to the left and 30 seconds to the right. Once liquefaction was complete, all canisters were cooled in an ice bath for approximately 20 minutes before proceeding to fermentation.

Simultaneous Saccharification and Fermentation (SSF): Penicillin was added to each mash to a final concentration of 3 ppm and adjusted to pH 5.0 with either 40% sulfuric acid or 45% potassium hydroxide as needed. Next, a portion of this mash was transferred to test tubes and represents “urea-free” fermentations, or ones which are considered to be nitrogen limited. Once the “urea-free” mashes were processed, the remaining mashes were dosed with urea up to a final concentration of 200 ppm and transferred to test tubes for fermentation. All test tubes were drilled with a 1/64” bit to allow CO_2 release. Furthermore, equivalent solids were maintained across all treatments through the addition of water as required to ensure that the urea versus urea-free mashes contained equal solids. Fermentation was initiated through the addition of Glucoamylase A (0.60 AGU/gDS), water and rehydrated yeast. Yeast rehydration took place by mixing 5.5 g of Fermentis’ ETHANOL RED™ into 100 mL of 32°C tap water for at least 15 minutes and dosing 100 µl per test tube.

Distillation: A Büchi Multivapor evaporation system was used for all distillations. The unit distilled 12 samples at a time. The parameters used are shown in Table 7. Tubes were weighed after distillation and weight lost during distillation was replaced with DI water. Tubes were weighed again after water addition. Three separate distillations were performed for this experiment which included a control each run.

Time	80 min
Temperature	75°C
Vacuum	200 - 153 mBar (40 min) 153 – 148 mBar (40 min)
RPM	8

Table 7. Distillation parameters for corn oil assay.

Oil Extraction: Hexane was added to each sample at a dose of 0.125 mL hexane/1 g starting material. Each tube was covered in Dura-seal to prevent sample leakage, and mixed thoroughly. Tubes were centrifuged at 3,000 x g for 10 minutes in an Avanti JE Series centrifuge with JS-5.3 rotor. After centrifugation, the oil/hexane layer (supernatant) was removed using a positive displacement pipette, transferred to a pre-weighed 5 mL flip-top tube, and reweighed. The density of the sample was measured using a Rudolph Research Analytical density meter. The density of the supernatant was then calculated using the standard curve equation to find the %oil in the supernatant. From this value the total %oil in the starting material was derived.

HPLC analysis: HPLC analysis used an Agilent 1100/1200 combined with a Bio-Rad HPX-87H Ion Exclusion column (300 mm x 7.8 mm) and a Bio-Rad Cation H guard cartridge. The mobile phase was 0.005 M sulfuric acid and processed samples at a flow rate of 0.6 ml/min, with column and RI detector temperatures of 65 and 55°C, respectively. Fermentation sampling took place after 54 hours by sacrificing 3 tubes per treatment. Each tube was processed by deactivation with 50 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. Samples were stored at 4°C prior to and during HPLC analysis. The method quantified analytes using calibration standards for DP4+, DP3, DP2, glucose, fructose, acetic acid, lactic acid, glycerol and ethanol (% w/v). A four point calibration including the origin is used for quantification.

Results (corn oil extraction): Terminology used in the example: For corn oil extraction, there are three separate controls (Control A, B and C) because each distillation processed a control for each run. However, each control was processed the same during liquefaction and fermentation: no protease during fermentation with Protease X addition to fermentation. Extraction of corn oil showed that the lowest dose of Protease Pfu added to liquefaction matched the dose of

Protease X (0.025% w/w) to fermentation (Fig. 1). Moreover, increasing Protease Pfu to 3 and 5 µg/gDS surpassed the oil recovered with Protease X alone, and there was no additional benefit from combining both Protease Pfu and Protease X at this dose, suggesting little to no synergy between these two proteases (Fig. 2 & 3).

Ethanol: Performance Of Various Proteases On Limited Dosage Of Exogenous Nitrogen Addition

The performance of various proteases using limiting dosage of exogenous nitrogen was also tested. It is investigates how the substrates produced from these proteases affect fermentation rate, carbohydrate consumption and glycerol formation.

Treating fermentation with Protease X and operating without supplemental nitrogen from urea did not lead to dryness, whereas delivered dryness by the 54 hour mark at all dosages (Fig. 4). Furthermore, increasing Protease Pfu to 3 or 5 µg/gDS from 1.5 µg/gDS led to a significant increase in final ethanol concentration (%w/v) (Fig. 4 & 5). These various protease treatments also demonstrate an effect on fermentation rate as the 24 hour ethanol concentrations are highest with the highest treatment of Protease Pfu.

Similar trends were observed when incorporating 200 ppm urea into fermentation, but Protease Pfu activity during liquefaction remained superior relative to Protease X by delivering the lowest residual glucose and yielding the highest final ethanol. Here, the lowest dose of Protease Pfu (1.5 µg/gDS) outperformed Protease X by supporting a faster fermentation through the first 24 hours, combined with statistically higher final ethanol concentrations (%w/v) (Fig. 6 & 7). In general, more Protease Pfu led to more ethanol production by the 54 hour mark, though the two highest doses were statistically equivalent in this example. All fermentations reached a state of dryness by the 54 hour mark. Higher doses of Protease Pfu also reduced the formation of glycerol during fermentation, and this shift in metabolism is part of the reason why the increase in ethanol is being observed (Fig. 8 - %w/v).

Conclusions

Oil Extraction:

- 1.5 µg/gDS of Protease Pfu action during a conventional corn based liquefaction (85°C, pH 5.0, 2 hours) working in combination with 2.1 µg EP/gDS Alpha-Amylase 369 matched the increase in oil extracted from fermentation treatment with Protease X (5

µg/gDS).

- An even higher Protease Pfu dose of 3 µg/gDS led to approximately 5% more oil versus Protease X alone and similar results were seen with 5 µg/gDS dose as well.
- There was no apparent synergy in oil recovery when combining Protease Pfu with Protease X, and there seems to be no improvement in running protease during liquefaction versus fermentation.

Ethanol Yield: Protease Pfu showed superior performance over Protease X

- No Urea:
    - 24 hour data showed Protease Pfu (1.5 µg/gDS) delivered a much more efficient fermentation than Protease X by having lower residual glucose and higher ethanol concentrations, where increasing to 3 or 5 µg/gDS led to even more ethanol production.
    - 54 hour data showed Protease Pfu (1.5 µg/gDS) outperformed Protease X by delivering low residual glucose and much higher ethanol concentrations. Fermentations reached dryness with all dosages of Protease Pfu, whereas Protease X finished with just over 1% w/v.
    - Glycerol concentrations were 10% lower than Protease X with Protease Pfu (5 µg/gDS).
- 200 ppm Urea:
    - 24 hour data showed that Protease Pfu (1.5 µg/gDS) outperformed Protease X by having lower residual glucose and higher ethanol concentrations with 200 ppm urea. In general, more Protease Pfu led to more ethanol by this time point, though the two highest doses (3 and 5 µg/gDS) were very similar.
    - 54 hour HPLC showed that Protease Pfu (1.5 µg/gDS) outperformed Protease X by delivering the lowest residual glucose while yielding the highest final ethanol. All fermentations reached dryness. Even the lowest dose of Protease Pfu (1.5 µg/gDS) yielded 1% more ethanol thanProtease X. While 3 and 5 µg/gDS Protease Pfu were statistically equivalent in final ethanol, they were both higher than the lowest Protease Pfu dose.
    - Glycerol concentrations were approximately 9% lower than Protease X with Protease Pfu (5 µg/gDS). Protease Pfu delivered the best final ethanol concentrations while also delivering the lowest final glycerol concentrations.

Example 5

Use Of High Protease Pfu Dose in Liquefaction In Ethanol Production Process

Liquefaction: Thirteen slurries of whole ground corn and tap water were prepared to a total weight of 125 g targeting 32.50% Dry Solids (DS); backset was blended at 30% weight of backset per weight of slurry. Initial slurry pH was approximately 6.0 and was adjusted to 5.0 with either 45% w/v potassium hydroxide or 40% v/v sulfuric acid. A fixed dose of Alpha-Amylase 1407 (1.73 µg EP/gDS) was applied to all slurries and was combined with Protease Pfu as follows to evaluate the effect of high protease treatment during liquefaction:

- Control: Alpha-amylase Only
- Alpha-amylase 1407 + 0.0355 µg/gDS Protease Pfu
- Alpha-amylase 1407 + 0.25 µg/gDS Protease Pfu
- Alpha-amylase 1047 + 0.5 µg/gDS Protease Pfu
- Alpha-amylase 1407 + 1 µg/gDS Protease Pfu
- Alpha-amylase 1407 + 10 µg/gDS Protease Pfu
- Alpha-amylase 1407 + 50 µg/gDS Protease Pfu

Water and enzymes were added to each canister, and then each canister was sealed and mixed well prior to loading into the Labomat. All samples were incubated in the Labomat set to the following conditions: 5°C/min. Ramp, 15 minute Ramp to 80°C, hold for 1 min, Ramp to 85°C at 1°C/min and holding for 103 min., 40 rpm for 30 seconds to the left and 30 seconds to the right. Once liquefaction was complete, all canisters were cooled in an ice bath for approximately 20 minutes before proceeding to fermentation.

Simultaneous Saccharification and Fermentation (SSF): Penicillin was added to each mash to a final concentration of 3 ppm and pH was adjusted to 5.0. Next, portions of this mash were transferred to test tubes to represent “urea-free” fermentations and are considered nitrogen limited. Once the “urea-free” mashes were processed, the remaining mashes were dosed with urea up to a final concentration of 800 ppm and also transferred to test tubes. All test tubes were drilled with a 1/64” bit to allow CO_2 release. Furthermore, equivalent solids were

maintained across all treatments through the addition of water as required to ensure that the urea versus urea-free mashes contained equal solids. Fermentation was initiated through the addition of Glucoamylase U (0.50 AGU/gDS), water and rehydrated yeast. Yeast rehydration took place by mixing 5.5 g of ETHANOL RED™ into 100 mL of 32°C tap water for at least 15 minutes and dosing 100 µl per test tube.

HPLC analysis: HPLC analysis used an Agilent 1100/1200 combined with a Bio-Rad HPX-87H Ion Exclusion column (300 mm x 7.8 mm) and a Bio-Rad Cation H guard cartridge. The mobile phase was 0.005 M sulfuric acid and processed samples at a flow rate of 0.6 ml/min, with column and RI detector temperatures of 65 and 55°C, respectively. Fermentation sampling took place after 54 hours by sacrificing 3 tubes per treatment. Each tube was processed by deactivation with 50 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. Samples were stored at 4°C prior to and during HPLC analysis. The method quantified analytes using calibration standards for DP4+, DP3, DP2, glucose, fructose, acetic acid, lactic acid, glycerol and ethanol (% w/v). A four point calibration including the origin is used for quantification.

Conclusions: Comparison of 54 hour ethanol concentrations showed that more than 1 µg/gDS Protease Pfu was required to support fermentation to dryness under nitrogen limited conditions (Fig. 9 & 10). Moreover, residual glucose was 0.4% w/v for the 1 µg/gDS dose, whereas no residual glucose was observed at Protease Pfu dosages of 10 or 50 µg/gDS. The most significant reduction in glycerol was observed at the two highest dosages of Protease Pfu, 10 and 50 µg/gDS. These results suggest that as much as 10 to 50 µg/gDS Protease Pfu may be required during liquefaction to achieve optimal performance as it relates to liquefaction and fermentation.

SUMMARY PARAGRAPHS

The present invention is defined in the claims and accompanying description. For convenience, other aspects of the present invention are presented herein by way of numbered paragraphs:

1. A process of recovering oil from a fermentation product production process comprising the steps of:

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

- an alpha-amylase;

- more than 0.5 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS);

b) saccharifying using a glucoamylase;

c) fermenting using a fermenting organism.

d) recovering the fermentation product to form whole stillage;

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

f) optionally concentrating the thin stillage into syrup;

wherein oil is recovered from the:

- liquefied starch-containing material after step a); and/or

- downstream from fermentation step c).

2. The process of paragraph 1, wherein oil is recovered during and/or after liquefying the starch-containing material.

3. The process of paragraph 1, wherein oil is recovered from the whole stillage.

4. The process of any of paragraphs 1-3, wherein oil is recovered from the thin stillage.

5. The process of any of paragraphs 1-4, wherein oil is recovered from the syrup.

6. The process of any of paragraph 1-5, wherein 0.5-100 micro gram *Pyrococcus furiosus* protease per gram DS, such as 1-50 micro gram *Pyrococcus furiosus* protease per gram DS, such as 1-10 micro gram *Pyrococcus furiosus* protease per gram DS, such as 1.5-5 micro gram *Pyrococcus furiosus* protease per gram DS, such as around or more than 1.5 micro gram *Pyrococcus furiosus* protease per gram DS are present and/or added in liquefaction step a).

7. The process of any of paragraphs 1-6 wherein 2-100 micro gram *Pyrococcus furiosus* protease per gram DS, such as 2.5-50 micro gram *Pyrococcus furiosus* protease per gram DS, such as 2.5-10 micro gram *Pyrococcus furiosus* protease per gram DS, such as 2.5-5 micro

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

8. The process of any of paragraphs 1-7, wherein the *Pyrococcus furiosus* protease is the mature sequence shown in SEQ ID NO: 13 herein.

9. The process of any of paragraphs 1-8, wherein the *Pyrococcus furiosus* protease is one having at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 13 herein.

10. The process of any of paragraph 1-9, wherein no nitrogen-compound is present and/or added in steps a)-c), such as during saccharification step b), fermentation step c), or simultaneous saccharification and fermentation (SSF).

11. The process of any of paragraph 1-10, wherein 10-1,000 ppm, such as 50-800 ppm, such as 100-600 ppm, such as 200-500 ppm nitrogen-compound, preferably urea, is present and/or added in steps a)-c), such as in saccharification step b) or fermentation step c) or in simultaneous saccharification and fermentation (SSF).

12. The process of any of paragraphs 1-11, wherein the alpha-amylase is from the genus *Bacillus*, such as a strain of *Bacillus stearothermophilus*, such as the sequence shown in SEQ ID NO: 1.

13. The process of any of paragraphs 1-12, wherein the alpha-amylase a *Bacillus stearothermophilus* alpha-amylase shown in SEQ ID NO: 1 herein, such as one having at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 1 herein.

14. The process of paragraph 11, wherein the *Bacillus stearothermophilus* alpha-amylase or variant thereof is truncated, preferably to have around 491 amino acids, such as from 480-495 amino acids.

15. The process of any of paragraphs 12-14, wherein the *Bacillus stearothermophilus* alpha-amylase has a double deletion at positions I181 + G182, and optionally a N193F substitution (using SEQ ID NO: 1 for numbering),

16. The process of any of paragraphs 12-14, wherein the *Bacillus stearothermophilus* alpha-amylase has a double deletion at positions R179 + G180 and optionally a N193F substitution (using SEQ ID NO: 1 for numbering).

17. The process of any of paragraphs 12-16, wherein the *Bacillus stearothermophilus* alpha-amylase has a substitution at position S242, preferably S242Q substitution.

18. The process of any of paragraphs 12-17, wherein the *Bacillus stearothermophilus* alpha-amylase has a substitution at position E188, preferably E188P substitution.

19. The process of any of paragraphs 1-18, wherein the alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) of at least 10.

20. The process of any of paragraphs 1-19, wherein the alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) of at least 15, such as at least 20, such as at least 25, such as at least 30, such as at least 40, such as at least 50, such as at least 60, such as between 10-70, such as between 15-70, such as between 20-70, such as between 25-70, such as between 30-70, such as between 40-70, such as between 50-70, such as between 60-70.

21. The process of any of paragraphs 1-20, wherein the alpha-amylase is selected from the group of *Bacillus stearothermophilus* alpha-amylase variants with the following mutations in addition to I181*+G182*, and optionally N193F:

- V59A+Q89R+G112D+E129V+K177L+R179E+K220P+N224L+Q254S;
- V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+D269E+D281N;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+I270L;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+H274K;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+Y276F;
- V59A+E129V+R157Y+K177L+R179E+K220P+N224L+S242Q+Q254S;
- V59A+E129V+K177L+R179E+H208Y+K220P+N224L+S242Q+Q254S;

- 59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+H274K;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+D281N;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+G416V;
- V59A+E129V+K177L+R179E+K220P+N224L+Q254S;
- V59A+E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
- A91L+M96I+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- E129V+K177L+R179E;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F+L427M;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+N376*+I377*;
- E129V+K177L+R179E+K220P+N224L+Q254S;
- E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
- E129V+K177L+R179E+S242Q;
- E129V+K177L+R179V+K220P+N224L+S242Q+Q254S;
- K220P+N224L+S242Q+Q254S;
- M284V;

- V59A Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V.

22. The process of any of paragraphs 1-21, wherein the alpha-amylase is selected from the group of *Bacillus stearothermophilus* alpha-amylase variants:

- I181*+G182*+N193F+E129V+K177L+R179E;

- I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S

- I181*+G182*+N193F +V59A Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V; and

- I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 for numbering).

23. The process of any of paragraphs 1-22, wherein the alpha-amylase variant has at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 1 herein.

24. The process of any of paragraphs 1-23, wherein the alpha-amylase is a *Bacillus licheniformis* alpha-amylase, or a variant thereof.

25. The process of paragraph 24, wherein the *Bacillus licheniformis* alpha-amylase is the one shown in SEQ ID NO: 21 herein.

26. The process of any of paragraphs 1-25, wherein the alpha-amylase has at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99% identity to the mature part of the polypeptide of SEQ ID NO: 21 herein.

27. The process of any of paragraphs 1-26, wherein the alpha-amylase is present and/or added in a concentration of 0.1-100 micro gram per gram DS, such as 0.5-50 micro gram per gram DS,

such as 1-25 micro gram per gram DS, such as 1-10 micro gram per gram DS, such as 2-5 micro gram per gram DS.

28. The process of any of paragraphs 1-27, wherein from 1-10 micro gram *Pyrococcus furiosus* protease and 1-10 micro gram *Bacillus stearothermophilus* alpha-amylase are present and/or added in liquefaction.

29. The process of any of paragraphs 1-28, wherein a glucoamylase is present and/or added in liquefaction step a).

30. The process of paragraph 29, wherein the glucoamylase present and/or added in liquefaction has a heat stability at 85°C, pH 5.3, of at least 20%, such as at least 30%, preferably at least 35%.

31. The process of paragraph 29 or 30, wherein the glucoamylase has a relative activity pH optimum at pH 5.0 of at least 90%, preferably at least 95%, preferably at least 97%.

32. The process of any of paragraphs 29-30, wherein the glucoamylase has a pH stability at pH 5.0 of at least at least 80%, at least 85%, at least 90%.

33. The process of any of paragraphs 29-32, wherein the glucoamylase present and/or added in liquefaction step a) is derived from a strain of the genus *Penicillium*, especially a strain of *Penicillium oxalicum* disclosed as SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NOs: 9 or 14 herein.

34. The process of paragraph 29-33, wherein the glucoamylase has at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99% or 100% identity to the mature polypeptide shown in SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NOs: 9 or 14 herein.

35. The process of any of paragraphs 29-34, wherein the glucoamylase is a variant of the *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 2 in WO 2011/127802 having a K79V substitution (using the mature sequence shown in SEQ ID NO: 14 for numbering), such as a variant disclosed in WO 2013/053801.

36. The process of any of paragraph 29-35, wherein the *Penicillium oxalicum* glucoamylase has a K79V substitution (using SEQ ID NO: 14 for numbering) and further one of the following:

T65A; or

Q327F; or

E501V; or

Y504T; or

Y504*; or

T65A + Q327F; or

T65A + E501V; or

T65A + Y504T; or

T65A + Y504*; or

Q327F + E501V; or

Q327F + Y504T; or

Q327F + Y504*; or

E501V + Y504T; or

E501V + Y504*; or

T65A + Q327F + E501V; or

T65A + Q327F + Y504T; or

T65A + E501V + Y504T; or

Q327F + E501V + Y504T; or

T65A + Q327F + Y504*; or

T65A + E501V + Y504*; or

Q327F + E501V + Y504*; or

T65A + Q327F + E501V + Y504T; or

T65A + Q327F + E501V + Y504*;

E501V + Y504T; or

T65A + K161S; or

T65A + Q405T; or

T65A + Q327W; or

T65A + Q327F; or

T65A + Q327Y; or

P11F + T65A + Q327F; or

R1K + D3W + K5Q + G7V + N8S + T10K + P11S + T65A + Q327F; or

P2N + P4S + P11F + T65A + Q327F; or

P11F + D26C + K33C + T65A + Q327F; or

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

R1E + D3N + P4G + G6R + G7A + N8A + T10D+ P11D + T65A + Q327F; or

P11F + T65A + Q327W; or

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

P11F + T65A + Q327W + E501V + Y504T; or

T65A + Q327F + E501V + Y504T; or

T65A + S105P + Q327W; or

T65A + S105P + Q327F; or

T65A + Q327W + S364P; or

T65A + Q327F + S364P; or

T65A + S103N + Q327F; or

P2N + P4S + P11F + K34Y + T65A + Q327F; or

P2N + P4S + P11F + T65A + Q327F + D445N + V447S; or

P2N + P4S + P11F + T65A + I172V + Q327F; or

P2N + P4S + P11F + T65A + Q327F + N502*; or

P2N + P4S + P11F + T65A + Q327F + N502T + P563S + K571E; or

P2N + P4S + P11F + R31S + K33V + T65A + Q327F + N564D + K571S; or

P2N + P4S + P11F + T65A + Q327F + S377T; or

P2N + P4S + P11F + T65A + V325T+ Q327W; or

P2N + P4S + P11F + T65A + Q327F + D445N + V447S + E501V + Y504T; or

P2N + P4S + P11F + T65A + I172V + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + S377T + E501V + Y504T; or

P2N + P4S + P11F + D26N + K34Y + T65A + Q327F; or

P2N + P4S + P11F + T65A + Q327F + I375A + E501V + Y504T; or

P2N + P4S + P11F + T65A + K218A + K221D + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + S103N + Q327F + E501V + Y504T; or

P2N + P4S + T10D + T65A + Q327F + E501V + Y504T; or

P2N + P4S + F12Y + T65A + Q327F + E501V + Y504T; or

K5A + P11F + T65A + Q327F + E501V + Y504T; or

P2N + P4S + T10E + E18N + T65A + Q327F + E501V + Y504T; or

P2N + T10E + E18N + T65A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T568N; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + K524T + G526A; or

P2N + P4S + P11F + K34Y + T65A + Q327F + D445N + V447S + E501V + Y504T; or

P2N + P4S + P11F + R31S + K33V + T65A + Q327F + D445N + V447S + E501V + Y504T; or

P2N + P4S + P11F + D26N + K34Y + T65A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + F80* + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K112S + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T516P + K524T + G526A; or

P2N + P4S + P11F + T65A + Q327F + E501V + N502T + Y504*; or

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

P2N + P4S + P11F + T65A + S103N + Q327F + E501V + Y504T; or

K5A + P11F + T65A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T516P + K524T + G526A; or

P2N + P4S + P11F + T65A + K79A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79G + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79I + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79L + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79S + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + L72V + Q327F + E501V + Y504T; or

S255N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + E74N + V79K + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + G220N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Y245N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q253N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + D279N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + S359N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + D370N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + V460S + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + V460T + P468T + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + T463N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + S465N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + T477N + E501V + Y504T.

37. The process of any of paragraphs 29-36, wherein the glucoamylase present and/or added in liquefaction is the *Penicillium oxalicum* glucoamylase having a K79V substitution (using SEQ ID NO: 14 for numbering) and further one of the following:

- P11F + T65A + Q327F;

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

38. The process of any of paragraphs 11-27, wherein the glucoamylase variant has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least

96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 14 herein.

39. The process of any of paragraphs 1-38, further wherein a glucoamylase is present and/or added in saccharification and/or fermentation.

40. The process of paragraph 39, wherein the glucoamylase present and/or added in saccharification and/or fermentation is of fungal origin, preferably from a stain of *Aspergillus*, preferably *A. niger*, *A. awamori*, or *A. oryzae*; or a strain of *Trichoderma*, preferably *T. reesei*; or a strain of *Talaromyces*, preferably *T. emersonii*, or a strain of *Pycnoporus*, or a strain of *Gloephyllum*, such as *G. serpiarium* or *G. trabeum,* or a strain of the *Nigrofomes.*

41. The process of any of paragraphs 39-40, wherein the glucoamylase is derived from *Talaromyces emersonii*, such as the one shown in SEQ ID NO: 19 herein,

42. The process of any of paragraphs 39-41, wherein the glucoamylase is selected from the group consisting of:

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

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 19 herein.

43. The process of any of paragraphs 39-42, wherein the glucoamylase is derived from *Gloephyllum serpiarium*, such as the one shown in SEQ ID NO: 15 herein.

44. The process of any of paragraphs 39-43, wherein the glucoamylase is selected from the group consisting of:

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

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 15 herein.

45. The process of any of paragraphs 39-44, wherein the glucoamylase is derived from *Gloeophyllum trabeum* such as the one shown in SEQ ID NO: 17 herein.

46. The process of any of paragraphs 39-45, wherein the glucoamylase 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.

47. The process of any of paragraphs 39-46, wherein the glucoamylase is present and/or added in saccharification and/or fermentation in combination with an alpha-amylase.

48. The process of paragraph 47, wherein the alpha-amylase present and/or added in saccharification and/or fermentation is of fungal or bacterial origin.

49. The process of paragraph 47 or 48, wherein the alpha-amylase present and/or added in saccharification and/or fermentation is derived from a strain of the genus *Rhizomucor*, preferably a strain the *Rhizomucor pusillus,* such as the one shown in SEQ ID NO: 3 in WO 2013/006756, such as a *Rhizomucor pusillus* alpha-amylase hybrid having an *Aspergillus niger* linker and starch-bonding domain, such as the one shown in SEQ ID NO: 16 herein, or a variant thereof.

50. The process of any of paragraphs 47-49, wherein the alpha-amylase present and/or added in saccharification and/or fermentation is selected from the group consisting of:

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

(ii) an alpha-amylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 16 herein.

51. The process of any of paragraphs 47-50, wherein the alpha-amylase is a variant of the alpha-amylase shown in SEQ ID NO: 13 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 (using SEQ ID NO: 16 for numbering).

52. The process of any of paragraphs 47-51, wherein the alpha-amylase is derived from a *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably disclosed as SEQ ID NO: 13 herein, preferably having one or more of the following substitutions: G128D, D143N, preferably G128D+D143N (using SEQ ID NO: 13 for numbering).

53. The process of amylase of paragraphs 47-52, wherein the alpha-amylase variant present and/or added in saccharification and/or fermentation has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 16 herein.

54. The process of any of paragraphs 1-53, further wherein a pullulanase is present and/or added in liquefaction and/or saccharification and/or fermentation.

55. The process of paragraph 54, wherein the pullulanase is a family GH57 pullulanase, wherein the pullulanase preferably includes an X47 domain as disclosed in WO 2011/087836.

56. The process of paragraphs 54-55, wherein the pullulanase is derived from a strain from the genus *Thermococcus*, including *Thermococcus litoralis* and *Thermococcus hydrothermalis* or a hybrid thereof.

57. The process of any of paragraphs 54-56, wherein the pullulanase is the truncated *Thermococcus hydrothermalis* pullulanase at site X4 or a *T. hydrothermalis/T. litoralis* hybrid enzyme with truncation site X4 disclosed in WO 2011/087836 or shown in SEQ ID NO: 12 herein.

58. The process of any of paragraphs 1-57, further comprises, prior to the liquefaction step a), the steps of:

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

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

59. The process of any of paragraphs 1-58, wherein at least 50%, preferably at least 70%, more preferably at least 80%, especially at least 90% of the starch-containing material fit through a sieve with # 6 screen.

60. The process of any of paragraphs 1-59, wherein the pH during liquefaction is between above 4.5-6.5, such as around 4.8, or a pH between 5.0-6.2, such as 5.0-6.0, such as between 5.0-5.5, such as around 5.2, such as around 5.4, such as around 5.6, such as around 5.8.

61. The process of any of paragraphs 1-60, wherein the temperature during liquefaction is above the initial gelatinization temperature, preferably in the range from 70-100°C, such as between 75-95°C, such as between 75-90°C, preferably between 80-90°C, especially around 85°C.

62. The process of any of paragraphs 1-61, wherein a jet-cooking step is carried out before liquefaction in step a).

63. The process of paragraph 62, wherein the jet-cooking is carried out at a temperature between 110-145°C, preferably 120-140°C, such as 125-135°C, preferably around 130°C for about 1-15 minutes, preferably for about 3-10 minutes, especially around about 5 minutes.

64. The process of any of paragraphs 1-63, wherein saccharification and fermentation is carried out sequentially or simultaneously.

65. The process of any of paragraphs 1-64, wherein saccharification is carried out at a temperature from 20-75°C, preferably from 40-70°C, such as around 60°C, and at a pH between 4 and 5.

66. The process of any of paragraphs 1-65, wherein fermentation or simultaneous saccharification and fermentation (SSF) is carried out carried out at a temperature from 25°C to 40°C, such as from 28°C to 35°C, such as from 30°C to 34°C, preferably around about 32°C. In an embodiment fermentation is ongoing for 6 to 120 hours, in particular 24 to 96 hours.

67. The process of any of paragraphs 1-66, wherein the fermentation product is recovered after fermentation, such as by distillation.

68. The process of any of paragraphs 1-67, wherein the fermentation product is an alcohol, preferably ethanol, especially fuel ethanol, potable ethanol and/or industrial ethanol.

69. The process of any of paragraphs 1-68, wherein the starch-containing starting material is whole grains.

70. The process of any of paragraphs 1-69, wherein the starch-containing material is selected from the group of corn, wheat, barley, rye, milo, sago, cassava, manioc, tapioca, sorghum, rice, and potatoes.

71. The process of any of paragraphs 1-70, wherein the fermenting organism is yeast, preferably a strain of *Saccharomyces*, especially a strain of *Saccharomyces cerevisae*.

72. The process of any of paragraphs 1-71, wherein the alpha-amylase is a bacterial or fungal alpha-amylase.

73. The process of any of paragraphs 1-72, wherein saccharification step b) and fermentation step c) are carried out simultaneously or sequentially.

74. The process of any of paragraphs 1-73, wherein the temperature in step (a) is above the initial gelatinization temperature, such as at a temperature between 80-90°C, such as around 85°C.

75. The process of any of paragraphs 1-74, further comprising a pre-saccharification step, before saccharification step b), carried out for 40-90 minutes at a temperature between 30-65°C.

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

77. The process of any of paragraphs 1-76, wherein fermentation step c) or simultaneous saccharification and fermentation (SSF) (i.e., steps b) and c)) are carried out carried out at a temperature from 25°C to 40°C, such as from 28°C to 35°C, such as from 30°C to 34°C, preferably around about 32°C.

78. The process of any of paragraphs 1-77, wherein fermentation step c) or simultaneous saccharification and fermentation (SSF) (i.e., steps b) and c)) are ongoing for 6 to 120 hours, in particular 24 to 96 hours.

79. The process of any of paragraphs 1-78, wherein separation in step e) is carried out by centrifugation, preferably a decanter centrifuge, filtration, preferably using a filter press, a screw press, a plate-and-frame press, a gravity thickener or decker.

80. The process of any of paragraphs 1-79, wherein the fermentation product is recovered by distillation.

81. A process of recovering oil of any of paragraphs 1-80, comprising the steps of:

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

- *Bacillus stearothermophilus* alpha-amylase comprising a double deletion at positions I181 + G182 using SEQ ID NO: 1 for numbering;

- more than 0.5 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS);

- *Penicillium oxalicum* shown in SEQ ID NO: 14 comprising a K79V substitution;

b) saccharifying using a glucoamylase;

c) fermenting using a fermenting organism.

d) recovering the fermentation product to form whole stillage;

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

f) optionally concentrating the thin stillage into syrup;

wherein oil is recovered from the:

- liquefied starch-containing material after step a); and/or

- downstream from fermentation step c).

82. A process of recovering oil of any of paragraphs 1-81 comprising the steps of:

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

- *Bacillus stearothermophilus* alpha-amylase comprising a double deletion at positions I181 + G182 and the following substitutions N193F+V59A+Q89R+E129V+K177L+R179E+Q254S+M284V truncated to 491 amino acids (using SEQ ID NO: 1 for numbering).

- more than 0.5 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS);

- *Penicillium oxalicum* glucoamylase having the following mutations: K79V+P2N+P4S+P11F+T65A+Q327F (using SEQ ID NO: 14 for numbering);

b) saccharifying using a glucoamylase;

c) fermenting using a fermenting organism.

d) recovering the fermentation product to form whole stillage;

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

f) optionally concentrating the thin stillage into syrup;

wherein oil is recovered from the:

- liquefied starch-containing material after step a); and/or

- downstream from fermentation step c).

83. The process of any of paragraphs 1-82, wherein the ratio between alpha-amylase and glucoamylase in liquefaction is between 1:1 and 1:10, such as around 1:2 (micro gram alpha-amylase per gram DS : micro gram glucoamylase per gram DS).

84. The process of any of paragraphs 1-83, wherein the ratio between alpha-amylase and protease in liquefaction is in the range between 1:1 and 1:25, such between 1:1.2 and as 1:10, such as around 1:1.4 (micro gram alpha-amylase per gram DS : micro gram protease per gram DS).

85. A process for producing fermentation products from starch-containing material comprising the steps of:

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

- an alpha-amylase;

- more than 2 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS);

b) saccharifying using a glucoamylase;

c) fermenting using a fermenting organism.

86. The process of paragraph 85, wherein 2-100 micro gram per gram DS, such as 2.5-50 micro gram per gram DS, such as 2.5-10 micro gram per gram DS, such as 2.5-5 micro gram per gram DS, especially around 3 micro gram per gram DS *Pyrococcus furiosus* protease.

87. The process of any of paragraph 85 or 86, wherein the *Pyrococcus furiosus* protease is the one shown in SEQ ID NO: 13 herein.

88. The process of any of paragraphs 85-87, wherein the *Pyrococcus furiosus* protease is one having at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 13 herein.

89. The process of any of paragraphs 85-88, wherein no nitrogen-compound is present and/or added in steps a)-c), such as during saccharification step b) or fermentation step c) or simultaneous saccharification and fermentation (SSF).

90. The process of any of paragraphs 85-89, wherein 10-1,000 ppm, such as 50-800 ppm, such as 100-600 ppm, such as 200-500 ppm nitrogen-compound, preferably urea, is present and/or added in steps a)-c), such as during saccharification step b) or fermentation step c) or simultaneous saccharification and fermentation (SSF).

91. The process of any of paragraphs 85-90, wherein the alpha-amylase is from the genus *Bacillus*, such as a strain of *Bacillus stearothermophilus,* in particular a variant of a *Bacillus*

stearothermophilus alpha-amylase, such as the one shown in SEQ ID NO: 3 in WO 99/019467 or SEQ ID NO: 1 herein, or a variant thereof or a strain of *Bacillus licheniformis*, such as the one shown in SEQ ID NO: 21 herein,.

92. The process of paragraph 91, wherein the *Bacillus stearothermophilus* alpha-amylase or variant thereof is truncated, preferably to have around 491 amino acids, such as from 480-495 amino acids.

93. The process of any of paragraphs 91 or 92, wherein the *Bacillus stearothermophilus* alpha-amylase has a double deletion at positions I181 + G182 and optionally a N193F substitution, or deletion of R179 and G180 (using SEQ ID NO: 1 for numbering).

94. The process of any of paragraphs 85-93 wherein the *Bacillus stearothermophilus* alpha-amylase has a substitution at position S242, preferably S242Q substitution.

95. The process of any of paragraphs 85-94, wherein the *Bacillus stearothermophilus* alpha-amylase has a substitution at position E188, preferably E188P substitution.

96. The process of any of paragraphs 85-95, wherein the alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) of at least 10, such as at least 15, such as at least 20, such as at least 25, such as at least 30, such as at least 40, such as at least 50, such as at least 60, such as between 10-70, such as between 15-70, such as between 20-70, such as between 25-70, such as between 30-70, such as between 40-70, such as between 50-70, such as between 60-70.

97. The process of any of paragraphs 85-96, wherein the alpha-amylase is selected from the group of *Bacillus stearothermophilus* alpha-amylase variants with the following mutations in addition to I181*+G182* and optionally N193F:

-V59A+Q89R+G112D+E129V+K177L+R179E+K220P+N224L+Q254S;
- V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+D269E+D281N;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+I270L;

- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+H274K;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+Y276F;
- V59A+E129V+R157Y+K177L+R179E+K220P+N224L+S242Q+Q254S;
- V59A+E129V+K177L+R179E+H208Y+K220P+N224L+S242Q+Q254S;
- 59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+H274K;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+D281N;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+G416V;
- V59A+E129V+K177L+R179E+K220P+N224L+Q254S;
- V59A+E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
- A91L+M96I+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- E129V+K177L+R179E;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F+L427M;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+N376*+I377*;
- E129V+K177L+R179E+K220P+N224L+Q254S;
- E129V+K177L+R179E+K220P+N224L+Q254S+M284T;

- E129V+K177L+R179E+S242Q;
- E129V+K177L+R179V+K220P+N224L+S242Q+Q254S;
- K220P+N224L+S242Q+Q254S;
- M284V;
- V59A Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V.

98. The process of any of paragraphs 85-97, wherein the alpha-amylase is selected from the group of *Bacillus stearothermophilus* alpha-amylase variants:

- I181*+G182*+N193F+E129V+K177L+R179E;

- I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S

- I181*+G182*+N193F +V59A Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V; and

- I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 for numbering).

99. The process of any of paragraphs 91-98, wherein the alpha-amylase variant has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 1 herein.

100. The process of any of paragraphs 85-98, wherein the alpha-amylase is a *Bacillus licheniformis* alpha-amylase, or a variant thereof.

101. The process of paragraph 100, wherein the *Bacillus licheniformis* alpha-amylase is the one shown in SEQ ID NO: 21 herein.

102. The process of any of paragraphs 100-101, wherein the alpha-amylase has at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more

preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99% identity to the mature part of the polypeptide of SEQ ID NO: 21 herein.

103. The process of any of paragraphs 85-102, wherein the alpha-amylase is present and/or added in a concentration of 0.1-100 micro gram per gram DS, such as 0.5-50 micro gram per gram DS, such as 1-25 micro gram per gram DS, such as 1-10 micro gram per gram DS, such as 2-5 micro gram per gram DS.

104. The process of any of paragraphs 85-103, wherein from 1-10 micro gram *Pyrococcus furiosus* protease and 1-10 micro gram *Bacillus stearothermophilus* alpha-amylase are present and/or added in liquefaction.

105. The process of any of paragraphs 85-104, wherein a glucoamylase is present and/or added in liquefaction step i).

106. The process of paragraph 105, wherein the glucoamylase present and/or added in liquefaction has a heat stability at 85°C, pH 5.3, of at least 20%, such as at least 30%, preferably at least 35%.

107. The process of paragraph 105 or 106, wherein the glucoamylase has a relative activity pH optimum at pH 5.0 of at least 90%, preferably at least 95%, preferably at least 97%.

108. The process of any of paragraphs 105-102, wherein the glucoamylase has a pH stability at pH 5.0 of at least at least 80%, at least 85%, at least 90%.

109. The process of any of paragraphs 105-103, wherein the glucoamylase present and/or added in liquefaction step i) is derived from a strain of the genus *Penicillium*, especially a strain of *Penicillium oxalicum* disclosed as SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NOs: 9 or 14 herein.

110. The process of paragraph 105-109, wherein the glucoamylase has at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99% or 100% identity to the mature polypeptide shown in SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NOs: 9 or 14 herein.

111. The process of any of paragraphs 105-110, wherein the glucoamylase is a variant of the *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 2 in WO 2011/127802 having a K79V substitution (using the mature sequence shown in SEQ ID NO: 14 for numbering), such as a variant disclosed in WO 2013/053801.

112. The process of any of paragraph 105-111, wherein the *Penicillium oxalicum* glucoamylase has a K79V substitution (using SEQ ID NO: 14 for numbering) and further one of the following:

T65A; or

Q327F; or

E501V; or

Y504T; or

Y504*; or

T65A + Q327F; or

T65A + E501V; or

T65A + Y504T; or

T65A + Y504*; or

Q327F + E501V; or

Q327F + Y504T; or

Q327F + Y504*; or

E501V + Y504T; or

E501V + Y504*; or

T65A + Q327F + E501V; or

T65A + Q327F + Y504T; or

T65A + E501V + Y504T; or

Q327F + E501V + Y504T; or

T65A + Q327F + Y504*; or

T65A + E501V + Y504*; or

Q327F + E501V + Y504*; or

T65A + Q327F + E501V + Y504T; or

T65A + Q327F + E501V + Y504*;

E501V + Y504T; or

T65A + K161S; or

T65A + Q405T; or

T65A + Q327W; or

T65A + Q327F; or

T65A + Q327Y; or

P11F + T65A + Q327F; or

R1K + D3W + K5Q + G7V + N8S + T10K + P11S + T65A + Q327F; or

P2N + P4S + P11F + T65A + Q327F; or

P11F + D26C + K33C + T65A + Q327F; or

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

R1E + D3N + P4G + G6R + G7A + N8A + T10D+ P11D + T65A + Q327F; or

P11F + T65A + Q327W; or

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

P11F + T65A + Q327W + E501V + Y504T; or

T65A + Q327F + E501V + Y504T; or

T65A + S105P + Q327W; or

T65A + S105P + Q327F; or

T65A + Q327W + S364P; or

T65A + Q327F + S364P; or

T65A + S103N + Q327F; or

P2N + P4S + P11F + K34Y + T65A + Q327F; or

P2N + P4S + P11F + T65A + Q327F + D445N + V447S; or

P2N + P4S + P11F + T65A + I172V + Q327F; or

P2N + P4S + P11F + T65A + Q327F + N502*; or

P2N + P4S + P11F + T65A + Q327F + N502T + P563S + K571E; or

P2N + P4S + P11F + R31S + K33V + T65A + Q327F + N564D + K571S; or

P2N + P4S + P11F + T65A + Q327F + S377T; or

P2N + P4S + P11F + T65A + V325T+ Q327W; or

P2N + P4S + P11F + T65A + Q327F + D445N + V447S + E501V + Y504T; or

P2N + P4S + P11F + T65A + I172V + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + S377T + E501V + Y504T; or

P2N + P4S + P11F + D26N + K34Y + T65A + Q327F; or

P2N + P4S + P11F + T65A + Q327F + I375A + E501V + Y504T; or

P2N + P4S + P11F + T65A + K218A + K221D + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + S103N + Q327F + E501V + Y504T; or

P2N + P4S + T10D + T65A + Q327F + E501V + Y504T; or

P2N + P4S + F12Y + T65A + Q327F + E501V + Y504T; or

K5A + P11F + T65A + Q327F + E501V + Y504T; or

P2N + P4S + T10E + E18N + T65A + Q327F + E501V + Y504T; or

P2N + T10E + E18N + T65A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T568N; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + K524T + G526A; or

P2N + P4S + P11F + K34Y + T65A + Q327F + D445N + V447S + E501V + Y504T; or

P2N + P4S + P11F + R31S + K33V + T65A + Q327F + D445N + V447S + E501V + Y504T; or

P2N + P4S + P11F + D26N + K34Y + T65A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + F80* + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K112S + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T516P + K524T + G526A; or

P2N + P4S + P11F + T65A + Q327F + E501V + N502T + Y504*; or

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

P2N + P4S + P11F + T65A + S103N + Q327F + E501V + Y504T; or

K5A + P11F + T65A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T516P + K524T + G526A; or

P2N + P4S + P11F + T65A + K79A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79G + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79I + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79L + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79S + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + L72V + Q327F + E501V + Y504T; or

S255N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + E74N + V79K + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + G220N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Y245N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q253N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + D279N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + S359N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + D370N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + V460S + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + V460T + P468T + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + T463N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + S465N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + T477N + E501V + Y504T.

113. The process of any of paragraphs 105-112, wherein the glucoamylase present and/or added in liquefaction is the *Penicillium oxalicum* glucoamylase having a K79V substitution (using SEQ ID NO: 14 for numbering) and further one of the following:

- P11F T65A Q327F

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

114. The process of any of paragraphs 105-113, wherein the glucoamylase variant has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 14 herein.

115. The process of any of paragraphs 85-114, further wherein a glucoamylase is present and/or added in saccharification and/or fermentation.

116. The process of paragraph 115, wherein the glucoamylase present and/or added in saccharification and/or fermentation is of fungal origin, preferably from a stain of *Aspergillus*, preferably *A. niger*, *A. awamori*, or *A. oryzae*; or a strain of *Trichoderma*, preferably *T. reesei*; or a strain of *Talaromyces*, preferably *T. emersonii*, or a strain of *Pycnoporus*, or a strain of *Gloephyllum*, such as *G. serpiarium* or *G. trabeum,* or a strain of the *Nigrofomes.*

117. The process of any of paragraphs 115-116, wherein the glucoamylase is derived from *Talaromyces emersonii*, such as the one shown in SEQ ID NO: 19 herein,

118. The process of any of paragraphs 115-117, wherein the glucoamylase is selected from the group consisting of:

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

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 19 herein.

119. The process of any of paragraphs 115-119, wherein the glucoamylase is derived from *Gloephyllum serpiarium*, such as the one shown in SEQ ID NO: 15 herein.

120. The process of any of paragraphs 115-119, wherein the glucoamylase is selected from the group consisting of:

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

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 15 herein.

121. The process of any of paragraphs 115-120, wherein the glucoamylase is derived from *Gloeophyllum trabeum* such as the one shown in SEQ ID NO: 17 herein.

122. The process of any of paragraphs 115-121, wherein the glucoamylase 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.

123. The process of any of paragraphs 115-122, wherein the glucoamylase is present in saccharification and/or fermentation in combination with an alpha-amylase.

124. The process of paragraph 123, wherein the alpha-amylase is present in saccharification and/or fermentation is of fungal or bacterial origin.

125. The process of paragraph 123 or 124, wherein the alpha-amylase present and/or added in saccharification and/or fermentation is derived from a strain of the genus *Rhizomucor*, preferably a strain the *Rhizomucor pusillus,* such as the one shown in SEQ ID NO: 3 in WO 2013/006756, such as a *Rhizomucor pusillus* alpha-amylase hybrid having an *Aspergillus niger* linker and starch-bonding domain, such as the one shown in SEQ ID NO: 16.

126. The process of any of paragraphs 123-125, wherein the alpha-amylase present in saccharification and/or fermentation is selected from the group consisting of:

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

(ii) an alpha-amylase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at

least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 16 herein.

127. The process of any of paragraphs 123-126, wherein the alpha-amylase is a variant of the alpha-amylase shown in SEQ ID NO: 13 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 (using SEQ ID NO: 16 for numbering).

128. The process of any of paragraphs 123-127, wherein the alpha-amylase is derived from a *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably disclosed as SEQ ID NO: 16 herein, preferably having one or more of the following substitutions: G128D, D143N, preferably G128D+D143N (using SEQ ID NO: 16 for numering).

129. The process of any of paragraphs 123-128, wherein the alpha-amylase variant has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 16 herein.

130. The process of any of paragraphs 85-129, further wherein a pullulanase is present and/or added in liquefaction and/or saccharification and/or fermentation.

131. The process of paragraph 130, wherein the pullulanase is a family GH57 pullulanase, wherein the pullulanase preferably includes an X47 domain as disclosed in WO 2011/087836.

132. The process of paragraphs 130-131, wherein the pullulanase is derived from a strain from the genus *Thermococcus*, including *Thermococcus litoralis* and *Thermococcus hydrothermalis* or a hybrid thereof.

133. The process of any of paragraphs 130-132, wherein the pullulanase is the truncated *Thermococcus hydrothermalis* pullulanase at site X4 or a *T. hydrothermalis/T. litoralis* hybrid enzyme with truncation site X4 disclosed in WO 2011/087836 or shown in SEQ ID NO: 12 herein.

134. The process of any of paragraphs 85-133, further comprises, prior to the liquefaction step i), the steps of:

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

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

135. The process of any of paragraphs 85-134, wherein at least 50%, preferably at least 70%, more preferably at least 80%, especially at least 90% of the starch-containing material fit through a sieve with # 6 screen.

136. The process of any of paragraphs 85-135, wherein the pH in liquefaction is between above 4.5-6.5, such as around 4.8, or a pH between 5.0-6.2, such as 5.0-6.0, such as between 5.0-5.5, such as around 5.2, such as around 5.4, such as around 5.6, such as around 5.8.

137. The process of any of paragraphs 85-136, wherein the temperature in liquefaction is above the initial gelatinization temperature, such as in the range from 70-100°C, such as between 75-95°C, such as between 75-90°C, preferably between 80-90°C, especially around 85°C.

138. The process of any of paragraphs 85-137, wherein a jet-cooking step is carried out before liquefaction in step a).

139. The process of paragraph 138, wherein the jet-cooking is carried out at a temperature between 110-145°C, preferably 120-140°C, such as 125-135°C, preferably around 130°C for about 1-15 minutes, preferably for about 3-10 minutes, especially around about 5 minutes.

140. The process of any of paragraphs 85-139, wherein saccharification and fermentation is carried out sequentially or simultaneously.

141. The process of any of paragraphs 85-140, wherein saccharification is carried out at a temperature from 20-75°C, preferably from 40-70°C, such as around 60°C, and at a pH between 4 and 5.

142. The process of any of paragraphs 85-141, wherein fermentation or simultaneous saccharification and fermentation (SSF) is carried out carried out at a temperature from 25°C to 40°C, such as from 28°C to 35°C, such as from 30°C to 34°C, preferably around about 32°C. In an embodiment fermentation is ongoing for 6 to 120 hours, in particular 24 to 96 hours.

143. The process of any of paragraphs 85-142, wherein the fermentation product is recovered after fermentation, such as by distillation.

144. The process of any of paragraphs 85-143, wherein the fermentation product is an alcohol, preferably ethanol, especially fuel ethanol, potable ethanol and/or industrial ethanol.

145. The process of any of paragraphs 85-144, wherein the starch-containing starting material is whole grains.

146. The process of any of paragraphs 85-145, wherein the starch-containing material is derived from corn, wheat, barley, rye, milo, sago, cassava, manioc, tapioca, sorghum, rice or potatoes.

147. The process of any of paragraphs 85-146, wherein the fermenting organism is yeast, preferably a strain of *Saccharomyces*, especially a strain of *Saccharomyces cerevisae*.

148. The process of any of paragraphs 85-147, wherein the alpha-amylase is a bacterial or fungal alpha-amylase.

149. The process of any of paragraphs 85-148, comprising the steps of:

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

- an alpha-amylase derived from *Bacillus stearothermophilus*;

- more than 2 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS); and

- optionally a *Penicillium oxalicum* glucoamylase;

b) saccharifying using a glucoamylase enzyme;

c) fermenting using a fermenting organism.

150. A process of paragraphs 85-149, comprising the steps of:

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

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

- more than 2 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS); and

- optionally a glucoamylase;

b) saccharifying using a glucoamylase enzyme;

c) fermenting using a fermenting organism.

151. A process of paragraphs 85-150, comprising the steps of:

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

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

- more than 2 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS); and

- a *Penicillium oxalicum* glucoamylasea;

b) saccharifying using a glucoamylase enzyme;

c) fermenting using a fermenting organism.

152. A process of paragraphs 85-151, comprising the steps of:

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

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

- E129V+K177L+R179E;

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

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

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

- more than 2 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS);

- a *Penicillium oxalicum* glucoamylase in SEQ ID NO: 14 having substitutions selected from the group of:

- K79V;

- K79V+ P11F + T65A + Q327F; or

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

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

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

- K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; or

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

b) saccharifying using a glucoamylase enzyme;

c) fermenting using a fermenting organism.

153. A process of paragraphs 85-152, comprising the steps of:

a) liquefying the starch-containing material at a pH in the range between from above 4.5-6.5 at a temperature between 80-90°C using:

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

  - E129V+K177L+R179E;

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

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

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

- more than 2 micro gram, such as between 2-5 micro gram, preferably around 3 micro gram *Pyrococcus furiosus* protease per gram DS dry solids (DS);

- a *Penicillium oxalicum* glucoamylase in SEQ ID NO: 14 having substitutions selected from the group of:

- K79V;

- K79V+ P11F + T65A + Q327F; or

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

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

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

- K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; or

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

b) saccharifying using a glucoamylase enzyme;

c) fermenting using a fermenting organism.

154. A process of paragraphs 85-153, comprising the steps of:

a) liquefying the starch-containing material at a pH in the range between from above 4.5-6.5 at a temperature between 80-90°C using:

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

  - E129V+K177L+R179E;

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

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

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

- more than 2 micro gram, such as between 2-5 micro gram, preferably around 3 micro gram *Pyrococcus furiosus* protease per gram DS dry solids (DS)

- a *Penicillium oxalicum* glucoamylase in SEQ ID NO: 14 having substitutions selected from the group of:

- K79V;

- K79V+ P11F + T65A + Q327F; or

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

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

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

- K79V +P2N + P4S + P11F + T65A + Q327F + E501V + Y504T; or

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

b) saccharifying using a *Rhizomucor pusillus* glucoamylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably disclosed as SEQ ID NO: 13

herein, preferably having one or more of the following substitutions: G128D, D143N, preferably G128D+D143N (using SEQ ID NO: 13 for numbering);

c) fermenting using a fermenting organism.

155. A process of any of paragraphs 85-154, wherein the ratio between alpha-amylase and glucoamylase in liquefaction is between 1:1 and 1:10, such as around 1:2 (micro gram alpha-amylase per g DS : micro gram glucoamylase per gram DS).

156. A process of any of paragraphs 85-155, wherein the ratio between alpha-amylase and protease in liquefaction is in the range between 1:1 and 1:25, such between 1:1.2 and as 1:10, such as around 1:1.4 (micro gram alpha-amylase per g DS : micro gram protease per gram DS).

157. An enzyme composition comprising:

i) *Bacillus* sp. alpha-amylase, or a variant thereof;

ii) *Pyrococcus furiosus* protease;

wherein the ratio between alpha-amylase and protease is in the range from 1:1 and 1:25 (micro gram alpha-amylase : micro gram protease).

158. The enzyme composition paragraph 157, wherein the ratio between alpha-amylase and protease is in the range between 1:1.2 and 1:10, such as around 1:1.4 (micro gram alpha-amylase : micro gram protease).

159. The enzyme composition of any of paragraphs 157-158, wherein the enzyme composition comprises a glucoamylase and the ratio between alpha-amylase and glucoamylase in liquefaction is between 1:1 and 1:10, such as around 1:2 (micro gram alpha-amylase : micro gram glucoamylase).

160. The enzyme composition of any of paragraphs 157-159, wherein the alpha-amylase is a bacterial or fungal alpha-amylase.

161, The enzyme composition of any of paragraphs 157-160, wherein the alpha-amylase is from the genus *Bacillus*, such as a strain of *Bacillus stearothermophilus,* in particular a variant of a *Bacillus stearothermophilus* alpha-amylase, such as the one shown in SEQ ID NO: 3 in WO 99/019467 or SEQ ID NO: 1 herein.

162. The enzyme composition of any of paragraphs 157-161, wherein the *Bacillus stearothermophilus* alpha-amylase or variant thereof is truncated, preferably to have around 491 amino acids, such as from 480-495 amino acids.

163. The enzyme composition of any of paragraphs 157-162, wherein the *Bacillus stearothermophilus* alpha-amylase has a double deletion, preferably at positions I181 + G182 and optionally a N193F substitution, or double deletion of R179 and G180 (using SEQ ID NO: 1 for numbering).

164. The enzyme composition of any of paragraphs 157-163 wherein the *Bacillus stearothermophilus* alpha-amylase has a substitution at position S242, preferably S242Q substitution.

165. The enzyme composition of any of paragraphs 157-164, wherein the *Bacillus stearothermophilus* alpha-amylase has a substitution at position E188, preferably E188P substitution.

166. The enzyme composition of any of paragraphs 157-165, wherein the alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) of at least 10, such as at least 15, such as at least 20, such as at least 25, such as at least 30, such as at least 40, such as at least 50, such as at least 60, such as between 10-70, such as between 15-70, such as between 20-70, such as between 25-70, such as between 30-70, such as between 40-70, such as between 50-70, such as between 60-70.

167. The enzyme composition of any of paragraphs 157-166, wherein the alpha-amylase is selected from the group of *Bacillus stearomthermphilus* alpha-amylase variants with the following mutations:

- I181*+G182*+N193F+E129V+K177L+R179E;

- I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

- I181*+G182*+N193F +V59A Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V; and

- I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 1 herein for numbering).

168. The enzyme composition of any of paragraphs 157-167, wherein the alpha-amylase variant has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 1 herein.

169. The enzyme composition of any of paragraphs 157-168, wherein the alpha-amylase is a *Bacillus licheniformis* alpha-amylase, or a variant thereof.

170. The enzyme composition of paragraph 169, wherein the *Bacillus licheniformis* alpha-amylase is the one shown in SEQ ID NO: 21 herein.

171. The enzyme composition of any of paragraphs 157-170, wherein the alpha-amylase has at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99% identity to the mature part of the polypeptide of SEQ ID NO: 21 herein.

172. The enzyme composition of any of paragraphs 157-171, wherein the enzyme composition comprises a *Bacillus licheniformis* alpha-amylase and a *Pyrococcus furiosus* protease.

173. The enzyme composition of any of paragraphs 157-172, wherein the enzyme composition further comprises a glucoamylase.

174. The composition of any of paragraphs 157-173, wherein the *Pyrococcus furiosus* is the one shown in SEQ ID NO: 13 herein.

175. The composition of any of paragraphs 157-174, wherein the *Pyrococcus furiosus* protease is one having at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 13 herein.

176. The composition of any of paragraphs 157-175, wherein the enzyme composition further comprises a glucoamylase shown in SEQ ID NO: 14, or a variant thereof.

177. The composition of paragraph 152-166, wherein the glucoamylase has a heat stability at 85°C, pH 5.3, of at least 20%, such as at least 30%, preferably at least 35%.

178. The composition of any of paragraphs 176-177, wherein the glucoamylase has a relative activity pH optimum at pH 5.0 of at least 90%, preferably at least 95%, preferably at least 97%.

179. The composition of any of paragraphs 176-178, wherein the glucoamylase has a pH stability at pH 5.0 of at least at least 80%, at least 85%, at least 90%.

180. The composition of any of paragraphs 176-179, wherein the glucoamylase is derived from a strain of the genus *Penicillium*, especially a strain of *Penicillium oxalicum* disclosed as SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NOs: 9 or 14 herein.

181. The composition of paragraph 176-170, wherein the glucoamylase has at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99% or 100% identity to the mature polypeptide shown in SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NOs: 9 or 14 herein.

182. The composition of any of paragraphs 176-181, wherein the glucoamylase is a variant of the *Penicillium oxalicum* glucoamylase disclosed as SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NO: 14 herein having a K79V substitution (using the mature sequence shown in SEQ ID NO: 14 for numbering) such as a variant disclosed in WO 2013/053801.

183. The composition of any of paragraph 176-182, wherein the *Penicillium oxalicum* glucoamylase has a K79V substitution (using SEQ ID NO: 14 for numbering) and further one of the following:

T65A; or

Q327F; or

E501V; or

Y504T; or

Y504*; or

T65A + Q327F; or

T65A + E501V; or

T65A + Y504T; or

T65A + Y504*; or

Q327F + E501V; or

Q327F + Y504T; or

Q327F + Y504*; or

E501V + Y504T; or

E501V + Y504*; or

T65A + Q327F + E501V; or

T65A + Q327F + Y504T; or

T65A + E501V + Y504T; or

Q327F + E501V + Y504T; or

T65A + Q327F + Y504*; or

T65A + E501V + Y504*; or

Q327F + E501V + Y504*; or

T65A + Q327F + E501V + Y504T; or

T65A + Q327F + E501V + Y504*;

E501V + Y504T; or

T65A + K161S; or

T65A + Q405T; or

T65A + Q327W; or

T65A + Q327F; or

T65A + Q327Y; or

P11F + T65A + Q327F; or

R1K + D3W + K5Q + G7V + N8S + T10K + P11S + T65A + Q327F; or

P2N + P4S + P11F + T65A + Q327F; or

P11F + D26C + K33C + T65A + Q327F; or

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

R1E + D3N + P4G + G6R + G7A + N8A + T10D+ P11D + T65A + Q327F; or

P11F + T65A + Q327W; or

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

P11F + T65A + Q327W + E501V + Y504T; or

T65A + Q327F + E501V + Y504T; or

T65A + S105P + Q327W; or

T65A + S105P + Q327F; or

T65A + Q327W + S364P; or

T65A + Q327F + S364P; or

T65A + S103N + Q327F; or

P2N + P4S + P11F + K34Y + T65A + Q327F; or

P2N + P4S + P11F + T65A + Q327F + D445N + V447S; or

P2N + P4S + P11F + T65A + I172V + Q327F; or

P2N + P4S + P11F + T65A + Q327F + N502*; or

P2N + P4S + P11F + T65A + Q327F + N502T + P563S + K571E; or

P2N + P4S + P11F + R31S + K33V + T65A + Q327F + N564D + K571S; or

P2N + P4S + P11F + T65A + Q327F + S377T; or

P2N + P4S + P11F + T65A + V325T+ Q327W; or

P2N + P4S + P11F + T65A + Q327F + D445N + V447S + E501V + Y504T; or

P2N + P4S + P11F + T65A + I172V + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + S377T + E501V + Y504T; or

P2N + P4S + P11F + D26N + K34Y + T65A + Q327F; or

P2N + P4S + P11F + T65A + Q327F + I375A + E501V + Y504T; or

P2N + P4S + P11F + T65A + K218A + K221D + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + S103N + Q327F + E501V + Y504T; or

P2N + P4S + T10D + T65A + Q327F + E501V + Y504T; or

P2N + P4S + F12Y + T65A + Q327F + E501V + Y504T; or

K5A + P11F + T65A + Q327F + E501V + Y504T; or

P2N + P4S + T10E + E18N + T65A + Q327F + E501V + Y504T; or

P2N + T10E + E18N + T65A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T568N; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + K524T + G526A; or

P2N + P4S + P11F + K34Y + T65A + Q327F + D445N + V447S + E501V + Y504T; or

P2N + P4S + P11F + R31S + K33V + T65A + Q327F + D445N + V447S + E501V + Y504T; or

P2N + P4S + P11F + D26N + K34Y + T65A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + F80* + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K112S + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T516P + K524T + G526A; or

P2N + P4S + P11F + T65A + Q327F + E501V + N502T + Y504*; or

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

P2N + P4S + P11F + T65A + S103N + Q327F + E501V + Y504T; or

K5A + P11F + T65A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + E501V + Y504T + T516P + K524T + G526A; or

P2N + P4S + P11F + T65A + K79A + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79G + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79I + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79L + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + K79S + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + L72V + Q327F + E501V + Y504T; or

S255N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + E74N + V79K + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + G220N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Y245N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q253N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + D279N + Q327F + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + S359N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + D370N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + V460S + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + V460T + P468T + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + T463N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + S465N + E501V + Y504T; or

P2N + P4S + P11F + T65A + Q327F + T477N + E501V + Y504T.

184. The composition of any of paragraphs 176-183, wherein the glucoamylase is the *Penicillium oxalicum* glucoamylase having a K79V substitution (using SEQ ID NO: 14 for numbering) and further one of the following substitutions:

- P11F + T65A + Q327F

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

185. The composition of any of paragraphs 182-184, wherein the glucoamylase variant has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 14 herein.

186. The composition of any of paragraphs 157-185, further comprising a pullulanase.

187. The composition of paragraph 186, wherein the pullulanase is a family GH57 pullulanase, wherein the pullulanase preferably includes an X47 domain as disclosed in WO 2011/087836.

188. The composition of paragraphs 186-187, wherein the pullulanase is derived from a strain from the genus *Thermococcus*, including *Thermococcus litoralis* and *Thermococcus hydrothermalis* or a hybrid thereof.

189. The composition of any of paragraphs 186-188, wherein the pullulanase is the truncated *Thermococcus hydrothermalis* pullulanase at site X4 or a *T. hydrothermalis/T. litoralis* hybrid enzyme with truncation site X4 disclosed in WO 2011/087836 or shown in SEQ ID NO: 12 herein.

190. The composition of any of paragraphs 157-189 comprising

- *Bacillus stearothermophilus* alpha-amylase, or a variant thereof;

- *Pyrococcus furiosus* protease; and

- *Penicillium oxalicum* glucoamylase,

wherein the ratio between alpha-amylase and protease is in the range from 1:1 and 1:25 (micro gram alpha-amylase : micro gram protease).

191. The composition of any of paragraphs 157-190, comprising

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

- *Pyrococcus furiosus* protease; and

- *Penicillium oxalicum* glucoamylase,

wherein the ratio between alpha-amylase and protease is in the range from 1:1 and 1:25 (micro gram alpha-amylase : micro gram protease).

192. The composition of any of paragraphs 157-191, comprising

- an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion I181 + G182 and substitution N193F; and further one of the following set of substitutions:
  - E129V+K177L+R179E;
  - V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
  - V59A+Q89R+E129V+K177L+R179E+Q254S+M284V;

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

- *Pyrococcus furiosus* protease; and

- *Penicillium oxalicum* glucoamylase in SEQ ID NO: 14 having substitutions selected from the group of:
- K79V;
- K79V+ P11F + T65A + Q327F; or
- K79V+P2N + P4S + P11F + T65A + Q327F; or
- K79V +P11F + D26C + K33C + T65A + Q327F; or
- K79V +P2N + P4S + P11F + T65A + Q327W + E501V+Y504T; or
- K79V +P2N + P4S + P11F + T65A + Q327F+E501V+Y504T; or
- K79V+P11F+T65A+Q327W+E501V+Y504T (using SEQ ID NO: 14 for numbering),

wherein the ratio between alpha-amylase and protease is in the range from 1:1 and 1:25 (micro gram alpha-amylase : micro gram protease).

193. The enzyme composition of any of paragraphs 190-192, wherein the ratio between alpha-amylase and protease is in the range between 1:1.2 and 1:10, such as around 1:1.4 (micro gram alpha-amylase : micro gram protease).

194. The enzyme composition of any of paragraphs 190-193, wherein the ratio between alpha-amylase and glucoamylase is between 1:1 and 1:10, such as around 1:2 (micro gram alpha-amylase : micro gram glucoamylase).

CLAIMS

1. A process of recovering oil from a fermentation product production process comprising the steps of:

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

- an alpha-amylase;

- more than 0.5 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS);

b) saccharifying using a glucoamylase;

c) fermenting using a fermenting organism.

d) recovering the fermentation product to form whole stillage;

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

f) optionally concentrating the thin stillage into syrup;

wherein oil is recovered from the:

- liquefied starch-containing material after step a); and/or

- downstream from fermentation step c).

2. The process of claim 1, wherein oil is recovered during and/or after liquefying the starch-containing material; from the whole stillage; from the thin stillage; or from the syrup.

3. The process of any of claim 1-2, wherein 0.5-100 micro gram *Pyrococcus furiosus* protease per gram DS, such as 1-50 micro gram *Pyrococcus furiosus* protease per gram DS, such as 1-10 micro gram *Pyrococcus furiosus* protease per gram DS, such as 1.5-5 micro gram *Pyrococcus furiosus* protease per gram DS, such as around or more than 1.5 micro gram *Pyrococcus furiosus* protease per gram DS are present and/or added in liquefaction step a).

4. The process of any of claims 1-8, wherein the *Pyrococcus furiosus* protease is one having at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least

96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 13 herein.

5. The process of any of claim 1-4, wherein no nitrogen-compound is present and/or added in steps a)-c), such as during saccharification step b), fermentation step c), or simultaneous saccharification and fermentation (SSF).

6. A process for producing fermentation products from starch-containing material comprising the steps of:

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

- an alpha-amylase;

- more than 2 micro gram *Pyrococcus furiosus* protease per gram dry solids (DS);

b) saccharifying using a glucoamylase;

c) fermenting using a fermenting organism.

7. The process of claim 6, wherein 2-100 micro gram per gram DS, such as 2.5-50 micro gram per gram DS, such as 2.5-10 micro gram per gram DS, such as 2.5-5 micro gram per gram DS, especially around 3 micro gram per gram DS *Pyrococcus furiosus* protease.

8. The process of any of claims 6-7, wherein the *Pyrococcus furiosus* protease is one having at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 13 herein.

9. The process of any of claims 6-8, wherein no nitrogen-compound is present and/or added in steps a)-c), such as during saccharification step b) or fermentation step c) or simultaneous saccharification and fermentation (SSF).

10. An enzyme composition comprising:

i) *Bacillus* sp. alpha-amylase, or a variant thereof;

ii) *Pyrococcus furiosus* protease;

wherein the ratio between alpha-amylase and protease is in the range from 1:1 and 1:25 (micro gram alpha-amylase : micro gram protease).

11. The enzyme composition of claim 10, wherein the ratio between alpha-amylase and protease is in the range between 1:1.2 and 1:10, such as around 1:1.4 (micro gram alpha-amylase : micro gram protease).

12. The enzyme composition of any of claims 10-11, wherein the enzyme composition comprises a glucoamylase and the ratio between alpha-amylase and glucoamylase in liquefaction is between 1:1 and 1:10, such as around 1:2 (micro gram alpha-amylase : micro gram glucoamylase).

13. The composition of any of claims 157-189 comprising

- *Bacillus stearothermophilus* alpha-amylase, or a variant thereof;

- *Pyrococcus furiosus* protease; and

- *Penicillium oxalicum* glucoamylase,

wherein the ratio between alpha-amylase and protease is in the range from 1:1 and 1:25 (micro gram alpha-amylase : micro gram protease).

14. The composition of any of claims 10-13, comprising

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

- *Pyrococcus furiosus* protease; and

- *Penicillium oxalicum* glucoamylase,

wherein the ratio between alpha-amylase and protease is in the range from 1:1 and 1:25 (micro gram alpha-amylase : micro gram protease).

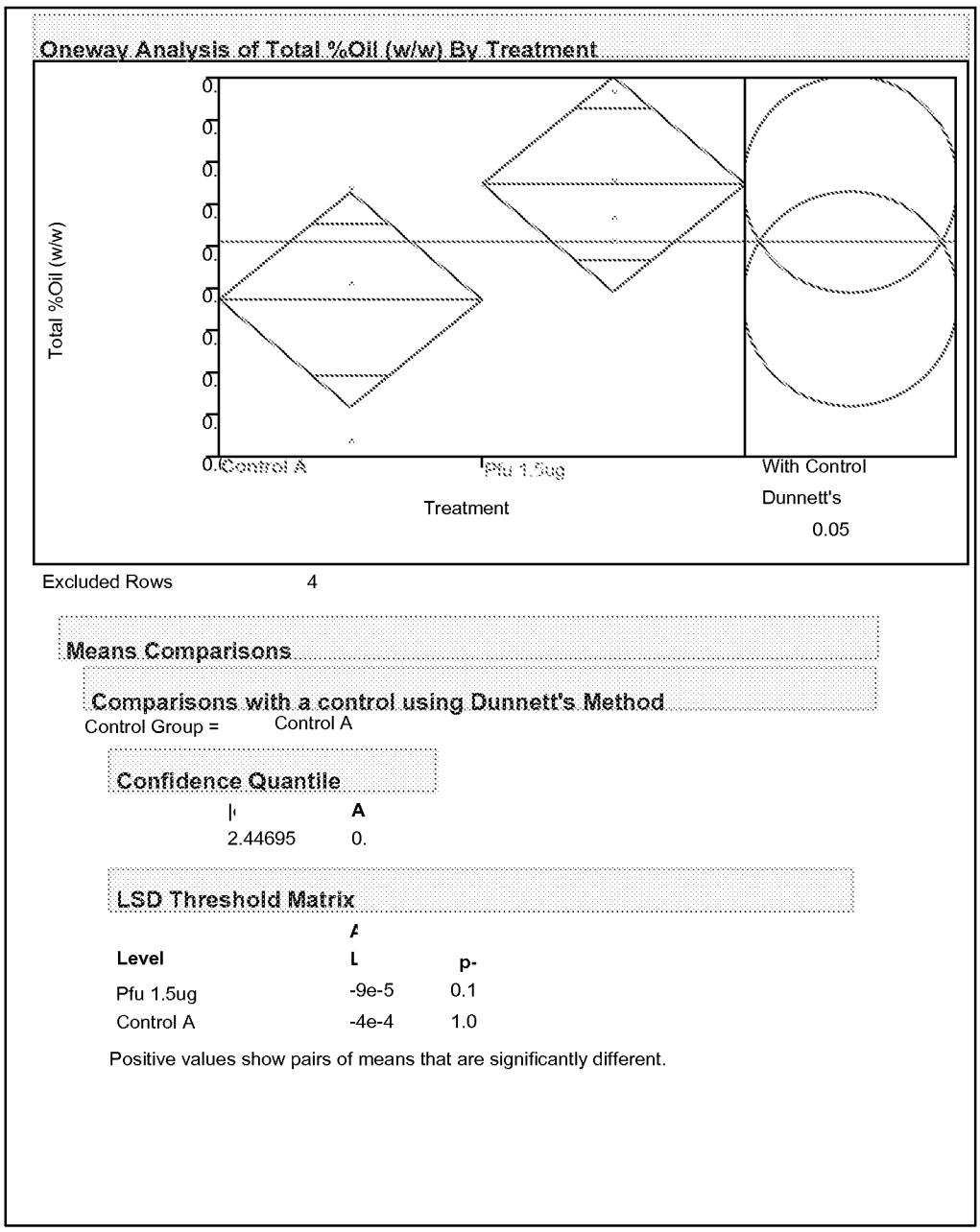

Oneway Analysis of Total %Oil (w/w) By Treatment
Total %Oil (w/w)
Control A
Pfu 1.5ug
Treatment
With Control
Dunnett's
0.05
Excluded Rows 4
Means Comparisons
Comparisons with a control using Dunnett's Method
Control Group = Control A
Confidence Quantile
2.44695 0.
LSD Threshold Matrix
Level p-
Pfu 1.5ug -9e-5 0.1
Control A -4e-4 1.0
Positive values show pairs of means that are significantly different.

Fig. 1

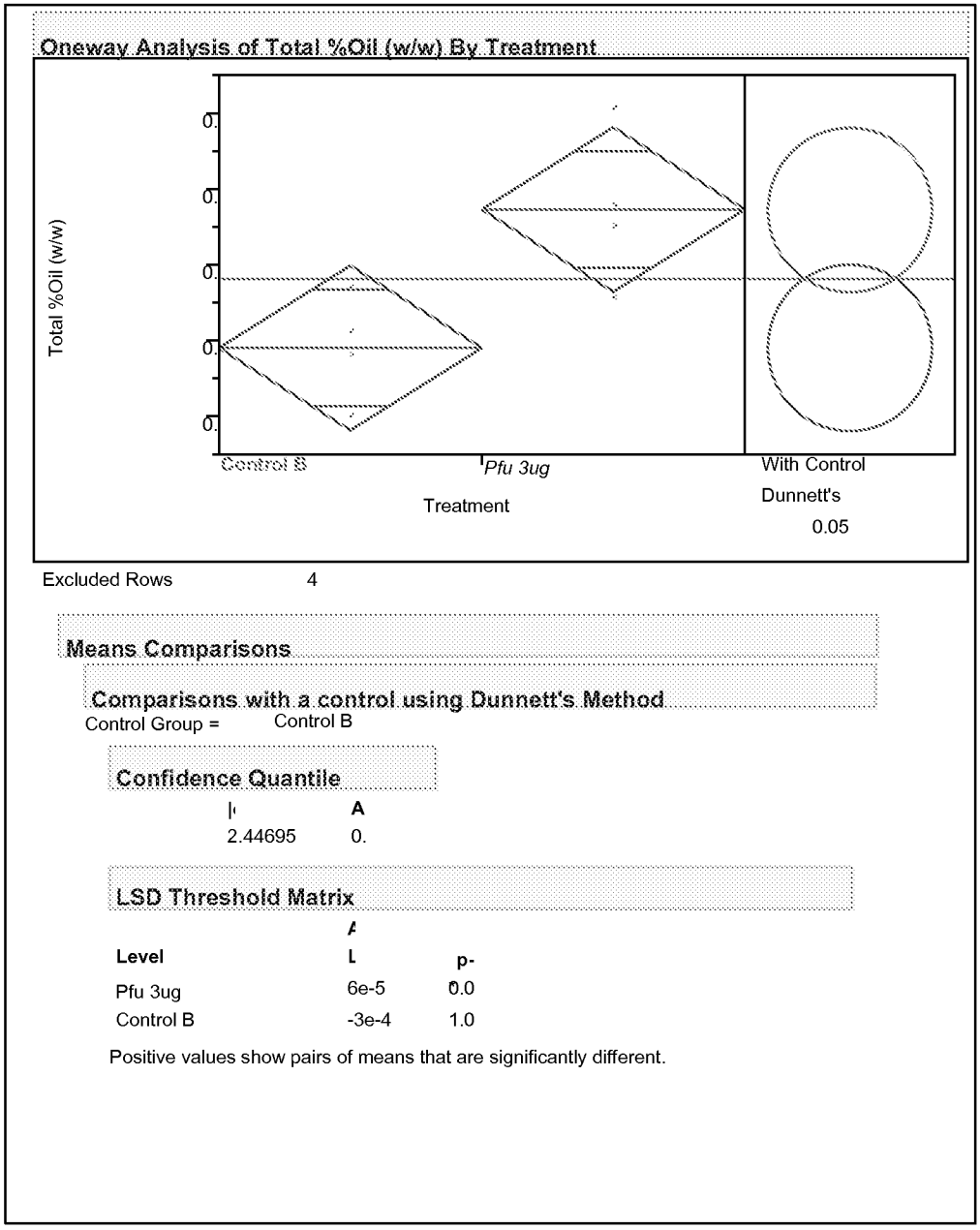

Oneway Analysis of Total %Oil (w/w) By Treatment
Total %Oil (w/w)
Control B
Pfu 3ug
Treatment
With Control
Dunnett's
0.05
Excluded Rows 4
Means Comparisons
Comparisons with a control using Dunnett's Method
Control Group = Control B
Confidence Quantile
2.44695
LSD Threshold Matrix
Level
Pfu 3ug 6e-5
Control B -3e-4
Positive values show pairs of means that are significantly different.

Fig. 2

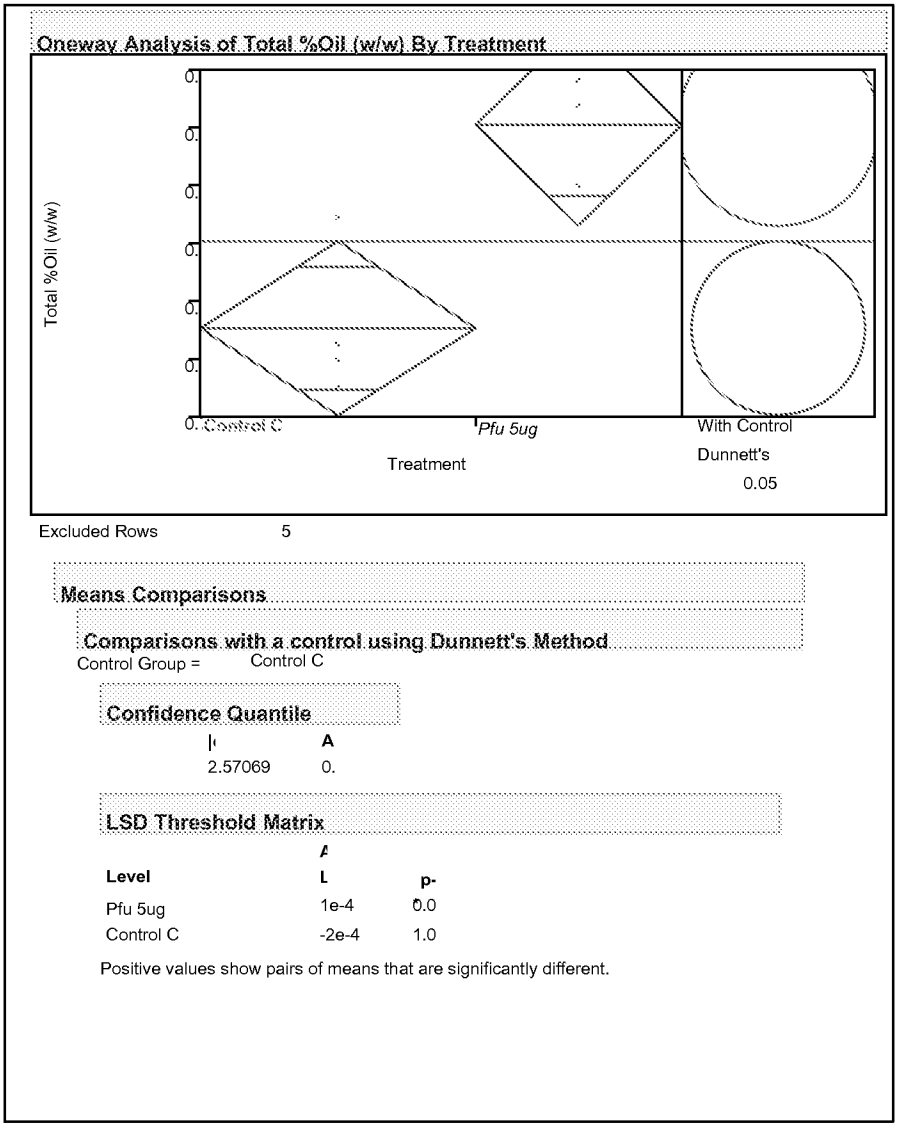

Oneway Analysis of Total %Oil (w/w) By Treatment
Total %Oil (w/w)
Control C
Pfu 5ug
With Control
Treatment
Dunnett's
0.05
Excluded Rows 5
Means Comparisons
Comparisons with a control using Dunnett's Method
Control Group = Control C
Confidence Quantile
2.57069 0.
LSD Threshold Matrix
Level
Pfu 5ug 1e-4 0.0
Control C -2e-4 1.0
Positive values show pairs of means that are significantly different.

Fig. 3

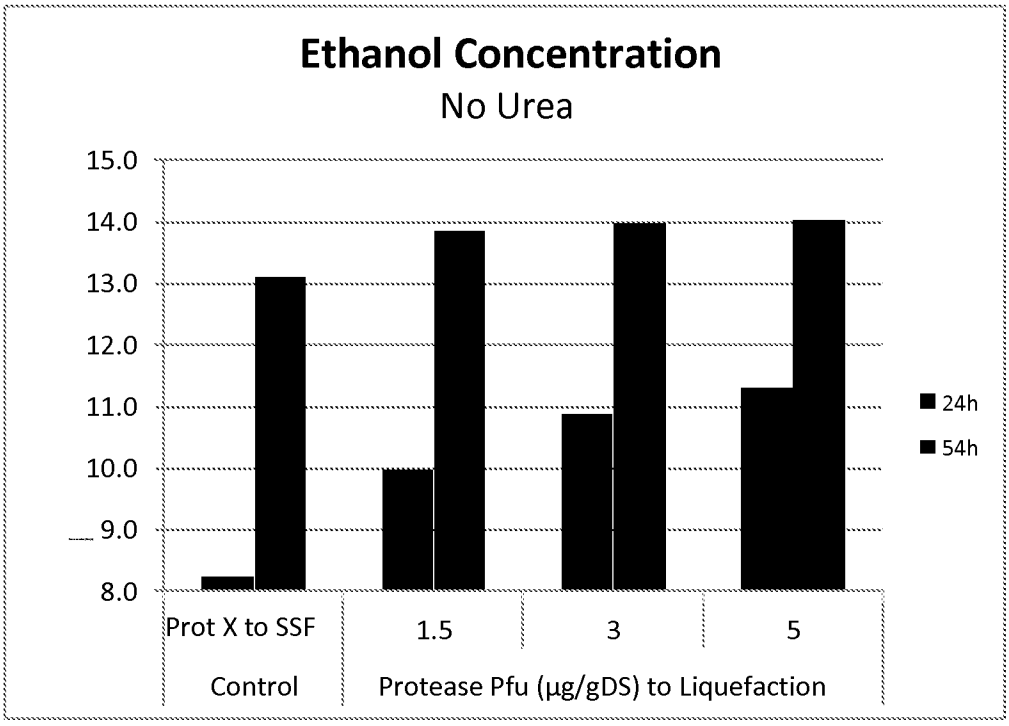

Ethanol Concentration
No Urea
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
24h
54h
Prot X to SSF
1.5
3
5
Control
Protease Pfu (µg/gDS) to Liquefaction

Fig. 4

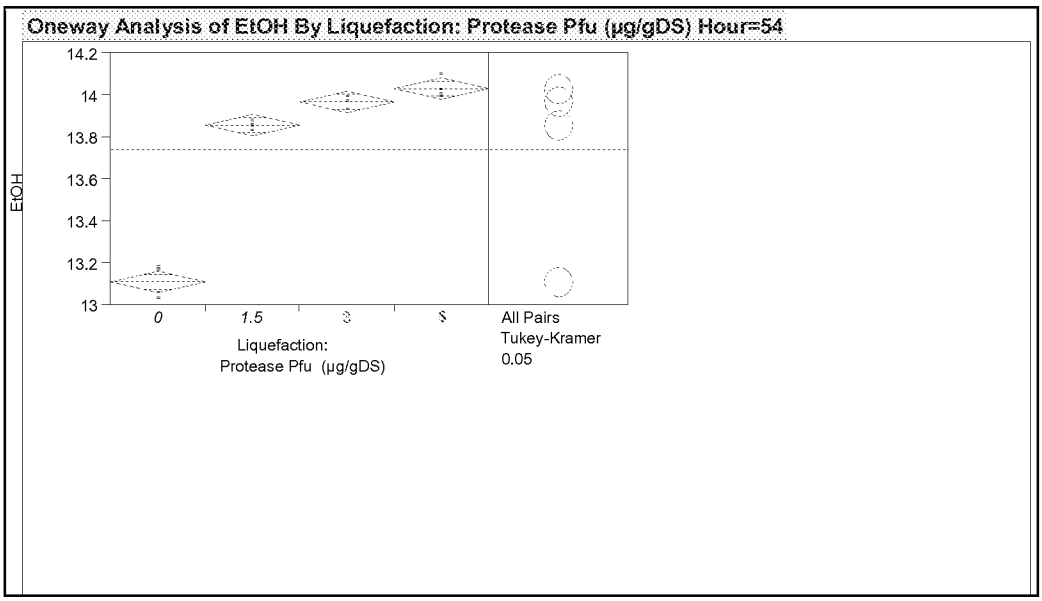

Oneway Analysis of EtOH By Liquefaction: Protease Pfu (µg/gDS) Hour=54
EtOH
14.2
14
13.8
13.6
13.4
13.2
13
0
1.5
3
5
Liquefaction:
Protease Pfu (µg/gDS)
All Pairs
Tukey-Kramer
0.05

Fig. 5

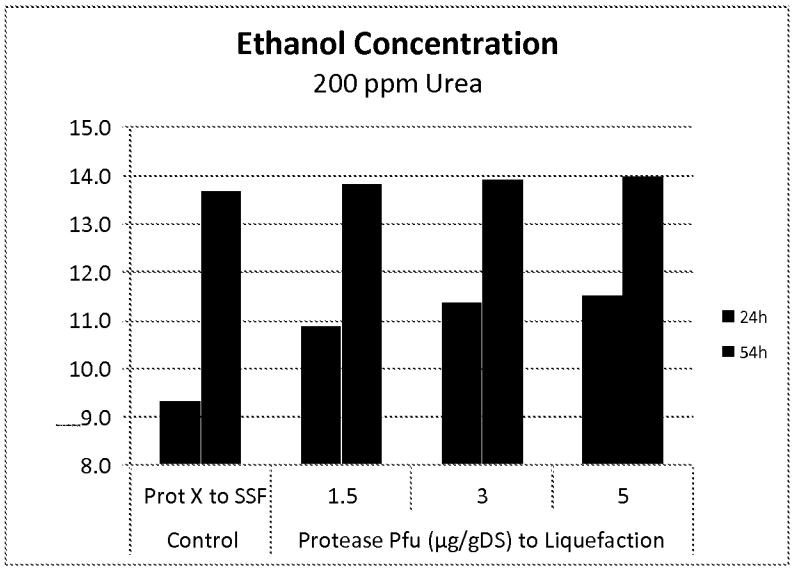

Ethanol Concentration
200 ppm Urea
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
24h
54h
Prot X to SSF
1.5
3
5
Control
Protease Pfu (µg/gDS) to Liquefaction

Fig. 6

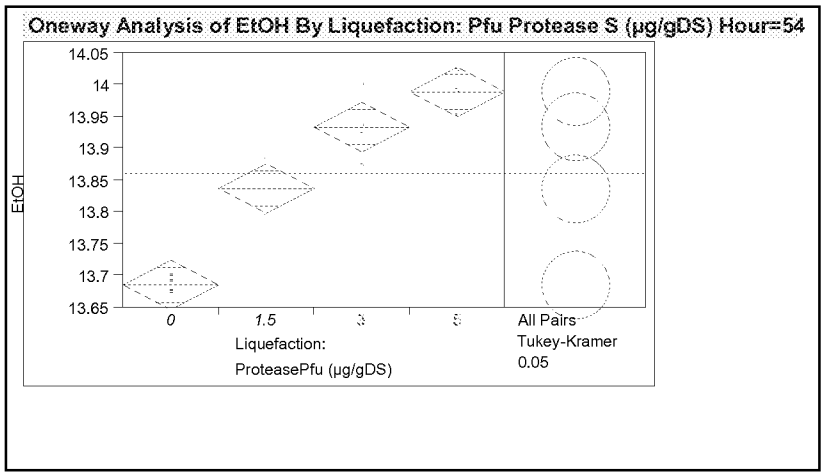

Oneway Analysis of EtOH By Liquefaction: Pfu Protease S (µg/gDS) Hour=54
EtOH
14.05
14
13.95
13.9
13.85
13.8
13.75
13.7
13.65
0
1.5
Liquefaction:
ProteasePfu (µg/gDS)
All Pairs
Tukey-Kramer
0.05

Fig. 7

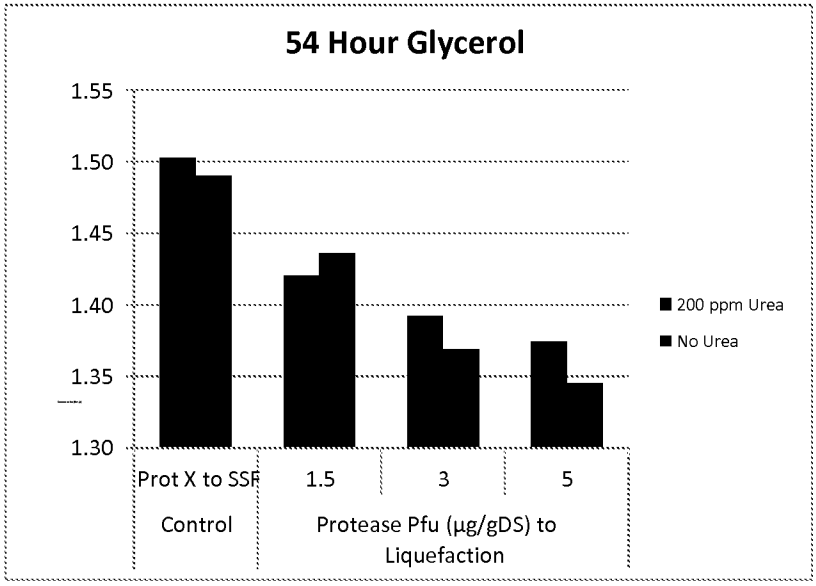

54 Hour Glycerol
1.55
1.50
1.45
1.40
1.35
1.30
200 ppm Urea
No Urea
Prot X to SSF
1.5
3
5
Control
Protease Pfu (µg/gDS) to Liquefaction

Fig. 8

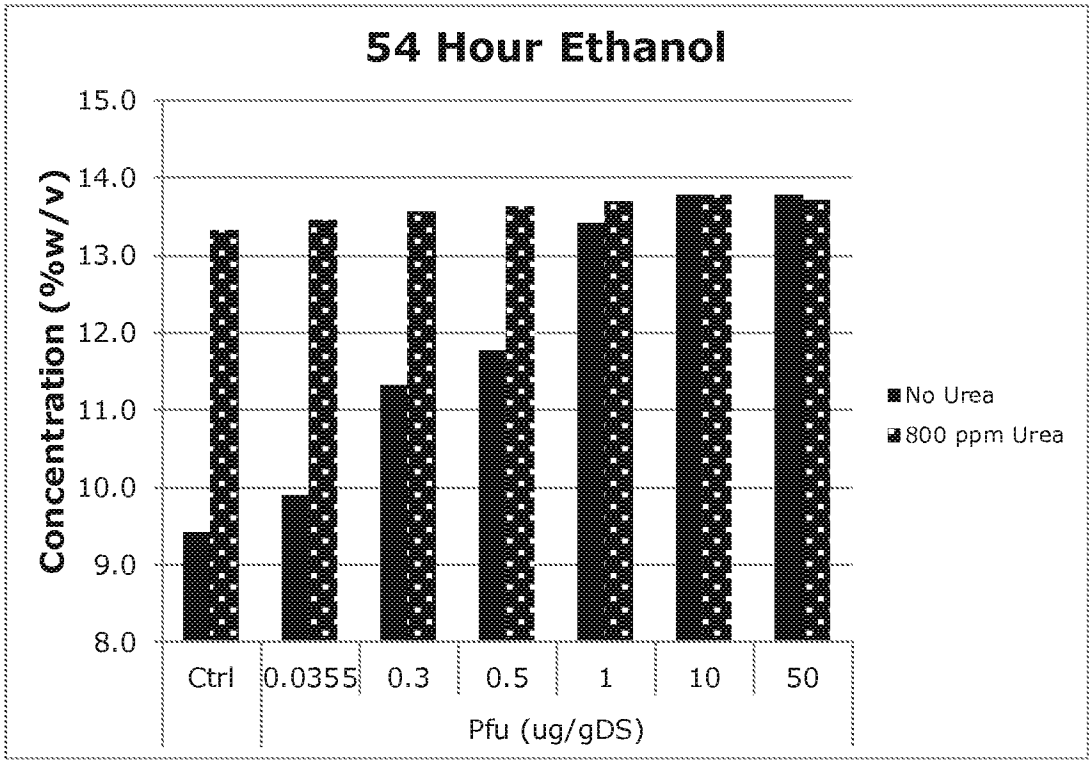

54 Hour Ethanol
Concentration (%w/v)
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
Ctrl
0.0355
0.3
0.5
1
10
50
Pfu (ug/gDS)
No Urea
800 ppm Urea

FIG. 9

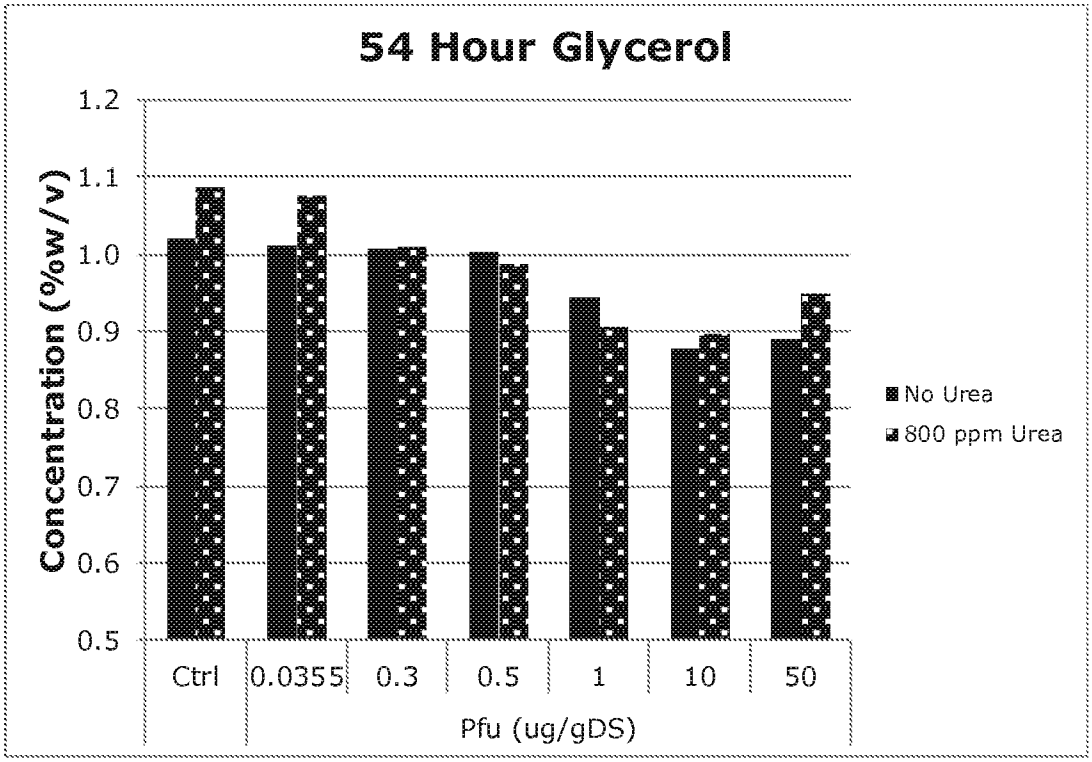

54 Hour Glycerol
Concentration (%w/v)
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
Ctrl
0.0355
0.3
0.5
1
10
50
Pfu (ug/gDS)
No Urea
800 ppm Urea

Fig. 10

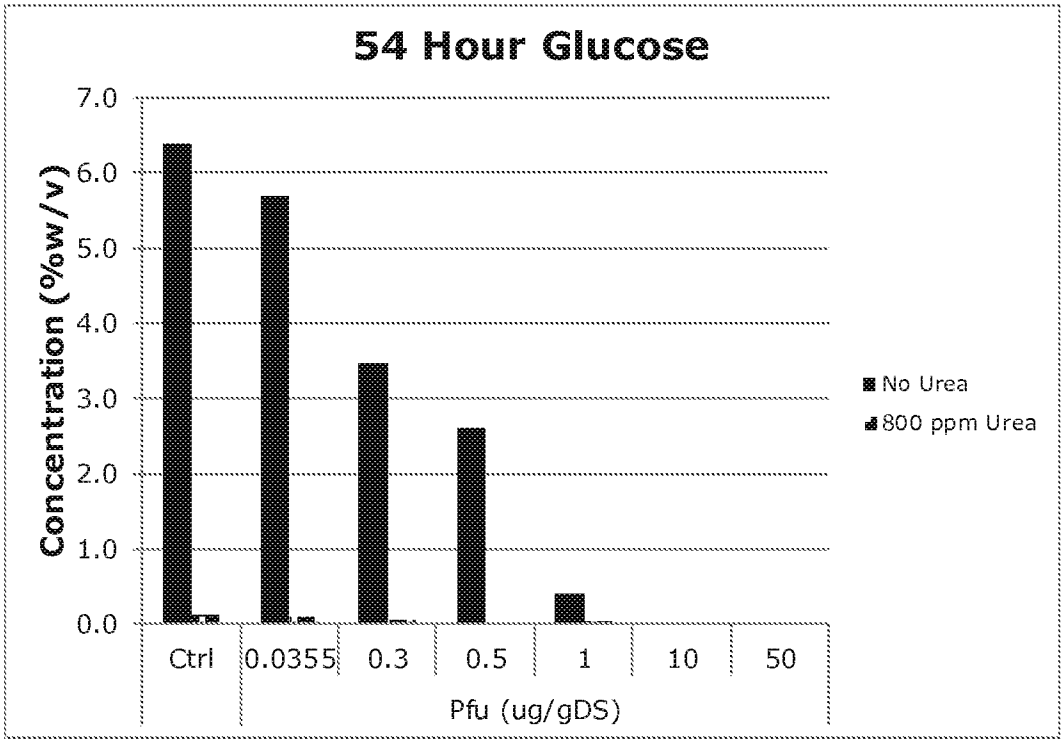

54 Hour Glucose
Concentration (%w/v)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Ctrl
0.0355
0.3
0.5
1
10
50
Pfu (ug/gDS)
No Urea
800 ppm Urea

Fig. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/043444

A. CLASSIFICATION OF SUBJECT MATTER

C12P 7/64(2006.01)i

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 7/64; C11B 1/00; C12P 7/10; A61B 7/02; C12P 1/04; C12N 9/50; C12P 7/06

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: recovering oil, alpha-amylase, protease, glucoamylase, thin stillage, syrup

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012-088303 A2 (NOVOZYMES NORTH AMERICA, INC. et al.) 28 June 2012 See abstract; claims 1-13, 25; page 21; and table 13.	6-7,10-12
Y		1-3,8
Y	WO 2011-126897 A2 (NOVOZYMES A/S et al.) 13 October 2011 See abstract; and claims 1-78.	1-3
Y	US 6358726 B1 (TAKAKURA, HIKARU et al.) 19 March 2002 See abstract; and sequence list.	8
A	WO 2013-082486 A1 (NOVOZYMES A/S et al.) 06 June 2013 See the whole document.	1-3,6-8,10-12
A	US 8008517 B2 (CANTRELL, DAVID FRED et al.) 30 August 2011 See the whole document.	1-3,6-8,10-12

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"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
23 October 2014 (23.10.2014)	**24 October 2014 (24.10.2014)**

Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701,
Republic of Korea
Facsimile No. +82-42-472-7140

Authorized officer
HEO, Joo Hyung
Telephone No. +82-42-481-8150

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/043444

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.: 13,14
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:

Since claim 13 is referring to claims 157 and 158 which do not exist in this application, said claim 13 does not clearly define the matter for which protection is sought. And claim 14 refers to said claim 13. Therefore, claims 13-14 are unclear (PCT Article 6).

3. ☒ Claims Nos.: 4,5,9,14
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:

1. ☐ As all required addtional 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 any 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.:

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.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/043444

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

a. a sequence listing filed or furnished

- ☐ on paper
- ☒ in electronic form

b. time of filing or furnishing

- ☒ contained in the international application as filed
- ☐ filed together with the international application in electronic form
- ☐ furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2014/043444

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012-088303 A2	28/06/2012	CA 2822637 A1	28/06/2012
		CN 103608460 A	26/02/2014
		EP 2654567 A2	30/10/2013
		US 2014-0017749 A1	16/01/2014
		WO 2012-088303 A3	31/10/2013
WO 2011-126897 A2	13/10/2011	CN 102834521 A	19/12/2012
		CN 102918160 A	06/02/2013
		EP 2553110 A1	06/02/2013
		EP 2553111 A2	06/02/2013
		US 2013-0034883 A1	07/02/2013
		US 2013-0052702 A1	28/02/2013
		WO 2011-123505 A1	06/10/2011
		WO 2011-126897 A3	19/01/2012
US 6358726 B1	19/03/2002	AU 7550098 A	30/12/1998
		EP 0994191 A1	19/04/2000
		JP 3601835 B2	15/12/2004
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