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DESCRIPTION

FIELD OF THE INVENTION

[0001] Disclosed are processes for producing fermentation products from starch-containing material and compositions suitable for use in a process of the invention.

REFERENCE TO A SEQUENCE LISTING

[0002] This patent contains a Sequence Listing.

BACKGROUND OF THE INVENTION

[0003] Production of fermentation products, such as ethanol, from starch-containing material is well-known in the art. Industrially two different kinds of processes are used today. The most commonly used process, often referred to as a "conventional process", including liquefying gelatinized starch at high temperature using typically a bacterial alpha-amylase, followed by simultaneous saccharification and fermentation carried out in the presence of a glucoamylase and a fermentation organism. Another well-known process, often referred to as a "raw starch hydrolysis"-process (RSH process) includes simultaneously saccharifying and fermenting granular starch below the initial gelatinization temperature typically in the presence of an acid fungal alpha-amylase and a glucoamylase.

[0004] Despite significant improvement of fermentation product production processes over the past decade a significant amount of residual starch material is not converted into the desired fermentation product, such as ethanol. At least some of the unconverted residual starch material, e.g., sugars and dextrins, is in the form of non-fermentable Maillard products.

[0005] Therefore, there is still a desire and need for providing processes for producing fermentation products, such as ethanol, from starch-containing material that can provide a higher fermentation product yield compared to a conventional process.

SUMMARY OF THE INVENTION

[0006] Disclosed are processes of producing fermentation products, such as ethanol from starch-containing material using a fermenting organism and compositions suitable for use in a process of the invention.

[0007] In the first aspect, the invention relates to processes for producing fermentation products, such as ethanol, from starch-containing material comprising the steps of:

1. i) liquefying the 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 variant of the *Bacillus stearothermophilus* alpha-amylase shown in SEQ ID NO: 1 with a double deletion in 1181 + G182 and substitution N193F, and further comprising mutations selected from the group of:
        - V59A+Q89R+G112D+E129V+K177L+R179E+K220P+N224L+Q254S;
        - V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
        - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+D269E+D281N;
        - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+I270L;
        - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+H274K;
        - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+Y276F;
        - V59A+E129V+R157Y+K177L+R179E+K220P+N224L+S242Q+Q254S;
        - V59A+E129V+K177L+R179E+H208Y+K220P+N224L+S242Q+Q254S;
        - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
        - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+H274K;
        - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F;
        - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+D281N;
        - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
        - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+G416V;
        - V59A+E129V+K177L+R179E+K220P+N224L+Q254S;
        - V59A+E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
        - A91L+M96I+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
        - E129V+K177L+R179E;
        - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
        - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F+L427M;
        - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
        - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+N376*+I377*;
        - E129V+K177L+R179E+K220P+N224L+Q254S;
        - E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
        - E129V+K177L+R179E+S242Q;
        - E129V+K177L+R179V+K220P+N224L+S242Q+Q254S;
        - K220P+N224L+S242Q+Q254S; and
        - M284V;

      which variant has at least 80% identity, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 1 and has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) of at least 10; and
    - a variant of a metallo protease which variant has at least 80% identity, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 3 herein and has a thermostability value of more than 20% determined as Relative Activity at 80°C/70°C; or
    - a *Pyrococcus furiosus*, protease which has at least 80% identity to SEQ ID NO: 13 herein and has a thermostability value of more than 20% determined as Relative Activity at 80°C/70°C ;
2. ii) saccharifying using a carbohydrate-source generating enzyme;
3. iii) fermenting using a fermenting organism.

[0008] In an embodiment, a carbohydrate-source generating enzyme, in particular a thermostable glucoamylase, and/or a pullulanase is(are) present and/or added during liquefaction in step i).

[0009] In a second aspect, the invention relates to compositions comprising an alpha-amylase and a protease, wherein the

1. i) alpha-amylase is a variant of the *Bacillus stearothermophilus* alpha-amylase shown in SEQ ID NO: 1 with a double deletion in 1181 + G182 and substitution N193F, and further comprising mutations selected from the group of:
    - V59A+Q89R+G112D+E129V+K177L+R179E+K220P+N224L+Q254S;
    - V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
    - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+D269E+D281N;
    - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+I270L;
    - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+H274K;
    - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+Y276F;
    - V59A+E129V+R157Y+K177L+R179E+K220P+N224L+S242Q+Q254S;
    - V59A+E129V+K177L+R179E+H208Y+K220P+N224L+S242Q+Q254S;
    - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
    - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+H274K;
    - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F;
    - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+D281N;
    - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
    - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+G416V;
    - V59A+E129V+K177L+R179E+K220P+N224L+Q254S;
    - V59A+E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
    - A91L+M96I+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
    - E129V+K177L+R179E;
    - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
    - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F+L427M;
    - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
    - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+N376*+I377*;
    - E129V+K177L+R179E+K220P+N224L+Q254S;
    - E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
    - E129V+K177L+R179E+S242Q;
    - E129V+K177L+R179V+K220P+N224L+S242Q+Q254S;
    - K220P+N224L+S242Q+Q254S; and
    - M284V;

    which variant has at least 80% identity, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 1 and has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) of at least 10; and
2. ii) a variant of a metallo protease which variant has at least 80% identity, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 3 herein and has a thermostability value of more than 20% determined as Relative Activity at 80°C/70°C; or
    - a *Pyrococcus furiosus*, protease which has at least 80% identity to SEQ ID NO: 13 herein and has a thermostability value of more than 20% determined as Relative

Activity at 80°C/70°C.

[0010] In an embodiment, the composition further comprises a carbohydrate-source generating enzyme, in particular a thermostable glucoamylase, and/or a pullulanase.

[0011] In an embodiment, a second alpha-amylase is present and/or added during liquefaction step i).

[0012] In an embodiment, the composition comprises a second alpha-amylase.

BRIEF DESCRIPTION OF THE FIGURES

[0013]

Fig. 1 shows a comparison of 54 hour ethanol for liquefactions (85°C) prepared with Alpha-Amylase 1407 with and without Protease Pfu or Glucoamylase PE001 at pH 4.8.

Fig. 2 shows the peak and break viscosity at 32% DS for the experiment in Example 10 comparing

- Alpha-Amylase A (1.4 micro g) (pH 5.8);
- Alpha-Amylase 1407 (1.4 micro g) + Glucoamylase PE001 (10 micro g) + Protease 196 (1 micro g) (pH 4.8);
- Alpha-Amylase A (0.35 micro g) + Alpha-Amylase 1407 (1.4 micro g) + Glucoamylase PE001 (10 micro g) + Protease 196 (1 micro g) (pH 4.8);
- Alpha-Amylase A (0.7 micro g) + Alpha-Amylase 1407 (1.4 micro g) + Glucoamylase PE001 (10 micro g) + Protease 196 (1 micro g) (pH 4.8);

Fig. 3 shows the final viscosity at 32% DS at 32°C for the experiment in Example 10 comparing

- Alpha-Amylase A (1.4 micro g) (pH 5.8);
- Alpha-Amylase 1407 (1.4 micro g) + Glucoamylase PE001 (10 micro g) + Protease 196 (1 micro g) (pH 4.8);
- Alpha-Amylase A (0.35 micro g) + Alpha-Amylase 1407 (1.4 micro g) + Glucoamylase PE001 (10 micro g) + Protease 196 (1 micro g) (pH 4.8);
- Alpha-Amylase A (0.7 micro g) + Alpha-Amylase 1407 (1.4 micro g) + Glucoamylase PE001 (10 micro g) + Protease 196 (1 micro g) (pH 4.8).

Fig. 4 shows the peak and break viscosity at 32% DS for the experiment in Example 10 comparing Alpha-Amylase A (1.4 micro g) (pH 5.8) and Alpha-Amylase A (1.4 micro g) + Glucoamylase PE001 (10 micro g) + Protease 196 (1 micro g) (pH 4.8).

Fig. 5 shows the final viscosity at 32% DS, at 32°C for the experiment in Example 10 comparing Alpha-Amylase A (1.4 micro g) (pH 5.8) and Alpha-Amylase A (1.4 micro g) + Glucoamylase PE001 (10 micro g) + Protease 196 (1 micro g) (pH 4.8).

DETAILED DESCRIPTION OF THE INVENTION

[0014] Disclosed are processes of producing fermentation products, such as ethanol from starch-containing material using a fermenting organism and compositions suitable for use in a process of the invention.

[0015] The inventors have shown that a process of the invention has a number of advantages. As shown in the Examples a process of the invention results in a higher ethanol yield. Other benefits, includes a reduced need for using H_2SO_4 for pH adjustment. This results in less sulfur downstream in the DDGS, less front-end fouling, less beerstone, and less phytate precipitation.

[0016] A process of the invention also results in reduced loss of sugars and dextrins to Maillard products. The DDGS color is improved and the heat exchanger lifetime (less solids) is extended. Furthermore, due to the higher thermostability of the enzymes used the enzyme dose may be reduced. A process of the invention requires limited changes to existing process and process equipment and thus limited capital investment.

[0017] By having a thermostable alpha-amylase and a second alpha-amylase as defined herein in liquefaction the peak viscosity, e.g., in slurry tank is (further) reduced. This results in less energy spent on mixing. Also having a lower average viscosity improves the mixing of the mash/starch in the slurry tank and its pumping through the liquefaction process.

[0018] In the first aspect, the invention relates to processes for producing fermentation products from starch-containing material comprising the steps of:

1. i) liquefying the 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 variant of the *Bacillus stearothermophilus* alpha-amylase shown in SEQ ID NO: 1 with a double deletion in 1181 + G182 and substitution N193F, and further comprising mutations selected from the group of:
        - V59A+Q89R+G112D+E129V+K177L+R179E+K220P+N224L+Q254S;
        - V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
        - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+D269E+D281N;
        - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+I270L;
        - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+H274K;
        - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+Y276F;
        - V59A+E129V+R157Y+K177L+R179E+K220P+N224L+S242Q+Q254S;
        - V59A+E129V+K177L+R179E+H208Y+K220P+N224L+S242Q+Q254S;
        - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
        - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+H274K;
        - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F;
        - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+D281N;
        - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
        - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+G416V;

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

which variant has at least 80% identity, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 1 and has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) of at least 10; and

- a variant of a metallo protease which variant has at least 80% identity, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 3 herein and has a thermostability value of more than 20% determined as Relative Activity at 80°C/70°C; or
- a *Pyrococcus furiosus*, protease which has at least 80% identity to SEQ ID NO: 13 herein and has a thermostability value of more than 20% determined as Relative Activity at 80°C/70°C ;

2. ii) saccharifying using a carbohydrate-source generating enzyme;
3. iii) fermenting using a fermenting organism.

[0019] In a preferred embodiment step ii) and iii) are carried out either sequentially or simultaneously. The thermostable alpha-amylase and thermostable protease defined above and optionally the carbohydrate-source generating enzyme, preferably thermostable glucoamylase, and/or optionally a pullulanase may be added before and/or during liquefaction step i). Examples of thermostable alpha-amylases can be found in the "Alpha-Amylase Present and/or Added During Liquefaction" section below. Examples of thermostable proteases can be found in the "Protease Present and/or Added During Liquefaction"-section below. A composition of the invention may suitably be used in a process of the invention. However, the enzyme components may also be added separately.

[0020] In a preferred embodiment, the pH during liquefaction is between 4.5-4.8.

[0021] In an embodiment, a carbohydrate-source generating enzyme is also present during liquefaction. In a preferred embodiment, the carbohydrate-source generating enzymes is a thermostable glucoamylase. In an embodiment, the carbohydrate-source generating enzyme is different from the one used during saccharification in step ii) and/or fermentation in step iii).

[0022] Examples of "carbohydrate-source generating enzymes", including in particular glucoamylases, can be found in the "Carbohydrate-Source Generating Enzyme Present and/or Added During Liquefaction"-section below.

[0023] In an embodiment, the process of the invention further comprises, prior to the step i), the steps of:

1. a) reducing the particle size of the starch-containing material, preferably by dry milling;
2. b) forming a slurry comprising the starch-containing material and water.

[0024] The starch-containing starting material, such as whole grains, may be reduced in particle size, e.g., by milling, in order to open up the structure and allowing for further processing. Generally there are two types of processes: wet and dry milling. In dry milling whole kernels are milled and used. Wet milling gives a good separation of germ and meal (starch granules and protein) and is often applied at locations where the starch hydrolysate is used in production of, e.g., syrups. Both dry and wet milling is well known in the art of starch processing. According to the invention dry milling is preferred. In an embodiment the particle size is reduced to between 0.05 to 3.0 mm, preferably 0.1-0.5 mm, or so that at least 30%, preferably at least 50%, more preferably at least 70%, even more preferably at least 90% of the starch-containing material fit through a sieve with a 0.05 to 3.0 mm screen, preferably 0.1-0.5 mm screen. In another embodiment, at least 50%, preferably at least 70%, more preferably at least 80%, especially at least 90% of the starch-containing material fit through a sieve with # 6 screen.

[0025] The aqueous slurry may contain from 10-55 w/w-% dry solids (DS), preferably 25-45 w/w-% dry solids (DS), more preferably 30-40 w/w-% dry solids (DS) of starch-containing material. The slurry is heated to above the gelatinization temperature, preferably for between 80-90°C, pH 4.5-4.8 for around 15-60 minutes.

[0026] The thermostable alpha-amylase, thermostable protease and optional carbohydrate-source generating enzyme, in particular thermostable glucoamylase, and/or optional pullulanase may be added to the aqueous slurry to initiate liquefaction (thinning). In an embodiment, only a part of the enzyme blend (composition of the invention) is added to the aqueous slurry, while the rest of the enzyme is added during liquefaction step i). Liquefaction step i) is typically carried out at 80-90°C, pH 4.5-4.8 for 1-3 hours.

[0027] The aqueous slurry may in an embodiment be jet-cooked to further gelatinize the slurry before being subjected to liquefaction in step i). The jet-cooking may be carried out at a temperature between 110-145°C, preferably 120-140°C, such as 125-135°C, preferably around 130°C for about 1-15 minutes, preferably for about 3-10 minutes, especially around about 5 minutes.

Saccharification and Fermentation

[0028] One or more carbohydrate-source generating enzymes, in particular glucoamylases, are

present and/or added during saccharification step ii) and/or fermentation step iii). The carbohydrate-source generating enzyme may preferably be a glucoamylase, but may also be an enzyme selected from the group consisting of: beta-amylase, maltogenic amylase and alpha-glucosidase.

[0029] Examples of carbohydrate-source generating enzyme, including glucoamylases, can be found in the "Carbohydrate-Source Generating Enzyme Present and/or Added During Saccharification and/or Fermentation"-section below.

[0030] When doing sequential saccharification and fermentation the saccharification step ii) may be carried out using conditions well-known in the art. For instance, the saccharification step ii) may last up to from about 24 to about 72 hours, however, it is common to do only a pre-saccharification of typically 40-90 minutes at a temperature between 30-65°C, typically about 60°C, followed by saccharification during fermentation in simultaneous saccharification and fermentation ("SSF). Saccharification is typically carried out at temperatures from 20-75°C, preferably from 40-70°C, typically around 60°C, and at a pH between 4 and 5, normally at about pH 4.5.

[0031] Simultaneous saccharification and fermentation ("SSF") is widely used in industrial scale fermentation product production processes, especially ethanol production processes. When doing SSF the saccharification step ii) and the fermentation step iii) are carried out simultaneously. There is no holding stage for the saccharification, meaning that a fermenting organism, such as yeast, and enzyme(s), may be added together. SSF is according to the invention typically carried out at a temperature from 25°C to 40°C, such as from 28°C to 35°C, such as from 30°C to 34°C, preferably around about 32°C. In an embodiment fermentation is ongoing for 6 to 120 hours, in particular 24 to 96 hours. In an embodiment, the pH is between 3.5-5, in particular between 3.8 and 4.3.

Fermentation Medium

[0032] "Fermentation media" or "fermentation medium" which refers to the environment in which fermentation is carried out and which includes the fermentation substrate, that is, the carbohydrate source that is metabolized by the fermenting organism. 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

[0033] The term "fermenting organism" refers to any organism, including bacterial and fungal organisms, 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*.

[0034] In one embodiment, the fermenting organism is added to the fermentation medium so that the viable fermenting organism, such as yeast, count per mL of fermentation medium is in the range from 10^5 to 10^{12}, preferably from 10^7 to 10^{10}, especially about $5x10^7$.

[0035] 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

[0036] 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 there from, or cereals. Contemplated are also waxy and non-waxy types of corn and barley.

Fermentation Products

[0037] 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); 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. Preferred fermentation processes used include alcohol fermentation processes. The fermentation product, such as ethanol, obtained according to the invention, may preferably be used as fuel that typically is blended with gasoline. However, in the case of ethanol it may also be used as potable ethanol.

Recovery

[0038] Subsequent to fermentation the fermentation product may be separated from the fermentation medium. The slurry may be distilled to extract the desired fermentation product.

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.

Alpha-Amylase Present and/or Added During Liquefaction

[0039] According to the invention the alpha-amylase is a variant of the *Bacillus stearothermophilus* alpha-amylase shown in SEQ ID NO: 1 with a double deletion in 1181 + G182 and substitution N193F, and further comprising mutations selected from the group of:

- V59A+Q89R+G112D+E129V+K177L+R179E+K220P+N224L+Q254S;
- V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+D269E+D281N;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+I270L;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+H274K;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+Y276F;
- V59A+E129V+R157Y+K177L+R179E+K220P+N224L+S242Q+Q254S;
- V59A+E129V+K177L+R179E+H208Y+K220P+N224L+S242Q+Q254S;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+H274K;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+D281N;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+G416V;
- V59A+E129V+K177L+R179E+K220P+N224L+Q254S;
- V59A+E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
- A91L+M96I+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- E129V+K177L+R179E;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F+L427M;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+N376*+I377*;
- E129V+K177L+R179E+K220P+N224L+Q254S;
- E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
- E129V+K177L+R179E+S242Q;
- E129V+K177L+R179V+K220P+N224L+S242Q+Q254S;
- K220P+N224L+S242Q+Q254S; and
- M284V;

which variant has at least 80% identity, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 1 and has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) of at least 10. According to the invention the alpha-amylase has high activity toward starch solubilisation in liquefaction at pH 4.5 to 5.0 and high thermostability at pH 4.5-5.0 and 80-90°C, preferably 4.5-4.8, around 85°C.

[0040] More specifically the alpha-amylase used in a process of the invention has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$ of at least 10 determined as described in Example 1.

[0041] In a preferred embodiment T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$, is 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.

[0042] In an embodiment the thermostable alpha-amylase is a *Bacillus stearothermophilus* alpha-amylase variant having 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.

[0043] The thermostable alpha-amylase may be a truncated *Bacillus stearothermophilus* alpha-amylase, preferably to have around 491 amino acids.

Second Alpha-amylase Present and/or Added During Liquefaction

[0044] When a second alpha-amylase is present and/or added during liquefaction step i) a positive viscosity reducing effect is obtained. As can be seen from Example 10 the combination of a thermostable alpha-amylase (e.g., Alpha-Amylase BE1407) with or without the presence of a thermostable protease (e.g., Protease 196) and thermostable glucoamylase (e.g., Glucoamylase PO) and further a second alpha-amylase (e.g. Alpha-amylase A) results in decreased peak viscosity and final viscosity.

[0045] Therefore, in this aspect of the invention a second alpha-amylase is added during liquefaction step i). The second alpha-amylase may be less thermostable and/or less efficient at pH 4.5, 85°C, 0.12 mM $CaCl_2$, or around pH 4.8, than a thermostable alpha-amylase defined herein added and/or present during liquefaction according to the invention.

[0046] In an embodiment, the second alpha-amylase is of bacterial origin.

[0047] In an embodiment, the second alpha-amylase is derived from a strain of 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. The second alpha-amylase may be a truncated *Bacillus stearothermophilus* alpha-amylase, preferably to have around 491 amino acids.

[0048] In an embodiment second 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%, or at least 100% identity to the

mature part of the polypeptide of SEQ ID NO: 3 disclosed in WO 99/019467 or SEQ ID NO: 1 herein.

[0049] In an embodiment, the second alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) of below 10 determined as described in Example 1.

[0050] In an embodiment, the second alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) of below 8, such as below 7, such as below 6, such as below 5.

[0051] In an embodiment, the second alpha-amylase has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) between 2 and 10, such as between 3 and 8, such as above 4 to 10, such as above 4 to 8.

[0052] In an embodiment, the second alpha-amylase may be derived from *Bacillus stearothermophilus* and may have the following mutations: I181*+G182* or I181*+G182*+N193F (using SEQ ID NO: 1 for numbering).

Protease Present and/or Added During Liquefaction

[0053] According to the invention the thermostable protease is a variant of a metallo protease which variant has at least 80% identity, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 3 herein and has a thermostability value of more than 20% determined as Relative Activity at 80°C/70°C; or a *Pyrococcus furiosus* protease which has at least 80% identity to SEQ ID NO: 13 herein and has a thermostability value of more than 20% determined as Relative Activity at 80°C/70°C.

[0054] In an embodiment, the protease has a thermostability value:

- of more than 30%, more than 40%, more than 50%, more than 60%, more than 70%, more than 80%, more than 90% determined as Relative Activity at 80°C/70°C.

[0055] Purified variants may have a themostability for above 90, above 100 at 85°C as determined using the Zein-BCA assay as disclosed in Example 3.

[0056] Determination of "Relative Activity" and "Remaining Activity" is determined as described in Example 2.

[0057] Proteases are classified on the basis of their catalytic mechanism into the following groups: Serine proteases (S), Cysteine proteases (C), Aspartic proteases (A), Metallo proteases (M), and Unknown, or as yet unclassified, proteases (U), see Handbook of Proteolytic Enzymes, A.J.Barrett, N.D.Rawlings, J.F.Woessner (eds), Academic Press (1998), in particular the general introduction part.

[0058] In a preferred embodiment, the thermostable protease used in a process of the invention is

a "metallo protease" defined as a protease belonging to EC 3.4.24 (metalloendopeptidases); preferably EC 3.4.24.39 (acid metallo proteinases).

[0059] To determine whether a given protease is a metallo protease or not, reference is made to the above "Handbook of Proteolytic Enzymes" and the principles indicated therein. Such determination can be carried out for all types of proteases, be it naturally occurring or wild-type proteases; or genetically engineered or synthetic proteases.

[0060] Protease activity can be measured using any suitable assay, in which a substrate is employed, that includes peptide bonds relevant for the specificity of the protease in question. Assay-pH and assay-temperature are likewise to be adapted to the protease in question. Examples of assay-pH-values are pH 6, 7, 8, 9, 10, or 11. Examples of assay-temperatures are 30, 35, 37, 40, 45, 50, 55, 60, 65, 70 or 80°C.

[0061] Examples of protease substrates are casein, such as Azurine-Crosslinked Casein (AZCL-casein). Two protease assays are described below in the "Materials & Methods"-section, of which the so-called "AZCL-Casein Assay" is the preferred assay.

[0062] In an embodiment, the protease used in a process of the invention is a fungal metallo protease derived from a strain of the genus *Thermoascus,* preferably a strain of *Thermoascus aurantiacus,* especially *Thermoascus aurantiacus* CGMCC No. 0670 (classified as EC 3.4.24.39).

[0063] In an embodiment, the protease variant has 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: 3 herein.

[0064] In an embodiment, the protease is a variant of the mature part of the metallo protease shown in SEQ ID NO: 2 disclosed in WO 2003/048353 or the mature part of SEQ ID NO: 1 in WO 2010/008841 and shown as SEQ ID NO: 3 herein with the following mutations:

- S5*+N26R+D79L+S87P+A112P+D142L;
- S5*+D79L+S87P+A112P+D142L;
- N26R+T46R+D79L+S87P+A112P+D142L;
- A27K+D79L+Y82F+S87G+D104P+A112P+A126V+D142L;
- A27K+D79L+Y82F+D104P+A112P+A126V+D142L;
- A27K+D79L+S87P+A112P+T124V+D142L;
- A27K+D79L+S87P+A112P+A126V+D142L;
- A27K+D79L+S87P+A112P+D142L;
- A27K+Y82F+S87G+D104P+A112P+A126V+D142L;
- A27K+Y82F+D104P+A112P+A126V+D142L;
- S36P+D79L+S87P+A112P+D142L;
- A37P+D79L+S87P+A112P+D142L;
- S38T+D79L+S87P+A112P+A126V+D142L;
- T46R+D79L+S87P+T116V+D142L;
- S49P+D79L+S87P+A112P+D142L;

- S50P+D79L+S87P+A112P+D142L;
- S70V+D79L+Y82F+S87G+Y97W+A112P+D142L;
- S70V+D79L+Y82F+S87G+A112P+D142L;
- D79L+P81R+S87P+A112P+D142L;
- D79L+Y82F+S87G+Y97W+D104P+A112P+D142L;
- D79L+Y82F+S87G+D104P+A112P+D142L;
- D79L+Y82F+S87G+A112P+A126V+D142L;
- D79L+Y82F+S87G+A112P+D142L;
- D79L+Y82F+S87P+A112P+T124V+D142L;
- D79L+Y82F+S87P+A112P+A126V+D142L;
- D79L+Y82F+S87P+A112P+D142L;
- D79L+S87P+N98C+A112P+G135C+D142L;
- D79L+S87P+D104P+A112P+D142L;
- D79L+S87P+A112P+T124V+A126V+D142L;
- D79L+S87P+A112P+T124V+D142L;
- D79L+S87P+A112P+D142L;
- D79L+S87P+A112P+D142L+T141C+M161C;
- Y82F+S87G+S70V+D79L+D104P+A112P+D142L;
- Y82F+S87G+D79L+D104P+A112P+A126V+D142L.

[0065] In an embodiment, the thermostable protease present and/or added during liquefaction step i) is derived from a strain of *Pyrococcus furiosus* having at least 80% identity, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 13 herein. The *Pyroccus furiosus* protease can be purchased from Takara Shuzo Co. Ltd, Japan.

[0066] The *Pyrococcus furiosus* protease is a thermostable protease. The commercial product *Pyrococcus furiosus* protease (Pfu S) was found to have a thermostability of 110% (80°C/70°C) and 103% (90°C/70°C) at pH 4.5 determined as described in Example 2 herein.

Carbohydrate-Source Generating Enzyme Present and/or Added During Liquefaction

[0067] According to the invention a carbohydrate-source generating enzyme, preferably a thermostable glucoamylase, may be present and/or added during liquefaction together with the thermostable alpha-amylase defined in claim 1 and the thermostable protease defined in claim 1. As mentioned above a pullulanase may also be present and/or added during liquefaction step i).

[0068] The term "carbohydrate-source generating enzyme" includes any enzymes generating fermentable sugars. A carbohydrate-source generating enzyme is capable of producing a carbohydrate that can be used as an energy-source by the fermenting organism(s) in question, for instance, when used in a process of the invention for producing a fermentation product, such as ethanol. The generated carbohydrates may be converted directly or indirectly to the desired fermentation product, preferably ethanol. According to the invention a mixture of carbohydrate-

source generating enzymes may be used. Specific examples include glucoamylase (being glucose generators), beta-amylase and maltogenic amylase (being maltose generators).

[0069] In a preferred embodiment, the carbohydrate-source generating enzyme is a thermostable glucoamylase. The carbohydrate-source generating enzyme, in particular thermostable glucoamylase, may be added together with or separately from the thermostable alpha-amylase and the thermostable protease.

[0070] In an embodiment, the carbohydrate-source generating enzyme, preferably a thermostable glucoamylase, has a Relative Activity heat stability at 85°C of at least 20%, at least 30%, preferably at least 35%. In an embodiment, the carbohydrate-generating enzyme is a glucoamylase having a relative activity at pH 4.5 of at least 80%, preferably at least 85%, preferably at least 90%, preferably at least 95%.

[0071] In a specific and preferred embodiment, the carbohydrate-source generating enzyme is a thermostable glucoamylase, preferably of fungal origin, preferably a filamentous fungi, such as from a strain of the genus *Penicillium,* especially a strain of *Penicillium oxalicum* disclosed as SEQ ID NO: 2 in PCT/CN10/071753 published as WO 2011/127802 and shown in SEQ ID NO: 9 or 14 herein.

[0072] In a preferred embodiment, the carbohydrate-source generating enzyme is a variant of the *Penicillium oxalicum* glucoamylase disclosed as SEQ ID NO: 2 in PCT/CN10/071753 published as WO 2011/127802 and shown in SEQ ID NOS: 9 and 14 herein, having a K79V substitution (using the mature sequence shown in SEQ ID NO: 14 for numbering). The K79V glucoamylase variant has reduced sensitivity to protease degradation relative to the parent as disclosed in co-pending US application No. 61/531,189.

[0073] In a specific embodiment, the carbohydrate-source generating enzyme is a glucoamylase, preferably derived from a strain of the genus *Penicillium,* especially a strain of *Penicillium oxalicum* disclosed in PCT/CN10/071753 published as WO 2011/127802. The glucoamylase may also be glucoamylase having 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 PCT/CN10/071753 published as WO 2011/127802 and shown as SEQ ID NO: 9 and 14 herein.

Pullulanase Present and/or Added During Liquefaction

[0074] Optionally a pullulanase may be present and/or added during liquefaction step i) together with the thermostable alpha-amylase defined in claim 1 and the thermostable protease defined in claim 1. As mentioned above a carbohydrate-source generating enzyme, preferably a thermostable glucoamylase, may also be present and/or added during liquefaction step i).

[0075] The pullulanase may be present and/or added during liquefaction step i) and/or

saccharification step ii) or simultaneous saccharification and fermentation.

[0076] 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.

[0077] Contemplated pullulanases according to the present invention include the pullulanases from *Bacillus amyloderamificans* disclosed in U.S. Patent No. 4,560,651, the pullulanase disclosed as SEQ ID NO: 2 in WO 01/151620, the *Bacillus deramificans* disclosed as SEQ ID NO: 4 in WO 01/151620, and the pullulanase from *Bacillus acidopullulyticus* disclosed as SEQ ID NO: 6 in WO 01/151620 and also described in FEMS Mic. Let. 115: 97-106 (1994).

[0078] Additional pullulanases contemplated according to the present invention included the pullulanases from *Pyrococcus woesei,* specifically from *Pyrococcus woesei* DSM No. 3773 disclosed in WO 92/02614, and the mature protein sequence disclosed as SEQ ID No: 6 herein.

[0079] In an embodiment, the pullulanase is a family GH57 pullulanase. In an embodiment the pullulanase includes an X47 domain as disclosed in US 61/289,040 published as WO 2011/087836. More specifically the pullulanase may be derived from a strain of the genus *Thermococcus,* including *Thermococcus litoralis* and *Thermococcus hydrothermalis,* such as the *Thermococcus hydrothermalis* pullulanase shown in SEQ ID NO: 11 truncated at site X4 right after the X47 domain (i.e., amino acids 1-782 in SEQ ID NOS: 11 and 12 herein). The pullulanase may also be a hybrid of the *Thermococcus litoralis* and *Thermococcus hydrothermalis* pullulanases or a *T. hydrothermalis*/*T. litoralis* hybrid enzyme with truncation site X4 disclosed in US 61/289,040 published as WO 2011/087836 and disclosed in SEQ ID NO: 12.

[0080] 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.

[0081] 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).

Carbohydrate-Source Generating Enzyme present and/or added during Saccharification and/or Fermentation

[0082] According to the invention a carbohydrate-source generating enzyme, preferably a glucoamylase, is present and/or added during saccharification and/or fermentation.

[0083] In a preferred embodiment, the carbohydrate-source generating enzyme is a glucoamylase, of fungal origin, preferably from a strain of *Aspergillus,* preferably *A. niger, A. awamori,* or *A. oryzae;* or a strain of *Trichoderma,* preferably *T. reesei*; or a strain of *Talaromyces,*

preferably *T. emersonii*.

Glucoamylase

[0084] According to the invention the glucoamylase present and/or added during saccharification and/or fermentation may be derived from any suitable source, e.g., derived from a microorganism or a plant. Preferred glucoamylases are of fungal or bacterial origin, selected from the group consisting of *Aspergillus* glucoamylases, in particular *Aspergillus nigerG1* or G2 glucoamylase (Boel et al., 1984, EMBO J. 3(5): 1097-1102), or variants thereof, such as those disclosed in WO 92/00381, WO 00/04136 and WO 01/04273 (from Novozymes, Denmark); the *A. awamori* glucoamylase disclosed in WO 84/02921, *Aspergillus oryzae* glucoamylase (Agric. Biol. Chem. 55(4): 941-949 (1991)), or variants or fragments thereof. Other *Aspergillus* glucoamylase variants include variants with enhanced thermal stability: G137A and G139A (Chen et al., 1996, Prot. Eng. 9: 499-505); D257E and D293E/Q (Chen et al., 1995, Prot. Eng. 8: 575-582); N182 (Chen et al., 1994, Biochem. J. 301: 275-281); disulphide bonds, A246C (Fierobe et al., 1996, Biochemistry 35: 8698-8704; and introduction of Pro residues in position A435 and S436 (Li et al., 1997, Protein Eng. 10: 1199-1204.

[0085] Other glucoamylases include *Athelia rolfsii* (previously denoted *Corticium rolfsii*) glucoamylase (see US patent no. 4,727,026 and (Nagasaka et al., 1998, "Purification and properties of the raw-starch-degrading glucoamylases from Corticium rolfsii, Appl. Microbiol. Biotechnol. 50:323-330), *Talaromyces* glucoamylases, in particular derived from *Talaromyces emersonii* (WO 99/28448), *Talaromyces leycettanus* (US patent no. Re. 32,153), *Talaromyces duponti, Talaromyces thermophilus* (US patent no. 4,587,215). In a preferred embodiment, the glucoamylase used during saccharification and/or fermentation is the *Talaromyces emersonii* glucoamylase disclosed in WO 99/28448.

[0086] Bacterial glucoamylases contemplated include glucoamylases from the genus *Clostridium,* in particular *C. thermoamylolyticum* (EP 135,138), and *C. thermohydrosulfuricum* (WO 86/01831) and *Trametes cingulata, Pachykytospora papyracea;* and *Leucopaxillus giganteus* all disclosed in WO 2006/069289; or *Peniophora rufomarginata* disclosed in WO 2007/124285; or a mixture thereof. Also hybrid glucoamylase are contemplated according to the invention. Examples the hybrid glucoamylases disclosed in WO 2005/045018. Specific examples include the hybrid glucoamylase disclosed in Tables 1 and 4 of Example 1.

[0087] In an embodiment the glucoamylase is derived from a strain of the genus *Pycnoporus,* in particular a strain of *Pycnoporus* as described in US 61/264,977 published as WO 2011/066576 (SEQ ID NO: 2, 4 or 6), or from a strain of the genus *Gloephyllum,* in particular a strain of *Gloephyllum* as described in US 61/406,741 published as WO 2011/068803 (SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16) or a strain of the genus *Nigrofomes,* in particular a strain of *Nigrofomes sp.* disclosed in US 61/411,044 or PCT/US10/058375 (SEQ ID NO: 2). Contemplated are also glucoamylases which exhibit a high identity to any of above mention glucoamylases, i.e., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or even 100% identity to any one of the mature parts of the enzyme sequences mentioned above.

[0088] Contemplated are also glucoamylases which exhibit a high identity to any of above mention glucoamylases, i.e., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or even 100% identity to the mature enzymes sequences mentioned above.

[0089] Glucoamylases may in an embodiment be added 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.

[0090] 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™ ECXEL and AMG™ E (from Novozymes A/S); OPTIDEX™ 300, GC480, GC417 (from Genencor Int.); AMIGASE™ and AMIGASE™ PLUS (from DSM); G-ZYME™ G900, G-ZYME™ and G990 ZR (from Genencor Int.).

Maltogenic Amylase

[0091] The carbohydrate-source generating enzyme present and/or added during saccharification and/or fermentation may also be a maltogenic alpha-amylase. A "maltogenic alpha-amylase" (glucan 1,4-alpha-maltohydrolase, E.C. 3.2.1.133) is able to hydrolyze amylose and amylopectin to maltose in the alpha-configuration. A maltogenic amylase from *Bacillus stearothermophilus* strain NCIB 11837 is commercially available from Novozymes A/S. Maltogenic alpha-amylases are described in US Patent nos. 4,598,048, 4,604,355 and 6,162,628. The maltogenic amylase may in a preferred embodiment be added in an amount of 0.05-5 mg total protein/gram DS or 0.05-5 MANU/g DS.

A Composition Comprising Alpha-amylase and Protease

[0092] In this aspect, the invention relates to composition comprising an alpha-amylase and a protease, wherein the

1. i) alpha-amylase is a variant of the *Bacillus stearothermophilus* alpha-amylase shown in SEQ ID NO: 1 with a double deletion in 1181 + G182 and substitution N193F, and further comprising mutations selected from the group of:
    - V59A+Q89R+G112D+E129V+K177L+R179E+K220P+N224L+Q254S;
    - V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
    - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+D269E+D281N;
    - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+I270L;
    - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+H274K;
    - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+Y276F;
    - V59A+E129V+R157Y+K177L+R179E+K220P+N224L+S242Q+Q254S;
    - V59A+E129V+K177L+R179E+H208Y+K220P+N224L+S242Q+Q254S;
    - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
    - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+H274K;

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

which variant has at least 80% identity, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 1 and has a T½ (min) at pH 4.5, 85°C, 0.12 mM $CaCl_2$) of at least 10; and

2. ii) a variant of a metallo protease which variant has at least 80% identity, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 3 herein and has a thermostability value of more than 20% determined as Relative Activity at 80°C/70°C; or
   - a *Pyrococcus furiosus*, protease which has at least 80% identity to SEQ ID NO: 13 herein and has a thermostability value of more than 20% determined as Relative Activity at 80°C/70°C.

[0093] The composition optionally further comprises a carbohydrate-source generating enzyme. Said carbohydrate-source generating enzyme may be a thermostable glucoamylase having a Relative Activity heat stability at 85°C of at least 20%, at least 30%, preferably at least 35%.

[0094] . Alpha-amylase variants are described further in the "Alpha-Amylase Present and/or Added During Liquefaction"-section above. The alpha- amylase may have 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.

[0095] In an embodiment, the alpha-amylase is selected from the group of *Bacillus stearomthermphilus* alpha-amylase variants, in particular truncated to be 491 amino acids long, with mutations selected from the group of:

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

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

[0096] The protease has a thermostability of:

1. i) more than 30%, more than 40%, more than 50%, more than 60%, more than 70%, more than 80%, more than 90% determined as Relative Activity at 80°C/70°C.

[0097] In a specific preferred embodiment the protease is a variant of the metallo protease derived from *Thermoascus aurantiacus* disclosed as the mature part of SEQ ID NO: 3 herein with mutations selected from the group of:

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

[0098] In another preferred embodiment, the protease is derived from a strain of *Pyrococcus furiosus* shown in SEQ ID NO: 13 herein having a thermostability value of more than 20% determined as Relative Activity at 80°C/70°C.

[0099] In another embodiment, the protease is one disclosed in SEQ ID NO: 13 herein or a protease having at least 80% identity, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 13 herein. The *Pyroccus furiosus* protease can be purchased from Takara Shuzo Co. Ltd, Japan.

[0100] The *Pyrococcus furiosus* protease is a thermostable protease. The commercial product *Pyrococcus furiosus* protease (Pfu S) was found to have a thermostability of 110% (80°C/70°C) and 103% (90°C/70°C) at pH 4.5 determined as described in Example 2 herein.

[0101] It should be understood that these proteases are only examples. Any protease disclosed above in the "Protease Present and/or Added During Liquefaction" section above may be used as the protease component in a composition of the invention.

[0102] A composition of the invention may optionally further comprise a carbohydrate-source generating enzyme, in particular a glucoamylase, which has a heat stability at 85°C, pH 5.3, of at least 30%, preferably at least 35%.

[0103] In a preferred embodiment, the carbohydrate-source generating enzyme is a glucoamylase having a relative activity of at least 80%, preferably at least 85%, preferably at least 90% at pH 4.5.

[0104] In a preferred embodiment carbohydrate-source generating enzyme is a glucoamylase

having a pH stability at pH 4.5 of at least at least 80%, at least 85%, at least 90%, at least 95%, at least 100%.

[0105] Determination heat stability, and pH stability is described in the Example 4.

[0106] In a specific embodiment, the carbohydrate-source generating enzyme is a glucoamylase, preferably derived from a strain of the genus *Penicillium,* especially a strain of *Penicillium oxalicum* disclosed in PCT/CN10/071753 published as WO 2011/127802. The glucoamylase may also be glucoamylase having 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 PCT/CN10/071753 published as WO 2011/127802.

[0107] In a preferred embodiment, the carbohydrate-source generating enzyme is a variant of the *Penicillium oxalicum* glucoamylase disclosed as SEQ ID NO: 2 in PCT/CN10/071753 published as WO 2011/127802 and shown in SEQ ID NO: 9 and 14 herein, having a K79V substitution (using the mature sequence shown in SEQ ID NO: 14 for numbering). The K79V glucoamylase variant has reduced sensitivity to protease degradation relative to the parent as disclosed in co-pending US application No. 61/531,189.

[0108] A composition of the invention may further comprise a pullulanase. In a preferred embodiment, the pullulanase includes an X47 domain as disclosed in US 61/289,040 published as WO 2011/087836.

[0109] Specifically, the pullulanase may be derived from a strain from the genus *Thermococcus,* including *Thermococcus litoralis* and *Thermococcus hydrothermalis* or a hybrid thereof.

[0110] The pullulanase may be *Thermococcus hydrothermalis* pullulanase truncated at site X4 or a *Thermococcus hydrothermalis*/*T. litoralis* hybrid enzyme with truncation site X4 as disclosed in US 61/289,040 published as WO 2011/087836 or shown in SEQ ID NO: 12 herein.

[0111] In an embodiment, the ratio of enzyme protein (weight basis) between the components in a composition of the invention may be:

Alpha-Amylase: Glucoamylase: Protease: 0.1-10 : 0.5-50 : 0.1-7, such as 0.5-3 : 1-30 : 0.5-2, such as 1-2 : 5-20 : 0.5-2.

Materials & Methods

Materials:

[0112]

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

Alpha-Amylase 1093: *Bacillus stearothermophilus* alpha-amylase with the mutations I181*+G182*+N193F+E129V+K177L+R179E truncated to 491 amino acids (SEQ ID NO: 1)

Alpha-Amylase 1407: *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 1236: *Bacillus stearothermophilus* alpha-amylase with the mutations I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S truncated to 491 amino acids (SEQ ID NO: 1)

Protease 136: 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 with the following mutations:

D79L+Y82F+S87P+A112P+A126V+D142L

Protease 196: 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 with the following mutations:

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

Protease 077: 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 with the following mutations: A27K+D79L+S87P+A112P+D142L.

Protease Pfu: Protease derived from *Pyrococcus furiosus* purchased from Takara Bio Inc. (Japan) as Pfu Protease S (activity 10.5 mg/mL) and also shown in SEQ ID NO: 13 herein.

Glucoamylase PO: Mature part of the *Penicillium oxalicum* glucoamylase disclosed as SEQ ID NO: 2 in PCT/CN10/071753 published as WO 2011/127802 and shown in SEQ ID NO: 9 and 14 herein.

Glucoamylase PE001: Variant of the *Penicillium oxalicum* glucoamylase having a K79V substitution using the mature sequence shown in SEQ ID NO: 14 for numbering.

Glucoamylase BL: Blend of *Tamaromyces emersonii* glucoamylase disclosed in WO 99/28448 as SEQ ID NO: 7 and *Trametes cingulata* glucoamylase disclosed in WO 06/069289 in a ratio of about 9:1.

Glucoamylase BL2: Blend comprising *Talaromyces emersonii* glucoamylase disclosed in WO 99/28448, *Trametes cingulata* glucoamylase disclosed in WO 06/69289, and *Rhizomucor pusillus* alpha-amylase with *Aspergillus niger* glucoamylase linker and SBD disclosed as V039 in Table 5 in WO 2006/069290 as side activities (ratio about 65:15:1)

Substrate in Example 9: Ground corn from Corn LP, Iowa, USA (84.19% DS) and backset (6.27%

DS).

Pullulanase TH: Pullulanase from *Thermococcus hydrothermalis* shown in SEQ ID NO: 11 herein.

Yeast: RED STAR ETHANOL RED™ available from Red Star/Lesaffre, USA.

Methods

[0113] Identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "identity".

[0114] 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.

[0115] "Align" is part of the FASTA package version v20u6 (see Pearson and Lipman, 1988, "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448, and 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", Smith and Waterman, 1981, J. Mol. Biol. 147:195-197).

Protease assays

AZCL-casein assay

[0116] 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 20 min) is used as a blank. After incubation the reaction is stopped by transferring the microtiter plate onto ice and the coloured solution is separated from the solid by centrifugation at 3000 rpm for 5 minutes at 4°C. 60 microL of supernatant is transferred to a microtiter plate and the absorbance at 595 nm is measured using a BioRad Microplate Reader.

pNA-assay

[0117] 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)

[0118] Glucoamylase activity may be measured in Glucoamylase Units (AGU).

[0119] 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.

[0120] 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

[0121] A folder (EB-SM-0131.02/01) describing this analytical method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Alpha-amylase activity (KNU)

[0122] 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.

[0123] 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.

[0124] 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)

[0125] 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.

[0126] 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.

EXAMPLES

Example 1

Stability of Alpha-Amylase Variants

[0127] 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)) 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).

[0128] 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.

[0129] 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.

[0130] Using this assay setup the half life time was determined for the reference alpha-amylase and variant thereof as shown in Table 1.

Table 1

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

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 $CaCl_2$)**
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+ N224L+S242Q+Q254S	141	41	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+H208Y+K220P+ N224L+S242Q+Q254S	>180	62	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S	>180	49	>480
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S+H274K	>180	53	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S+Y276F	>180	57	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S+D281N	>180	37	ND
Reference Alpha-Amylase A with the substitutions V59A+E129V+ K177L+R179E+K220P+N224L+ S242Q+Q254S+M284T	>180	51	ND
Reference Alpha-Amylase A with the			

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 $CaCl_2$)**
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 A91 L+M96I+ E129V+K177L+R179E+K220P+ N224L+S242Q+Q254S	>180	38	ND
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E	57	11	402
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+S242Q+ Q254S	174	44	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+S242Q+ Q254S+Y276F+L427M	>180	49	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+S242Q+ Q254S+M284T	>180	49	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+S242Q+ Q254S+N376*+I377*	177	36	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+Q254S	94	13	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+K220P+N224L+Q254S+ M284T	129	24	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179E+S242Q	148	30	>480
Reference Alpha-Amylase A with the substitutions E129V+K177L+ R179V	78	9	>480
Reference Alpha-Amylase A with the			

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 $CaCl_2$)
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			

[0131] The results demonstrate that the alpha-amylase variants have a significantly greater half-life and stability than the reference alpha-amylase.

Example 2

Preparation of Protease Variants and Test of Thermostability

[0132] Chemicals used were commercial products of at least reagent grade.

Strains and plasmids:

[0133] *E. coli* DH12S (available from Gibco BRL) was used for yeast plasmid rescue. pJTP000 is a *S. cerevisiae* and *E. coli* shuttle vector under the control of TPI promoter, constructed from pJC039 described in WO 01/92502, in which the *Thermoascus aurantiacus* M35 protease gene (WO 03/048353) has been inserted.

[0134] *Saccharomyces cerevisiae* YNG318 competent cells: MATa Dpep4[cir+] ura3-52, leu2-D2, his 4-539 was used for protease variants expression. It is described in J. Biol. Chem. 272(15): 9720-9727 (1997).

Media and substrates

[0135]

10X Basal solution: Yeast nitrogen base w/o amino acids (DIFCO) 66.8 g/L, succinate 100 g/l, NaOH 60 g/l.

SC-glucose: 20% glucose (i.e., a final concentration of 2% = 2 g/100 mL)) 100 mL/L, 5% threonine 4 mL/L, 1% tryptophan10 ml/l, 20% casamino acids 25 ml/l, 10 X basal solution 100 ml/l. The solution is sterilized using a filter of a pore size of 0.20 micrometer. Agar (2%) and H_2O (approx. 761 mL) is autoclaved together, and the separately sterilized SC-glucose solution is added to the agar solution.

YPD: Bacto peptone 20 g/l, yeast extract 10 g/L, 20 % glucose 100 mL/L.

YPD+Zn: YPD+0.25 mM $ZnSO_4$.

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

96 well Zein micro titre plate:

[0136] Each well contains 200 microL of 0.05-0.1 % of zein (Sigma), 0.25 mM $ZnSO_4$ and 1 % of agar in 20 mM sodium acetate buffer, pH 4.5.

DNA manipulations

[0137] Unless otherwise stated, DNA manipulations and transformations were performed using standard methods of molecular biology as described in Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab. Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology", John Wiley and Sons, 1995; Harwood, C. R. and Cutting, S. M. (Eds.).

Yeast transformation

[0138] Yeast transformation was performed using the lithium acetate method. 0.5 microL of vector (digested by restriction endnucleases) and 1 microL of PCR fragments is mixed. The DNA mixture, 100 microL of YNG318 competent cells, and 10 microL of YEAST MAKER carrier DNA (Clontech) is added to a 12 mL polypropylene tube (Falcon 2059). Add 0.6 mL PEG/LiAc solution and mix gently. Incubate for 30 min at 30°C, and 200 rpm followed by 30 min at 42°C (heat shock). Transfer to an eppendorf tube and centrifuge for 5 sec. Remove the supernatant and resolve in 3 mL of YPD. Incubate the cell suspension for 45 min at 200 rpm at 30°C. Pour the suspension to SC-glucose plates and incubate 30°C for 3 days to grow colonies. Yeast total DNA are extracted by Zymoprep Yeast Plasmid Miniprep Kit (ZYMO research).

DNA sequencing

[0139] *E. coli* transformation for DNA sequencing was carried out by electroporation (BIO-RAD Gene Pulser). DNA Plasmids were prepared by alkaline method (Molecular Cloning, Cold Spring Harbor) or with the Qiagen® Plasmid Kit. DNA fragments were recovered from agarose gel by the Qiagen gel extraction Kit. PCR was performed using a PTC-200 DNA Engine. The ABI PRISMTM 310 Genetic Analyzer was used for determination of all DNA sequences.

Construction of protease expression vector

[0140] The *Themoascus* M35 protease gene was amplified with the primer pair Prot F (SEQ ID NO: 4) and Prot R (SEQ ID NO: 5). The resulting PCR fragments were introduced into *S. cerevisiae* YNG318 together with the pJC039 vector (described in WO 2001/92502) digested with restriction enzymes to remove the *Humicola insolens* cutinase gene.

[0141] The Plasmid in yeast clones on SC-glucose plates was recovered to confirm the internal sequence and termed as pJTP001.

Construction of yeast library and site-directed variants

[0142] Library in yeast and site-directed variants were constructed by SOE PCR method (Splicing by Overlap Extension, see "PCR: A practical approach", p. 207-209, Oxford University press, eds. McPherson, Quirke, Taylor), followed by yeast *in vivo* recombination.

General primers for amplification and sequencing

[0143] The primers AM34 (SEQ ID NO: 6) and AM35 (SEQ ID NO:7) were used to make DNA fragments containing any mutated fragments by the SOE method together with degenerated primers (AM34 + Reverse primer and AM35 + forward primer) or just to amplify a whole protease gene (AM34 + AM35).

PCR reaction system:	Conditions:	
48.5 microL H_2O	1	94°C 2 min
2 beads puRe Taq Ready-To-Go PCR (Amersham Biosciences)	2	94°C 30 sec
0.5 microL X 2 100 pmole/microL of primers	3	55°C 30 sec
0.5 microL template DNA	4	72°C 90 sec
	2-4	25 cycles
	5	72°C 10 min

[0144] DNA fragments were recovered from agarose gel by the Qiagen gel extraction Kit. The resulting purified fragments were mixed with the vector digest. The mixed solution was introduced into *Saccharomyces cerevisiae* to construct libraries or site-directed variants by *in vivo* recombination.

Relative activity assay

[0145] Yeast clones on SC-glucose were inoculated to a well of a 96-well micro titre plate containing YPD+Zn medium and cultivated at 28°C for 3 days. The culture supernatants were applied to a 96-well zein micro titer plate and incubated at at least 2 temperatures (ex., 70°C and 80°C) for more than 4 hours or overnight. The turbidity of zein in the plate was measured as A630 and the relative activity (higher/lower temperatures) was determined as an indicator of thermoactivity improvement. The clones with higher relative activity than the parental variant were selected and the sequence was determined.

Remaining activity assay

[0146] Yeast clones on SC-glucose were inoculated to a well of a 96-well micro titre plate and cultivated at 28°C for 3 days. Protease activity was measured at 65°C using azo-casein (Megazyme) after incubating the culture supernatant in 20 mM sodium acetate buffer, pH 4.5, for 10 min at a certain temperature (80°C or 84°C with 4°C as a reference) to determine the remaining activity. The clones with higher remaining activity than the parental variant were selected and the sequence was determined.

Azo-casein assay

[0147] 20 microL of samples were mixed with 150 microL of substrate solution (4 mL of 12.5% azo-casein in ethanol in 96 mL of 20 mM sodium acetate, pH 4.5, containing 0.01 % triton-100 and 0.25 mM $ZnSO_4$) and incubated for 4 hours or longer.

[0148] After adding 20 microL/well of 100 % trichloroacetic acid (TCA) solution, the plate was centrifuge and 100 microL of supernatants were pipette out to measure A440.

Expression of protease variants in *Aspergillus oryzae*

[0149] The constructs comprising the protease variant genes were used to construct expression vectors for *Aspergillus*. The *Aspergillus* expression vectors consist of an expression cassette based on the *Aspergillus niger* neutral amylase II promoter fused to the *Aspergillus nidulans* triose phosphate isomerase non translated leader sequence (Pna2/tpi) and the *Aspergillus niger* amyloglycosidase terminator (Tamg). Also present on the plasmid was the *Aspergillus selective*

marker amdS from *Aspergillus nidulans* enabling growth on acetamide as sole nitrogen source. The expression plasmids for protease variants were transformed into *Aspergillus* as described in Lassen et al., 2001, Appl. Environ. Microbiol. 67: 4701-4707. For each of the constructs 10-20 strains were isolated, purified and cultivated in shake flasks.

Purification of expressed variants

[0150]

1. 1. Adjust pH of the 0.22 µm filtered fermentation sample to 4.0.
2. 2. Put the sample on an ice bath with magnetic stirring. Add $(NH_4)_2SO_4$ in small aliquots (corresponding to approx. 2.0-2.2 M $(NH_4)_2SO_4$ not taking the volume increase into account when adding the compound).
3. 3. After the final addition of $(NH_4)_2SO_4$, incubate the sample on the ice bath with gentle magnetic stirring for min. 45 min.
4. 4. Centrifugation: Hitachi himac CR20G High-Speed Refrigerated Centrifuge equipped with R20A2 rotor head, 5°C, 20,000 rpm, 30 min.
5. 5. Dissolve the formed precipitate in 200 mL 50 mM Na-acetate pH 4.0.
6. 6. Filter the sample by vacuum suction using a 0.22 micro m PES PLUS membrane (IWAKI).
7. 7. Desalt/buffer-exchange the sample to 50 mM Na-acetate pH 4.0 using ultrafiltration (Vivacell 250 from Vivascience equipped with 5 kDa MWCO PES membrane) overnight in a cold room. Dilute the retentate sample to 200 ml using 50 mM Na-acetate pH 4.0.
   The conductivity of sample is preferably less than 5 mS/cm.
8. 8. Load the sample onto a cation-exchange column equilibrated with 50 mM Na-acetate pH 4.0. Wash unbound sample out of the column using 3 column volumes of binding buffer (50 mM Na-acetate pH 4.0), and elute the sample using a linear gradient, 0-100% elution buffer (50 mM Na-acetate + 1 M NaCl pH 4.0) in 10 column volumes.
9. 9. The collected fractions are assayed by an endo-protease assay (cf. below) followed by standard SDS-PAGE (reducing conditions) on selected fractions. Fractions are pooled based on the endo-protease assay and SDS-PAGE.

Endo-protease assay

[0151]

1. 1. Protazyme OL tablet/5 ml 250 mM Na-acetate pH 5.0 is dissolved by magnetic stirring (substrate: endo-protease Protazyme AK tablet from Megazyme - cat. # PRAK 11/08).
2. 2. With stirring, 250 microL of substrate solution is transferred to a 1.5 mL Eppendorf tube.
3. 3. 25 microL of sample is added to each tube (blank is sample buffer).
4. 4. The tubes are incubated on a Thermomixer with shaking (1000 rpm) at 50°C for 15 minutes.
5. 5. 250 microL of 1 M NaOH is added to each tube, followed by vortexing.

6. 6. Centrifugation for 3 min. at 16,100 × G and 25°C.
7. 7. 200 microL of the supernatant is transferred to a MTP, and the absorbance at 590 nm is recorded.

Table 2. Relative Activity of protease variants. Numbering of substitution(s) starts from N-terminal of the mature peptide in amino acids 1 to 177 of SEQ ID NO: 3.

Variant	**Substitution(s) and/or deletion(s)**	**Remaining Activity**	
		80°C	**84°C**
JTP082	ΔS5/D79L/S87P/A112P/D142L		53%
JTP091	D79L/S87P/A112P/T124V/D142L	43%	
JTP092	ΔS5/N26R/D79L/S87P/A112P/D142L		60%
JTP095	N26R/T46R/D79L/S87P/A112P/D142L		62%
JTP096	T46R/D79L/S87P/T116V/D142L		67%
JTP099	D79L/P81 R/S87P/A112P/D142L		80%
JTP101	A27K/D79L/S87P/A112P/T124V/D142L	81%	
JTP116	D79L/Y82F/S87P/A112P/T124V/D142L	59%	
JTP117	D79L/Y82F/S87P/A112P/T124V/D142L	94%	
JTP127	D79L/S87P/A112P/T124V/A126V/D142L	53%	

Table 3 Relative Activity of protease variants. Numbering of substitution(s) starts from N-terminal of the mature peptide in amino acids 1 to 177 of SEQ ID NO: 3.

Variant	**Substitutions**	**Relative Activity**	
		80°C/70°C	**85°C/70°C**
JTP050	D79L S87P A112P D142L	23%	9%
JTP134	D79LY82F S87P A112P D142L	40%	
JTP135	S38T D79LS87P A112P A126V D142L	62%	
JTP136	D79LY82F S87P A112P A126V D142L	59%	
JTP137	A27K D79L S87P A112P A126V D142L	54%	
JTP145	S49P D79L S87P A112P D142L	59%	
JTP146	S50P D79L S87P A112P D142L	63%	
JTP148	D79L S87P D104P A112P D142L	64%	
JTP161	D79L Y82F S87G A112P D142L	30%	12%
JTP180	S70V D79L Y82F S87G Y97W A112P D142L	52%	
JTP181	D79L Y82F S87G Y97W D104P A112P D142L	45%	
JTP187	S70V D79L Y82F S87G A112P D142L	45%	

Table 3 Relative Activity of protease variants. Numbering of substitution(s) starts from N-terminal of the mature peptide in amino acids 1 to 177 of SEQ ID NO: 3.

Variant	Substitutions	Relative Activity	
		80°C/70°C	85°C/70°C
JTP188	D79L Y82F S87G D104P A112P D142L	43%	
JTP189	D79L Y82F S87G A112P A126V D142L	46%	
JTP193	Y82F S87G S70V D79L D104P A112P D142L		15%
JTP194	Y82F S87G D79L D104P A112P A126V D142L		22%
JTP196	A27K D79L Y82F S87G D104P A112P A126V D142L		18%

Table 4 Relative Activity of protease variants. Numbering of substitution(s) starts from N-terminal of the mature peptide in amino acids 1 to 177 of SEQ ID NO: 3.

		Relative Activity
Variant	Substitutions	80°C/70°C
JTP196	A27K D79L Y82F S87G D104P A112P A126V D142L	55%
JTP210	A27K Y82F S87G D104P A112P A126V D142L	36%
JTP211	A27K D79L Y82F D104P A112P A126V D142L	44%
JTP213	A27K Y82F D104P A112P A126V D142L	37%

Example 3

Temperature Profile of Selected Protease Variants Using Purified Enzymes

[0152] Selected protease variants showing good thermostability were purified and the purified enzymes were used in a zein-BCA assay as described below. The remaining protease activity was determined at 60°C after incubation of the enzyme at elevated temperatures as indicated for 60 min.

Zein-BCA assay:

[0153] Zein-BCA assay was performed to detect soluble protein quantification released from zein

by variant proteases at various temperatures.

Protocol:

[0154]

1. 1) Mix 10 microL of 10 micro g/mL enzyme solutions and 100 microL of 0.025% zein solution in a micro titer plate (MTP).
2. 2) Incubate at various temperatures for 60 min.
3. 3) Add 10 microL of 100% trichloroacetic acid (TCA) solution.
4. 4) Centrifuge MTP at 3500rpm for 5 min.
5. 5) Take out 15 microL to a new MTP containing 100 microL of BCA assay solution (Pierce Cat#:23225, BCA Protein Assay Kit).
6. 6) Incubate for 30 min. at 60°C.
7. 7) Measure A562.

[0155] The results are shown in Table 5. All of the tested protease variants showed an improved thermostability as compared to the wild type (WT) protease.

Table 5 Zein-BCA assay

WT/Variant	Sample incubated 60 min at indicated temperatures (°C) (micro g/mL Bovine serum albumin equivalent peptide released)						
	60°C	70°C	75°C	80°C	85°C	90°C	95°C
WT	94	103	107	93	58	38	
JTP050 (D79L+S87P+A112P+D142L)	86	101	107	107	104	63	36
JTP077 (A27K+D79L+S87P+A112P+D142L)	82	94	104	105	99	56	31
JTP188 (D79L+Y82F+S87G+D104P+A112P+ D142L)	71	83	86	93	100	75	53
JTP196 (A27K+D79L+Y82F+S87G+D104P+ A112P+A126V+D142L)	87	99	103	106	117	90	38

Example 4

Characterization of *Penicillium oxalicum* glucoamylase

[0156] The *Penicillium oxalicum* glucoamylase is disclosed WO 2011/127802 and in SEQ ID NO: 9 herein.

[0157] **Substrate.** Substrate: 1% soluble starch (Sigma S-9765) in deionized water Reaction buffer: 0.1 M Acetate buffer at pH 5.3

[0158] Glucose concentration determination kit: Wako glucose assay kit (LabAssay glucose, WAKO, Cat# 298-65701).

[0159] **Reaction condition.** 20 microL soluble starch and 50 microL acetate buffer at pH5.3 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.

[0160] The glucose concentration was determined by Wako kits.

[0161] All the work carried out in parallel.

[0162] **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 6.

Table 6 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

[0163] 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.

[0164] **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.

[0165] The results are shown in Table 7.

Table 7 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

[0166] 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.

[0167] **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, 1 mM $CaCl_2$, 150 mM 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.

[0168] The results are shown in Table 8.

Table 8 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

[0169] 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.

[0170] **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.

[0171] The results are shown in Table 9.

Table 9 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

[0172] From the results it can be seen that *Penicillium oxalicum* glucoamylase, is stable from pH 3 to pH 7 after preincubation for 20 hours and it decreases its activity at pH 8.

Example 5

Improved Ethanol Production Process

[0173] Mash Preparation: Corn mashes were prepared through liquefaction in an 85°C water bath for 2 hours. The dry solids (DS) content was around 30-33% and the backset ratio around 30%. Mash Preparation: Corn slurries were prepared for liquefaction by weighing out the specified amounts of ground corn, backset, and tap water into Nalgene bottles. Slurries were pH-adjusted to

either 5.80 (Control) using 50% w/w NaOH or 4.50 (Study A, B) or 4.80 (Study C, D, E, F) using 40% v/v H_2SO_4. Control mashes using Alpha-Amylase A were made at pH 5.8. Aliquots of enzyme stock solutions were added. Bottles were tightly capped and placed into the water bath. Slurries were shaken vigorously once every 5 minutes for the first 30 minutes and then once every 30 minutes thereafter for a total of 2 hours. Mashes were immediately cooled in an ice bath. Urea and penicillin were then added to each mash to reach concentrations of 500 and 3 ppm, respectively.

[0174] Fermentation Setup: Mashes were adjusted to pH 5.0 using 40% H_2SO_4 or 50% NaOH. Approximately 5 g of each mash was transferred into pre-weighed 15 mL plastic Falcon centrifuge tubes for fermentation. Typically, five replicate fermentations were prepared for each treatment. A small hole was drilled into the lid of each tube to allow for CO_2 release during fermentation. Following mash transfer, all tubes were reweighed to obtain their initial sample weights. Into each tube was then added 100 microL of rehydrated RED STAR ETHANOL RED yeast (rehydrated by weighing 5.5 g of dry yeast into a 150 mL Erlenmeyer flask, adding 100 mL of tap water and stirring in a 32°C water bath for 30 minutes), an aliquot of diluted Glucoamylase BL (diluted in deionized water) needed to reach starting concentrations of 0.50 AGU/g DS. Deionized water was added to each tube such that the total volume of liquid added to each tube relative to the sample weight was the same. All tubes were then reweighed and then placed into a water bath set at 32°C. Fermentation was typically allowed to progress for 54 hours (if nothing else is stated). Tubes were vigorously vortexed after approximately 7 hours and then vortexed and reweighed twice per day for the remaining fermentation time. The grams of ethanol produced per gram of dry solids in each tube were calculated from the weight loss data according to the following equation:

$$g\ ethanol\ /\ g\ DS = \frac{g\ CO_2\ weight\ loss \times \frac{1\ mol\ CO_2}{44.0098\ g\ CO_2} \times \frac{1\ mol\ ethanol}{1\ mol\ CO_2} \times \frac{46.094\ g\ ethanol}{1\ mol\ ethanol}}{g\ corn\ in\ tube \times \%DS\ of\ corn}$$

[0175] Typically, 4 replicate tubes for each treatment were pulled after 54 hours of fermentation for HPLC analysis. Pulled samples were treated with 50 microL of 40% H_2SO_4 to stop fermentation and vortexed thoroughly. The samples were then centrifuged at 1460xg for 10 minutes and then filtered into HPLC vials through 0.45 micro m syringe filters. HPLC analysis was finally conducted on the samples to quantify the amounts of ethanol.

RESULTS

[0176] An overview of the results is provided in Table 10.

Table 10: The doses of enzymes are listed in parentheses for each and are expressed as micro g EP/g DS.

Study	pH	Enzymes In liquefaction step i).	Glucoamylase In SSF	HPLC EtOH vs Reference Alpha-Amylase A (Control)
A	4.5	Alpha-Amylase 1093 (1.4)	Glucoamylase BL	3.0%
		Protease 077 (2)		
		Pullulanase TH (2)		

Study	pH	Enzymes In liquefaction step i).	Glucoamylase In SSF	HPLC EtOH vs Reference Alpha-Amylase A (Control)
B	4.5	Alpha-amylase 1093	Glucoamylase BL	1.6%
		(2.75) Protease 077 (5)		
		Pullulanase TH (2)		
C	4.8	Alpha-amylase 1236 (2)	Glucoamylase BL	4.7%
		Protease 136 (2)		
		Glucoamylase PO (15)		
D	4.8	Alpha-Amylase 1093 (2)	Glucoamylase BL	4.2% (48 hrs)
		Protease 180 (2,1)		
		Glucoamylase PO (10)		
E	4.8	Alpha-Amylase 1236 (2)	Glucoamylase BL	7.1%
		Protease 188 (2)		
		Glucoamylase PO (15)		
F	4.8	Alpha-Amylase 1407 (1)	Glucoamylase BL	4.8% (at 72 hrs)
		Protease 196 (2)		
		Glucoamylase PO (2)		
*measured at 54 hours unless otherwise noted.				

Example 6

Whole corn liquefaction and SSF process using the *P.oxalicum* AMG variant (PE001)

[0177] The *Penicillium oxalicum* glucoamylase (Glucoamylase PO) variant, Glucoamylase PE001, showing reduced sensitivity to protease degradation, was tested in both whole corn liquefaction and starch saccharification (shown in next section). For the whole corn liquefactions, the Glucoamylase PE001 enzyme was added in different doses with a low pH amylase variant, Alpha-Amylase 1407. In some liquefactions, the Glucoamylase PE001 variant was tested with both the low pH amylase Alpha-Amylase 1407 and the thermostable protease Protease 196. In all experiments, the liquefactions were done using the automated system called the "Lab-O-Mat". This instrument

controls the temperature and provides constant mixing. The other experimental conditions were: pH was 4.8 (for the liquefacts containing the Alpha-Amylase 1407 low pH amylase) or 5.8 (for the Alpha-Amylase A control), 32% dry solids, 85°C, 2 hours total time. The enzyme dosing schemes are shown in Table 11. The liquefied mashes were saccharified and fermented using Glucoamylase BL2 (at a dose of 0.5 AGU/gram dry solids for 54 hours at 32°C).

Table 11. Enzyme dosing scheme for the three whole corn liquefaction experiments done using Glucoamylase PO protease nicking stable variant, i.e., Glucoamylase PE001.

Alpha-Amylase (Dose)	Protease (Dose)	Glucoamylase (Dose)
Alpha-Amylase A	None	None
(0.02% w/w corn)		
Alpha-Amylase 1407	None	None
(1.4 µg EP/g DS)		
Alpha-Amylase 1407	None	Glucoamylase PO
(1.4 µg EP/g DS)		(P3HK)
		(10 µg EP/g DS)
Alpha-Alpha 1407	None	Glucoamylase PE001
(1.4 µg EP/g DS)		(10 µg EP/g DS)
Alpha-Amylase 1407	Protease 196	Glucoamylase PO
(1.4 µg EP/g DS)	(1 µg EP/g DS)	(P3HK)
		(10 µg EP/g DS)
Alpha-Amylase 1407	Protease 196	Glucoamylase PE001
(1.4 µg EP/g DS)	(1 µg EP/g DS)	(10 µg EP/g DS)

[0178] The HPLC quantified ethanol titers (in grams per liter) are shown in Table 12.

Table 12. Average ethanol titers and associated standard deviations, in grams per liter. The Protease196 is a temperature stable protease described in WO 2011/072191 and Alpha-Amylase 1407 is a low pH amylase described in WO 2011/082425.

Treatment	Ethanol (Average ± Standard deviation; grams/liter)
Alpha-Amylase A control	126.4 ± 0.3
Alpha-Amylase 1407 (low pH alpha-amylase variant) control	126.7 ± 0.3
Glucoamylase PO (wild-type) P3HK (10 µg EP/g DS)	127.2 ± 0.4
Glucoamylase PE001 variant (10 µg EP/g DS)	127.1 ± 0.5
Glucoamylase PO (wild-type) P3HK (10 µg EP/g DS) + Protease 196 (1 µg EP/g DS)	127.6 ± 0.4
Glucoamylase PE001 variant (10 µg EP/g DS) + Protease 196 (1 µg EP/g DS)	127.7 ± 0.2

Example 7

Thermostability of Protease Pfu

[0179] The thermostability of the *Pyrococcus furiosus* protease (Pfu S) purchased from Takara Bio, (Japan) was tested using the same methods as in Example 2. It was found that the thermostability (Relative Activity) was 110% at (80°C/70°C) and 103% (90°C/70°C) at pH 4.5.

Example 8

Ethanol Production Using Alpha-Amylase 1407 and Pfu protease for Liquefaction

[0180] The purpose of this experiment was to evaluate application performance of Protease Pfu derived from *Pyrococcus furiosus* at pH 4.8 during liquefaction at 85°C for 2 hours.

Liquefaction (Labomat)

[0181] Each liquefaction received ground corn (84.19% DS), backset (6.27% DS), and tap water targeting a total weight of 100 g at 32.50% Dry Solids (DS). Backset was blended at 30% w/w of total slurry weight. Initial slurry pH was approximately 5.2 and was adjusted to pH 4.8 with 40% v/v sulfuric acid prior to liquefaction. All enzymes were added according to the experimental design listed in Table 13 below. Liquefaction took place in a Labomat using the following conditions: 5°C/min. Ramp, 17 minute Ramp, 103 minute hold time, 40 rpm for the entire run, 200 mL stainless steel canisters. After liquefaction, all canisters were cooled in an ice bath and prepared for fermentation based on the protocol listed below under SSF.

Simultaneous Saccharification and Fermentation (SSF)

[0182] Each mash was adjusted to pH 5.0 with 50% w/w Sodium Hydroxide or 40% v/v sulfuric acid. Penicillin was applied to each mash to a total concentration of 3 ppm. The tubes were prepared with mash by aliquoting approximately 4.5 g of mash per 15 mL pre-drilled test tubes to allow CO_2 release. The test tubes sat, overnight, at 4°C until the next morning.

[0183] All test tubes of mash were removed from cold storage and warmed up to 32°C in the walk-in incubation chamber. Once warmed, Glucoamylase BL2, was dosed to each tube of mash at 0.50 AGU/g DS, water was added so that all tubes received 120 µL of liquid and each mash sample received 100 µL of rehydrated yeast. Rehydrated yeast was prepared by mixing 5.5 g of Fermentis

RED STAR into 100 mL of 32°C tap water for at least 15 minutes.

[0184] In monitoring CO_2 weight-loss over time, each unit of CO_2 generated and lost is converted to gram ethanol produced per gram of dry solids (g EtOH/gDS) by the following:

$$\text{g ethanol/ g DS} = \text{g CO2 weight loss} \times \frac{1\text{mol CO2}}{44.0098\text{g CO2}} \times \frac{1\text{mol ethanol}}{1\text{mol CO2}} \times \frac{46.094\text{g ethanol}}{1\text{mol ethanol}} \times \frac{\text{g mash in tube}}{\%\text{DS of mash}}$$

HPLC analysis

[0185] Fermentation sampling took place after 54 hours of fermentation by taking 3 tubes per treatment. Each sample was deactivated 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. 54 hour samples were analyzed under HPLC without further dilution. Samples were stored at 4°C prior to and during HPLC analysis.

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

[0186] The method quantified analyte(s) using calibration standard for ethanol (% w/v). A four point calibration including the origin is used for quantification.

[0187] Where applicable, data were analyzed using JMP software (Cary, NC) with Oneway ANOVA of pairs using Tukey-Kramer HSD or Dunnett's. Error bars denoting the 95% confidence level were established by multiplying the standard error of Oneway Anova analysis by 1.96.

Table 13. Experimental Plan.

Liquefaction at 85°C (pH 4.8)					
Alpha-amylase	Dose µg/g DS	Protease	Dose µg/g DS	Glucoamylase	Dose µg/g DS
1407	1.4	-	-	-	-
1407	1.4	Pfu	2	-	-
1407	1.4	Pfu	2	PE001	10

Table 14 and Fig. 1 below show the results:

Treatment	pH	EtOH (%w/v)	EtOH (%Δ)	JMP Std Error	95%CI
Control	4.8	9.2	100%	0.022	0.042
Pfu	4.8	11.0	120%	0.022	0.042
Pfu+PE001	4.8	11.0	120%	0.022	0.042

Example 9

Ethanol Production Using Alpha-Amylase 1407 and Pfu protease for Liquefaction

[0188] The purpose of this experiment was to evaluate application performance of Protease Pfu derived from *Pyrococcus furiosus* at pH 4.8 during liquefaction at 85°C for 2 hours.

Liquefaction (Labomat)

[0189] Each liquefaction received ground corn (84.19% DS), backset (6.27% DS), and tap water targeting a total weight of 100 g at 32.50% Dry Solids (DS). Backset was blended at 30% w/w of total slurry weight. Initial slurry pH was approximately 5.2 and was adjusted to pH 4.8 with 40% v/v sulfuric acid prior to liquefaction. All enzymes were added according to the experimental design listed in Table 13 below. Liquefaction took place in a Labomat using the following conditions: 5°C/min. Ramp, 17 minute Ramp, 103 minute hold time, 40 rpm for the entire run, 200 mL stainless steel canisters. After liquefaction, all canisters were cooled in an ice bath and prepared for fermentation based on the protocol listed below under SSF.

Simultaneous Saccharification and Fermentation (SSF)

[0190] Each mash was adjusted to pH 5.0 with 50% w/w Sodium Hydroxide or 40% v/v sulfuric acid. Penicillin was applied to each mash to a total concentration of 3 ppm. The tubes were prepared with mash by aliquoting approximately 4.5 g of mash per 15 mL pre-drilled test tubes to allow CO_2 release. The test tubes sat, overnight, at 4°C until the next morning.

[0191] All test tubes of mash were removed from cold storage and warmed up to 32°C in the walk-in incubation chamber. Once warmed, Glucoamylase BL2, was dosed to each tube of mash at 0.50 AGU/g DS, water was added so that all tubes received 120 µL of liquid and each mash sample received 100 µL of rehydrated yeast. Rehydrated yeast was prepared by mixing 5.5 g of Fermentis RED STAR into 100 mL of 32°C tap water for at least 15 minutes.

[0192] In monitoring CO_2 weight-loss over time, each unit of CO_2 generated and lost is converted to gram ethanol produced per gram of dry solids (g EtOH/gDS) by the following:

$$\frac{1\text{mol } CO_2}{} \quad \frac{1\text{mol ethanol}}{} \quad \frac{46.094\text{g ethanol}}{}$$

$$\text{g ethanol/ g DS} = \frac{\text{g CO2 weight loss} \times \frac{44.0098\text{g CO2}}{\text{1mol CO2}} \times \frac{\text{1mol ethanol}}{\text{g mash in tube}}}{\%\text{DS of mash}}$$

HPLC analysis

[0193] Fermentation sampling took place after 54 hours of fermentation by taking 3 tubes per treatment. Each sample was deactivated 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. 54 hour samples were analyzed under HPLC without further dilution. Samples were stored at 4°C prior to and during HPLC analysis.

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

[0194] The method quantified analyte(s) using calibration standard for ethanol (% w/v). A four point calibration including the origin is used for quantification.

[0195] Where applicable, data were analyzed using JMP software (Cary, NC) with Oneway ANOVA of pairs using Tukey-Kramer HSD or Dunnett's. Error bars denoting the 95% confidence level were established by multiplying the standard error of Oneway Anova analysis by 1.96.

Table 15. Experimental Plan.

Liquefaction at 85°C (pH 4.8)					
Alpha-amylase	Dose µg/g DS	Protease	Dose µg/g DS	Glucoamylase	Dose µg/g DS
1407	1.4	-	-	-	-
1407	1.4	Pfu	2	-	-
1407	1.4	Pfu	2	PE001	10

Table 16 below shows the results:

Treatment	pH	EtOH (%w/v)	EtOH (%Δ)	JMP Std Error	95%CI+I
Control	4.8	9.2	100%	0.022	0.042
Pfu	4.8	11.0	120%	0.022	0.042
Pfu+ PE001	4.8	11.0	120%	0.022	0.042

Example 10

Improved Lower Viscosity in the Ethanol Production Process

[0196] Corn Flour Preparation: Corn flour from Corn LP, Iowa, USA, was sieved and its particle size distribution (PSD) defined. U.S. Standard Test Sieves with ASTM E-11 Specifications for number 12, 16, 20, 30, 40, and 60 sieves were used. The dry-solids (DS) content of the received flour was around 87.4%. Each experimental run was prepared to have the same PSD.

[0197] Viscosity Profile Setup and Determination in Rapid Visco Analyzer: A Perten RVA-4 unit was used for measuring the viscosity profile during liquefaction. Corn slurries were prepared for liquefaction by weighing out specified amounts of sieved corn flour into a Perten metal cup that replicated the PSD of the received flour. A 40 gram slurry was made to 32% DS by adding tap water and the pH-adjusted to either 5.80 (Control) using 50% w/w NaOH or 4.80 using 40% v/v H_2SO_4. Aliquots of enzyme stock solutions were added prior to each run in the Perten RVA-4 and the amounts were also considered for getting the desired solids. The control slurry used Alpha-Amylase A at pH 5.8. The Perten RVA-4 was programmed to mix the slurry for 1 minute at 25°C, increase the slurry temperature from 25°C to 85°C in 6 minutes, hold the temperature at 85°C constant for 2 hours, cool the liquefied mash temperature from 85°C down to 32°C in 7 minutes, and maintain the liquefied mash temperature at 32°C for 5 minutes. During each run, the mixing was maintained constant at 210 rpm.

RESULTS

[0198] An overview of the results is provided in Table 17 and shown in Figs. 2-5.

Table 17: The doses of enzymes are listed in parentheses for each and are expressed as micro g EP/g DS.

Experiment No.	Enzyme Description	Peak Viscosity	Average Viscosity Peak-to-Final	Final Viscosity	% Reduction of Peak Viscosity vs. Experiment 2	% Reduction of Average Viscosity Peak-to-Final vs. Experiment 2	% Reduction of Final Viscosity vs. Experiment 2
1	Alpha-Amylase A (1.4) at pH=5.8	12769	535	1078			

Experiment No.	Enzyme Description	Peak Viscosity	Average Viscosity Peak-to-Final	Final Viscosity	% Reduction of Peak Viscosity vs. Experiment 2	% Reduction of Average Viscosity Peak-to-Final vs. Experiment 2	% Reduction of Final Viscosity vs. Experiment 2
2	Alpha-Amylase 1407 (1.4) + Glucoamylase PE001 (10) + Protease 196 (1) at pH=4.8	15050	659	816			
3	Alpha-Amylase A (1.4) + Glucoamylase PE001 (10) + Protease 196 (1) at pH=4.8	11848	728	1831			
4	Alpha-Amylase A (0.35) + Alpha-Amylase 1407 (1.4) + Glucoamylase PE001 (10) + Protease 196 (1) at pH=4.8	12927	527	689	14%	20%	16%
5	Alpha-Amylase A (0.7) + Alpha-Amylase 1407 (1.4) + Glucoamylase PE001 (10) + Protease 196 (1) at pH=4.8	11454	423	682	24%	36%	16%

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Deinhammer, Randy
Clark, Suzanne
Quiros, Mauricio
Matthews, John
Hjulmand, Anne Glud
Soong, Chee-Leong
Matsui, Tomoko
Takagi, Shinobu

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        35                  40                  45

Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
    50                  55                  60

Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr
65                  70                  75                  80

Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
                85                  90                  95

Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
            100                 105                 110

Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
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Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu
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Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys
225                 230                 235                 240

Phe Ser Phe Phe Pro Asp Trp Leu Ser Tyr Val Arg Ser Gln Thr Gly
                245                 250                 255

Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys
            260                 265                 270

Leu His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu Phe Asp
        275                 280                 285

Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Ala
    290                 295                 300

Phe Asp Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp Gln Pro
305                 310                 315                 320

Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln
                325                 330                 335

Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala
            340                 345                 350

Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp
        355                 360                 365

Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile
    370                 375                 380

Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His
385                 390                 395                 400

Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Val
                405                 410                 415

Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
            420                 425                 430

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Val Val Gln Asn  Thr Gly Ser Asp Glu  Leu Ser Phe Val His  Leu
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                30                  35                  40

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        60                  65                  70

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caa gcg acc act gcc ctt cac gag ttc acc cat gcg cct ggc gtc tac        945
Gln Ala Thr Thr Ala Leu His Glu Phe Thr His Ala Pro Gly Val Tyr
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                30                  35                  40

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            45                  50                  55

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        60                  65                  70

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    75                  80                  85

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90                  95                  100                 105

Phe Tyr Thr Tyr Leu Pro Ala Leu Thr Ser Thr Cys His Ala Gln Asp
                110                 115                 120

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            125                 130                 135

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            20                  25                  30

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        35                  40                  45

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    50                  55                  60

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65                  70                  75                  80

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Ser Arg Ala Lys Phe Pro Ile Asp Arg Lys Tyr Leu Glu Thr Gly Ile
            100                 105                 110

cgg gac tac gtg tcg tcc caa gca atc ctc cag agt gtg tct aat cct         384
Arg Asp Tyr Val Ser Ser Gln Ala Ile Leu Gln Ser Val Ser Asn Pro
        115                 120                 125

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Ser Gly Thr Leu Lys Asp Gly Ser Gly Leu Gly Glu Pro Lys Phe Glu
    130                 135                 140

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Ile Asp Leu Asn Pro Phe Ser Gly Ala Trp Gly Arg Pro Gln Arg Asp
145                 150                 155                 160

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Gly Pro Ala Leu Arg Ala Thr Ala Met Ile Thr Tyr Ala Asn Tyr Leu
                165                 170                 175

ata tcc cat ggt cag aaa tcg gat gtg tca cag gtc atg tgg ccg att         576
Ile Ser His Gly Gln Lys Ser Asp Val Ser Gln Val Met Trp Pro Ile
            180                 185                 190

att gcc aat gat cta gca tat gtt ggt caa tac tgg aat aat acc gga         624
Ile Ala Asn Asp Leu Ala Tyr Val Gly Gln Tyr Trp Asn Asn Thr Gly
        195                 200                 205

ttt gac ctg tgg gaa gag gtg gat ggg tca agc ttt ttc acg att gcg         672
Phe Asp Leu Trp Glu Glu Val Asp Gly Ser Ser Phe Phe Thr Ile Ala
    210                 215                 220

gtc cag cac cga gcc ctt gtt gaa ggc tcg caa ctg gcg aaa aag ctc         720
Val Gln His Arg Ala Leu Val Glu Gly Ser Gln Leu Ala Lys Lys Leu
225                 230                 235                 240

ggc aag tcc tgc gat gcc tgt gat tct cag cct ccc cag ata ttg tgt         768
Gly Lys Ser Cys Asp Ala Cys Asp Ser Gln Pro Pro Gln Ile Leu Cys
                245                 250                 255

ttc ctg cag agt ttc tgg aac gga aag tac atc acc tcc aac atc aac         816
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Phe Leu Gln Ser Phe Trp Asn Gly Lys Tyr Ile Thr Ser Asn Ile Asn

            260                 265                 270

acg caa gca agc cgc tct ggt atc gac ctg gac tct gtc ctg gga agc        864
Thr Gln Ala Ser Arg Ser Gly Ile Asp Leu Asp Ser Val Leu Gly Ser
        275                 280                 285

att cat acc ttt gat ccc gaa gca gcc tgt gac gat gca act ttc cag        912
Ile His Thr Phe Asp Pro Glu Ala Ala Cys Asp Asp Ala Thr Phe Gln
    290                 295                 300

cct tgt tct gcc cgc gct ctg gcg aac cac aag gtc tat gtg gat tcc        960
Pro Cys Ser Ala Arg Ala Leu Ala Asn His Lys Val Tyr Val Asp Ser
305                 310                 315                 320

ttc cgc tct atc tac aag att aat gcg ggt ctt gca gag gga tcg gct       1008
Phe Arg Ser Ile Tyr Lys Ile Asn Ala Gly Leu Ala Glu Gly Ser Ala
                325                 330                 335

gcc aac gtt ggc cgc tac ccc gag gat gtt tac caa gga ggc aat cca       1056
Ala Asn Val Gly Arg Tyr Pro Glu Asp Val Tyr Gln Gly Gly Asn Pro
            340                 345                 350

tgg tat ctc gcc acc cta ggc gca tct gaa ttg ctt tac gac gcc ttg       1104
Trp Tyr Leu Ala Thr Leu Gly Ala Ser Glu Leu Leu Tyr Asp Ala Leu
        355                 360                 365

tac cag tgg gac aga ctt ggc aaa ctt gaa gtc tcg gag acc tcg ttg       1152
Tyr Gln Trp Asp Arg Leu Gly Lys Leu Glu Val Ser Glu Thr Ser Leu
    370                 375                 380

tca ttc ttc aaa gac ttt gac gcg acc gtg aaa att ggc tcg tac tcg       1200
Ser Phe Phe Lys Asp Phe Asp Ala Thr Val Lys Ile Gly Ser Tyr Ser
385                 390                 395                 400

agg aac agc aag acc tac aag aaa ttg acc cag tcc atc aag tcg tac       1248
Arg Asn Ser Lys Thr Tyr Lys Lys Leu Thr Gln Ser Ile Lys Ser Tyr
                405                 410                 415

gcg gac ggg ttc atc cag tta gtg cag cag tac act cct tct aat gga       1296
Ala Asp Gly Phe Ile Gln Leu Val Gln Gln Tyr Thr Pro Ser Asn Gly
            420                 425                 430

tct ctg gcc gag caa tac gat cgc aat acg gct gct cct ctc tct gca       1344
Ser Leu Ala Glu Gln Tyr Asp Arg Asn Thr Ala Ala Pro Leu Ser Ala
        435                 440                 445

aac gat ctg act tgg tca ttt gcc tct ttc ttg acg gct acg caa cgc       1392
Asn Asp Leu Thr Trp Ser Phe Ala Ser Phe Leu Thr Ala Thr Gln Arg
    450                 455                 460

cgc gat gcc gtg gtt cct ccc tcc tgg ggc gca aag tcg gca aac aaa       1440
Arg Asp Ala Val Val Pro Pro Ser Trp Gly Ala Lys Ser Ala Asn Lys
465                 470                 475                 480

gtc cca acc act tgt tca gcc tcc cct gtt gtg ggt act tat aag gcg       1488
Val Pro Thr Thr Cys Ser Ala Ser Pro Val Val Gly Thr Tyr Lys Ala
                485                 490                 495

ccc acg gca act ttc tca tcc aag act aag tgc gtc ccc gct aaa gat       1536
Pro Thr Ala Thr Phe Ser Ser Lys Thr Lys Cys Val Pro Ala Lys Asp
            500                 505                 510

att gtg cct atc acg ttc tac ctg att gag aac act tac tat gga gag       1584

Ile Val Pro Ile Thr Phe Tyr Leu Ile Glu Asn Thr Tyr Tyr Gly Glu
        515                 520                 525

aac gtc ttc atg agt ggc aac att act gcg ctg ggt aac tgg gac gcc       1632
Asn Val Phe Met Ser Gly Asn Ile Thr Ala Leu Gly Asn Trp Asp Ala
    530                 535                 540

aag aaa ggc ttc cca ctc acc gca aac ctc tac acg caa gat caa aac       1680
Lys Lys Gly Phe Pro Leu Thr Ala Asn Leu Tyr Thr Gln Asp Gln Asn
545                 550                 555                 560

ttg tgg ttc gcc agt gtc gag ttc atc cca gca ggc aca ccc ttt gag       1728
Leu Trp Phe Ala Ser Val Glu Phe Ile Pro Ala Gly Thr Pro Phe Glu
```

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                565                 570                 575

tac aag tac tac aag gtc gag ccc aat ggc gat att act tgg gag aag      1776
Tyr Lys Tyr Tyr Lys Val Glu Pro Asn Gly Asp Ile Thr Trp Glu Lys
            580                 585                 590

ggt ccc aac cgg gtg ttc gtc gct ccc acg gga tgc cca gtt cag cct      1824
Gly Pro Asn Arg Val Phe Val Ala Pro Thr Gly Cys Pro Val Gln Pro
        595                 600                 605

cac tcc aac gac gtg tgg cag ttt tga                                  1851
His Ser Asn Asp Val Trp Gln Phe
    610                 615
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<210> 9

<211> 616

<212> PRT

<213> Penicillium oxalicum

<400> 9

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Met Arg Leu Thr Leu Leu Ser Gly Val Ala Gly Val Leu Cys Ala Gly
1               5                   10                  15

Gln Leu Thr Ala Ala Arg Pro Asp Pro Lys Gly Gly Asn Leu Thr Pro
            20                  25                  30

Phe Ile His Lys Glu Gly Glu Arg Ser Leu Gln Gly Ile Leu Asp Asn
        35                  40                  45

Leu Gly Gly Arg Gly Lys Lys Thr Pro Gly Thr Ala Ala Gly Leu Phe
    50                  55                  60

Ile Ala Ser Pro Asn Thr Glu Asn Pro Asn Tyr Tyr Tyr Thr Trp Thr
65                  70                  75                  80

Arg Asp Ser Ala Leu Thr Ala Lys Cys Leu Ile Asp Leu Phe Glu Asp
                85                  90                  95

Ser Arg Ala Lys Phe Pro Ile Asp Arg Lys Tyr Leu Glu Thr Gly Ile
            100                 105                 110

Arg Asp Tyr Val Ser Ser Gln Ala Ile Leu Gln Ser Val Ser Asn Pro
        115                 120                 125

Ser Gly Thr Leu Lys Asp Gly Ser Gly Leu Gly Glu Pro Lys Phe Glu
    130                 135                 140

Ile Asp Leu Asn Pro Phe Ser Gly Ala Trp Gly Arg Pro Gln Arg Asp
145                 150                 155                 160

Gly Pro Ala Leu Arg Ala Thr Ala Met Ile Thr Tyr Ala Asn Tyr Leu
                165                 170                 175

Ile Ser His Gly Gln Lys Ser Asp Val Ser Gln Val Met Trp Pro Ile
            180                 185                 190

Ile Ala Asn Asp Leu Ala Tyr Val Gly Gln Tyr Trp Asn Asn Thr Gly
        195                 200                 205

Phe Asp Leu Trp Glu Glu Val Asp Gly Ser Ser Phe Phe Thr Ile Ala
    210                 215                 220
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Val Gln His Arg Ala Leu Val Glu Gly Ser Gln Leu Ala Lys Lys Leu
225                 230                 235                 240

Gly Lys Ser Cys Asp Ala Cys Asp Ser Gln Pro Pro Gln Ile Leu Cys
                245                 250                 255

Phe Leu Gln Ser Phe Trp Asn Gly Lys Tyr Ile Thr Ser Asn Ile Asn
            260                 265                 270

Thr Gln Ala Ser Arg Ser Gly Ile Asp Leu Asp Ser Val Leu Gly Ser
        275                 280                 285

Ile His Thr Phe Asp Pro Glu Ala Ala Cys Asp Asp Ala Thr Phe Gln
    290                 295                 300

Pro Cys Ser Ala Arg Ala Leu Ala Asn His Lys Val Tyr Val Asp Ser
305                 310                 315                 320

Phe Arg Ser Ile Tyr Lys Ile Asn Ala Gly Leu Ala Glu Gly Ser Ala
                325                 330                 335

Ala Asn Val Gly Arg Tyr Pro Glu Asp Val Tyr Gln Gly Gly Asn Pro
            340                 345                 350

Trp Tyr Leu Ala Thr Leu Gly Ala Ser Glu Leu Leu Tyr Asp Ala Leu
        355                 360                 365

Tyr Gln Trp Asp Arg Leu Gly Lys Leu Glu Val Ser Glu Thr Ser Leu
    370                 375                 380

Ser Phe Phe Lys Asp Phe Asp Ala Thr Val Lys Ile Gly Ser Tyr Ser
385                 390                 395                 400

Arg Asn Ser Lys Thr Tyr Lys Lys Leu Thr Gln Ser Ile Lys Ser Tyr
                405                 410                 415

Ala Asp Gly Phe Ile Gln Leu Val Gln Gln Tyr Thr Pro Ser Asn Gly
            420                 425                 430

Ser Leu Ala Glu Gln Tyr Asp Arg Asn Thr Ala Ala Pro Leu Ser Ala
        435                 440                 445

Asn Asp Leu Thr Trp Ser Phe Ala Ser Phe Leu Thr Ala Thr Gln Arg
    450                 455                 460

Arg Asp Ala Val Val Pro Pro Ser Trp Gly Ala Lys Ser Ala Asn Lys
465                 470                 475                 480

Val Pro Thr Thr Cys Ser Ala Ser Pro Val Val Gly Thr Tyr Lys Ala
                485                 490                 495

Pro Thr Ala Thr Phe Ser Ser Lys Thr Lys Cys Val Pro Ala Lys Asp
            500                 505                 510

Ile Val Pro Ile Thr Phe Tyr Leu Ile Glu Asn Thr Tyr Tyr Gly Glu
        515                 520                 525

Asn Val Phe Met Ser Gly Asn Ile Thr Ala Leu Gly Asn Trp Asp Ala
    530                 535                 540
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Lys Lys Gly Phe Pro Leu Thr Ala Asn Leu Tyr Thr Gln Asp Gln Asn
545                 550                 555                 560

Leu Trp Phe Ala Ser Val Glu Phe Ile Pro Ala Gly Thr Pro Phe Glu
                565                 570                 575

Tyr Lys Tyr Tyr Lys Val Glu Pro Asn Gly Asp Ile Thr Trp Glu Lys
            580                 585                 590

Gly Pro Asn Arg Val Phe Val Ala Pro Thr Gly Cys Pro Val Gln Pro
        595                 600                 605

His Ser Asn Asp Val Trp Gln Phe
    610                 615
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<210> 10

<211> 4014

<212> DNA

<213> Thermococcus hydrothermalis

<220>

<221> CDS

<222> (1)..(4011)

<220>

<221> misc_signal

<222> (1)..(81)

<220>

<221> mat_peptide

<222> (82)..(4014)

<400> 10

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atg agg cgg gtg gtt gcc ctc ttc att gca att ttg atg ctt gga agc         48
Met Arg Arg Val Val Ala Leu Phe Ile Ala Ile Leu Met Leu Gly Ser
        -25                 -20                 -15

atc gtt gga gcg aac gtt aag agc gtt ggc gcg gcg gag ccg aag ccg         96
Ile Val Gly Ala Asn Val Lys Ser Val Gly Ala Ala Glu Pro Lys Pro
    -10                 -5                  -1  1               5

ctc aac gtc ata ata gtc tgg cac cag cac cag ccc tac tac tac gac        144
Leu Asn Val Ile Ile Val Trp His Gln His Gln Pro Tyr Tyr Tyr Asp
                10                  15                  20

cct gtc cag gac gtc tac acc agg ccc tgg gtc agg ctc cac gcg gcg        192
Pro Val Gln Asp Val Tyr Thr Arg Pro Trp Val Arg Leu His Ala Ala
            25                  30                  35

aac aac tac tgg aag atg gcc cac tac ctg agc cag tac ccg gag gtt        240
Asn Asn Tyr Trp Lys Met Ala His Tyr Leu Ser Gln Tyr Pro Glu Val
        40                  45                  50

cac gcc acc att gac ctc tcg ggt tcg ctg ata gcc cag ctt gcc gac        288
His Ala Thr Ile Asp Leu Ser Gly Ser Leu Ile Ala Gln Leu Ala Asp
    55                  60                  65

tac atg aac ggc aag aag gac acc tac cag ata atc acc gag aag ata        336
Tyr Met Asn Gly Lys Lys Asp Thr Tyr Gln Ile Ile Thr Glu Lys Ile
70                  75                  80                  85

gcc aac ggg gaa ccc ctc acc gtc gac gag aag tgg ttc atg ctc cag        384
Ala Asn Gly Glu Pro Leu Thr Val Asp Glu Lys Trp Phe Met Leu Gln
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                90                  95                  100

gca ccg gga ggg ttc ttc gac aac acc atc ccc tgg aac ggt gaa ccg        432
Ala Pro Gly Gly Phe Phe Asp Asn Thr Ile Pro Trp Asn Gly Glu Pro
            105                 110                 115

ata acc gac ccc aac ggc aac ccg ata agg gac ttc tgg gac cgc tac        480
Ile Thr Asp Pro Asn Gly Asn Pro Ile Arg Asp Phe Trp Asp Arg Tyr
        120                 125                 130

acg gag ctg aag aac aag atg ctc agc gca aag gcc aag tac gca aac        528
Thr Glu Leu Lys Asn Lys Met Leu Ser Ala Lys Ala Lys Tyr Ala Asn

    135                 140                 145

ttc gtg act gag agc cag aag gtc gct gtg acg aac gag ttc aca gag        576
Phe Val Thr Glu Ser Gln Lys Val Ala Val Thr Asn Glu Phe Thr Glu
150                 155                 160                 165

cag gac tac ata gac cta gcg gtt ctc ttc aat ctc gct tgg att gac        624
Gln Asp Tyr Ile Asp Leu Ala Val Leu Phe Asn Leu Ala Trp Ile Asp
                170                 175                 180

tac aat tac atc acg agc acg ccg gag ttc aag gcc ctc tac gac aag        672
Tyr Asn Tyr Ile Thr Ser Thr Pro Glu Phe Lys Ala Leu Tyr Asp Lys
            185                 190                 195

gtt gac gag ggc ggc tat aca agg gcg gac gtc aaa acc gtt ctc gac        720
Val Asp Glu Gly Gly Tyr Thr Arg Ala Asp Val Lys Thr Val Leu Asp
        200                 205                 210

gcc cag atc tgg ctt ctc aac cac acc ttc gag gag cac gag aag ata        768
Ala Gln Ile Trp Leu Leu Asn His Thr Phe Glu Glu His Glu Lys Ile
    215                 220                 225

aac ctc ctc ctc gga aac ggc aac gtc gag gtc acg gtc gtt ccc tac        816
Asn Leu Leu Leu Gly Asn Gly Asn Val Glu Val Thr Val Val Pro Tyr
230                 235                 240                 245

gcc cac ccg ata ggc ccg ata ctc aac gac ttc ggc tgg gac agc gac        864
Ala His Pro Ile Gly Pro Ile Leu Asn Asp Phe Gly Trp Asp Ser Asp
                250                 255                 260

ttc aac gac cag gtc aag aag gcc gac gaa ctg tac aag ccg tac ctc        912
Phe Asn Asp Gln Val Lys Lys Ala Asp Glu Leu Tyr Lys Pro Tyr Leu
            265                 270                 275

ggc ggc ggc acc gcg gtt cca aaa ggc gga tgg gcg gct gag agc gcc        960
Gly Gly Gly Thr Ala Val Pro Lys Gly Gly Trp Ala Ala Glu Ser Ala
        280                 285                 290

ctc aac gac aaa act ctg gag atc ctc gcc gag aac ggc tgg gag tgg       1008
Leu Asn Asp Lys Thr Leu Glu Ile Leu Ala Glu Asn Gly Trp Glu Trp
    295                 300                 305

gtc atg acc gac cag atg gtt ctc gga aag ctc ggc att gag gga acc       1056
Val Met Thr Asp Gln Met Val Leu Gly Lys Leu Gly Ile Glu Gly Thr
310                 315                 320                 325

gtc gag aac tac cac aag ccc tgg gtg gcc gag ttc aac gga aag aag       1104
Val Glu Asn Tyr His Lys Pro Trp Val Ala Glu Phe Asn Gly Lys Lys
                330                 335                 340

ata tac ctc ttc cca aga aat cac gat cta agt gac aga gtt ggc ttt       1152
Ile Tyr Leu Phe Pro Arg Asn His Asp Leu Ser Asp Arg Val Gly Phe
            345                 350                 355

acc tac agc gga atg aac cag cag cag gcc gtt gag gac ttc gtc aac       1200
Thr Tyr Ser Gly Met Asn Gln Gln Gln Ala Val Glu Asp Phe Val Asn
        360                 365                 370

gag ctc ctc aag ctc cag aag cag aac tac gat ggc tcg ctg gtt tac       1248
Glu Leu Leu Lys Leu Gln Lys Gln Asn Tyr Asp Gly Ser Leu Val Tyr
    375                 380                 385

gtg gtc acg ctc gac ggc gag aac ccc gtg gag aac tac ccc tac gac       1296

Val Val Thr Leu Asp Gly Glu Asn Pro Val Glu Asn Tyr Pro Tyr Asp
390                 395                 400                 405
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ggg gag ctc ttc ctc acc gaa ctc tac aag aag ctg acc gaa ctc cag       1344
Gly Glu Leu Phe Leu Thr Glu Leu Tyr Lys Lys Leu Thr Glu Leu Gln
                410                 415                 420

gag cag ggt ctc ata aga acc ctc acc ccg agc gag tac atc cag ctc       1392
Glu Gln Gly Leu Ile Arg Thr Leu Thr Pro Ser Glu Tyr Ile Gln Leu
            425                 430                 435

tac ggc gac aag gcc aac aag ctc aca cct cgg atg atg gag cgc ctt       1440
Tyr Gly Asp Lys Ala Asn Lys Leu Thr Pro Arg Met Met Glu Arg Leu
        440                 445                 450

gac ctc acc gga gac aac gtt aac gcc ctc ctc aag gcc cag agc ctc       1488
Asp Leu Thr Gly Asp Asn Val Asn Ala Leu Leu Lys Ala Gln Ser Leu
    455                 460                 465

ggc gaa ctc tac gac atg acc ggc gtt aag gag gag atg cag tgg ccc       1536
Gly Glu Leu Tyr Asp Met Thr Gly Val Lys Glu Glu Met Gln Trp Pro
470                 475                 480                 485

gag agc agc tgg ata gac gga acc ctc tcc acg tgg ata ggc gag ccc       1584
Glu Ser Ser Trp Ile Asp Gly Thr Leu Ser Thr Trp Ile Gly Glu Pro
                490                 495                 500

cag gag aac tac ggc tgg tac tgg ctc tac atg gcc agg aag gcc ctt       1632
Gln Glu Asn Tyr Gly Trp Tyr Trp Leu Tyr Met Ala Arg Lys Ala Leu
            505                 510                 515

atg gag aac aag gat aaa atg agc cag gcg gac tgg gag aag gcc tac       1680
Met Glu Asn Lys Asp Lys Met Ser Gln Ala Asp Trp Glu Lys Ala Tyr
        520                 525                 530

gag tac ctg ctc cgc gcc gag gca agc gac tgg ttc tgg tgg tac gga       1728
Glu Tyr Leu Leu Arg Ala Glu Ala Ser Asp Trp Phe Trp Trp Tyr Gly
    535                 540                 545

agc gac cag gac agc ggc cag gac tac acc ttc gac cgc tac ctg aag       1776
Ser Asp Gln Asp Ser Gly Gln Asp Tyr Thr Phe Asp Arg Tyr Leu Lys
550                 555                 560                 565

acc tac ctc tac gag atg tac aag ctg gca gga gtc gag ccg ccg agc       1824
Thr Tyr Leu Tyr Glu Met Tyr Lys Leu Ala Gly Val Glu Pro Pro Ser
                570                 575                 580

tac ctc ttc ggc aac tac ttc ccg gac gga gag ccc tac acc acg agg       1872
Tyr Leu Phe Gly Asn Tyr Phe Pro Asp Gly Glu Pro Tyr Thr Thr Arg
            585                 590                 595

ggc ctg gtc gga ctc aag gac ggc gag atg aag aac ttc tcc agc atg       1920
Gly Leu Val Gly Leu Lys Asp Gly Glu Met Lys Asn Phe Ser Ser Met
        600                 605                 610

tcc ccg ctg gca aag ggc gtg agc gtc tat ttc gac ggc gag ggg ata       1968
Ser Pro Leu Ala Lys Gly Val Ser Val Tyr Phe Asp Gly Glu Gly Ile
    615                 620                 625

cac ttc ata gtg aaa ggg aac ctg gac agg ttc gag gtg agc atc tgg       2016
His Phe Ile Val Lys Gly Asn Leu Asp Arg Phe Glu Val Ser Ile Trp
630                 635                 640                 645

gag aag gat gag cgc gtt ggc aac acg ttc acc cgc ctc caa gag aag       2064
Glu Lys Asp Glu Arg Val Gly Asn Thr Phe Thr Arg Leu Gln Glu Lys
                650                 655                 660

ccg gac gag ttg agc tat ttc atg ttc cca ttc tca agg gac agc gtt       2112
Pro Asp Glu Leu Ser Tyr Phe Met Phe Pro Phe Ser Arg Asp Ser Val
            665                 670                 675

ggt ctc ctc ata acc aag cac gtc gtg tac gag aac gga aag gcc gag       2160
Gly Leu Leu Ile Thr Lys His Val Val Tyr Glu Asn Gly Lys Ala Glu
        680                 685                 690

ata tac ggc gcc acc gac tac gag aag agc gag aag ctt ggg gaa gcc       2208
Ile Tyr Gly Ala Thr Asp Tyr Glu Lys Ser Glu Lys Leu Gly Glu Ala
    695                 700                 705

acc gtc aag aac acg agc gaa gga atc gaa gtc gtc ctt ccc ttt gac       2256
Thr Val Lys Asn Thr Ser Glu Gly Ile Glu Val Val Leu Pro Phe Asp
710                 715                 720                 725
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710                 715                 720                 725

tac ata gaa aac ccc tcc gac ttc tac ttc gct gtc tcg acg gtc aaa      2304
Tyr Ile Glu Asn Pro Ser Asp Phe Tyr Phe Ala Val Ser Thr Val Lys
                730                 735                 740

gat gga gac ctt gag gtg ata agc act cct gtg gag ctc aag ctc ccg      2352
Asp Gly Asp Leu Glu Val Ile Ser Thr Pro Val Glu Leu Lys Leu Pro
            745                 750                 755

acc gag gtc aag gga gtc gtc ata gcc gat ata acc gac cca gaa ggc      2400
Thr Glu Val Lys Gly Val Val Ile Ala Asp Ile Thr Asp Pro Glu Gly
        760                 765                 770

gac gac cat ggg ccc gga aac tac act tat ccc acg gac aag gtc ttc      2448
Asp Asp His Gly Pro Gly Asn Tyr Thr Tyr Pro Thr Asp Lys Val Phe
    775                 780                 785

aag cca ggt gtt ttc gac ctc ctc cgc ttc agg atg ctc gaa cag acg      2496
Lys Pro Gly Val Phe Asp Leu Leu Arg Phe Arg Met Leu Glu Gln Thr
790                 795                 800                 805

gag agc tac gtc atg gag ttc tac ttc aag gac cta ggt ggt aac ccg      2544
Glu Ser Tyr Val Met Glu Phe Tyr Phe Lys Asp Leu Gly Gly Asn Pro
                810                 815                 820

tgg aac gga ccc aac ggc ttc agc ctc cag ata atc gag gtc tac ctc      2592
Trp Asn Gly Pro Asn Gly Phe Ser Leu Gln Ile Ile Glu Val Tyr Leu
            825                 830                 835

gac ttc aag gac ggt gga aac agt tcg gcc att aag atg ttc ccc gac      2640
Asp Phe Lys Asp Gly Gly Asn Ser Ser Ala Ile Lys Met Phe Pro Asp
        840                 845                 850

gga ccg gga gcc aac gtc aac ctc gac ccc gag cat cca tgg gac gtt      2688
Gly Pro Gly Ala Asn Val Asn Leu Asp Pro Glu His Pro Trp Asp Val
    855                 860                 865

gcc ttc agg ata gcg ggc tgg gac tac gga aac ctc atc atc ctg ccg      2736
Ala Phe Arg Ile Ala Gly Trp Asp Tyr Gly Asn Leu Ile Ile Leu Pro
870                 875                 880                 885

aac gga acg gcc atc cag ggc gag atg cag att tcc gca gat ccg gtt      2784
Asn Gly Thr Ala Ile Gln Gly Glu Met Gln Ile Ser Ala Asp Pro Val
                890                 895                 900

aag aac gcc ata ata gtc aag gtt cca aag aag tac atc gcc ata aac      2832
Lys Asn Ala Ile Ile Val Lys Val Pro Lys Lys Tyr Ile Ala Ile Asn
            905                 910                 915

gag gac tac ggc ctc tgg gga gac gtc ctc gtc ggc tcg cag gac ggc      2880
Glu Asp Tyr Gly Leu Trp Gly Asp Val Leu Val Gly Ser Gln Asp Gly
        920                 925                 930

tac ggc ccg gac aag tgg aga acg gcg gca gtg gat gcg gag cag tgg      2928
Tyr Gly Pro Asp Lys Trp Arg Thr Ala Ala Val Asp Ala Glu Gln Trp
    935                 940                 945

aag ctt gga ggt gcg gac ccg cag gca gtc ata aac ggc gtg gcc ccg      2976
Lys Leu Gly Gly Ala Asp Pro Gln Ala Val Ile Asn Gly Val Ala Pro
950                 955                 960                 965

cgc gtc att gat gag ctg gtt ccg cag ggc ttt gaa ccg acc cag gag      3024
Arg Val Ile Asp Glu Leu Val Pro Gln Gly Phe Glu Pro Thr Gln Glu
                970                 975                 980

gag cag ctg agc agc tac gat gca aac gac atg aag ctc gcc act gtc      3072
Glu Gln Leu Ser Ser Tyr Asp Ala Asn Asp Met Lys Leu Ala Thr Val
            985                 990                 995

aag gcg ctg  cta ctc ctc aag cag  ggc atc gtt gtg acc  gac ccg      3117
Lys Ala Leu  Leu Leu Leu Lys Gln  Gly Ile Val Val Thr  Asp Pro
        1000                1005                1010

gag gga gac  gac cac ggg ccg gga  acg tac acc tat ccg  acg gac      3162
Glu Gly Asp  Asp His Gly Pro Gly  Thr Tyr Thr Tyr Pro  Thr Asp
        1015                1020                1025

aaa gtt ttc  aag ccc ggt gtt ttc  gac ctc ctc aag ttc  aag gtg      3207
Lys Val Phe  Lys Pro Gly Val Phe  Asp Leu Leu Lys Phe  Lys Val
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        1030                1035                1040

acc gag gga  agc gac gac tgg acg  ctg gag ttc cac ttc  aaa gac        3252
Thr Glu Gly  Ser Asp Asp Trp Thr  Leu Glu Phe His Phe  Lys Asp
        1045                1050                1055

ctc ggt gga  aac ccg tgg aac ggg  ccg aac ggc ttc agc  ctg cag        3297
Leu Gly Gly  Asn Pro Trp Asn Gly  Pro Asn Gly Phe Ser  Leu Gln
        1060                1065                1070

ata atc gag  gta tac ttc gac ttc  aag gag ggc ggg aac  gtc tcg        3342
Ile Ile Glu  Val Tyr Phe Asp Phe  Lys Glu Gly Gly Asn  Val Ser
        1075                1080                1085

gcc att aag  atg ttc ccg gat ggg  ccc gga agc aac gtc  cgt ctt        3387
Ala Ile Lys  Met Phe Pro Asp Gly  Pro Gly Ser Asn Val  Arg Leu
        1090                1095                1100

gat cca aat  cac cca tgg gac ctg  gcg ctt agg ata gcc  ggc tgg        3432
Asp Pro Asn  His Pro Trp Asp Leu  Ala Leu Arg Ile Ala  Gly Trp
        1105                1110                1115

gac tac gga  aac ctg ata att ctg  ccc gac gga acc gcc  tac caa        3477
Asp Tyr Gly  Asn Leu Ile Ile Leu  Pro Asp Gly Thr Ala  Tyr Gln
        1120                1125                1130

ggc gag atg  cag att tcc gca gat  ccg gtt aag aac gcc  ata ata        3522
Gly Glu Met  Gln Ile Ser Ala Asp  Pro Val Lys Asn Ala  Ile Ile


        1135                1140                1145

gtc aag gtt  cca aag aag tac ctg  aac ata tcc gac tac  gga ctc        3567
Val Lys Val  Pro Lys Lys Tyr Leu  Asn Ile Ser Asp Tyr  Gly Leu
        1150                1155                1160

tac acc gcc  gtc atc gtg ggt tcc  caa gac ggg tac ggc  ccg gac        3612
Tyr Thr Ala  Val Ile Val Gly Ser  Gln Asp Gly Tyr Gly  Pro Asp
        1165                1170                1175

aag tgg agg  ccc gtg gcc gct gag  gcc gag cag tgg aag  ctc gga        3657
Lys Trp Arg  Pro Val Ala Ala Glu  Ala Glu Gln Trp Lys  Leu Gly
        1180                1185                1190

ggc gca gac  ccc cag gcg gtc ata  gac aac ctc gta cca  agg gtc        3702
Gly Ala Asp  Pro Gln Ala Val Ile  Asp Asn Leu Val Pro  Arg Val
        1195                1200                1205

gtt gat gaa  ctc gtg ccg gag ggc  ttc aag cca acg cag  gag gag        3747
Val Asp Glu  Leu Val Pro Glu Gly  Phe Lys Pro Thr Gln  Glu Glu
        1210                1215                1220

cag ctg agc  agc tac gac ctt gag  aag aag acc ctg gcg  acg gtg        3792
Gln Leu Ser  Ser Tyr Asp Leu Glu  Lys Lys Thr Leu Ala  Thr Val
        1225                1230                1235

ctc atg gta  ccg ctc gtc aat ggg  act ggc ggc gag gaa  cca acg        3837
Leu Met Val  Pro Leu Val Asn Gly  Thr Gly Gly Glu Glu  Pro Thr
        1240                1245                1250

ccg acg gag  agc cca acg gaa acg  acg aca acc aca ccc  agc gaa        3882
Pro Thr Glu  Ser Pro Thr Glu Thr  Thr Thr Thr Thr Pro  Ser Glu
        1255                1260                1265

aca acc acc  aca act tca acg acc  acc ggc cca agc tca  acg acc        3927
Thr Thr Thr  Thr Thr Ser Thr Thr  Thr Gly Pro Ser Ser  Thr Thr
        1270                1275                1280

acc agc aca  ccc ggc gga gga atc  tgc ggc cca ggc att  ata gcg        3972
Thr Ser Thr  Pro Gly Gly Gly Ile  Cys Gly Pro Gly Ile  Ile Ala
        1285                1290                1295

ggc ctg gcc  ctg ata ccg ctc ctc  ctc aag agg agg aac  tga           4014
Gly Leu Ala  Leu Ile Pro Leu Leu  Leu Lys Arg Arg Asn
        1300                1305                1310
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<210> 11

<211> 1337

<212> PRT

<213> Thermococcus hydrothermalis

<400> 11

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Met Arg Arg Val Val Ala Leu Phe Ile Ala Ile Leu Met Leu Gly Ser
        -25                 -20                 -15

Ile Val Gly Ala Asn Val Lys Ser Val Gly Ala Ala Glu Pro Lys Pro
    -10                 -5              -1  1               5

Leu Asn Val Ile Ile Val Trp His Gln His Gln Pro Tyr Tyr Tyr Asp

               10                  15                  20

Pro Val Gln Asp Val Tyr Thr Arg Pro Trp Val Arg Leu His Ala Ala
            25                  30                  35

Asn Asn Tyr Trp Lys Met Ala His Tyr Leu Ser Gln Tyr Pro Glu Val
        40                  45                  50

His Ala Thr Ile Asp Leu Ser Gly Ser Leu Ile Ala Gln Leu Ala Asp
    55                  60                  65

Tyr Met Asn Gly Lys Lys Asp Thr Tyr Gln Ile Ile Thr Glu Lys Ile
70                  75                  80                  85

Ala Asn Gly Glu Pro Leu Thr Val Asp Glu Lys Trp Phe Met Leu Gln
               90                  95                  100

Ala Pro Gly Gly Phe Phe Asp Asn Thr Ile Pro Trp Asn Gly Glu Pro
            105                 110                 115

Ile Thr Asp Pro Asn Gly Asn Pro Ile Arg Asp Phe Trp Asp Arg Tyr
        120                 125                 130

Thr Glu Leu Lys Asn Lys Met Leu Ser Ala Lys Ala Lys Tyr Ala Asn
    135                 140                 145

Phe Val Thr Glu Ser Gln Lys Val Ala Val Thr Asn Glu Phe Thr Glu
150                 155                 160                 165

Gln Asp Tyr Ile Asp Leu Ala Val Leu Phe Asn Leu Ala Trp Ile Asp
               170                 175                 180

Tyr Asn Tyr Ile Thr Ser Thr Pro Glu Phe Lys Ala Leu Tyr Asp Lys
            185                 190                 195

Val Asp Glu Gly Gly Tyr Thr Arg Ala Asp Val Lys Thr Val Leu Asp
        200                 205                 210

Ala Gln Ile Trp Leu Leu Asn His Thr Phe Glu Glu His Glu Lys Ile
    215                 220                 225

Asn Leu Leu Leu Gly Asn Gly Asn Val Glu Val Thr Val Val Pro Tyr
230                 235                 240                 245

Ala His Pro Ile Gly Pro Ile Leu Asn Asp Phe Gly Trp Asp Ser Asp
```

```
                250                 255                 260

Phe Asn Asp Gln Val Lys Lys Ala Asp Glu Leu Tyr Lys Pro Tyr Leu
            265                 270                 275

Gly Gly Gly Thr Ala Val Pro Lys Gly Gly Trp Ala Ala Glu Ser Ala
        280                 285                 290

Leu Asn Asp Lys Thr Leu Glu Ile Leu Ala Glu Asn Gly Trp Glu Trp
    295                 300                 305

Val Met Thr Asp Gln Met Val Leu Gly Lys Leu Gly Ile Glu Gly Thr
310                 315                 320                 325

Val Glu Asn Tyr His Lys Pro Trp Val Ala Glu Phe Asn Gly Lys Lys
                330                 335                 340

Ile Tyr Leu Phe Pro Arg Asn His Asp Leu Ser Asp Arg Val Gly Phe
            345                 350                 355

Thr Tyr Ser Gly Met Asn Gln Gln Gln Ala Val Glu Asp Phe Val Asn
        360                 365                 370

Glu Leu Leu Lys Leu Gln Lys Gln Asn Tyr Asp Gly Ser Leu Val Tyr
    375                 380                 385

Val Val Thr Leu Asp Gly Glu Asn Pro Val Glu Asn Tyr Pro Tyr Asp
390                 395                 400                 405

Gly Glu Leu Phe Leu Thr Glu Leu Tyr Lys Lys Leu Thr Glu Leu Gln
                410                 415                 420

Glu Gln Gly Leu Ile Arg Thr Leu Thr Pro Ser Glu Tyr Ile Gln Leu
            425                 430                 435

Tyr Gly Asp Lys Ala Asn Lys Leu Thr Pro Arg Met Met Glu Arg Leu
        440                 445                 450

Asp Leu Thr Gly Asp Asn Val Asn Ala Leu Leu Lys Ala Gln Ser Leu
    455                 460                 465

Gly Glu Leu Tyr Asp Met Thr Gly Val Lys Glu Glu Met Gln Trp Pro
470                 475                 480                 485

Glu Ser Ser Trp Ile Asp Gly Thr Leu Ser Thr Trp Ile Gly Glu Pro
                490                 495                 500

Gln Glu Asn Tyr Gly Trp Tyr Trp Leu Tyr Met Ala Arg Lys Ala Leu
            505                 510                 515

Met Glu Asn Lys Asp Lys Met Ser Gln Ala Asp Trp Glu Lys Ala Tyr
        520                 525                 530

Glu Tyr Leu Leu Arg Ala Glu Ala Ser Asp Trp Phe Trp Trp Tyr Gly
    535                 540                 545

Ser Asp Gln Asp Ser Gly Gln Asp Tyr Thr Phe Asp Arg Tyr Leu Lys
550                 555                 560                 565

Thr Tyr Leu Tyr Glu Met Tyr Lys Leu Ala Gly Val Glu Pro Pro Ser
```

570 575 580

Tyr Leu Phe Gly Asn Tyr Phe Pro Asp Gly Glu Pro Tyr Thr Thr Arg
585 590 595

Gly Leu Val Gly Leu Lys Asp Gly Glu Met Lys Asn Phe Ser Ser Met
600 605 610

Ser Pro Leu Ala Lys Gly Val Ser Val Tyr Phe Asp Gly Glu Gly Ile
615 620 625

His Phe Ile Val Lys Gly Asn Leu Asp Arg Phe Glu Val Ser Ile Trp
630 635 640 645

Glu Lys Asp Glu Arg Val Gly Asn Thr Phe Thr Arg Leu Gln Glu Lys
650 655 660

Pro Asp Glu Leu Ser Tyr Phe Met Phe Pro Phe Ser Arg Asp Ser Val
665 670 675

Gly Leu Leu Ile Thr Lys His Val Val Tyr Glu Asn Gly Lys Ala Glu
680 685 690

Ile Tyr Gly Ala Thr Asp Tyr Glu Lys Ser Glu Lys Leu Gly Glu Ala
695 700 705

Thr Val Lys Asn Thr Ser Glu Gly Ile Glu Val Val Leu Pro Phe Asp
710 715 720 725

Tyr Ile Glu Asn Pro Ser Asp Phe Tyr Phe Ala Val Ser Thr Val Lys
730 735 740

Asp Gly Asp Leu Glu Val Ile Ser Thr Pro Val Glu Leu Lys Leu Pro
745 750 755

Thr Glu Val Lys Gly Val Val Ile Ala Asp Ile Thr Asp Pro Glu Gly
760 765 770

Asp Asp His Gly Pro Gly Asn Tyr Thr Tyr Pro Thr Asp Lys Val Phe
775 780 785

Lys Pro Gly Val Phe Asp Leu Leu Arg Phe Arg Met Leu Glu Gln Thr
790 795 800 805

Glu Ser Tyr Val Met Glu Phe Tyr Phe Lys Asp Leu Gly Gly Asn Pro
810 815 820

Trp Asn Gly Pro Asn Gly Phe Ser Leu Gln Ile Ile Glu Val Tyr Leu
825 830 835

Asp Phe Lys Asp Gly Gly Asn Ser Ser Ala Ile Lys Met Phe Pro Asp
840 845 850

Gly Pro Gly Ala Asn Val Asn Leu Asp Pro Glu His Pro Trp Asp Val
855 860 865

Ala Phe Arg Ile Ala Gly Trp Asp Tyr Gly Asn Leu Ile Ile Leu Pro
870 875 880 885

Asn Gly Thr Ala Ile Gln Gly Glu Met Gln Ile Ser Ala Asp Pro Val

```
                890                 895                 900

Lys Asn Ala Ile Ile Val Lys Val Pro Lys Lys Tyr Ile Ala Ile Asn
            905                 910                 915

Glu Asp Tyr Gly Leu Trp Gly Asp Val Leu Val Gly Ser Gln Asp Gly
        920                 925                 930

Tyr Gly Pro Asp Lys Trp Arg Thr Ala Ala Val Asp Ala Glu Gln Trp
    935                 940                 945

Lys Leu Gly Gly Ala Asp Pro Gln Ala Val Ile Asn Gly Val Ala Pro
950                 955                 960                 965

Arg Val Ile Asp Glu Leu Val Pro Gln Gly Phe Glu Pro Thr Gln Glu
                970                 975                 980

Glu Gln Leu Ser Ser Tyr Asp Ala Asn Asp Met Lys Leu Ala Thr Val
            985                 990                 995

Lys Ala Leu  Leu Leu Leu Lys Gln  Gly Ile Val Val Thr  Asp Pro
        1000                1005                1010

Glu Gly Asp  Asp His Gly Pro Gly  Thr Tyr Thr Tyr Pro  Thr Asp

        1015                1020                1025

Lys Val Phe  Lys Pro Gly Val Phe  Asp Leu Leu Lys Phe  Lys Val
        1030                1035                1040

Thr Glu Gly  Ser Asp Asp Trp Thr  Leu Glu Phe His Phe  Lys Asp
        1045                1050                1055

Leu Gly Gly  Asn Pro Trp Asn Gly  Pro Asn Gly Phe Ser  Leu Gln
        1060                1065                1070

Ile Ile Glu  Val Tyr Phe Asp Phe  Lys Glu Gly Gly Asn  Val Ser
        1075                1080                1085

Ala Ile Lys  Met Phe Pro Asp Gly  Pro Gly Ser Asn Val  Arg Leu
        1090                1095                1100

Asp Pro Asn  His Pro Trp Asp Leu  Ala Leu Arg Ile Ala  Gly Trp
        1105                1110                1115

Asp Tyr Gly  Asn Leu Ile Ile Leu  Pro Asp Gly Thr Ala  Tyr Gln
        1120                1125                1130

Gly Glu Met  Gln Ile Ser Ala Asp  Pro Val Lys Asn Ala  Ile Ile
        1135                1140                1145

Val Lys Val  Pro Lys Lys Tyr Leu  Asn Ile Ser Asp Tyr  Gly Leu
        1150                1155                1160

Tyr Thr Ala  Val Ile Val Gly Ser  Gln Asp Gly Tyr Gly  Pro Asp
        1165                1170                1175

Lys Trp Arg  Pro Val Ala Ala Glu  Ala Glu Gln Trp Lys  Leu Gly
        1180                1185                1190
```

```
Gly Ala Asp  Pro Gln Ala Val Ile  Asp Asn Leu Val Pro  Arg Val
        1195                 1200                 1205

Val Asp Glu  Leu Val Pro Glu Gly  Phe Lys Pro Thr Gln  Glu Glu
        1210                 1215                 1220

Gln Leu Ser  Ser Tyr Asp Leu Glu  Lys Lys Thr Leu Ala  Thr Val
        1225                 1230                 1235

Leu Met Val  Pro Leu Val Asn Gly  Thr Gly Gly Glu Glu  Pro Thr
        1240                 1245                 1250

Pro Thr Glu  Ser Pro Thr Glu Thr  Thr Thr Thr Thr Pro  Ser Glu
        1255                 1260                 1265

Thr Thr Thr  Thr Thr Ser Thr Thr  Thr Gly Pro Ser Ser  Thr Thr
        1270                 1275                 1280

Thr Ser Thr  Pro Gly Gly Gly Ile  Cys Gly Pro Gly Ile  Ile Ala
        1285                 1290                 1295

Gly Leu Ala  Leu Ile Pro Leu Leu  Leu Lys Arg Arg Asn
        1300                 1305                 1310
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<210> 12

<211> 809

<212> PRT

<213> Artificial Sequence

<220>

<223> Hybrid pullulanase of Thermoccus hydrothermalis and Thermococcus litoralis

<220>

<221> SIGNAL

<222> (1)..(27)

<220>

<221> mat_peptide

<222> (28)..(809)

<400> 12

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Met Lys Lys Pro Leu Gly Lys Ile Val Ala Ser Thr Ala Leu Leu Ile
        -25                 -20                 -15

Ser Val Ala Phe Ser Ser Ser Ile Ala Ser Ala Glu Glu Pro Lys Pro
    -10                 -5                  -1  1                5

Leu Asn Val Ile Ile Val Trp His Gln His Gln Pro Tyr Tyr Tyr Asp
                10                  15                  20

Pro Ile Gln Asp Ile Tyr Thr Arg Pro Trp Val Arg Leu His Ala Ala
            25                  30                  35

Asn Asn Tyr Trp Lys Met Ala Asn Tyr Leu Ser Lys Tyr Pro Asp Val
        40                  45                  50
```

```
His Val Ala Ile Asp Leu Ser Gly Ser Leu Ile Ala Gln Leu Ala Asp
    55                  60                  65

Tyr Met Asn Gly Lys Lys Asp Thr Tyr Gln Ile Val Thr Glu Lys Ile
70                  75                  80                  85

Ala Asn Gly Glu Pro Leu Thr Leu Glu Asp Lys Trp Phe Met Leu Gln
                90                  95                  100

Ala Pro Gly Gly Phe Phe Asp His Thr Ile Pro Trp Asn Gly Glu Pro
            105                 110                 115

Val Ala Asp Glu Asn Gly Asn Pro Tyr Arg Glu Gln Trp Asp Arg Tyr
        120                 125                 130

Ala Glu Leu Lys Asp Lys Arg Asn Asn Ala Phe Lys Lys Tyr Ala Asn
    135                 140                 145

Leu Pro Leu Asn Glu Gln Lys Val Lys Ile Thr Ala Glu Phe Thr Glu
150                 155                 160                 165

Gln Asp Tyr Ile Asp Leu Ala Val Leu Phe Asn Leu Ala Trp Ile Asp
                170                 175                 180

Tyr Asn Tyr Ile Ile Asn Thr Pro Glu Leu Lys Ala Leu Tyr Asp Lys
            185                 190                 195

Val Asp Val Gly Gly Tyr Thr Lys Glu Asp Val Ala Thr Val Leu Lys
        200                 205                 210

His Gln Met Trp Leu Leu Asn His Thr Phe Glu Glu His Glu Lys Ile
    215                 220                 225

Asn Tyr Leu Leu Gly Asn Gly Asn Val Glu Val Thr Val Val Pro Tyr
230                 235                 240                 245

Ala His Pro Ile Gly Pro Leu Leu Asn Asp Phe Gly Trp Tyr Glu Asp
                250                 255                 260

Phe Asp Ala His Val Lys Lys Ala His Glu Leu Tyr Lys Lys Tyr Leu
            265                 270                 275

Gly Asp Asn Arg Val Glu Pro Gln Gly Gly Trp Ala Ala Glu Ser Ala
        280                 285                 290

Leu Asn Asp Lys Thr Leu Glu Ile Leu Thr Asn Asn Gly Trp Lys Trp
    295                 300                 305

Val Met Thr Asp Gln Met Val Leu Asp Ile Leu Gly Ile Pro Asn Thr
310                 315                 320                 325

Val Glu Asn Tyr Tyr Lys Pro Trp Val Ala Glu Phe Asn Gly Lys Lys
                330                 335                 340

Ile Tyr Leu Phe Pro Arg Asn His Asp Leu Ser Asp Arg Val Gly Phe
            345                 350                 355

Arg Tyr Ser Gly Met Asn Gln Tyr Gln Ala Val Glu Asp Phe Val Asn
        360                 365                 370
```

```
Glu Leu Leu Lys Val Gln Lys Glu Asn Tyr Asp Gly Ser Leu Val Tyr
    375                 380                 385

Val Val Thr Leu Asp Gly Glu Asn Pro Trp Glu His Tyr Pro Phe Asp
390                 395                 400                 405

Gly Lys Ile Phe Leu Glu Glu Leu Tyr Lys Lys Leu Thr Glu Leu Gln
                410                 415                 420

Lys Gln Gly Leu Ile Arg Thr Val Thr Pro Ser Glu Tyr Ile Gln Met
            425                 430                 435

Tyr Gly Asp Lys Ala Asn Lys Leu Thr Pro Arg Met Met Glu Arg Leu
        440                 445                 450

Asp Leu Thr Gly Asp Asn Val Asn Ala Leu Leu Lys Ala Gln Ser Leu
    455                 460                 465

Gly Glu Leu Tyr Asp Met Thr Gly Val Lys Glu Glu Met Gln Trp Pro
470                 475                 480                 485

Glu Ser Ser Trp Ile Asp Gly Thr Leu Ser Thr Trp Ile Gly Glu Pro
                490                 495                 500

Gln Glu Asn Tyr Gly Trp Tyr Trp Leu Tyr Met Ala Arg Lys Ala Leu
            505                 510                 515

Met Glu Asn Lys Asp Lys Met Ser Gln Ala Asp Trp Glu Lys Ala Tyr
        520                 525                 530

Glu Tyr Leu Leu Arg Ala Glu Ala Ser Asp Trp Phe Trp Trp Tyr Gly
    535                 540                 545

Ser Asp Gln Asp Ser Gly Gln Asp Tyr Thr Phe Asp Arg Tyr Leu Lys
550                 555                 560                 565

Thr Tyr Leu Tyr Glu Met Tyr Lys Leu Ala Gly Val Glu Pro Pro Ser
                570                 575                 580

Tyr Leu Phe Gly Asn Tyr Phe Pro Asp Gly Glu Pro Tyr Thr Thr Arg

            585                 590                 595

Gly Leu Val Gly Leu Lys Asp Gly Glu Met Lys Asn Phe Ser Ser Met
        600                 605                 610

Ser Pro Leu Ala Lys Gly Val Ser Val Tyr Phe Asp Gly Glu Gly Ile
    615                 620                 625

His Phe Ile Val Lys Gly Asn Leu Asp Arg Phe Glu Val Ser Ile Trp
630                 635                 640                 645

Glu Lys Asp Glu Arg Val Gly Asn Thr Phe Thr Arg Leu Gln Glu Lys
                650                 655                 660

Pro Asp Glu Leu Ser Tyr Phe Met Phe Pro Phe Ser Arg Asp Ser Val
            665                 670                 675
```

```
Gly Leu Leu Ile Thr Lys His Val Val Tyr Glu Asn Gly Lys Ala Glu
        680                 685                 690

Ile Tyr Gly Ala Thr Asp Tyr Glu Lys Ser Glu Lys Leu Gly Glu Ala
    695                 700                 705

Thr Val Lys Asn Thr Ser Glu Gly Ile Glu Val Val Leu Pro Phe Asp
710                 715                 720                 725

Tyr Ile Glu Asn Pro Ser Asp Phe Tyr Phe Ala Val Ser Thr Val Lys
                730                 735                 740

Asp Gly Asp Leu Glu Val Ile Ser Thr Pro Val Glu Leu Lys Leu Pro
            745                 750                 755

Thr Glu Val Lys Gly Val Val Ile Ala Asp Ile Thr Asp Pro Glu Gly
        760                 765                 770

Asp Asp His Gly Pro Gly Asn Tyr Thr
    775                 780
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<210> 13

<211> 412

<212> PRT

<213> Pyrococcus furiosus

<220>

<221> mat_peptide

<222> (1)..(412)

<223> Pyrococcus furiosus protease (Pfu)

<400> 13

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Ala Glu Leu Glu Gly Leu Asp Glu Ser Ala Ala Gln Val Met Ala Thr
1               5                   10                  15

Tyr Val Trp Asn Leu Gly Tyr Asp Gly Ser Gly Ile Thr Ile Gly Ile
            20                  25                  30

Ile Asp Thr Gly Ile Asp Ala Ser His Pro Asp Leu Gln Gly Lys Val
        35                  40                  45

Ile Gly Trp Val Asp Phe Val Asn Gly Arg Ser Tyr Pro Tyr Asp Asp
    50                  55                  60

His Gly His Gly Thr His Val Ala Ser Ile Ala Ala Gly Thr Gly Ala
65                  70                  75                  80

Ala Ser Asn Gly Lys Tyr Lys Gly Met Ala Pro Gly Ala Lys Leu Ala
                85                  90                  95

Gly Ile Lys Val Leu Gly Ala Asp Gly Ser Gly Ser Ile Ser Thr Ile
            100                 105                 110

Ile Lys Gly Val Glu Trp Ala Val Asp Asn Lys Asp Lys Tyr Gly Ile
        115                 120                 125

Lys Val Ile Asn Leu Ser Leu Gly Ser Ser Gln Ser Ser Asp Gly Thr
```

```
    130                 135                 140

Asp Ala Leu Ser Gln Ala Val Asn Ala Ala Trp Asp Ala Gly Leu Val
145                 150                 155                 160

Val Val Val Ala Ala Gly Asn Ser Gly Pro Asn Lys Tyr Thr Ile Gly
                165                 170                 175

Ser Pro Ala Ala Ala Ser Lys Val Ile Thr Val Gly Ala Val Asp Lys
            180                 185                 190

Tyr Asp Val Ile Thr Ser Phe Ser Ser Arg Gly Pro Thr Ala Asp Gly
        195                 200                 205

Arg Leu Lys Pro Glu Val Val Ala Pro Gly Asn Trp Ile Ile Ala Ala
    210                 215                 220

Arg Ala Ser Gly Thr Ser Met Gly Gln Pro Ile Asn Asp Tyr Tyr Thr
225                 230                 235                 240

Ala Ala Pro Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ile Ala
                245                 250                 255

Ala Leu Leu Leu Gln Ala His Pro Ser Trp Thr Pro Asp Lys Val Lys
            260                 265                 270

Thr Ala Leu Ile Glu Thr Ala Asp Ile Val Lys Pro Asp Glu Ile Ala
        275                 280                 285

Asp Ile Ala Tyr Gly Ala Gly Arg Val Asn Ala Tyr Lys Ala Ile Asn
    290                 295                 300

Tyr Asp Asn Tyr Ala Lys Leu Val Phe Thr Gly Tyr Val Ala Asn Lys
305                 310                 315                 320

Gly Ser Gln Thr His Gln Phe Val Ile Ser Gly Ala Ser Phe Val Thr
                325                 330                 335

Ala Thr Leu Tyr Trp Asp Asn Ala Asn Ser Asp Leu Asp Leu Tyr Leu
            340                 345                 350

Tyr Asp Pro Asn Gly Asn Gln Val Asp Tyr Ser Tyr Thr Ala Tyr Tyr
        355                 360                 365

Gly Phe Glu Lys Val Gly Tyr Tyr Asn Pro Thr Asp Gly Thr Trp Thr
    370                 375                 380

Ile Lys Val Val Ser Tyr Ser Gly Ser Ala Asn Tyr Gln Val Asp Val
385                 390                 395                 400

Val Ser Asp Gly Ser Leu Ser Gln Pro Gly Ser Ser
                405                 410
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<210> 14

<211> 595

<212> PRT

<213> Penicillium oxalicum

<220>

<221> mat_peptide

<222> (1)..(595)

<223> mature Penicillium oxalicum glucoamylase sequence

<400> 14

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Arg Pro Asp Pro Lys Gly Gly Asn Leu Thr Pro Phe Ile His Lys Glu
1               5                  10                  15

Gly Glu Arg Ser Leu Gln Gly Ile Leu Asp Asn Leu Gly Gly Arg Gly
            20                  25                  30

Lys Lys Thr Pro Gly Thr Ala Ala Gly Leu Phe Ile Ala Ser Pro Asn
        35                  40                  45

Thr Glu Asn Pro Asn Tyr Tyr Tyr Thr Trp Thr Arg Asp Ser Ala Leu
    50                  55                  60

Thr Ala Lys Cys Leu Ile Asp Leu Phe Glu Asp Ser Arg Ala Lys Phe
65                  70                  75                  80

Pro Ile Asp Arg Lys Tyr Leu Glu Thr Gly Ile Arg Asp Tyr Lys Ser
                85                  90                  95

Ser Gln Ala Ile Leu Gln Ser Val Ser Asn Pro Ser Gly Thr Leu Lys
            100                 105                 110

Asp Gly Ser Gly Leu Gly Glu Pro Lys Phe Glu Ile Asp Leu Asn Pro
        115                 120                 125

Phe Ser Gly Ala Trp Gly Arg Pro Gln Arg Asp Gly Pro Ala Leu Arg
    130                 135                 140

Ala Thr Ala Met Ile Thr Tyr Ala Asn Tyr Leu Ile Ser His Gly Gln
145                 150                 155                 160

Lys Ser Asp Val Ser Gln Val Met Trp Pro Ile Ile Ala Asn Asp Leu
                165                 170                 175

Ala Tyr Val Gly Gln Tyr Trp Asn Asn Thr Gly Phe Asp Leu Trp Glu
            180                 185                 190

Glu Val Asp Gly Ser Ser Phe Phe Thr Ile Ala Val Gln His Arg Ala
        195                 200                 205

Leu Val Glu Gly Ser Gln Leu Ala Lys Lys Leu Gly Lys Ser Cys Asp
    210                 215                 220

Ala Cys Asp Ser Gln Pro Pro Gln Ile Leu Cys Phe Leu Gln Ser Phe
225                 230                 235                 240

Trp Asn Gly Lys Tyr Ile Thr Ser Asn Ile Asn Thr Gln Ala Ser Arg
                245                 250                 255

Ser Gly Ile Asp Leu Asp Ser Val Leu Gly Ser Ile His Thr Phe Asp
            260                 265                 270

Pro Glu Ala Ala Cys Asp Asp Ala Thr Phe Gln Pro Cys Ser Ala Arg
        275                 280                 285
```

```
Ala Leu Ala Asn His Lys Val Tyr Val Asp Ser Phe Arg Ser Ile Tyr
    290                 295                 300

Lys Ile Asn Ala Gly Leu Ala Glu Gly Ser Ala Ala Asn Val Gly Arg
305                 310                 315                 320

Tyr Pro Glu Asp Val Tyr Gln Gly Gly Asn Pro Trp Tyr Leu Ala Thr
                325                 330                 335

Leu Gly Ala Ser Glu Leu Leu Tyr Asp Ala Leu Tyr Gln Trp Asp Arg
            340                 345                 350

Leu Gly Lys Leu Glu Val Ser Glu Thr Ser Leu Ser Phe Phe Lys Asp
        355                 360                 365

Phe Asp Ala Thr Val Lys Ile Gly Ser Tyr Ser Arg Asn Ser Lys Thr
    370                 375                 380

Tyr Lys Lys Leu Thr Gln Ser Ile Lys Ser Tyr Ala Asp Gly Phe Ile
385                 390                 395                 400

Gln Leu Val Gln Gln Tyr Thr Pro Ser Asn Gly Ser Leu Ala Glu Gln
                405                 410                 415

Tyr Asp Arg Asn Thr Ala Ala Pro Leu Ser Ala Asn Asp Leu Thr Trp
            420                 425                 430

Ser Phe Ala Ser Phe Leu Thr Ala Thr Gln Arg Arg Asp Ala Val Val
        435                 440                 445

Pro Pro Ser Trp Gly Ala Lys Ser Ala Asn Lys Val Pro Thr Thr Cys
    450                 455                 460

Ser Ala Ser Pro Val Val Gly Thr Tyr Lys Ala Pro Thr Ala Thr Phe
465                 470                 475                 480

Ser Ser Lys Thr Lys Cys Val Pro Ala Lys Asp Ile Val Pro Ile Thr
                485                 490                 495

Phe Tyr Leu Ile Glu Asn Thr Tyr Tyr Gly Glu Asn Val Phe Met Ser
            500                 505                 510

Gly Asn Ile Thr Ala Leu Gly Asn Trp Asp Ala Lys Lys Gly Phe Pro
        515                 520                 525

Leu Thr Ala Asn Leu Tyr Thr Gln Asp Gln Asn Leu Trp Phe Ala Ser
    530                 535                 540

Val Glu Phe Ile Pro Ala Gly Thr Pro Phe Glu Tyr Lys Tyr Tyr Lys
545                 550                 555                 560

Val Glu Pro Asn Gly Asp Ile Thr Trp Glu Lys Gly Pro Asn Arg Val
                565                 570                 575

Phe Val Ala Pro Thr Gly Cys Pro Val Gln Pro His Ser Asn Asp Val
            580                 585                 590

Trp Gln Phe
        595
```

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Patentkrav

1. Fremgangsmåde til fremstilling af fermenteringsprodukter ud fra stivelsesholdigt materiale, hvilken fremgangsmåde omfatter følgende trin:

i) at gøre det stivelsesholdige materiale flydende ved en pH i intervallet fra 4,5 til 5,0 ved en temperatur i intervallet fra 80 til 90°C ved anvendelse af:

- en variant af *Bacillus stearothermophilus*-alfa-amylasen, som er vist i SEQ ID NO: 1, hvilken variant har en dobbeltdeletion i I181 + G182 og substitutionen N193F og endvidere omfatter mutationer valgt fra gruppen bestående af:
  - V59A+Q89R+G112D+E129V+K177L+R179E+K220P+N224L+Q254S;
  - V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
  - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+D269E+D281N;
  - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+I270L;
  - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+H274K;
  - V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+Y276F;
  - V59A+E129V+R157Y+K177L+R179E+K220P+N224L+S242Q+Q254S;
  - V59A+E129V+K177L+R179E+H208Y+K220P+N224L+S242Q+Q254S;
  - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
  - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+H274K;
  - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F;
  - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+D281N;
  - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
  - V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+G416V;
  - V59A+E129V+K177L+R179E+K220P+N224L+Q254S;
  - V59A+E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
  - A91L+M96I+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
  - E129V+K177L+R179E;
  - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
  - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F+L427M;
  - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
  - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+N376*+I377*;
  - E129V+K177L+R179E+K220P+N224L+Q254S;
  - E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
  - E129V+K177L+R179E+S242Q;
  - E129V+K177L+R179V+K220P+N224L+S242Q+Q254S;
  - K220P+N224L+S242Q+Q254S og

- M284V;

hvilken variant udviser mindst 80% identitet, men mindre end 100% identitet, med den modne del af polypeptidet ifølge SEQ ID NO: 1 og har en T½ (min) ved pH 4,5, 85°C, 0,12 mM $CaCl_2$) på mindst 10; og

- en variant af en metalloprotease, hvilken variant udviser mindst 80% identitet, men mindre end 100% identitet, med den modne del af polypeptidet ifølge SEQ ID NO: 3 heri og har en termostabilitetsværdi på mere end 20%, bestemt som relativ aktivitet ved 80°C/70°C; eller

- en *Pyrococcus furiosus*-protease, som udviser mindst 80% identitet med SEQ ID NO: 13 heri og har en termostabilitetsværdi på mere end 20%, bestemt som relativ aktivitet ved 80°C/70°C;

ii) at forsukre ved anvendelse af et carbohydratkildefrembringende enzym;

iii) at fermentere ved anvendelse af en fermenterende organisme.

2. Fremgangsmåde ifølge krav 1, hvor varianten af *Bacillus stearothermophilus*-alfa-amylasen, som er vist i SEQ ID NO: 1 heri, er trunkeret, fortrinsvis til at have omkring 491 aminosyrer.

3. Fremgangsmåde ifølge krav 2, hvor alfa-amylasevarianten udviser mindst 85%, mere fortrinsvis mindst 90%, mere fortrinsvis mindst 91%, mere fortrinsvis mindst 92%, endnu mere fortrinsvis mindst 93%, mest fortrinsvis mindst 94% og allermest fortrinsvis mindst 95%, såsom endda mindst 96%, mindst 97%, mindst 98%, mindst 99%, men mindre end 100%, identitet med den modne del af polypeptidet ifølge SEQ ID NO: 1 heri.

4. Fremgangsmåde ifølge et hvilket som helst af kravene 1-3, hvor alfa-amylasen er afledt af *Bacillus stearothermophilus*-alfa-amylase trunkeret til at have omkring 491 aminosyrer og med mutationerne valgt fra gruppen bestående af:

- I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
- I181*+G182*+N193F+E129V+K177L+R179E og
- I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S.

5. Fremgangsmåde ifølge et hvilket som helst af kravene 1-4, hvor en anden alfa-amylase tilsættes under forflydningstrin i).

6. Fremgangsmåde ifølge et hvilket som helst af kravene 1-5, hvor proteasen er en variant af metalloproteasen, der er beskrevet som den modne del af SEQ ID NO: 3 heri,

hvilken proteasevariant udviser mindst 80%, mere fortrinsvis mindst 85%, mere fortrinsvis mindst 90%, mere fortrinsvis mindst 91%, mere fortrinsvis mindst 92%, endnu mere fortrinsvis mindst 93%, mest fortrinsvis mindst 94% og allermest fortrinsvis mindst 95%, såsom endda mindst 96%, mindst 97%, mindst 98%, mindst 99%, men mindre end 100%, identitet med den modne del af polypeptidet ifølge SEQ ID NO: 3 heri.

7. Fremgangsmåde ifølge et hvilket som helst af kravene 1-6, hvor proteasen er en variant af *Thermoascus aurantiacus*-proteasen, som er vist i SEQ ID NO: 3, hvilken variant har mutationerne valgt fra gruppen bestående af:

- A27K+D79L+Y82F+S87G+D104P+A112P+A126V+D142L;
- D79L+Y82F+S87G+A112P+D142L;
- Y82F+S87G+S70V+D79L+D104P+A112P+D142L og
- Y82F+S87G+D79L+D104P+A112P+A126V+D142L.

8. Fremgangsmåde ifølge et hvilket som helst af kravene 1-7, hvor proteasen stammer fra en *Pyrococcus furiosus*-stamme, som udviser mindst 85%, såsom mindst 90%, såsom mindst 95%, såsom mindst 96%, såsom mindst 97%, såsom mindst 98%, såsom mindst 99%, identitet med SEQ ID NO: 13 heri.

9. Fremgangsmåde ifølge et hvilket som helst af kravene 1-8, hvor der endvidere tilsættes og/eller er et carbohydratkildefrembringende enzym til stede under forflydningstrin i), fortrinsvis en glucoamylase, navnlig hvor det carbohydratkildefrembringende enzym er en glucoamylase med en varmestabilitet ved 85°C, pH 5,3, på mindst 30%, fortrinsvis mindst 35%.

10. Fremgangsmåde ifølge krav 9, hvor det carbohydratkildefrembringende enzym er en glucoamylase, som fortrinsvis stammer fra en stamme af slægten *Penicillium*, især en*Penicillium oxalicum*-stamme, der er beskrevet som SEQ ID NO: 9 eller 14 heri, navnlig hvor det carbohydratkildefrembringende enzym er en variant af glucoamylasen, som stammer fra en *Penicillium oxalicum*-stamme, og som har en K79V-substitution i SEQ ID NO: 9 eller 14 (ved anvendelse af den modne sekvens, som er vist i SEQ ID NO: 14, til nummerering), såsom hvor glucoamylasen udviser mindst 80%, mere fortrinsvis mindst 85%, mere fortrinsvis mindst 90%, mere fortrinsvis mindst 91%, mere fortrinsvis mindst 92%, endnu mere fortrinsvis mindst 93%, mest fortrinsvis mindst 94% og allermest fortrinsvis mindst 95%, såsom endda mindst 96%, mindst 97%, mindst 98%, mindst 99% eller 100% identitet med det modne polypeptid, som er vist i SEQ ID NO: 9 eller 14 heri.

11. Fremgangsmåde ifølge et hvilket som helst af kravene 1-10, hvor der endvidere tilsættes og/eller er en glucoamylase til stede under forsukring og/eller fermentering, fortrinsvis hvor glucoamylasen er af svampeoprindelse, fortrinsvis hvor den stammer fra en *Aspergillus*-stamme, fortrinsvis *A. niger*, *A. awamori* eller *A. oryzae*; eller en *Trichoderma*-stamme, fortrinsvis *T. reesei*; eller en *Talaromyces*-stamme, fortrinsvis *T. emersonii*, eller en *Pycnoporus*-stamme eller en *Gloephyllum*-stamme.

12. Fremgangsmåde ifølge et hvilket som helst af kravene 1-11, hvor der endvidere er en pullulanase til stede under forflydning og/eller forsukring.

13. Sammensætning, som omfatter en alfa-amylase og en protease, hvor

i) alfa-amylasen er en variant af *Bacillus stearothermophilus*-alfa-amylasen, som er vist i SEQ ID NO: 1, hvilken variant har en dobbeltdeletion i I181 + G182 og substitutionen N193F og endvidere omfatter mutationer valgt fra gruppen bestående af:

- V59A+Q89R+G112D+E129V+K177L+R179E+K220P+N224L+Q254S;
- V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
-V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+D269E+D281N;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+I270L;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+H274K;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+Y276F;
- V59A+E129V+R157Y+K177L+R179E+K220P+N224L+S242Q+Q254S;
- V59A+E129V+K177L+R179E+H208Y+K220P+N224L+S242Q+Q254S;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+H274K;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+D281N;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+G416V;
- V59A+E129V+K177L+R179E+K220P+N224L+Q254S;
- V59A+E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
- A91L+M96I+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- E129V+K177L+R179E;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F+L427M;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;

- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+N376*+I377*;
- E129V+K177L+R179E+K220P+N224L+Q254S;
- E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
- E129V+K177L+R179E+S242Q;
- E129V+K177L+R179V+K220P+N224L+S242Q+Q254S;
- K220P+N224L+S242Q+Q254S og
- M284V;

hvilken variant udviser mindst 80% identitet, men mindre end 100% identitet, med den modne del af polypeptidet ifølge SEQ ID NO: 1 og har en T½ (min) ved pH 4,5, 85°C, 0,12 mM $CaCl_2$) på mindst 10; og

ii) en variant af en metalloprotease, hvilken variant udviser mindst 80% identitet, men mindre end 100% identitet, med den modne del af polypeptidet ifølge SEQ ID NO: 3 heri og har en termostabilitetsværdi på mere end 20%, bestemt som relativ aktivitet ved 80°C/70°C; eller

- en *Pyrococcus furiosus*-protease, som udviser mindst 80% identitet med SEQ ID NO: 13 heri og har en termostabilitetsværdi på mere end 20%, bestemt som relativ aktivitet ved 80°C/70°C.

14. Sammensætning ifølge krav 13, hvor varianten af *Bacillus stearothermophilus*-alfa-amylasen, som er vist i SEQ ID NO: 1 heri, er trunkeret, fortrinsvis til at have omkring 491 aminosyrer, navnlig hvor alfa-amylasevarianten udviser mindst 85%, mere fortrinsvis mindst 90%, mere fortrinsvis mindst 91%, mere fortrinsvis mindst 92%, endnu mere fortrinsvis mindst 93%, mest fortrinsvis mindst 94% og allermest fortrinsvis mindst 95%, såsom endda mindst 96%, mindst 97%, mindst 98%, mindst 99%, men mindre end 100%, identitet med den modne del af polypeptidet ifølge SEQ ID NO: 1 heri.

15. Sammensætning ifølge krav 13 eller 14, hvor alfa-amylasen er afledt af *Bacillus stearothermophilus*-alfa-amylase trunkeret til at have omkring 491 aminosyrer og med mutationerne valgt fra gruppen bestående af:

- I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
- I181*+G182*+N193F+E129V+K177L+R179E og
- I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S.

16. Sammensætning ifølge et hvilket som helst af kravene 13-15, hvor sammensætningen endvidere omfatter en anden alfa-amylase, som stammer fra *Bacillus stearothermophilus*.

17. Sammensætning ifølge et hvilket som helst af kravene 13-16, hvor proteasen har en termostabilitet på mere end 30%, mere end 40%, mere end 50%, mere end 60%, mere end 70%, mere end 80%, mere end 90% mere end 100%, såsom mere end 105%, såsom mere end 110%, såsom mere end 115%, såsom mere end 120% bestemt som relativ aktivitet ved 80°C/70°C.

18. Sammensætning ifølge et hvilket som helst af kravene 13-17, hvor proteasen er en variant af *Thermoascus aurantiacus*-proteasen, som er vist i SEQ ID NO: 3 heri, hvilken variant har mutationerne valgt fra gruppen bestående af:

- A27K+D79L+Y82F+S87G+D104P+A112P+A126V+D142L;
- D79L+Y82F+S87G+A112P+D142L;
- Y82F+S87G+S70V+D79L+D104P+A112P+D142L;
- Y82F+S87G+D79L+D104P+A112P+A126V+D142L.

19. Sammensætning ifølge et hvilket som helst af kravene 13-18, hvor *Pyrococcus furiosus*-proteasen er en, som udviser mindst 85%, såsom mindst 90%, såsom mindst 95%, såsom mindst 96%, såsom mindst 97%, såsom mindst 98%, såsom mindst 99%, identitet med SEQ ID NO: 13 heri.

20. Sammensætning ifølge et hvilket som helst af kravene 13-19, som endvidere omfatter et carbohydratkildefrembringende enzym, navnlig hvor det carbohydratkildefrembringende enzym er en glucoamylase, fortrinsvis med en varmestabilitet udtrykt som relativ aktivitet ved 85°C, pH 5,3, på mindst 20%, mindst 30%, fortrinsvis mindst 35%, navnlig hvor glucoamylasen stammer fra en stamme af slægten *Penicillium*, især en *Penicillium oxalicum*-stamme, der er beskrevet som SEQ ID NO: 9 eller 14 heri, navnlig hvor det carbohydratkildefrembringende enzym er en variant af glucoamylasen, som stammer fra en *Penicillium oxalicum*-stamme, og som har en K79V-substitution i SEQ ID NO: 9 eller 14 (ved anvendelse af den modne sekvens, som er vist i SEQ ID NO: 14, til nummerering), navnlig hvor glucoamylasen udviser mindst 80%, mere fortrinsvis mindst 85%, mere fortrinsvis mindst 90%, mere fortrinsvis mindst 91%, mere fortrinsvis mindst 92%, endnu mere fortrinsvis mindst 93%, mest fortrinsvis mindst 94% og allermest fortrinsvis mindst 95%, såsom endda mindst 96%, mindst 97%, mindst 98%, mindst 99% eller 100% identitet med det modne polypeptid, som er vist i SEQ ID NO: 9 eller 14 heri.

21. Sammensætning ifølge krav 13-20, som endvidere omfatter en pullulanase.

22. Sammensætning ifølge et hvilket som helst af kravene 13-21, som omfatter:

i) en variant af *Bacillus stearothermophilus*-alfa-amylasen, som er vist i SEQ ID NO: 1, hvilken variant har en dobbeltdeletion i I181 + G182 og substitutionen N193F, og hvilken variant udviser mindst 80% identitet, men mindre end 100% identitet, med den modne del af polypeptidet ifølge SEQ ID NO: 1 og har en T½ (min) ved pH 4,5, 85°C, 0,12 mM $CaCl_2$) på mindst 10;

ii) en variant af en metalloprotease, hvilken variant udviser mindst 80% identitet, men mindre end 100% identitet, med den modne del af polypeptidet ifølge SEQ ID NO: 3 heri og har en termostabilitetsværdi på mere end 20%, bestemt som relativ aktivitet ved 80°C/70°C; eller

- en *Pyrococcus furiosus*-protease, som udviser mindst 80% identitet med SEQ ID NO: 13 heri og har en termostabilitetsværdi på mere end 20%, bestemt som relativ aktivitet ved 80°C/70°C; og

iii) en glucoamylase, som stammer fra *Penicillium oxalicum*, der er vist i SEQ ID NO: 9 eller 14 heri, eller en glucoamylase, som udviser mindst 80% identitet med det modne polypeptid, der er vist i SEQ ID NO: 9 eller 14 heri.

DRAWINGS

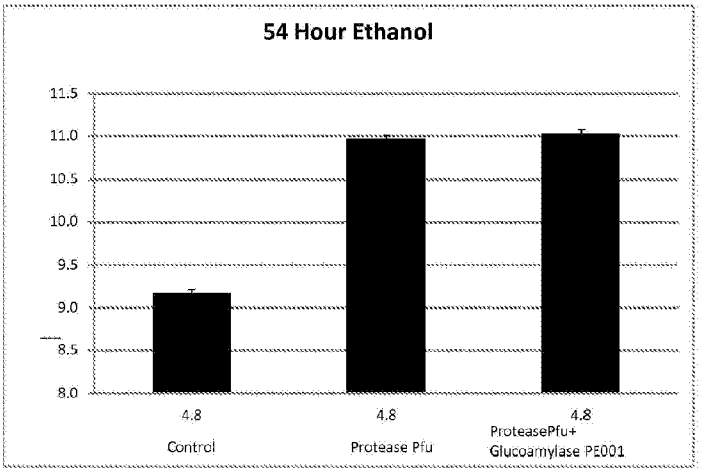

Fig. 1

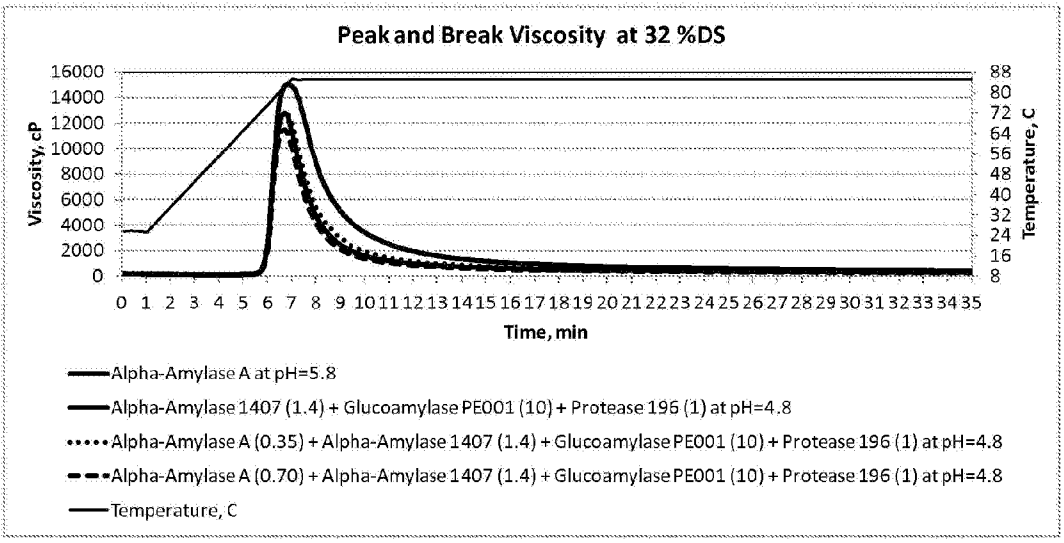
Peak and Break Viscosity at 32 %DS
Viscosity, cP
Temperature, C
Time, min
Alpha-Amylase A at pH=5.8
Alpha-Amylase 1407 (1.4) + Glucoamylase PE001 (10) + Protease 196 (1) at pH=4.8
Alpha-Amylase A (0.35) + Alpha-Amylase 1407 (1.4) + Glucoamylase PE001 (10) + Protease 196 (1) at pH=4.8
Alpha-Amylase A (0.70) + Alpha-Amylase 1407 (1.4) + Glucoamylase PE001 (10) + Protease 196 (1) at pH=4.8
Temperature, C

Fig. 2

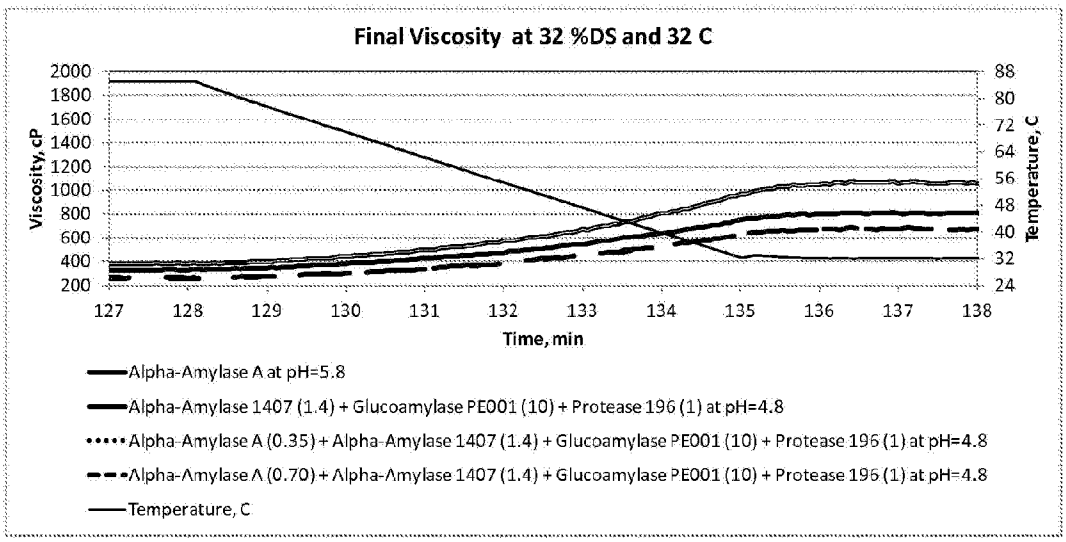
Final Viscosity at 32 %DS and 32 C
Viscosity, cP
2000
1800
1600
1400
1200
1000
800
600
400
200
Temperature, C
88
80
72
64
56
48
40
32
24
127
128
129
130
131
132
133
134
135
136
137
138
Time, min
Alpha-Amylase A at pH=5.8
Alpha-Amylase 1407 (1.4) + Glucoamylase PE001 (10) + Protease 196 (1) at pH=4.8
Alpha-Amylase A (0.35) + Alpha-Amylase 1407 (1.4) + Glucoamylase PE001 (10) + Protease 196 (1) at pH=4.8
Alpha-Amylase A (0.70) + Alpha-Amylase 1407 (1.4) + Glucoamylase PE001 (10) + Protease 196 (1) at pH=4.8
Temperature, C

Fig. 3

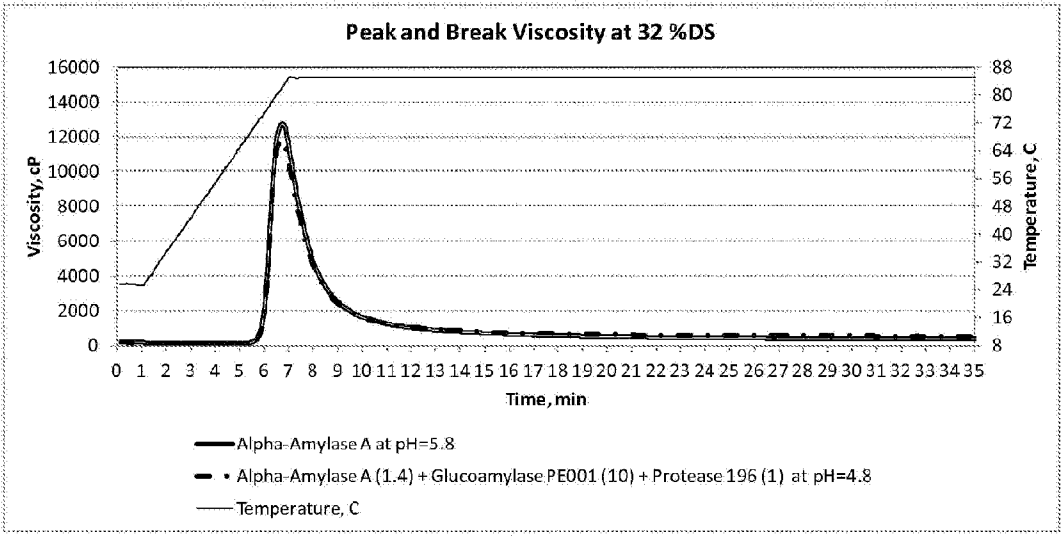
Peak and Break Viscosity at 32 %DS
Viscosity, cP
16000
14000
12000
10000
8000
6000
4000
2000
0
Temperature, C
88
80
72
64
56
48
40
32
24
16
8
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35
Time, min
Alpha-Amylase A at pH=5.8
Alpha-Amylase A (1.4) + Glucoamylase PE001 (10) + Protease 196 (1) at pH=4.8
Temperature, C

Fig. 4

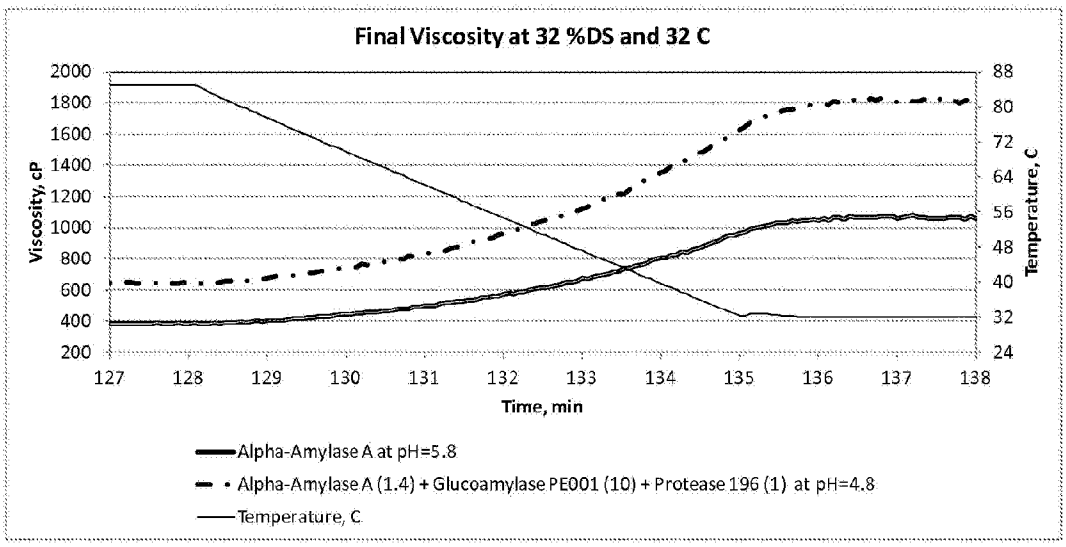
Final Viscosity at 32 %DS and 32 C
Viscosity, cP
2000
1800
1600
1400
1200
1000
800
600
400
200
Temperature, C
88
80
72
64
56
48
40
32
24
127
128
129
130
131
132
133
134
135
136
137
138
Time, min
Alpha-Amylase A at pH=5.8
Alpha-Amylase A (1.4) + Glucoamylase PE001 (10) + Protease 196 (1) at pH=4.8
Temperature, C

Fig. 5