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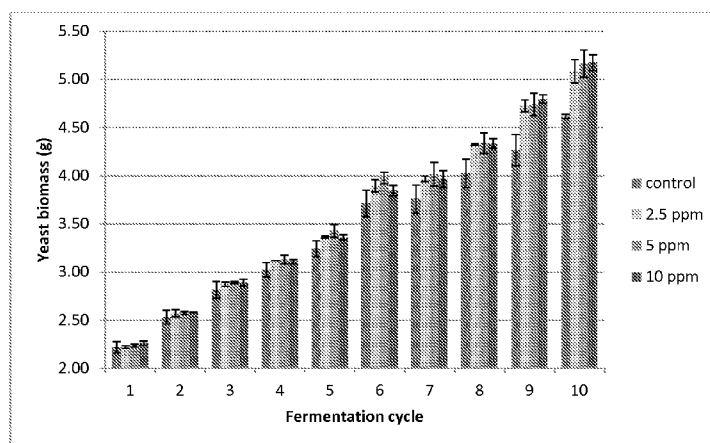
(54) **Title:** FERMENTATION PROCESSES WITH REDUCED FOAMING

Fig. 1

(57) **Abstract:** The present invention relates to processes of producing a fermentation product from readily fermentable sugar-material in a fermentation vat comprising a fermentation medium, comprising: feeding the readily fermentable sugar-material into the fermentation vat comprising a slurry of fermenting organism; fermenting the readily fermentable sugar material into a desired fermentation product, wherein protease is added during or after feeding of the readily fermentable sugar-material into fermentation vat or during fermentation of the readily fermentable sugar-material into the desired fermentation product. The invention also related to the use of protease for reducing foaming in the fermentation vells generating by the fermenting organism during fermentation of the readily fermentable sugar-material.

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Fermentation Processes with Reduced Foaming

FIELD OF THE INVENTION

The present invention relates to reducing foaming in fermentation processes for producing fermentation products, such as ethanol, from readily fermentable sugar materials.

REFERENCE TO A SEQUENCE LISTING

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

BACKGROUND OF THE INVENTION

Fermentation products, such as ethanol, can be produced from a wide range of renewable feedstocks. These can be classified in three main groups: (1) readily fermentable sugar materials, such as sugar cane (i.e., sugar cane juice and molasses), sugar beets, sweet sorghum; (2) starchy materials, such as corn, potatoes, rice, wheat, agave; and (3) cellulosic materials, such as stover, grasses, corn cobs, wood and sugar cane bagasse. The readily fermentable sugar material contains simple sugars, such as sucrose, glucose and fructose, that can readily be fermented by yeast. In contrast to starchy and cellulosic feedstocks there is no need for prior hydrolysis of polysaccharides such as starch and/or cellulose/hemicellulose.

Readily fermentable sugar materials, such as sugar cane juice and molasses, are used as substrates in, e.g., Brazilian ethanol production. Yeast, such as especially *Saccharomyces cerevisiae*, is used as the fermentation organism. Often a yeast recycling system is used where up to 90-95% of the yeast is reused from one fermentation cycle to the next. This results in very high cell densities inside the fermentation vat (e.g., 8-17% w/v, wet basis) and in a very short fermentation time. Ethanol concentrations of 8-11% (v/v) are achieved within a period of 6-11 hours at around 32°C. After every batch fermentation, yeast cells are collected by centrifugation, acid washed (e.g., sulfuric acid at pH 1.5-3.0 for 1-2 hours) and sent back to the fermentation vat. Today a chemical defoamer (dispersant) is added during acid wash at a fixed dosage after each cycle and another chemical defoamer (antifoam) is added directly into the fermentation vat automatically (when foam reaches a level sensor) or manually until foam is fully controlled.

US 3,959,175 discloses an aqueous defoamer composition containing liquid polybutene. The defoamer composition can further comprise in part hydrophobic silica and silicone oils.

US 5,288,789 discloses the use of a condensate of alkylphenol and aldehyde that has been polyoxyalkylated to reduce foam in a fermentation broth.

US 6,083,998 concerns defoamer compositions for alcoholic fermentations which as aqueous based and comprise polydimethylsiloxane oils, ethylene oxide/propylene oxide block copolymers and a silicone/silica blend.

When producing ethanol from readily fermentable sugar materials, such as sugar cane juice and molasses, foam generated by the fermenting organism is a serious problem.

Even though chemical defoamers can be used there is still a desire and need for providing processes for producing fermentation products, such as ethanol, where the foam generation is reduced/controlled.

10 SUMMARY OF THE INVENTION

When producing fermentation products, such as especially ethanol, from readily fermentable sugar-materials, such as sugar cane juice and molasses, foam generated by the fermenting organism is a serious problem. Thus, the object of the present invention is to reduce foam generated by fermenting organisms during fermentation when producing fermentation products, such as especially ethanol, from readily fermentable sugar materials, such as sugar cane molasses. The inventors surprisingly found that proteases can be used to effectively solve the foaming problem.

The invention related to processes of producing a fermentation product from readily fermentable sugar-material in a fermentation vat comprising a fermentation medium using a fermenting organism, comprising

i) feeding the readily fermentable sugar-material into the fermentation vat comprising a slurry of fermenting organism;

ii) fermenting the readily fermentable sugar-material into a desired fermentation product,

wherein a protease is added

a) before, during and/or after feeding in step i), and/or

b) during fermentation in step ii).

In an embodiment the readily fermentable sugar material is feed into the fermentation vat as a feeding stream. The protease may be mixed with the feeding stream of the readily fermentable sugar-material. In a preferred embodiment the protease is mixed with the feeding stream before feeding step i).

After fermentation in step ii) the (used) fermenting organisms are collected/isolated, e.g., by centrifugation. The collected fermenting organism are then acid washed, e.g., with sulfuric acid, at pH 1.5–3.0, such as 2.0-2.5 for 1–2 hours. Thereafter, the fermenting organisms are

returned to the fermentation vat, and (re-)used for fermentation in one or more subsequent fermentation cycles. Thus, in an embodiment the slurry of fermentation organisms, such as yeast slurry, may be prepared by resuspending acid treated yeast biomass in water. In an embodiment of the invention the protease may be added to, or mixed with, the readily fermentable sugar-material, such as sugar cane molasses, before feeding it into the slurry of fermenting organisms in step i). In another embodiment the protease may be added to the slurry of fermentation organisms during feeding of the readily fermentable sugar-material into the slurry of fermentation organisms in step i). In another embodiment the protease may be added during fermentation in step ii). In a preferred embodiment the fermentation is carried out as a batch or fed batch process. However, the fermentation may also be carried out as a semi-continuous or continuous process.

In a preferred embodiment the protease is a bacterial protease, such as a bacterial serine protease, such as a bacterial serine protease derived from a strain of *Pyrococcus*, such as a strain of *Pyrococcus furiosus*, especially the one shown in SEQ ID NO: 2 herein, or a protease having at least 60%, such as at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to in SEQ ID NO: 1 in US 6,358,726 or SEQ ID NO: 2 herein.

In another preferred embodiment the protease is a serine protease, such as a peptidase family S53 protease. Serine proteases of the peptidase family S53 comprises two different types of peptidases: tripeptidyl aminopeptidases (exo-type) and endo-peptidases; as described in 1993, *Biochem. J.* 290:205-218 and in MEROPS protease database, release, 9.4 (31 January 2011) (www.merops.ac.uk). The database is described in Rawlings, N.D., Barrett, A.J. and Bateman, A., 2010, "MEROPS: the peptidase database", *Nucl. Acids Res.* 38: D227-D233. In a preferred embodiment the protease is a peptidase family S53 protease derived from a strain of *Meripilus*, preferably a strain of *Meripilus giganteus*. In an embodiment the protease is the mature sequence from *Meripilus giganteus* protease 3 (peptidase family S53 protease) concerned in Example 2 in WO 2014/037438 and shown as SEQ ID NO: 7 herein. In an embodiment the protease is the mature protease 3 sequence from *Meripilus giganteus* shown as SEQ ID NO: 6 herein and SEQ ID NO: 5 in WO 2014/037438. In an embodiment the protease, such as serine protease, is capable of cutting cell wall protein on the surface of the fermenting organism, such as yeast, in particular *Saccharomyces cerevisiae* yeast, fermenting the readily fermentable sugar-material into a desired fermentation product, in particular ethanol, in step ii). In an embodiment the protease, such as serine protease, is capable of cutting cell

5 wall protein, such as mannoprotein, on the surface of the fermenting organism. In an embodiment the protease, such as serine protein, is capable of degrading protein, such as mannoprotein, released from the fermenting organism. In an embodiment the protease, such as serine protease, is capable of hydrolysing protein in fermented readily fermentable sugar material such as fermented molasses (wine). The invention also relates to the use of a protease for reducing foam generated by fermenting organisms when producing a desired fermentation product from readily fermentable sugars, e.g., as defined in the claims. In a preferred embodiment the protease is a serine protease, in particular *Pyrococcus furiosus* protease or a family S53 protease, especially *Meripilus giganteus* protease 3.

10 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a comparison between the average biomass in the Control (no enzyme addition) and the average biomass of protease treated samples (2.5, 5 and 10 ppm; 1.9, 3.7 and 7.5 ppm on cycles 9 and 10) along with 9 cycles of fermentation. Point 10 was taken after last fermentation batch.

Fig. 2 shows a comparison between the average viability in the Control (no enzyme addition) samples and the average viability of treated samples (2.5, 5 and 10 ppm; 1.9, 3.7 and 7.5 ppm on cycles 9 and 10) along 9 cycles of fermentation.

Fig. 3 shows a comparison between the average ethanol productivity [g EtOH] of a Control (neither antifoam nor enzyme added), two industrial antifoam agents (antifoam 1 and antifoam 2), and protease (PfuS) over 5 cycles of fermentation.

Fig. 4 shows a comparison between the average yeast cell viability [%] of a Control (neither antifoam nor enzyme added), two industrial antifoam agents (antifoam 1 and antifoam 2), and protease PfuS over 5 cycles of fermentation.

Fig. 5 shows a SDS-PAGE analysis of protein fragments from foaming yeast (BRT) and non-foaming yeast (CAT-1) fermentation supernatants (wines), treated with different proteases. Markers are shown as Kda and some protein fragments are indicated by arrows. The lanes are as follows:

Lane	Description
1	CAT-1, control wine
2	CAT-1, PfuS treated wine

3	CAT-1, Mg Prot 3 treated wine
4	CAT-1, Protease Z5S17 treated wine
5	CAT-1, Protease Z6Z6A treated wine
6	BRT, control wine
7	BRT, PfuS treated wine
8	BRT, Mg Prot 3 treated wine
9	BRT, Protease Z5S17 treated wine
10	BRT, Protease Z6Z6A treated wine

DETAILED DESCRIPTION OF THE INVENTION

The object of the present invention is to reduce foaming generated by fermenting organisms, especially foaming yeast, such as of the genus *Saccharomyces*, in particular *Saccharomyces cerevisiae* yeast, during fermentation when producing a desired fermentation product, such as especially ethanol, from readily fermentable sugar material, such as especially sugar cane molasses. In a preferred embodiment the invention relates to a Brazilian-type ethanol fermentation process, e.g., as describe by Basso et al in (2011) in “Ethanol Production in Brazil: The Industrial Process and Its Impact on Yeast Fermentation, Biofuel Production-Recent Developments and Prospects, Dr. Marco Aurelio Dos Santos Bernardes (Ed.), ISBN: 978-953-307-478-8, InTech.” Generally Brazilian ethanol processes include recycling of the fermenting organisms, especially foaming fermenting yeast, such as *Saccharomyces cerevisiae* yeast, and are carried out as batch or fed batch processes. However, some plants do semi-continuous and continuous fermentation processes.

The inventors have found a number of surprising advantages of adding protease in accordance with the invention.

The amount of foam generated during fermentation by some foaming yeast (i.e., foaming *Saccharomyces cerevisiae* yeast) when producing ethanol from sugar cane molasses was reduced by adding serine protease from *Pyrococcus furiosus*. (Protease PfuS). This is described in Examples 1 and 2.

The inventors also found that the ethanol yield was increased when using a protease in accordance with the present invention as defoamer compared to using a commercial chemical defoamer. Example 3 and Fig. 3 compare the average ethanol productivity of a Control (neither antifoam nor enzyme added), two industrial antifoam agents (antifoam 1 and antifoam 2), and
5 Protease PfuS over 5 cycles of fermentation. The ethanol productivity was higher for recycled *Saccharomyces cerevisiae* yeast in a process of the invention when compared with two commercial antifoam products.

Fig. 4 compares the average yeast cell viability of a Control (neither antifoam nor enzyme added), two industrial antifoam agents (antifoam 1 and antifoam 2), and a Protease
10 PfuS over 5 cycles of fermentation. The viability of the yeast was higher for recycled *Saccharomyces cerevisiae* yeast in a process of the invention when compared with two commercial antifoam products. Samples treated with industrial antifoam agents showed an increased drop in viability when compared to Control and treatment with protease in accordance with the invention. The inventors also found that when Protease PfuS and Mg Protease 3 were
15 added in cane sugar molasses fermentation no foam was generated. Example 4 shows that certain proteins (likely mannoproteins), release by the yeast cells in the fermentation medium, were degraded. When adding Snapalysin protease and EVERLASE™ protease in synthetic cane sugar molasses fermentation foam was generated and the same proteins (likely mannoproteins) were not hydrolysed. More specifically the invention related to processes of
20 producing a fermentation product from readily fermentable sugar-material, especially sugar cane molasses, in a fermentation vat comprising a fermentation medium using a fermenting organism, comprising:

i) feeding the readily fermentable sugar material into the fermentation vat comprising a slurry of fermenting organism;

25 ii) fermenting the readily fermentable sugar material into a desired fermentation product,

wherein a protease is added

a) before, during and/or after feeding in step i), and/or

b) during fermentation in step ii).

30 The fermentation is done with a foaming fermenting organism, such as foaming yeast such as a foaming strain of the genus *Saccharomyces*, such as a foaming strain of *Saccharomyces cerevisiae*. In an embodiment the readily fermentable sugar material is feed into the fermentation vat as a feeding stream. The protease, such as serine protease, especially

Pyrococcus furiosus protease or a family S53 protease, especially the *Meripilus giganteus* protease 3, may be mixed with the feeding stream of the readily fermentable sugar-material. In a preferred embodiment the protease is mixed with the feeding stream before feeding step i).

5 According to the invention the term "readily fermentable sugar-material" means that the sugar-containing starting material to be converted/fermented into a desired fermentation product, such as especially ethanol, is of the kind which contains simple sugars, such as sucrose, glucose and fructose, that can be readily fermented by the fermenting organism, such as especially yeast strains derived from *Saccharomyces cerevisiae*.

10 According to the invention the term "fermentation vat" means and includes any type of fermentation vat, fermentation vessel, fermentation tank, or fermentation container, or the like, in which fermentation is carried out.

15 According to the invention in steps i) and ii) may be carried out simultaneously or sequentially. The fermentation may be 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 32°C. In an embodiment the fermentation is ongoing for 2 to 120 hours, in particular 4 to 96 hours. In an embodiment the fermentation may be done in less than 24 hours, such as less than 12 hours, such as between 6 and 12 hours.

20 In contrast to starch-containing feedstocks, such as corn, wheat, rye, milo, sorghum etc, and cellulosic feedstocks, such corn cobs, corn stover, bagasse, wheat straw, wood etc. there is no need for pretreatment and/or (prior) hydrolysis before fermentation. In a preferred embodiment the readily fermentable sugar-material is selected from the group consisting of sugar cane juice, sugar cane molasses, sweet sorghum, sugar beets, and mixture thereof. However, according to the invention the fermentation medium may also further comprise other by-products of sugar cane, in particular hydrolysate from sugar cane bagasse. In an
25 embodiment the fermentation medium may include separate streams comprising, e.g., C5-liquor, etc. According to the invention the readily fermentable sugar-material (substrate) does not include a substantial content of polysaccharide, such as starch and/or cellulose/hemicellulose.

30 In a preferred embodiment the fermenting organism used in a process of the invention may be a foaming fermenting organism capable of fermenting readily fermentable sugar-material into a desired fermentation product, such as especially ethanol. Many commercial yeast strains, including especially strains of *Saccharomyces cerevisiae*, used commercially, e.g., in Brazil, today, e.g., for producing ethanol from sugar cane molasses generate foam during fermentation. In an embodiment the fermenting organism is a yeast, e.g., from a strain of the

genus *Saccharomyces*, such as a strain of *Saccharomyces cerevisiae*. Thus, in a preferred embodiment the fermenting organism is a foaming fermenting organism, such as a foaming strain of *Saccharomyces*, such as especially a strain of *Saccharomyces cerevisiae* generating foam during fermentation. According to the invention the density of yeast in the fermentation medium is high, such as from 8–17% w/v, wet basis of the fermentation medium. In an embodiment, the fermentation occurs at non-aseptically conditions, e.g., where wild yeast strains with a foaming phenotype may also be introduced to the fermentation vat and incorporated into the yeast population.

In a preferred embodiment of the invention the fermenting organisms are recycled after fermentation in step ii). According to the invention from 50-100%, such as 70-95%, such as about 90% of the fermentation organisms are recycled. The fermenting organisms, such as yeast, are collected after fermentation in step ii), acid washed, and recycled to the fermentation vat. The fermenting organisms are acid washed with sulfuric acid, e.g., at pH 1.5–3.0, such as 2.0-2.5, e.g., for 1–2 hours. The process of the invention may be carried out as a batch or fed-batch fermentation. However, the process of the invention may also be done as a semi-continuous or continuous process.

The terms “fermentation product” and “desired fermentation product” mean a product produced by fermentation 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., H₂ and CO₂); antibiotics (e.g., penicillin and tetracycline); enzymes; vitamins (e.g., riboflavin, B12, 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. According to the invention the preferred fermentation product is ethanol. The desired fermentation product, such as ethanol, obtained according to the invention, may preferably be used as fuel, e.g., for vehicles, such as cars. Fuel ethanol may be blended with gasoline. Ethanol it may also be used as potable ethanol.

Subsequent to fermentation in step ii) the desired fermentation product, such as ethanol, may be separated from the fermentation medium, e.g., by distillation, or another separation technology. 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.

Enzymes Addition During Fermentation

A process of the invention, as defined above, includes addition of a protease. In an embodiment the protease is a serine protease capable of cutting protein, such as mannoprotein, on the surface of the foaming fermenting yeast.

In an embodiment the protease is a serine protease capable of cutting cell wall protein, such as mannoprotein, on the surface of the fermenting organism, in particular foaming *Saccharomyces cerevisiae* yeast and/or is capable of hydrolyzing protein, such as mannoprotein, in fermented readily fermentable sugar material, such as fermented molasses (wine).

According to an embodiment of the invention the protease may, e.g., be added in a dosage from 0.2 to 25 mg Enzyme Protein (EP)/L fermentation medium.

In an embodiment the protease may be added in dosages from 0.01-100 ppm EP (Enzyme Protein) protease, such as 0.1-50 ppm, such as 1-25 ppm.

The protease used in a process of the invention may preferably be selected from the group of serine proteases, metallo proteases, and aspartic acid proteases. The protease may in one embodiment be the only enzyme added (i.e., no other enzymes added). In a preferred embodiment the protease is of bacterial origin, e.g., a serine protease, such as a bacterial serine protease derived from a strain of *Pyrococcus*, preferably from a strain of *Pyrococcus furiosus*, especially the protease shown in SEQ ID NO: 1 in US 6,358,726 or SEQ ID NO: 2 herein.

The protease may be one having at least 60%, such as at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to in SEQ ID NO: 1 in US 6,358,726 or SEQ ID NO: 2 herein.

In another embodiment the protease used in a process of the invention defined above is of fungal origin.

In another embodiment the protease is a metallo protease. In a preferred embodiment the metallo protease may be derived from a strain of the genus *Thermoascus*, preferably a strain of *Thermoascus aurantiacus*, especially *Thermoascus aurantiacus* CGMCC No. 0670, such as the mature part of SEQ ID NO: 2 disclosed in WO 2003/048353 or SEQ ID NO: 1 herein, or a variant thereof.

In an embodiment the protease or variant thereof has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least

91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 1.

5 In another preferred embodiment the protease is a fungal serine protease, such as a serine peptidase family S53 protease ("S53 protease"), such as a family S53 protease derived from a strain of *Meripilus*, preferably a strain of *Meripilus giganteus*. In an embodiment the protease is the mature sequence from *Meripilus giganteus* protease 3 concerned in Example 2 in WO 2014/037438 and shown as SEQ ID NO: 7 herein. In an embodiment the protease is the
10 mature sequence from *Meripilus giganteus* protease 3 shown as SEQ ID NO: 7 herein.

In an embodiment the protease is one having at least 60%, such as at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 7 herein.

15 In an embodiment the protease is one having at least 60%, such as at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID: 6 herein.

Examples of suitable proteases can be found in the "Proteases"-section below.

20 In an embodiment the protease is added together with (simultaneously with) one or more enzymes selected from the group consisting of: cellulase, glucoamylase, alpha-amylase, oxidase, peroxidase, catalase, laccase, beta-glucosidase, other carbohydrases, and oxidases.

In an embodiment the protease is added before and/or after the other enzymes.

25 According to the process of the invention adding a protease results in increased yields, e.g., ethanol yield, compared to a corresponding process where no protease is present or added. The process of the invention may also reduce the residual sugars present in the fermentation medium. However, most importantly foaming in the fermentation vat is reduced compared to a corresponding process where no protease is added.

30 According to the invention an alpha-amylase may be added together with the protease or present and/or added during fermentation. The alpha-amylase may be of microbial origin, e.g., fungal or bacterial origin. In an embodiment the alpha-amylase is of fungal origin. In an embodiment the fungal alpha-amylase is derived from a strain of *Rhizomucor*, such as a strain of *Rhizomucor pusillus*, such as a hybrid of the *Rhizomucor pusillus* alpha-amylase shown in

SEQ ID NO: 3 herein further comprising a starch-binding module, such as a CBM20 starch-binding module, such as the sequence shown in SEQ ID NO: 4 herein.

In another embodiment the alpha-amylase may be of bacterial origin. In a preferred embodiment the bacterial alpha-amylase may be derived from the genus *Bacillus*, such as a strain of the species *Bacillus stearothermophilus* or variant thereof. The alpha-amylase may be a *Bacillus stearothermophilus* alpha-amylase, e.g., the mature part of the one shown in SEQ ID NO: 5 herein, or a mature alpha-amylase or a corresponding mature alpha-amylase having at least 60%, such as 70%, such as 80% identity, such as at least 90% identity, such as at least 95% identity, such as at least 96% identity, such as at least 97% identity, such as at least 99% identity to the SEQ ID NO: 5 herein. In an embodiment the mature *Bacillus stearothermophilus* alpha-amylase, or variant thereof, is truncated, preferably to have around 485-496 amino acids, such as around 491 amino acids. In an embodiment the *Bacillus stearothermophilus* alpha-amylase has a double deletion in positions I181 + G182, and optionally a N193F substitution, or deletion of R179 + G180 (using SEQ ID NO: 5 herein for numbering).

Examples of suitable alpha-amylase can be found in the "Alpha-Amylases"-section below.

According to the invention a glucoamylase may be added together with the protease or be present and/or added during fermentation. In an embodiment the glucoamylase may be from a strain of *Aspergillus*, preferably *A. niger*, *A. awamori*, or *A. oryzae*; or a strain of *Trichoderma*, preferably from *Trichoderma reesei*; or a strain of *Talaromyces*, preferably *Talaromyces emersonii*; or a strain of *Trametes*, such as *Trametes cingulate*; or a strain of *Pycnoporus*, or a strain of *Gloeophyllum*, or a strain of the *Nigrofores*.

Examples of suitable glucoamylases can be found in the "Glucoamylases"-section below

In an embodiment of the process of the invention a desired fermentation product, such as especially ethanol, is produced from readily fermentable sugar-material by fermentation in a fermentation vat, the process comprises adding protease to the readily fermentable sugar material before feeding; feeding the protease-containing readily fermentable sugar material into the fermentation vat comprising the slurry of fermenting organisms; fermenting the readily fermentable sugar material into the desired fermentation product.

In a preferred embodiment ethanol is produced in a batch or fed-batch fermentation process in a fermentation vat comprising sugar cane molasses, comprising adding protease to the sugar cane molasses before feeding; feeding the protease-containing sugar cane molasses into the fermentation vat comprising a slurry of *Saccharomyces cerevisiae* yeast; and fermenting the sugar cane molasses into ethanol.

In another embodiment a desired fermentation product, such as especially ethanol, is produced from readily fermentable sugar-material by fermentation in a fermentation vat, wherein the process comprises: feeding readily fermentable sugar material into the fermentation vat comprising a slurry of fermenting organisms; feeding protease into the fermentation vat comprising the slurry of readily fermentable sugars and fermenting organisms before fermentation; fermenting the readily fermentable sugar material into the desired fermentation product.

In a preferred embodiment ethanol is produced in a batch or fed-batch fermentation process in a fermentation vat comprising sugar cane molasses, wherein the process comprises: feeding sugar cane molasses into the fermentation vat comprising a slurry of *Saccharomyces cerevisiae* yeast; feeding protease into the fermentation vat comprising the slurry of *Saccharomyces cerevisiae* yeast and the sugar cane molasses before fermentation; fermenting the sugar cane molasses into ethanol.

In a further embodiment of the invention a desired fermentation product is produced from readily fermentable sugar material by fermentation in a fermentation vat, wherein the process comprises: feeding readily fermentable sugar-material into the fermentation vat comprising a slurry of fermenting organisms; adding protease into the fermentation vat during fermentation of the readily fermentable sugar material into the desired fermentation product.

In a preferred embodiment ethanol is produced as a batch or fed-batch fermentation process in a fermentation vat comprising sugar cane molasses, wherein the process comprises: feeding sugar cane molasses into the fermentation vat comprising a slurry of *Saccharomyces cerevisiae* yeast; adding protease into the fermentation vat during fermentation of the sugar cane molasses into ethanol.

In a preferred specific embodiment the process of the invention, comprises

i) feeding the readily fermentable sugar material into the fermentation vat comprising a slurry of fermenting organism;

ii) fermenting the readily fermentable sugar material into a desired fermentation product,

wherein feeding of the readily fermentable sugar-material is done by introducing a feeding stream into the fermentation vat; wherein

- protease is mixed with the feeding stream before in step i); or
- protease is added to fermentation vat after feeding.

In a preferred embodiment the protease is *Pyrococcus furiosus* protease as described above. In a preferred embodiment the protease is *Meripilus giganteus* protease 3 as described above.

5 The fermentation is done with a foaming fermenting organism, such as foaming yeast such as a foaming strain of the genus *Saccharomyces*, such as a foaming strain of *Saccharomyces cerevisiae*.

Use Of Protease for Foam Reduction

10 In this aspect the invention relates to the use of a protease for reducing foam generated by fermenting organisms when producing a desired fermentation product from readily fermentable sugars. In a preferred embodiment the desired fermentation product is produced according to a process of the invention.

15 **ENZYMES**

One or more of the following enzyme activities may be used according to the invention.

Proteases

A process of the invention, as defined above, includes addition of a protease.

20 The protease may be of any origin. In an embodiment the protease is of fungal origin. In another embodiment the protease is of bacterial origin.

The protease may be a protease selected from the group of serine proteases, metalloproteases, and aspartic acid proteases.

25 In an embodiment the protease is capable of cutting protein, such as mannoprotein, on the cell wall surface of the foaming fermenting organism, preferably foaming yeast, such as foaming *Saccharomyces cerevisiae* yeast. In a preferred embodiment the protease is capable of hydrolyzing protein, in particular mannoprotein, in fermented readily fermentable sugar material, such as fermented molasses (wine).

30 Fungal Proteases

In an embodiment the protease is of fungal origin.

In a preferred embodiment the protease is a metallo protease, such as one derived from a strain of the genus *Thermoascus*, preferably a strain of *Thermoascus aurantiacus*, especially *Thermoascus aurantiacus* CGMCC No. 0670, or a variant thereof.

In an embodiment the protease, or protease variant, is the metallo protease disclosed as the mature part of SEQ ID NO: 2 disclosed in WO 2003/048353 or SEQ ID NO: 1 herein.

In an embodiment the protease has at least 60%, such as at least 70%, such as at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, such as least 100% identity to the mature part of the polypeptide of SEQ ID NO: 2 disclosed in WO 2003/048353 or SEQ ID NO: 1 herein.

In an embodiment the protease variant has at least 70%, such as at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 2 disclosed in WO 2003/048353 or SEQ ID NO: 1 herein.

The protease may be a protease variant of the protease shown in SEQ ID NO: 1 herein, having the following substitutions:

D79L+S87P+A112P+D142L

D79L+Y82F+S87P+A112P+D142L

S38T+D79L+S87P+A112P+A126V+D142L

D79L+Y82F+S87P+A112P+A126V+D142L

A27K+D79L+S87P+A112P+A126V+D142L

S49P+D79L+S87P+A112P+D142L

S50P+D79L+S87P+A112P+ D142L

D79L+S87P+D104P+A112P+D142L

D79L+Y82F+S87G+ A112P+D142L

S70V+D79L+Y82F+S87G+Y97W+A112P+D142L

D79L+Y82F+S87G+Y97W+D104P+A112P+ D142L

S70V+D79L+Y82F+S87G+A112P+D142L

D79L+Y82F+S87G+D104P+A112P+D142L

D79L+Y82F+S87G+A112P+A126V+D142L

Y82F+S87G+S70V+D79L+D104P+A112P+D142L

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

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

A27K Y82F S87G D104P A112P A126V D142L

A27K D79L Y82F D104P A112P A126V D142L

A27K Y82F D104P A112P A126V D142L

In a preferred embodiment the protease is a variant of the *Thermoascus aurantiacus* protease shown in SEQ ID NO: 1 herein with mutations selected from the group consisting of:

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

- D79L+Y82F+S87G+A112P+D142L;

5 - Y82F+S87G+S70V+D79L+D104P+A112P+D142L;

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

In an additional embodiment the protease may be a filamentous fungus protease, e.g., derived from a strain of *Rhizomucor*, such as *Rhizomucor miehei*, such as the protease shown in SEQ ID NO: 3 herein, or one having a sequence identity thereto of at least 60%, at least 70%,
10 at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%.

In a preferred embodiment the protease is a fungal serine protease, such as a serine peptidase family S53 protease ("S53 protease"), such as a family S53 protease derived from a strain of *Meripilus*, preferably a strain of *Meripilus giganteus*. In an embodiment the family S53 protease is the mature sequence from *Meripilus giganteus* protease 3 concerned in Example 2
15 in WO 2014/037438 and shown as SEQ ID NO: 7 herein. In an embodiment the protease is the mature sequence from *Meripilus giganteus* protease 3 shown as SEQ ID NO: 6 herein.

In an embodiment the S53 protease is one having at least 60%, such as at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to in
20 SEQ ID NO: 7 herein.

In an embodiment the S53 protease is one having at least 60%, such as at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to in
25 SEQ ID NO: 6 herein.

Bacterial Proteases

In an embodiment the protease is of bacterial origin.

In a preferred embodiment the protease is derived from a strain of *Pyrococcus*, preferably a strain of *Pyrococcus furiosus*.

In a preferred embodiment the protease is the one shown in SEQ ID NO: 1 in US 6,258,726 or SEQ ID NO: 2 herein.

In an embodiment the protease has at least 60%, such as at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%,
5 such as at least 97%, such as at least 98%, such as at least 99% sequence identity to SEQ ID NO: 1 in US 6,258,726.

In an embodiment the protease has at least 60%, such as at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%,
10 such as at least 97%, such as at least 98%, such as at least 99% sequence identity to SEQ ID NO: 2 herein.

According to an embodiment of the invention the protease may be added in a dosage from 0.2 to 25 mg Enzyme Protein(EP)/L fermentation medium. In an embodiment the protease may be added in a dosage in the range from 0.01-100 ppm EP (Enzyme Protein) protease, such as 0.1-50 ppm, such as 1-25 ppm.

Alpha-Amylases

According to the invention an alpha-amylase may be added together with the protease or present and/or added during fermentation. The alpha-amylase may be of, e.g., bacterial or fungal origin.

Bacterial Alpha-Amylases

Examples of suitable bacterial alpha-amylases include the below mentioned. Preferred bacterial alpha-amylases may be derived from a strain the genus *Bacillus* (sometimes referred to as *Geobacillus*), including a strain of *Bacillus licheniformis*, *Bacillus amyloliquefaciens*,
25 *Bacillus stearothermophilus*, or *Bacillus subtilis*. Other bacterial alpha-amylases include alpha-amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described in detail in WO 95/26397, and the alpha-amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31 (hereby incorporated by reference).

The *Bacillus* alpha-amylase may also be a variant and/or hybrid, especially one described in any of WO 96/23873, WO 96/23874, WO 97/41213, WO 99/19467, WO 00/60059, and WO 02/10355 (all documents hereby incorporated by reference). Specifically contemplated alpha-amylase variants are disclosed in US patent nos. 6,093,562, 6,297,038 or US patent no. 6,187,576 (hereby incorporated by reference) and include *Bacillus stearothermophilus* alpha-

amylase (BSG alpha-amylase) variants having a deletion of one or two amino acid in positions R179 to G182, preferably a double deletion disclosed in WO 1996/023873 – see e.g., page 20, lines 1-10 (hereby incorporated by reference), preferably corresponding to delta(181-182) compared to the wild-type BSG alpha-amylase amino acid sequence set forth in SEQ ID NO:3 disclosed in WO 99/19467 or deletion of amino acids R179 and G180 using SEQ ID NO:3 in WO 99/19467 for numbering (which reference is hereby incorporated by reference). Even more preferred are *Bacillus* alpha-amylases, especially *Bacillus stearothermophilus* alpha-amylase, which have a double deletion corresponding to delta(181-182) and further comprise a N193F substitution (also denoted I181* + G182* + N193F) compared to the wild-type BSG alpha-amylase amino acid sequence set forth in SEQ ID NO:3 disclosed in WO 99/19467.

In an embodiment the *Bacillus stearothermophilus* alpha-amylase is one disclosed in WO 2011/082425, or SEQ ID NO: 5 herein, such as one selected from the group of:

- I181*+G182*+N193F+E129V+K177L+R179E;
- I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
- I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+Q254S+M284V; and
- I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 5 herein for numbering).

In an embodiment the *Bacillus stearothermophilus* alpha-amylase has the following mutations: 181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+Q254S+M284V (SEQ ID NO: 5).

The truncated *Bacillus stearothermophilus* alpha-amylase is typically naturally truncated to be about from 485-495 amino acids long, such as 491 amino acids. In a preferred embodiment the truncation is at the C-terminal. A hybrid alpha-amylase specifically contemplated comprises 445 C-terminal amino acid residues of the *Bacillus licheniformis* alpha-amylase (shown in SEQ ID NO: 4 of WO 99/19467) and the 37 N-terminal amino acid residues of the alpha-amylase derived from *Bacillus amyloliquefaciens* (shown in SEQ ID NO: 5 of WO 99/19467), with the following substitution: G48A+T49I+G107A+H156Y+A181T+N190F+I201F+A209V+Q264S (using the numbering in SEQ ID NO: 4 in WO 99/19467). Especially preferred are variants having one or more of the mutations H154Y, A181T, N190F, A209V and Q264S and/or deletion of two residues between positions 176 and 179, preferably deletion of E178 and G179 (using the SEQ ID NO: 5 numbering of WO 99/19467).

Commercially available bacterial alpha-amylase products and products containing alpha-amylases include TERMAMYL™ SC, LIQUOZYME™ SC, BAN (Novozymes A/S, Denmark)

DEX-LO™, SPEZYME™ XTRA, SPEZYME™ AA, SPEZYME FRED-L, SPEZYME™ ALPHA, GC358, SPEZYME RSL, SPEZYME HPA and SPEZYME™ DELTA AA (from DuPont, USA), FUELZYME™ (Verenium, USA).

Bacterial alpha-amylase may be added in concentrations well-known in the art. When measured in KNU units (described below in the Materials & Methods"-section) the alpha-amylase activity is preferably present in the range from 0.5-50 KNU/ L fermentation medium, such as 1-25 KNU/L fermentation medium, or more preferably in an amount of 2-10 KNU/L fermentation medium.

Fungal Alpha-Amylases

Fungal alpha-amylases (EC 3.2.1.1) are preferably of filamentous fungus origin. The fungal alpha-amylase may be a fungal acid alpha-amylase.

Fungal acid alpha-amylases include acid alpha-amylases derived from a strain of the genus *Aspergillus*, such as *Aspergillus oryzae* and *Aspergillus niger* alpha-amylases.

A preferred fungal alpha-amylase is a Fungamyl-like alpha-amylase which is preferably derived from a strain of *Aspergillus oryzae*. In the present disclosure, the term "Fungamyl-like alpha-amylase" indicates an alpha-amylase which exhibits a high identity, i.e. more than 70%, more than 75%, more than 80%, more than 85% more than 90%, more than 95%, more than 96%, more than 97%, more than 98%, more than 99% or even 100% identity to the mature part of the amino acid sequence shown in SEQ ID NO: 10 in WO 96/23874.

Another preferred acid alpha-amylase is derived from a strain *Aspergillus niger*. In a preferred embodiment the acid fungal alpha-amylase is the one from *A. niger* disclosed as "AMYA_ASPNG" in the Swiss-prot/TrEMBL database under the primary accession no. P56271 and described in more detail in WO 89/01969 (Example 3). The acid *Aspergillus niger* acid alpha-amylase is also shown as SEQ ID NO: 1 in WO 2004/080923 (Novozymes) which is hereby incorporated by reference. Also variants of said acid fungal amylase having at least 70% identity, such as at least 80% or even at least 90% identity, such as at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO: 1 in WO 2004/080923 are contemplated. A suitable commercially available acid fungal alpha-amylase derived from *Aspergillus niger* is SP288 (available from Novozymes A/S, Denmark).

The fungal acid alpha-amylase may also be a wild-type enzyme comprising a carbohydrate-binding module (CBM) and an alpha-amylase catalytic domain (i.e., a non-hybrid), or a variant thereof. In an embodiment the wild-type acid fungal alpha-amylase is derived from a strain of *Aspergillus kawachii*.

Commercial available compositions comprising fungal alpha-amylase include FUNGAMYL™ and the acid fungal alpha-amylase sold under the trade name SP288 (available from Novozymes A/S, Denmark).

In an embodiment the fungal acid alpha-amylase is a hybrid alpha-amylase. Preferred examples of fungal hybrid alpha-amylases include the ones disclosed in WO 2005/003311 or U.S. Patent Publication no. 2005/0054071 (Novozymes) or US patent application no. 60/638,614 (Novozymes) which is hereby incorporated by reference. A hybrid alpha-amylase may comprise an alpha-amylase catalytic domain (CD) and a carbohydrate-binding domain/module (CBM), such as a starch binding domain, and optional a linker.

Specific examples of contemplated hybrid alpha-amylases include those disclosed in Table 1 to 5 of the examples in co-pending US patent application no. 60/638,614, including Fungamyl variant with catalytic domain JA118 and *Athelia rolfsii* SBD (SEQ ID NO: 2 herein and SEQ ID NO:100 in US 60/638,614), *Rhizomucor pusillus* alpha-amylase with *Athelia rolfsii* AMG linker and SBD (SEQ ID NO: 3 herein and SEQ ID NO:101 in US 60/638,614), *Rhizomucor pusillus* alpha-amylase with *Aspergillus niger* glucoamylase linker and SBD (which is disclosed in Table 5 as a combination of amino acid sequences SEQ ID NO:20 SEQ ID NO:72 and SEQ ID NO:96 in US application no. 11/316,535 and further as SEQ ID NO: 13 herein), and *Meripilus giganteus* alpha-amylase with *Athelia rolfsii* glucoamylase linker and SBD (SEQ ID NO: 4 herein and SEQ ID NO:102 in US 60/638,614). Other specifically contemplated hybrid alpha-amylases are any of the ones listed in Tables 3, 4, 5, and 6 in Example 4 in US application no. 11/316,535 or (WO 2006/069290) (hereby incorporated by reference). Other specific examples of contemplated hybrid alpha-amylases include those disclosed in U.S. Patent Publication no. 2005/0054071, including those disclosed in Table 3 on page 15, such as *Aspergillus niger* alpha-amylase with *Aspergillus kawachii* linker and starch binding domain.

An acid alpha-amylases may be added in an amount of 0.1 to 250 FAU(F)/L fermentation medium, preferably 1 to 100 FAU(F)/L fermentation medium.

Glucoamylase

According to the invention an alpha-amylase may be added together with the protease or present and/or added during fermentation. The alpha-amylase may be of, e.g., bacterial or fungal origin.

Contemplated glucoamylases include those from the group consisting of *Aspergillus* glucoamylases, in particular *A. niger* G1 or G2 glucoamylase (Boel et al. (1984), EMBO J. 3 (5), p. 1097-1102), or variants thereof, such as those disclosed in WO 92/00381, WO 00/04136 and

WO 01/04273 (from Novozymes, Denmark); the *A. awamori* glucoamylase disclosed in WO 84/02921, *A. oryzae* glucoamylase (Agric. Biol. Chem. (1991), 55 (4), p. 941-949), or variants or fragments thereof. Other *Aspergillus* glucoamylase variants include variants with enhanced thermal stability: G137A and G139A (Chen et al. (1996), Prot. Eng. 9, 499-505); D257E and
 5 D293E/Q (Chen et al. (1995), Prot. Eng. 8, 575-582); N182 (Chen et al. (1994), Biochem. J. 301, 275-281); disulphide bonds, A246C (Fierobe et al. (1996), Biochemistry, 35, 8698-8704; and introduction of Pro residues in position A435 and S436 (Li et al. (1997), Protein Eng. 10, 1199-1204).

Other glucoamylases contemplated include glucoamylase derived from a strain of
 10 *Athelia*, preferably a strain of *Athelia rolfsii* (previously denoted *Corticium rolfsii*) glucoamylase (see US patent no. 4,727,026 and (Nagasaka, Y. 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*,
 15 *Talaromyces thermophilus* (US patent no. 4,587,215). Also contemplated are the *Trichoderma reesei* glucoamylases disclosed as SEQ ID NO: 4 in WO 2006/060062 and glucoamylases being at least 80% or at least 90% identical thereto and further the glucoamylase derived from *Humicola grisea* disclosed as SEQ ID NO: 3 in US 7,262,041-B2 (US10/992,187) (hereby incorporated by reference) or sequences having at least 80% or at least 90% identity thereto.

20 In a preferred embodiment the glucoamylase is derived from a strain of *Aspergillus*, preferably *A. niger*, *A. awamori*, or *A. oryzae*; or a strain of *Trichoderma*, preferably *T. reesei*; or a strain of *Talaromyces*, preferably *T. emersonii*.

Other contemplated glucoamylases include glucoamylase derived from a strain of *Trametes*, preferably a strain of *Trametes cingulata* disclosed in WO 2006/069289 (which is
 25 hereby incorporated by reference). 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 Table 1 and 4 of Example 1 (which hybrids are hereby incorporated by reference.).

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

Commercially available compositions comprising glucoamylase include AMG 200L; AMG 300 L; SAN™ SUPER, SAN™ EXTRA L, SPIRIZYME™ PLUS, SPIRIZYME™ FUEL, SPIRIZYME ULTRA, SPIRIZYME EXCEL, SPIRIZYME™ B4U and AMG™ E (from Novozymes

A/S); OPTIDEX™ 300 (from Genencor Int.); AMIGASE™ and AMIGASE™ PLUS (from DSM); G-ZYME™ G900, G-ZYME™ and G990 ZR (from Genencor Int.).

Glucoamylases may in an embodiment be added in an amount of 1-5,000 AGU/L fermentation medium, preferably 10-1,000 AGU/L fermentation medium.

5

Materials & Methods

Materials:

Protease PfuS: Protease derived from *Pyrococcus furiosus* shown in SEQ ID NO: 2 herein.

10 Mg Protease 3: Serine peptidase family S53 protease from a strain of *Meripilus giganteus* concerned in Example 2 in WO 2014/037438.

Protease Z5S17: Purified sample of Snapalysin (Example 4).

Protease Z6Z6A: EVERLASE™ (protease available from Novozymes A/S) (Example 4)

15 Chemical antifoamer (Examples 1 and 2): AD5520GA (condensed co-polymer from ethylene and propylene oxydes) from Alcolina, Brazil.

Antifoam 1: antifoam ART DISP 904 S and dispersant ART DISP 8000, Aratrop Industrial, Brazil (Example 3)

Antifoam 2: antifoam AD4415 and dispersant AD5520GA, Alcolina, Brazil (Example 3)

20 Yeast (BRT): Brazilian foaming *Saccharomyces cerevisiae* strain

Yeast CAT-1: purchased from LNF, Brazil (www.lnf.com.br) and described in Babrzadeh et al., 2012, "Whole-genome sequencing of the efficient industrial fuel-ethanol fermentative *Saccharomyces cerevisiae* strain CAT-1", Molecular genetics and genomics: MGG 287(6), 485–494.

25 Substrate: Sugarcane molasses (80° Brix) obtained from Santa Helena mill (Piracicaba, SP, Brazil) (Examples 1 and 2):

Sugar cane molasses samples from São José mill, Brazil (Example 3)

Methods

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

For purposes of the present invention the degree of identity between two amino acid sequences, as well as the degree of identity between two nucleotide sequences, may be

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

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

Alpha-Amylase activity (KNU)

15 The amylolytic 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
20 standard.

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

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

Determination of Acid Amylolytic Activity (FAU)

One Fungal Alpha-Amylase Unit (1 FAU) is defined as the amount of enzyme, which breaks down 5.26 g starch (Merck Amylum solubile Erg. B.6, Batch 9947275) per hour at No-
30 vozymes' standard method for determination of alpha-amylase based upon the following standard conditions:

Substrate Soluble starch
Temperature 37°C

pH 4.7

Reaction time 7-20 minutes

A detailed description of Novozymes' method for determining KNU and FAU is available on request as standard method EB-SM-0009.02/01.

Determination of acid alpha-amylase activity (AFAU)

Acid alpha-amylase activity is measured in AFAU (Acid Fungal Alpha-amylase Units), which are determined relative to an enzyme standard.

The standard used is AMG 300 L (wild type *A. niger* G1 AMG sold by Novozymes A/S). The neutral alpha-amylase in this AMG falls after storage at room temperature for 3 weeks from approx. 1 FAU/mL to below 0.05 FAU/mL.

The acid alpha-amylase activity in this AMG standard is determined in accordance with AF 9 1/3 (Novo method for the determination of fungal alpha-amylase). In this method, 1 AFAU is defined as the amount of enzyme, which degrades 5.260 mg starch dry matter per hour under standard conditions.

Iodine forms a blue complex with starch but not with its degradation products. The intensity of colour is therefore directly proportional to the concentration of starch. Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under specified analytic conditions.

Alpha-amylase

Starch + Iodine -----> Dextrins + Oligosaccharides

40°C, pH 2.5

Blue/violet t=23 sec. Decolouration

Standard conditions/reaction conditions: (per minute)

Substrate:	starch, approx. 0.17 g/L
Buffer:	Citrate, approx. 0.03 M
Iodine (I ₂):	0.03 g/L
CaCl ₂ :	1.85 mM
pH:	2.50 +/- 0.05
Incubation temperature:	40°C
Reaction time:	23 seconds

Wavelength: Lambda=590nm
 Enzyme concentration: 0.025 AFAU/mL
 Enzyme working range: 0.01-0.04 AFAU/mL

Further details can be found in standard method document EB-SM-0259.02/01 available
 5 on request from Novozymes A/S, which folder is hereby incorporated by reference.

Determination of FAU(F)

FAU(F) Fungal Alpha-Amylase Units (Fungamyl) is measured relative to an enzyme standard of a declared strength.

Reaction conditions	
Temperature	37°C
pH	7.15
Wavelength	405 nm
Reaction time	5 min
Measuring time	2 min

10

A folder (EB-SM-0216.02) describing this standard method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Glucoamylase activity (AGU)

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

An autoanalyzer system may be used. Mutarotase is added to the glucose dehydrogenase reagent so that any alpha-D-glucose present is turned into beta-D-glucose.
 20 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.

<u>AMG incubation:</u>	
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

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

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

5

Protease Assay method - AU(RH)

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

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

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

Protease assays

AZCL-casein assay

A solution of 0.2% of the blue substrate AZCL-casein is suspended in Borax/NaH₂PO₄ buffer pH9 while stirring. The solution is distributed while stirring to microtiter plate (100 microL to each well), 30 microL enzyme sample is added and the plates are incubated in an Eppendorf

Thermomixer for 30 minutes at 45°C and 600 rpm. Denatured enzyme sample (100°C boiling for 20min) is used as a blank. After incubation the reaction is stopped by transferring the microtiter plate onto ice and the coloured solution is separated from the solid by centrifugation at 3000rpm for 5 minutes at 4°C. 60 microL of supernatant is transferred to a microtiter plate and the absorbance at 595nm is measured using a BioRad Microplate Reader.

pNA-assay

50 microL protease-containing sample is added to a microtiter plate and the assay is started by adding 100 microL 1mM pNA substrate (5 mg dissolved in 100 microL DMSO and further diluted to 10 mL with Borax/NaH₂PO₄ buffer pH 9.0). The increase in OD₄₀₅ at room temperature is monitored as a measure of the protease activity.

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

EXAMPLES

Example 1

Comparison between Chemical Antifoamer and PfuS Protease on foam control in sugarcane molasses fermentation

Fermentation trials were performed at 32°C in 50 mL centrifuge vials (TPP), simulating an industrial ethanol fermentation process as performed in Brazil. A foaming Brazilian yeast strain was used. A fermentation substrate containing 22° Brix (composed of diluted molasses) was fed into a yeast slurry (prepared by resuspending acid treated yeast biomass in water). The yeast slurry represented 30% of the total fermentation volume, similar to industrial conditions. After fermentation for around 6 hours, yeast cells were collected by centrifugation (4000 rpm for 10 min), weighed, diluted with fermented molasses (wine) and water (to 35% w/v yeast wet weight), and treated with sulfuric acid (pH from 2.0 to 2.5 for 1 hour) and reused in subsequent 9 fermentation cycles. Samples were run in triplicate at each condition. Wet weight yeast biomass was determined gravimetrically after centrifugation (4000 rpm for 10 min) of the samples. Foam was registered every hour after feeding by recording the foam height in tubes and/or by taking pictures of representative 50mL tubes. Conditions were: control – no enzyme. No Chemical antifoamer was added during acid wash.

chemical antifoamer addition. Chemical antifoamer was added during acid wash, with increasing dosages after each cycle, until foam was controlled.

Protease PfuS addition. Protease PfuS was added after new molasses was added. No Chemical antifoamer was added during acid wash.

- 5 The dosages used were according Table 1. Antifoamer was added in the yeast slurry. Protease PfuS was added after the fermentation vat was fed with fresh molasses.

Table 1: Dosages of antifoam oil and PfuS used for controlling foaming

Product	Concentration (mg/L)
Chemical antifoamer	10 to 30
PfuS Protease	3.7 and 7.5

- 10 Foam measurements resulted in the following data, showing the more significant results, summarized in Table 2:

Table 2: Foam control results from each treatment. Data are presented on total volume increase compared to medium volume, due to foam formation.

Treatment	Total volume increase (foam formation)
Control	+ 94%
Chemical AntifoamerI (10 ppm)	+ 94%
Chemical AntifoamerI (20 ppm)	+29%
Chemical Antifoamer (30 ppm)	+23%
Protease PfuS (3.7 ppm)	+ 8%
Protease PfuS (7.5 ppm)	+ 0%

- 15 After Chemical antifoamer and Protease PfuS addition had ceased, the foam height was measured in the next three subsequent fermentation cycles. Tubes where Protease PfuS had been added presented a residual antifoam effect on the two sequential batches after last Protease PfuS addition. Foam formation, presented in total volume increase, is summarized in Table 3:

- 20 Table 3: Foam formation in two batches (cycle 1 and cycle 2) after Antifoam oil and Protease PfuS addition had ceased. Data are presented on total volume increase compared to medium volume, due to foam formation.

Treatment	Cycle 1	Cycle 2
Control	+ 100%	+ 101%

Chemical Antifoamer	+ 98%	+ 101%
Protease PfuS (3.7 ppm)	+17%	+ 13%
Protease PfuS (7.5 ppm)	+ 10%	+ 24%
Protease PfuS (3.7 ppm, single addition)*	+ 11%	+ 21%

* A single dosage of Protease PfuS (3.7 ppm) was added to this tube and its foam was measured up to three cycles after single dosage addition.

5 Example 2

Protease PfuS effect on Yeast Biomass production and Viability Maintenance in Sugarcane Molasses Fermentation, Using a Cell Recycle Ssystem

Fermentation trials were performed at 32°C in 50 mL centrifuge vials (TPP), simulating an industrial ethanol fermentation process as performed in Brazil. The same native foaming Brazilian yeast strain used in Example 1 was used. A fermentation substrate containing 22° Brix (diluted molasses) was fed into a yeast slurry (prepared by resuspending acid treated yeast biomass in water). The yeast slurry represented 30% of the total fermentation volume, similar to industrial conditions. After fermentation, yeast cells were collected by centrifugation (4000 rpm for 10 min), weighed, diluted with fermented molasses (wine) and water (to 35% w/v yeast wet weight), and treated with sulfuric acid (pH from 2.0 to 2.5 for 1 hour) and reused in subsequent 9 fermentation cycles. Samples were run in triplicate at each condition. Wet weight yeast biomass was determined gravimetrically after centrifugation (4000 rpm for 10 min) of the samples. After yeast biomass centrifugation, wines had their pHs determined by the end of each cycle. The yeast viability was assayed by microscopically cell count, with erythrosine red dye.

20 Conditions were:

control – no enzyme; No Chemical antifoamer was added during acid wash.

Protease PfuS addition. Protease PfuS was added after new molasses was added. No Chemical antifoamer was added during acid wash.

25 The dosages were according Table 1. Protease PfuS was added after the vat was fed with fresh molasses.

Table 1: Protease dosage addition for each treatment.

Treatment	Protease dosage (mg/L)
Control	0

Protease PfuS 2.5 ppm	2.5 to 1.9
Protease PfuS 5 ppm	5 to 3.7
Protease PfuS 10 ppm	10 to 7.5

During the experiment, yeast biomass (wet basis) measurements for each treatment showed a significant increase in treated samples, when compared to the Control, as depicted on Fig. 1:

Fig. 1 shows a comparison between the average yeast biomass of a Control (no enzyme addition) and the average yeast biomass of treated samples (2.5 ppm, 5 ppm and 10 ppm; 1.9 ppm, 3.7 ppm and 7.5 ppm in cycles 9 and 10) over 9 cycles of fermentation. Point 10 was taken after last fermentation batch. As can be seen from Fig. 1, for treated samples the average increase in yeast biomass is about 12% when compared to the Control. Viability results are presented in Fig. 2.

Fig. 2 shows a comparison between the average yeast viability of a Control (no enzyme addition) and the average yeast viability of treated samples (2.5 ppm, 5 ppm and 10 ppm; 1.9 ppm, 3.7 ppm and 7.5 ppm in cycles 9 and 10) over 9 fermentation cycles. Treated samples with higher protease dosage (i.e. 3.7 ppm to 10 ppm) resulted in an average of 5% more viable cells than the Control after 9 sequential fermentation cycles.

Example 3

Ethanol Productivity And Viability Effect of Chemical Antifoams And Protease

Fed-batch fermentations, simulating an industrial fuel ethanol fermentation process as performed in Brazil, were carried out in 50 mL falcon tubes. A non-foaming yeast strain CAT-1 (*Saccharomyces cerevisiae* yeast) was used. For the first cycle, yeast cells from the propagation culture were added to each tube in an amount corresponding to 8 % (w/v) of the final volume. Cells were fed with 25 mL sugar cane must (sugar cane molasses diluted to 20 Brix in (Brazilian) tap water, centrifuged to remove solids, and autoclaved) in three equal sized portions with 1.5 hours interval. Cultures were incubated for 7 hours at 32°C without agitation and left at room temperature overnight. The following day, cells were separated from the fermentation wine by centrifugation (3220 rcf, 5 min) and re-suspended in wine [30 % (wet w/w)] to simulate the industrial centrifuge efficiency. Cells were further diluted in demineralized water (1:1) before addition of 1 M sulphuric acid to a final pH of 2.5. After incubation in acid at room temperature for 1 hour, feeding was initiated restarting the process.

Industrial antifoam 1 (antifoam ART DISP 904 S and dispersant ART DISP 8000, Aratrop Industrial, Brazil) and antifoam 2 (antifoam AD4415 and dispersant AD5520GA,

Alcolina, Brazil) were administered as in the industry, with the dispersant added during the acid wash and antifoam after 1 hour of fermentation. PfuS was added straight to the feeding medium in a concentration of 5 ppm EP (Enzyme Protein).

Samples from sugar cane fermentations were diluted 1000 times in milliQ water to a final concentration of approximately 500 cells/mL. Cells were dyed with propidium iodide (PI) (215 nmol/mL) and incubated for 5-10 min in the dark before applying the samples on a flow cytometer (BD accuri C6) according to the manufacturers recommendations. Threshold of FSC-H gate was set to 200,000 to avoid large particles. Quantification of viability was done by separating two populations generated from the histogram of the PI fluorophore detector FL3-H.

Fermentation kinetics was also monitored by weighing the tubes hourly. Considering that cumulative CO₂ evolution is proportional to ethanol formation at a rate of ~ 1:1 in terms of moles, it is possible to infer ethanol cumulative evolution, which is directly correlated to ethanol productivity (g ethanol.L⁻¹.h⁻¹).

Ethanol productivity was monitored throughout all fermentation cycles and used as a measure for metabolic activity and ethanol formation. Fig 3 shows a comparison between the average ethanol productivity of a control (neither antifoam nor enzyme added), two industrial antifoam agents (antifoam 1 and antifoam 2), and protease PfuS over 5 cycles of fermentation. Samples treated with industrial antifoam agents showed less ethanol productivity than the control and treatment with protease.

Another important parameter of the fermentations was the viability which was monitored after each cycle. Fig 4 shows a comparison between the average yeast cell viability of a control (neither antifoam nor enzyme added), two industrial antifoam agents (antifoam 1 and antifoam 2), and a protease (PfuS) over 5 cycles of fermentation. Samples treated with industrial antifoam agents showed an increased drop in viability when compared to control and treatment with protease.

EXAMPLE 4

Effect Of Protease Addition To Protein Levels In Foaming-Yeast Fermentation

Fed-batch fermentation, simulating an industrial fuel ethanol fermentation process as performed in Brazil, were carried out in 50 mL falcon tubes. A foaming Brazilian yeast strain (BRT) was used (*Saccharomyces cerevisiae* yeast) and compared to a non-foaming yeast (CAT-1. Yeast cells were added to each tube in an amount corresponding to 10 % (w/v) of the final volume. Cells were fed with an adequate volume of chemically defined media (YNB media without amino acids and 10mM citrate buffer pH 5.5; Sigma-Aldrich), with glucose as the sole

carbon source (16% w/v). Cultures were incubated for 7 hours at 32°C without agitation and left at room temperature overnight. The following day, cells were separated from the fermentation wine by centrifugation (3220 rcf, 10 min) and re-suspended in wine [30 % (wet w/w)] to simulate the industrial centrifuge efficiency. Cells were further diluted in demineralized water (1:1). After that dilution, fresh media was added to the slurry and the process was started all over again. Protease was added to treatment tubes at a concentration of 10 ppm (10 mg EP/L). Control tubes received no enzyme.

Protein precipitation with TCA solution

Wines from the above fermentation set up were initially pooled together and filtered in 0.22 µm. Samples from each pool were collected and 0.11 volumes of ice-cold 100% TCA solution were added for protein precipitation. Tubes were placed on ice for 10 min and after that 0.500 mL of ice-cold 10% TCA was added to the sample. Tubes were placed on ice for additional 20 min and centrifuged at 20,000g for 30 min. Supernatant was removed and 0.500 mL of acetone was added to the pellet. Tubes were gently rocked and centrifuged at 20,000g for 10 min. Supernatant was removed. Pellets were left for 15 minutes to dry.

SDS-PAGE gel electrophoresis

Protein pellets from TCA precipitation were re-suspended directly into Laemmli loading buffer (according to the manufacturer's instruction) for SDS-PAGE analysis. Samples were then heated at 100°C for 5 minutes on a heat block. 45 µL of each sample was injected in separate wells of the gel (Criterion TGX Stain-Free 4-20%, 12 wells). 0.010 mL of the marker (Bio-Rad Precision Plus Protein Unstained Standard) were added to a separate well. Gel was run at 120V (constant voltage) for approximately 1 hour. The gel was transferred to a Bio-Rad Stain-Free Sample Tray and analyzed on a Gel Doc EZ Imager, using the ImageLab software.

A photo of the SDS-Page gel is shown in Fig. 5

Lane#	Description	Foam control
	Yeast/enzyme/medium	
1	CAT-1, control wine	No foam
2	CAT-1, Protease PfuS treated wine	No foam

3	CAT-1, Mg Prot 3 treated wine	No foam
4	CAT-1, Protease Z5S17 treated wine	No foam
5	CAT-1, Protease Z6Z6A treated wine	No foam
6	BRT, control wine	Foam
7	BRT, Protease PfuS treated wine	No foam
8	BRT, Mg Prot 3 treated wine	No foam
9	BRT, Protease Z5S17 treated wine	Foam
10	BRT, Protease Z6Z6A treated wine,	Foam

Conclusion

- Foaming yeast strain (BRT) releases significantly more protein fragments into the fermenting media (wine) when compared to non-foaming strain (CAT-1).

- The Protease PfuS and Mg Prot 3 degrade one of these protein bands (indicated by the arrow in Fig. 5), while the other two proteases (Protease Z5S17 and Protease Z6Z6A) that do not control foam are not able to degrade this band (see band in the range of 25-50 KDa).

References:

Cold Spring Harb Protoc; 2011; doi:10.1101/pdb.prot5651

10 Adapted from Proteomics: A Cold Spring Harbor Laboratory Course Manual, by Andrew J. Link and Joshua LaBaer. CSHL Press, Cold Spring Harbor, NY, USA, 2009.

SUMMARY PARAGRAPHS

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

1. A process of producing a fermentation product from readily fermentable sugar-material in a fermentation vat comprising a fermentation medium using a fermenting organism, comprising
- 5 i) feeding the readily fermentable sugar material into the fermentation vat comprising a slurry of fermenting organism;
- ii) fermenting the readily fermentable sugar material into a desired fermentation product,
- wherein a protease is added
- 10 a) before, during and/or after feeding in step i), and/or
- b) during fermentation in step ii).
2. The process of paragraph 1, wherein the readily fermentable sugar material is feed into the fermentation vat as a feeding stream.
- 15 3. The process of paragraph 2, wherein the protease is mixed with the feeding stream of the readily fermentable sugar-material.
4. The process of paragraph 3, wherein the protease is mixed with the feeding stream of readily fermentable sugar-material before feeding step i).
- 20 5. The process of any of paragraphs paragraphs 1-4, wherein the readily fermentable sugars-material is selected from the group consisting of sugar cane juice, sugar cane molasses, sweet sorghum, sugar beets, and mixture thereof.
- 25 6. The process of any of paragraphs 1-5, wherein the fermentation medium further comprises other by-products of sugar cane, in particular hydrolysate from sugar cane bagasse and its streams.
- 30 7. The process of any one of paragraphs 1-6, wherein the fermenting organism is a yeast, foaming yeast, e.g., from a strain of the genus *Saccharomyces*, such as a strain of *Saccharomyces cerevisiae*, especially a strain of *Saccharomyces cerevisiae* generating foam when fermented.

8. The process of any of paragraphs 1-7, wherein the fermenting organisms are recycled after fermentation in step ii).

9. The process of paragraph 8, wherein from 50-100%, such as 70-95%, such as about
5 90% of the fermentation organisms are recycled.

10. The process of any of paragraphs 1-9, wherein the fermentation organism cell densities during fermentation is 8–17% w/v (wet basis).

10 11. The process of any of paragraphs 9 or 10, wherein the fermenting organisms, such as yeast, are collected after fermentation in step ii), acid washed, and recycled to the fermentation vat.

12. The process of paragraph 11, wherein fermenting organisms, such as yeast, are
15 collected after fermentation in step ii), and acid washed with sulfuric acid, e.g., at pH 1.5–3.0, such as 2.0-2.5, e.g., for 1–2 hours.

13. The process of any of paragraphs 1-12, wherein the fermentation is carried out as batch, fed-batch or continuous fermentation.

20

14. The process of any of paragraphs 1-13, wherein the fermentation product is an alcohol, preferably ethanol, especially fuel ethanol, potable ethanol and/or industrial ethanol.

15. The process of any of paragraphs 1-14, wherein the protease is added in a dosage of
25 0.2 to 25 mg Enzyme Protein/L fermentation medium or 0.01-100 ppm EP (Enzyme Protein), such as 0.1-50 ppm EP, in particular 1-25 ppm EP.

16. The process of any of paragraphs 1-15, wherein the protease is the only enzyme added.

30 17. The process of any of paragraphs 1-16, wherein the protease is added together with one or more enzymes selected from the group consisting of: cellulase, glucoamylase, alpha-amylase, oxidase, peroxidase, catalase, laccase, beta-glucosidase, other carbohydrases, and oxidases.

18. The process of any of paragraphs 1-17, wherein foaming in the fermentation vat is reduced compared to a corresponding process where no protease is added.

19. The process of paragraph 18, wherein the protease is added after the other enzymes,
5 preferably one or more of the enzymes in paragraph 17.

20. The process of any of paragraph 1-19, wherein adding protease result in increased yields, e.g., ethanol yield, compared to a corresponding process where no protease is present or added.

10 21. The process of any of paragraphs 1-20, wherein the the residual sugars present in the fermentation medium is reduced.

22. The process of any of paragraphs 1-21, wherein step i) and ii) are carried out
15 simultaneously or sequentially.

23. The process of any of paragraphs 1-22, wherein fermentation is 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.

20 24. The process of any of paragraphs 1-23, wherein fermentation is ongoing for 2 to 120 hours, in particular 4 to 96 hours, such as between 6 and 12 hours.

25 25. The process of any of paragraphs 1-24, wherein the fermentation product is recovered after fermentation, such as by distillation.

26. The process of any of paragraphs 1-25, wherein the protease is selected from the group of serine proteases, metalloproteases, and aspartic acid proteases.

30 27. The process of any of paragraphs 1-26, wherein the protease is capable of cutting cell wall protein, in particular mannoprotein, on the surface of the fermenting organism, in particular *Saccharomyces cerevisiae* yeast and/or is capable of hydrolyzing protein, in particular mannoprotein, in fermented readily fermentable sugar material such as fermented molasses (wine).

28. The process of any of paragraphs 1-27, wherein the protease of microbial origin, e.g., fungal or bacterial origin.

5 29. The process of any of paragraphs 1-28, wherein the protease is of fungal origin.

30. The process of any of paragraphs 1-29, wherein the protease is a metallo protease, such as one derived from a strain of the genus *Thermoascus*, preferably a strain of *Thermoascus aurantiacus*, especially *Thermoascus aurantiacus* CGMCC No. 0670, such as the
10 mature part of SEQ ID NO: 2 disclosed in WO 2003/048353 or the mature part of SEQ ID NO: 1 in WO 2010/008841 or SEQ ID NO: 1 herein, or a variant thereof.

31. The process of any of paragraphs 1-30, wherein the protease or variant thereof has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at
15 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.

20 32. The process of any of paragraphs 1-31, wherein the protease is of bacterial origin.

33. The process of any of paragraphs 1-32 wherein the protease is a serine protease, such as a protease derived from a strain of *Pyrococcus*, preferably a strain of *Pyrococcus furiosus*.

25 34. The process of any of paragraphs 1-33, wherein the protease is the one shown in SEQ ID NO: 1 in US 6,358,726 or SEQ ID NO: 2 herein.

35. The process of any of paragraphs 1-34, wherein the protease is one having at least 60%, such as at least 70%, such as at least 80%, such as at least 85%, such as at least 90%,
30 such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to in SEQ ID NO: 1 in US 6,358,726 or SEQ ID NO: 2 herein.

36. The process of any of paragraphs 1-35, wherein the protease is a serine protease, such as a serine peptidase family S53 protease, such as a S53 protease derived from a strain of *Meripilus*, preferably a strain of *Meripilus giganteus*.

5 37. The process of any of paragraphs 1-36, wherein the protease is the mature sequence from *Meripilus giganteus* protease 3 from example 2 in WO 2014/037438 or SEQ ID NO: 6 herein or SEQ ID NO: 7 herein.

10 38. The process of any of paragraphs 1-37, wherein the protease is one having at least 60%, such as at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to in SEQ ID NO: 6 herein or SEQ ID NO: 7 herein.

15 39. The process of any of paragraphs 1-38, further wherein an alpha-amylase may be added together with the protease or present and/or added during fermentation.

40. The process of paragraph 39, wherein the alpha-amylase is of microbial origin, e.g., fungal or bacterial origin.

20 41. The process of paragraph 40, wherein the alpha-amylase is of fungal origin.

25 42. The process of paragraph 41, wherein the fungal alpha-amylase is derived from a strain of *Rhizomucor*, such as a strain of *Rhizomucor pusillus*, such as a hybrid of the *Rhizomucor pusillus* alpha-amylase shown in SEQ ID NO: 3 further comprising a starch-binding module, such as a CBM20 starch-binding module, such as the sequence shown in SEQ ID NO: 4.

43. The process of paragraph 42, wherein the alpha-amylase is of bacterial origin.

30 44. The process of paragraph 43, wherein the bacterial alpha-amylase is derived from the genus *Bacillus*, such as a strain of the species *Bacillus stearothermophilu*, or variant thereof.

45. The process of paragraph 44, wherein the alpha-amylase is a mature *Bacillus stearothermophilus* alpha-amylase or corresponding mature alpha-amylases having at least 60%, such as at least 70%, such as at least 80% identity, such as at least 90% identity, such as

at least 95% identity, such as at least 96% identity, such as at least 97% identity, such as at least 99% identity to SEQ ID NO: 5 herein.

46. The process of paragraph 44 or 45, wherein the mature *Bacillus stearothermophilus* alpha-amylase, or variant thereof, is truncated, preferably to have around 485-496 amino acids, such as around 491 amino acids

47. The process of any of paragraphs 44-46, wherein the *Bacillus stearothermophilus* alpha-amylase has a double deletion in positions I181 + G182, and optionally a N193F substitution, or deletion of R179 + G180 (using SEQ ID NO: 5 herein for numbering).

48. The process of any of paragraphs 1-47, further wherein a glucoamylase is added together with the protease or be present and/or added during fermentation.

49. The process of paragraph 48, wherein the glucoamylase is a strain of *Aspergillus*, preferably *A. niger*, *A. awamori*, or *A. oryzae*; or a strain of *Trichoderma*, preferably *T. reesei*; or a strain of *Talaromyces*, preferably *T. emersonii*, or a strain of *Trametes*, such as *Trametes cingulate*, or a strain of *Pycnoporus*, or a strain of *Gloeophyllum*, or a strain of the *Nigrofomes*.

50. The process of any of paragraphs 1-49, wherein the readily fermentable sugar-material substrate is not containing polysaccharide, such as starch and/or cellulose/hemicellulose.

51. The process of any of paragraphs 1-50, wherein the desired fermentation product is produced from readily fermentable sugar material by fermentation in a fermentation vat, the process comprises adding protease to the readily fermentable sugar material before feeding; feeding the protease-containing readily fermentable sugar material into the fermentation vat comprising a slurry of fermenting organisms; fermenting the readily fermentable sugar material into the desired fermentation product.

52. The process of any of paragraphs 1-51, wherein ethanol is produced in a batch, fed batch, semi-continuous or continuous fermentation process in a fermentation vat comprising sugar cane molasses, comprising adding protease to the sugar cane molasses before feeding; feeding the protease-containing sugar cane molasses into the fermentation vat comprising a slurry of *Saccharomyces cerevisiae* yeast; and fermenting the sugar cane molasses into ethanol.

53. The process of any of paragraphs 1-52, wherein ethanol is produced from sugar cane molasses by fermentation in a fermentation vat, the process comprises adding *Pyrococcus furiosus* protease to the sugar cane molasses before feeding; feeding the *Pyrococcus furiosus* protease-containing sugar cane molasses into the fermentation vat comprising a slurry of *Saccharomyces cerevisiae* yeast; fermenting the sugar cane molasses into ethanol.

54. The process of any of paragraphs 1-53, wherein ethanol is produced from sugar cane molasses by fermentation in a fermentation vat, the process comprises adding a peptidase family S53 protease, in particular *Meripilus giganteus* protease 3 to the sugar cane molasses before feeding; feeding peptidase family S53 protease, in particular *Meripilus giganteus* protease 3, containing sugar cane molasses into the fermentation vat comprising a slurry of *Saccharomyces cerevisiae* yeast; fermenting the sugar cane molasses into ethanol.

55. The process of any of paragraphs 1-54, wherein the desired fermentation product is produced from readily fermentable sugar material by fermentation in a fermentation vat, wherein the process comprises: feeding readily fermentable sugar material into the fermentation vat comprising a slurry of fermenting organisms; feeding protease into the fermentation vat comprising a slurry of readily fermentable sugars and fermenting organisms before fermentation; fermenting the readily fermentable sugar material into the desired fermentation product.

56. The process of any of paragraphs 1-55, wherein ethanol is produced in a batch or fed batch fermentation process in a fermentation vat comprising sugar cane molasses, wherein the process comprises: feeding sugar cane molasses into the fermentation vat comprising a slurry of *Saccharomyces cerevisiae* yeast; feeding protease, preferably serine protease, into the fermentation vat comprising a slurry of *Saccharomyces cerevisiae* yeast and the sugar cane molasses before fermentation; fermenting the sugar cane molasses into ethanol.

57. The process of any of paragraphs 1-56, wherein ethanol is produced from sugar cane molasses by fermentation in a fermentation vat, wherein the process comprises: feeding sugar cane molasses into the fermentation vat comprising a slurry of fermenting organisms; feeding *Pyrococcus furiosus* protease into the fermentation vat comprising a slurry of sugar cane

molasses and *Saccharomyces cerevisiae* yeast before fermentation; fermenting the sugar cane molasses into ethanol.

58. The process of any of paragraphs 1-57, wherein ethanol is produced from sugar cane molasses by fermentation in a fermentation vat, wherein the process comprises: feeding sugar cane molasses into the fermentation vat comprising a slurry of fermenting organisms; feeding a peptidase family S53 protease, in particular *Meripilus giganteus* protease 3, into the fermentation vat comprising a slurry of sugar cane molasses and *Saccharomyces cerevisiae* yeast before fermentation; fermenting the sugar cane molasses into ethanol.

59. The process of any of paragraphs 1-58, wherein the desired fermentation product is produced from readily fermentable sugar material by fermentation in a fermentation vat, wherein the process comprises: feeding readily fermentable sugar material into the fermentation vat comprising a slurry of fermenting organisms; adding protease into the fermentation vat during fermentation of the readily fermentable sugar-material into the desired fermentation product.

60. The process of any of paragraphs 1-59, wherein ethanol is produced as a batch, fed batch, semi-continuous or continuous fermentation process in a fermentation vat comprising sugar cane molasses, wherein the process comprises: feeding sugar cane molasses into the fermentation vat comprising a slurry of *Saccharomyces cerevisiae* yeast; adding protease, in particular serine protease, into the fermentation vat during fermentation of the sugar cane molasses into ethanol.

61. The process of any of paragraphs 1-60, wherein ethanol is produced from sugar cane molasses by fermentation in a fermentation vat, wherein the process comprises: feeding sugar cane molasses into the fermentation vat comprising a slurry of *Saccharomyces cerevisiae* yeast; adding *Pyrococcus furiosus* protease into the fermentation vat during fermentation of the sugar cane molasses into ethanol.

62. The process of any of paragraphs 1-61, wherein ethanol is produced from sugar cane molasses by fermentation in a fermentation vat, wherein the process comprises: feeding sugar cane molasses into the fermentation vat comprising a slurry of *Saccharomyces cerevisiae* yeast; adding a peptidase family S53 protease, in particular *Meripilus giganteus* protease 3, into the fermentation vat during fermentation of the sugar cane molasses into ethanol.

63. The process of any of paragraphs 1-62, comprising

i) feeding the readily fermentable sugar material into the fermentation vat comprising a slurry of fermenting organism;

5 ii) fermenting the readily fermentable sugar material into a desired fermentation product,

wherein feeding of the readily fermentable sugar-material is done by introducing a feeding stream into the fermentation vat; wherein

- protease is mixed with the feeding stream before in step i); or
- 10 - protease is added to fermentation vat after feeding.

64. The process of paragraph 63, wherein the protease is *Pyrococcus furiosus* protease.

65. The process of paragraph 63, wherein the protease is *Meripilus giganteus* protease 3.

15 66. Use of a protease for reducing foam generated by fermenting organisms when producing a desired fermentation product from readily fermentable sugars.

67. The use according to paragraph 66, wherein the desired fermentation product is
20 produced as defined in any of paragraphs 1-65.

Claims

1. A process of producing a fermentation product from readily fermentable sugar-material in a fermentation vat comprising a fermentation medium using a fermenting organism,
5 comprising
- i) feeding the readily fermentable sugar material into the fermentation vat comprising a slurry of fermenting organism;
 - ii) fermenting the readily fermentable sugar material into a desired fermentation product,
- 10 wherein a protease is added
- a) before, during and/or after feeding in step i), and/or
 - b) during fermentation in step ii).
2. The process of claim 1, wherein the readily fermentable sugar material is feed into the
15 fermentation vat as a feeding stream.
3. The process of claim 2, wherein the protease is mixed with the feeding stream of the readily fermentable sugar-material.
- 20 4. The process of any of claims 1-3, wherein the protease is mixed with the feeding stream of readily fermentable sugar-material before feeding step i).
5. The process of any of claims claims 1-4, wherein the readily fermentable sugars-material is selected from the group consisting of sugar cane juice, sugar cane molasses, sweet sorghum,
25 sugar beets, and mixture thereof.
6. The process of any one of claims 1-5, wherein the fermenting organism is yeast, such as foaming yeast, e.g., from a strain of the genus *Saccharomyces*, such as a strain of *Saccharomyces cerevisiae*, especially a strain of *Saccharomyces cerevisiae* generating foam
30 when fermented.
7. The process of any of claims 1-6, wherein the fermenting organisms are recycled after fermentation in step ii).

8. The process of any of claims 6-7, wherein the fermenting organisms, such as foaming yeast, are collected after fermentation in step ii), acid washed, and recycled to the fermentation vat.

5 9. The process of any of claims 1-8, wherein the protease is selected from the group of serine proteases, metalloproteases, and aspartic acid proteases.

10. The process of any of claims 1-9, wherein the protease is capable of cutting cell wall protein, in particular mannoprotein, on the surface of the fermenting organism, such as foaming yeast, in particular *Saccharomyces cerevisiae* yeast and/or is capable of hydrolyzing protein, in particular mannoprotein, in fermented readily fermentable sugar material such as fermented molasses (wine).

11. The process of any of claims 1-10, wherein the protease is a serine protease, such as a protease derived from a strain of *Pyrococcus*, preferably a strain of *Pyrococcus furiosus*.

12. The process of any of claims 1-10, wherein the protease is a serine protease, such as a serine peptidase family S53 protease, such as a family S53 protease derived from a strain of *Meripilus*, preferably a strain of *Meripilus giganteus*, such as of *Meripilus giganteus* Protease 3.

13. The process of any of claims 1-12, wherein the readily fermentable sugar-material substrate is not containing polysaccharide, such as starch and/or cellulose/hemicellulose.

14. The process of any of claims 1-13, wherein the desired fermentation product is produced from readily fermentable sugar material by fermentation in a fermentation vat, the process comprises adding protease to the readily fermentable sugar material before feeding; feeding the protease-containing readily fermentable sugar material into the fermentation vat comprising a slurry of fermenting organisms; fermenting the readily fermentable sugar material into the desired fermentation product.

15. The process of any of claims 1-14, wherein ethanol is produced in a batch, fed batch, semi-continuous or continuous fermentation process in a fermentation vat comprising sugar cane molasses, comprising adding protease to the sugar cane molasses before feeding; feeding

the protease-containing sugar cane molasses into the fermentation vat comprising a slurry of *Saccharomyces cerevisiae* yeast; and fermenting the sugar cane molasses into ethanol.

16. The process of any of claims 1-15, wherein the desired fermentation product is produced
5 from readily fermentable sugar material by fermentation in a fermentation vat, wherein the process comprises: feeding readily fermentable sugar material into the fermentation vat comprising a slurry of fermenting organisms; feeding protease into the fermentation vat comprising a slurry of readily fermentable sugars and fermenting organisms before fermentation; fermenting the readily fermentable sugar material into the desired fermentation
10 product.

17. The process of any of claims 1-16, wherein ethanol is produced in a batch or fed batch fermentation process in a fermentation vat comprising sugar cane molasses, wherein the process comprises: feeding sugar cane molasses into the fermentation vat comprising a slurry
15 of *Saccharomyces cerevisiae* yeast; feeding protease, preferably serine protease, into the fermentation vat comprising a slurry of *Saccharomyces cerevisiae* yeast and the sugar cane molasses before fermentation; fermenting the sugar cane molasses into ethanol.

18. The process of any of claims 1-17, wherein the desired fermentation product is produced
20 from readily fermentable sugar material by fermentation in a fermentation vat, wherein the process comprises: feeding readily fermentable sugar material into the fermentation vat comprising a slurry of fermenting organisms; adding protease into the fermentation vat during fermentation of the readily fermentable sugar-material into the desired fermentation product.

25 19. The process of any of claims 1-18, wherein ethanol is produced as a batch, fed batch, semi-continuous or continuous fermentation process in a fermentation vat comprising sugar cane molasses, wherein the process comprises: feeding sugar cane molasses into the fermentation vat comprising a slurry of *Saccharomyces cerevisiae* yeast; adding protease, in particular serine protease, into the fermentation vat during fermentation of the sugar cane
30 molasses into ethanol.

20. The process of any of claims 1-19, comprising

i) feeding the readily fermentable sugar material into the fermentation vat comprising a slurry of fermenting organism;

ii) fermenting the readily fermentable sugar material into a desired fermentation product,

wherein feeding of the readily fermentable sugar-material is done by introducing a feeding stream into the fermentation vat; wherein

- 5 - protease is mixed with the feeding stream before in step i); or
 - protease is added to fermentation vat after feeding.

21. The process of claim 63, wherein the protease is *Pyrococcus furiosus* protease or *Meripilus giganteus* protease 3.

10

22. Use of a protease for reducing foam generated by fermenting organisms when producing a desired fermentation product from readily fermentable sugars.

23. The use according to claim 22, wherein the desired fermentation product is produced as
15 defined in any of claims 1-21.

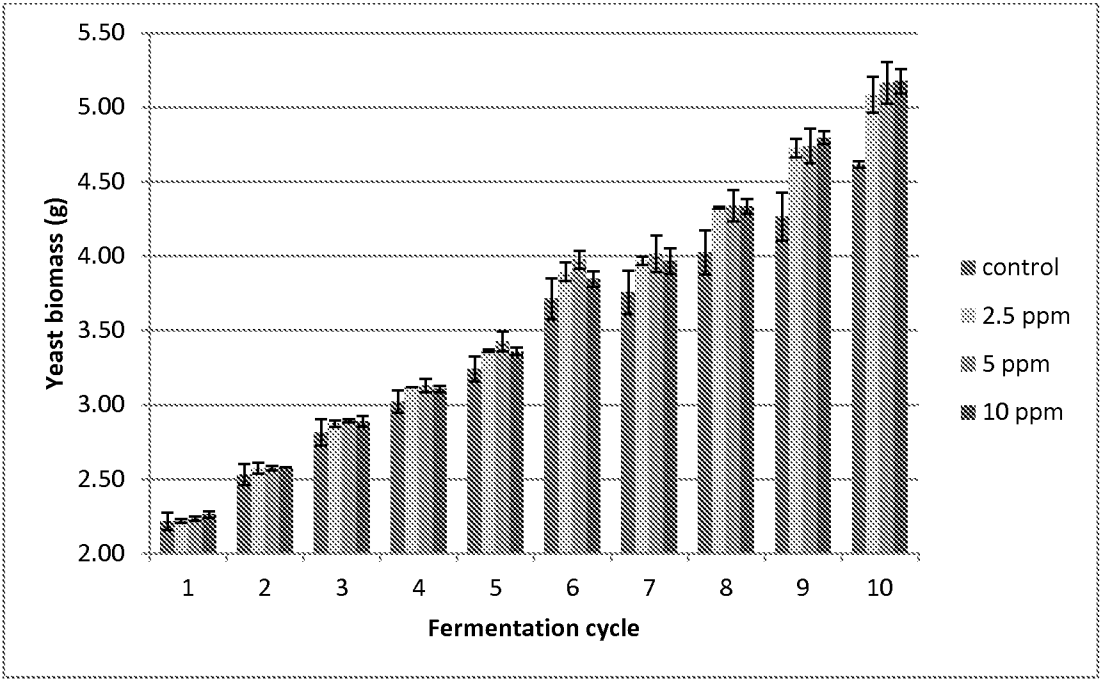


Fig. 1

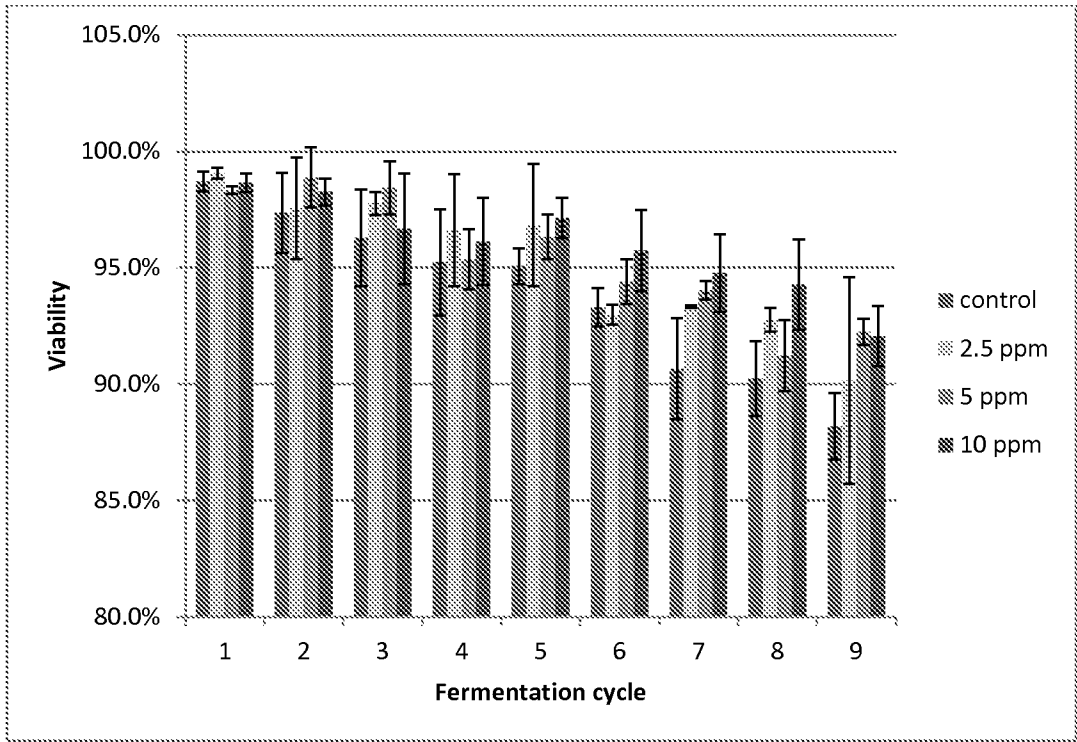


Fig. 2

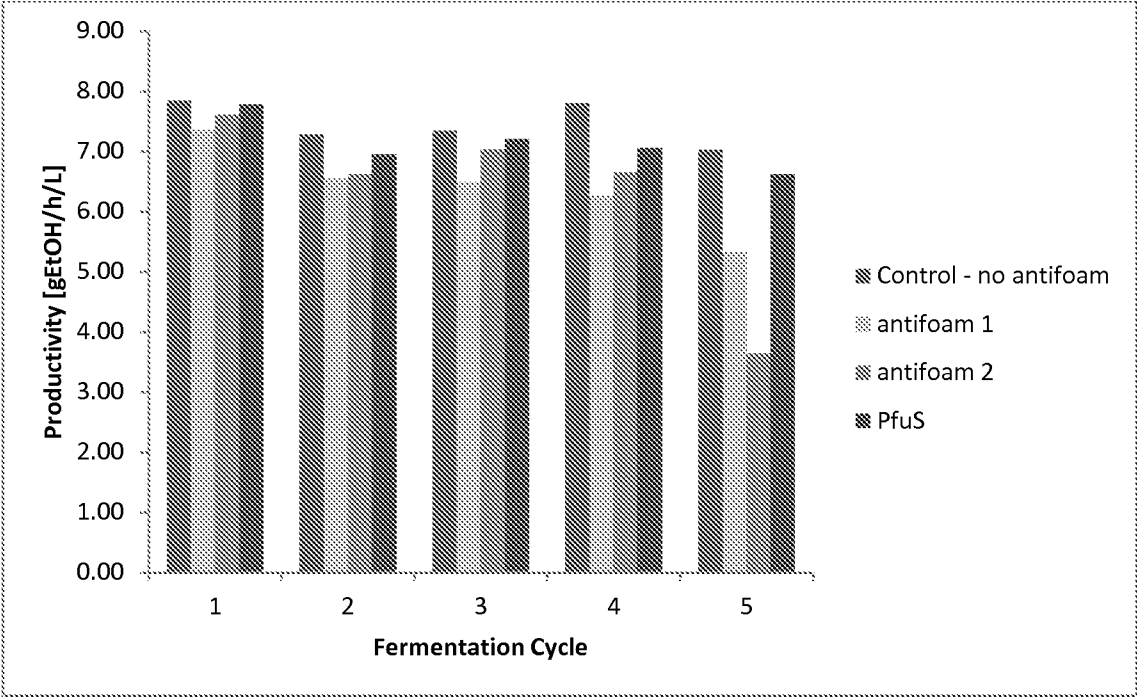


Fig. 3

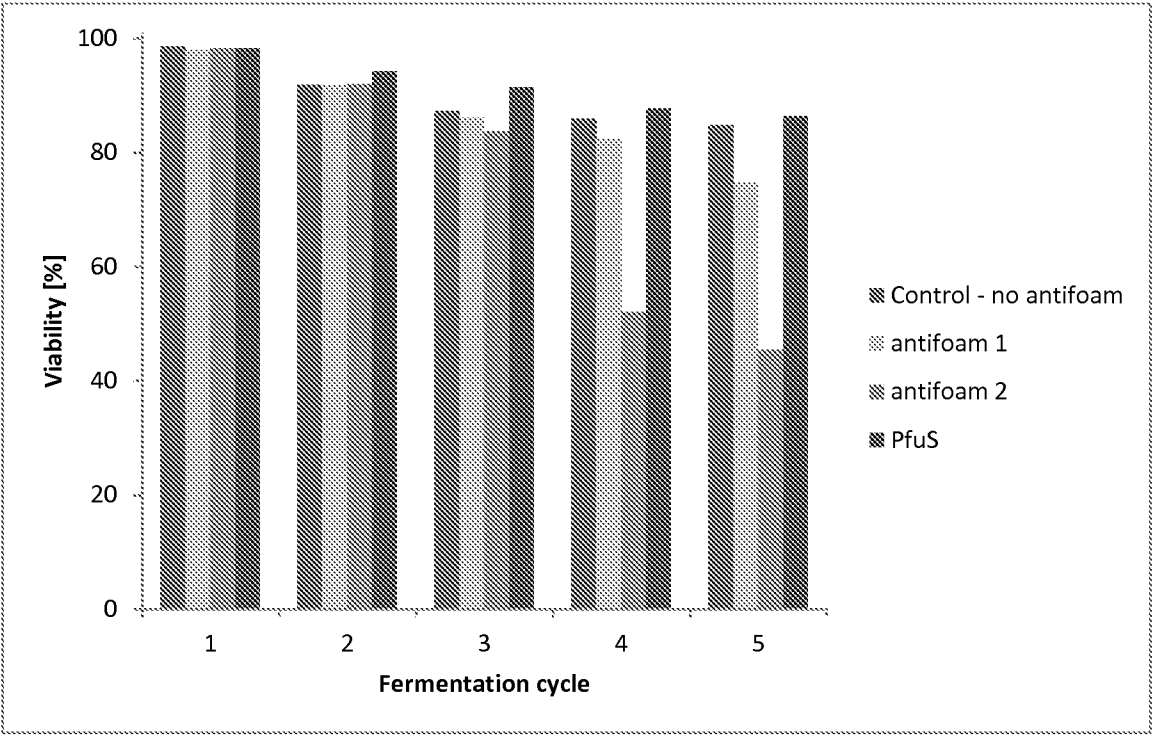


Fig. 4

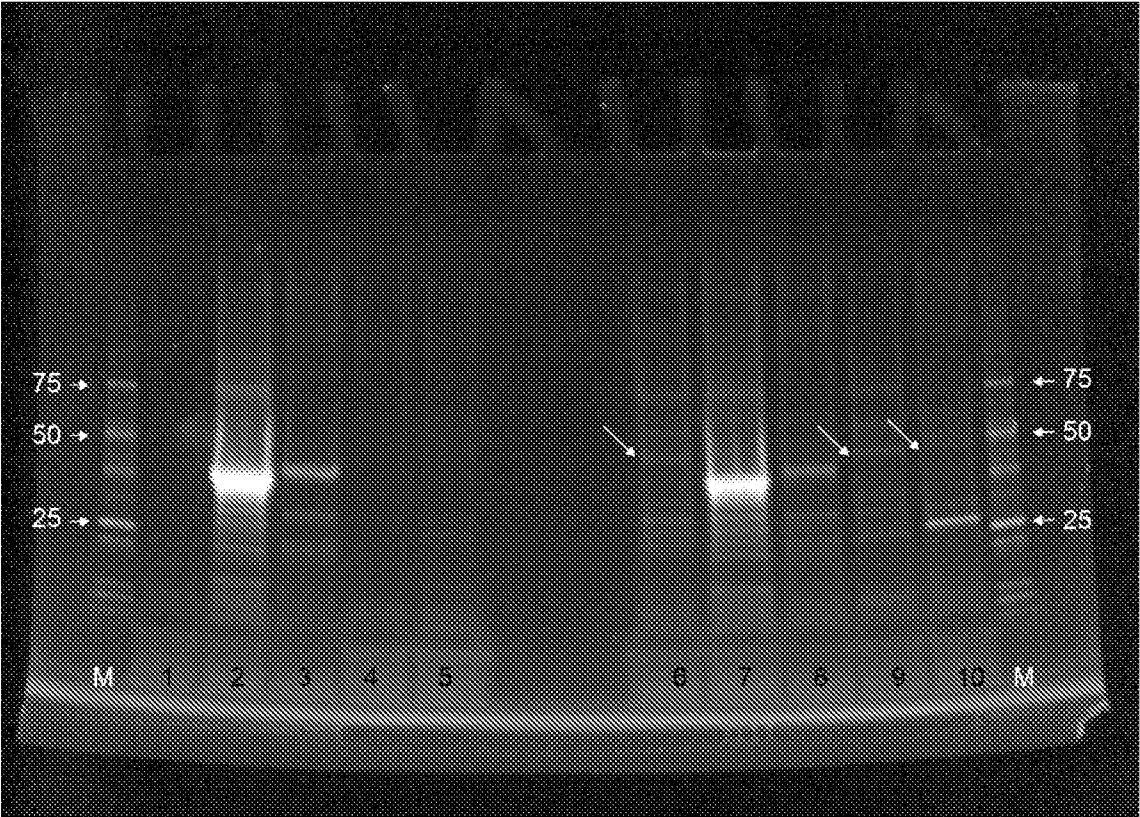


Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/043152**A. CLASSIFICATION OF SUBJECT MATTER****C12P 7/06(2006.01)i, C12N 9/52(2006.01)i**

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12P 7/06; C12N 1/00; C08F 283/06; B01D 19/04; C12P 39/00; C12P 7/20; C09K 3/00; C12N 9/52

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & keywords: fermentation, fermentable sugar-material, fermenting organism, sugar cane, molasses, protease, foam

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010-0227367 A1 (BORDIN, E. M. et al.) 9 September 2010 See paragraph [0035]; claims 1-10 and 16-21.	1-4, 22
A	US 4769324 A (MACKINTOSH, T. D. et al.) 6 September 1988 See abstract; claims 1-2.	1-4, 22
A	WO 96-13600 A1 (GENENCOR INTERNATIONAL, INC.) 9 May 1996 See abstract; claims 1-7.	1-4, 22
A	US 5288789 A (MCCARTHY, K. J.) 22 February 1994 See claim 1.	1-4, 22
A	US 6083998 A (ROMUALDO, R. et al.) 4 July 2000 See claim 1.	1-4, 22



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

27 October 2014 (27.10.2014)

Date of mailing of the international search report

27 October 2014 (27.10.2014)

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INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US2014/043152**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 21
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claim 21 is dependent on claim 63. However, claim 63 does not exist. Therefore, claim 21 does not meet PCT Article 6.
3. ☒ Claims Nos.: 5-20,23
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2014/043152

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US 05288789 A	22/02/1994	None	
US 06083998 A	04/07/2000	AR 015013 A1 AU 1999-11099 A AU 744552 B2 BR 9909858 A EP 1079908 A1 EP 1079908 A4 US 6448298 B1 WO 99-55439 A1	11/04/2001 16/11/1999 28/02/2002 19/12/2000 07/03/2001 26/07/2006 10/09/2002 04/11/1999