



(51) International Patent Classification:

C12P 1/00 (2006.01) *C12N 1/20* (2006.01)
C12N 1/14 (2006.01) *C12R 1/865* (2006.01)
C12N 1/16 (2006.01)

(21) International Application Number:

PCT/US2014/057997

(22) International Filing Date:

29 September 2014 (29.09.2014)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/885,299 1 October 2013 (01.10.2013) US

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

(72) Inventor; and

(71) Applicant (for BW only): HOLMES, Jason [US/US]; 77
 Perry Chapel Church Rd., P.O. Box 576, Franklinton,
 North Carolina 27525 (US).

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

(81) Designated States (unless otherwise indicated, for every
 kind of national protection available): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
 BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
 DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
 HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
 KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,
 MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM,
 PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,
 SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
 TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
 kind of regional protection available): ARIPO (BW, GH,
 GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,
 TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,
 TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,
 DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,
 LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
 SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
 GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— as to applicant's entitlement to apply for and be granted a
 patent (Rule 4.17(ii))

Published:

— with international search report (Art. 21(3))

(54) Title: PROCESSES OF PRODUCING FERMENTATION PRODUCTS

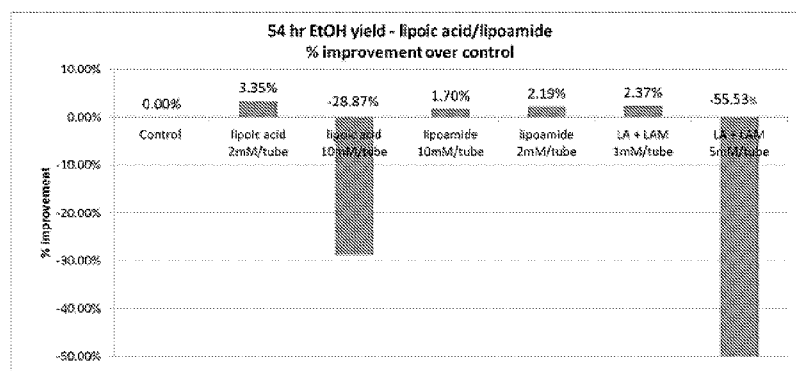


Fig. 1

(57) Abstract: The invention relates to a process of fermenting sugars derived from plant materials into fermentation products using a fermenting organism, wherein lipoic acid, lipoate, and/or lipoamide, or blends thereof, is(are) present in fermentation and/or added before and/or during fermentation. The invention also relates to processes of producing fermentation products; compositions comprising lipoic acid, lipoate, and/or lipoamide, or blends thereof; and the use of such compounds and blends comprising such compounds.

PROCESSES OF PRODUCING FERMENTATION PRODUCTS

TECHNICAL FIELD

The present invention relates to processes of producing fermentation products from plant material. The invention also relates to processes of fermenting sugars derived from plant materials using a fermenting organism. The invention also relates to compositions that can be used in processes of the invention.

BACKGROUND ART

A vast number of commercial products that are difficult to produce synthetically are today produced by fermenting organisms. Such products including alcohols (e.g., ethanol, methanol, butanol, 1,3-propanediol); organic acids (e.g., citric acid, acetic acid, itaconic acid, lactic acid, gluconic acid, gluconate, lactic acid, succinic acid, 2,5-diketo-D-gluconic acid); ketones (e.g., acetone); amino acids (e.g., glutamic acid); gases (e.g., H₂ and CO₂), and more complex compounds, including, for example, antibiotics (e.g., penicillin and tetracycline); enzymes; vitamins (e.g., riboflavin, B₁₂, beta-carotene); and hormones. Fermentation is also commonly used in the consumable alcohol (e.g., beer and wine), dairy (e.g., in the production of yogurt and cheese), leather, and tobacco industries.

A vast number of processes of producing fermentation products, such as ethanol, by fermentation of sugars provided by degradation of starch-containing and/or lignocellulose-containing material are known in the art.

However, production of fermentation products, such as ethanol, from plant materials, is still too costly. Therefore, there is a need for providing processes that can boost the yield of the fermentation product and thereby reducing the production costs.

SUMMARY OF THE INVENTION

The present invention relates to processes of producing fermentation products from plant materials. The invention provides processes of producing fermentation products, such as especially ethanol, in increased yield, from sugars derived from plant material by fermentation using a fermenting organism.

According to the invention the starting material may be any plant material or a part or constituent thereof. In one embodiment the starting material is starch-containing material. In another embodiment the starting material is lignocellulose-containing material. The sugars, to

12675-WO-PCT

be fermented into a desired fermentation product, may be derived from starch-containing material (i.e. mainly starch) or lignocellulose-containing material (i.e. mainly cellulose or hemicellulose).

5 Fermentation process of the invention

In the first aspect the invention relates to processes of fermenting sugars derived from plant material into fermentation products using a fermenting organism, wherein lipoic acid, lipoate and/or lipoamide, or blends thereof, is(are) present in fermentation and/or added before and/or during fermentation.

10 In an embodiment lipoic acid or lipoate, or blends thereof, is(are) present and/or dosed into fermentation in a concentration the range from 0.001-1.8 mM/g mash, preferably 0.001- 1.5 mM/g mash, such as 0.01-1.2 mM/g mash, such as 0.02-1.0 mM/g mash, preferably 0.03-0.8 mM/g mash, such as 0.04-0.6 mM/g mash, such as around 0.4 mM/g mash.

15 In an embodiment lipoamide is present and/or dosed into fermentation in a concentration in the range from 0.001-10 mM/g mash, preferably 0.001- 8 mM/g mash, such as 0.01-6 mM/g mash, such as 0.02-4 mM/g mash, preferably 0.03-2 mM/g mash, such as 0.04-1 mM/g mash, such as around 0.4 mM/g mash.

20 In an embodiment lipoic acid and/or lipoate together with lipoamide are present and/or dosed into fermentation in a concentration in the range from 0.001-0.80 mM/g mash, preferably 0.001- 0.7 mM/g mash, such as 0.01-0.6 mM/g mash, such as 0.02-0.5 mM/g mash, preferably 0.03-0.4 mM/g mash, such as 0.04-0.3 mM/g mash, such as around 0.2 mM/g mash.

In an embodiment the ratio between lipoic acid or lipoate and lipoamide is between 1:9 and 9:1, such as between 1:5 and 5:1, such as around 1:1.

25 Therefore, according to the invention lipoic acid, lipoate and/or lipoamide is(are) present in an increased concentration in fermentation compared to a similar fermentation wherein no lipoic acid, lipoate and/or lipoamide is(are) present and/or has been added.

When using lipoic acid, lipoate and/or lipoamide, in accordance with the invention, the fermentation product yield, such as especially ethanol yield, is boosted, i.e., increased compared to when no lipoic acid, lipoate and/or lipoamide is(are) present or added.

30 Lipoic acid, lipoate and/or lipoamide may be added before and/or during fermentation. In an embodiment lipoic acid, lipoate and/or lipoamide is(are) added into the fermentation medium, e.g., in form of a composition of the invention further comprising one or more enzymes, preferably glucoamylase and/or one or more fermenting organisms, preferably yeast.

12675-WO-PCT

Lipoic acid, lipoate or lipoamide, or blends thereof, is(are) according to the invention used as fermentation product yield boosting compounds, which means compounds that when present in fermentation, using a fermenting organism, result in increased yield of the desired fermentation product compared to a corresponding fermentation process where no lipoic acid, lipoate and/or lipoamide is(are) present and/or has been added.

In an aspect the invention relates to processes of producing fermentation products, preferably ethanol, from starch-containing material comprising the steps of:

- i) liquefying starch-containing material using an alpha-amylase;
- ii) saccharifying the liquefied material using a carbohydrate-source generating enzyme;
- iii) fermenting using a fermenting organism;

wherein fermentation is carried out in accordance with a fermentation process of the invention, i.e., wherein lipoic acid, lipoate and/or lipoamide, or blends thereof, is(are) present in fermentation and/or added before and/or during fermentation.

In another aspect the invention relates to processes of producing fermentation products, preferably ethanol, from starch-containing material, preferably granular starch, comprising the steps of:

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

- (b) fermenting using a fermenting organism;

wherein fermentation is carried out in accordance with a fermentation process of the invention, i.e., wherein lipoic acid, lipoate and/or lipoamide, or blends thereof, is(are) present in fermentation and/or added before and/or during fermentation.

In a preferred embodiment the saccharification and fermentation steps are carried out simultaneously.

In a further embodiment the invention relates to processes of producing fermentation products, preferably ethanol, from lignocellulose-containing material, comprising the steps of:

- (a) pre-treating lignocellulose-containing material;
- (b) hydrolyzing the material obtain in step (a);
- (c) fermenting using a fermenting organism;

wherein fermentation is carried out in accordance with a fermentation process of the invention, i.e., wherein lipoic acid, lipoate and/or lipoamide, or blends thereof, is(are) present in fermentation and/or added before and/or during fermentation.

In an aspect the invention also relates to compositions comprising lipoic acid, lipoate or lipoamide, or blends thereof, and further one or more enzymes and/or one or more fermenting organisms.

In invention also relates to the use of lipoic acid, lipoate or lipoamide, or blends thereof
5 for propagating fermenting organisms and for use in a fermentation process.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows the percentage (%) ethanol yield improvement over a Control after 54
hours corn mash fermentation in the presence of 2 mM and 10 mM lipoic acid or lipoamide,
10 respectively, and 1 mM and 5 mM lipoic acid and lipoamide, and 0.6 AGU/g TS Glucoamylase
E.

Fig. 2 shows the percentage (%) glycerol produced over a Control after 54 hours corn
mash fermentation in the presence of 2 mM and 10mM lipoic acid or lipoamide, respectively,
and 1 mM and 5 mM lipoic acid and lipoamide and 0.6 AGU/g TS Glucoamylase E.

Fig. 3 shows the percentage (%) residual glucose over a Control after 54 hours corn
mash fermentation in the presence of 2 mM and 10mM lipoic acid or lipoamide, respectively,
15 and 1 mM and 5 mM lipoic acid and lipoamide and 0.6 AGU/g TS Glucoamylase E.

DETAILED DESCRIPTION OF THE INVENTION

20 The present invention relates to processes of producing fermentation products from
plant material, including a fermentation step using a fermenting organism. The present
invention provides a means of increasing the fermentation product production yield, such as
especially ethanol production yield, in fermenting organisms through the alteration of the
metabolic pathway. This is done by adding one or more enzymatic cofactors selected from the
25 group of lipoic acid, lipoate and/or lipoamide, or blends thereof, in fermentation.

Lipoic Acid/Lipoate and Lipoamide

Lipoic acid, lipoate and lipoamide are enzymatic cofactors involved in the metabolic
pathway of fermenting organisms, such as yeast.

30 Lipoic acid (6,8-dithiooctanoic acid) is the free form of a catalytic cofactor important in
the generation and transfer of acyl groups formed by the pyruvate dehydrogenase (PDH) and
alpha-ketoglutarate dehydrogenase complexes, which carry out two central oxidative
decarboxylations in metabolism. The carboxyl group of lipoic acid is joined to dihydrolipoamide

transacetylase via an amide bond linking the carboxyl group of lipoic acid to a lysine ϵ -amino group of the enzyme. The resulting reactive species is an amide, called lipoamide (6,8-dithiooctanoic amide). Lipoate is the conjugate base of lipoic acid, i.e., base formed by removing a proton from lipoic acid.

5 The presence of or addition of one or more of these cofactors in fermentation is believed to ease a rate limiting factor found in the synthesis of ATP.

 As described in Example 1 and shown in Fig. 1 the ethanol yield is increased when 2 mM (i.e., 2 mmol) lipoic acid (LA); 2 mM or 10 mM lipoamide (LAM); or 1mM LA and LAM is added to 5 g corn mash (35.74% TS (Total Solids)) together with 0.6 AGU/g TS glucoamylase, and fermented for 54 hours, compared to when no lipoic acid, lipoate and/or lipoamide is(are) added. A dose of 10mM lipoic acid/5g mash caused a marked reduction in ethanol production. Lipoamide did not show a decrease in ethanol production at 10mM, however when lipoic acid and lipoamide were combined at 5mM a strong reduction in ethanol production was noted.

15 Fermentation Process of the Invention

 In the first aspect the invention relates to processes of fermenting sugars derived from plant material into fermentation products using a fermenting organism, wherein lipoic acid, lipoate, or lipoamide, or blends thereof, is(are) present and/or added before and/or during fermentation.

20 Lipoic acid, lipoate and/or lipoamide, or blends thereof, may according to the invention be added before and/or during fermentation in an effective amount/concentration. As lipoic acid, lipoate and/or lipoamide, or blends thereof, work(s) by altering the metabolic pathway these compounds are according to the invention present in fermentation by the fermenting organism. The lipoic acid, lipoate and/or lipoamide concentration is according to the invention higher compared to when no lipoic acid, lipoate and/or lipoamide is present or added in fermentation. When added in an effective amount in accordance with the invention lipoic acid, lipoate and/or lipoamide has(have) a yield boosting effect when producing fermentation products, such as especially ethanol, from starch-containing material in a process including a fermentation step, such as a conventional simultaneous saccharification and fermentation step (SSF step).

30 According to the invention effective amounts include embodiments where lipoic acid or lipoate, or blends thereof, is(are) present and/or dosed into fermentation in a concentration in the range from 0.001-1.8 mM/g mash, preferably 0.001-1.5 mM/g mash, such as 0.01-1.2 mM/g

12675-WO-PCT

mash, such as 0.02-1.0 mM/g mash, preferably 0.03-0.8 mM/g mash, such as 0.04-0.6 mM/g mash, such as around 0.4 mM/g mash.

According to the invention effective amounts include embodiments where lipoamide is present and/or dosed into fermentation in a concentration in the range from 0.001-10 mM/g mash, preferably 0.001- 8 mM/g mash, such as 0.01-6 mM/g mash, such as 0.02-4 mM/g mash, preferably 0.03-2 mM/g mash, such as 0.04-1 mM/g mash, such as around 0.4 mM/g mash.

According to the invention effective amounts include embodiments where lipoic acid and/or lipoate together with lipoamide are present and/or dosed into fermentation in a concentration in the range from 0.001-0.80 mM/g mash, preferably 0.001- 0.7 mM/g mash, such as 0.01-0.6 mM/g mash, such as 0.02-0.5 mM/g mash, preferably 0.03-0.4 mM/g mash, such as 0.04-0.3 mM/g mash, such as around 0.2 mM/g mash.

In an embodiment the ratio between lipoic acid or lipoate and lipoamide is between 1:9 and 9:1, such as between 1:5 and 5:1, such as around 1:1.

In an embodiment the fermenting organism is yeast, filamentous fungus, or a bacterium.

Examples of contemplated fermenting organisms can be found in the "Fermentation Organisms"-section below. In a preferred embodiment the fermenting organism is derived from a strain of *Saccharomyces*, such as a strain of *Saccharomyces cerevisiae*.

In an embodiment the sugars derived from plant materials are derived from starch-containing material. Examples of starch-containing materials can be found in the "Starch-Containing Materials"-section below. In a preferred embodiment the starch-containing material is corn. In another preferred embodiment the starch-containing material is a small grain such as wheat.

In an embodiment the sugars derived from plant materials are derived from lignocellulose-containing material. Examples of lignocellulose-containing materials can be found in the "Lignocellulose-Containing Materials (Biomass)"-section below. In a preferred embodiment the lignocellulose-containing material is selected from the group of corn cobs, corn stover, wheat fiber, and bagasse.

In a preferred embodiment the fermentation product is an alcohol, preferably ethanol, especially fuel ethanol, potable ethanol and/or industrial ethanol. Examples of contemplated fermentation products can be found in the "Fermentation Products"-section below.

Fermenting Organisms

The term "fermenting organism" refers to any organism, including bacterial and fungal organisms, including yeast and filamentous fungi, suitable for producing a desired fermentation product. Especially suitable fermenting organisms according to the invention are able to ferment, *i.e.*, convert sugars, such as glucose, fructose, maltose, xylose, mannose and/or arabinose, directly or indirectly into the desired fermentation product. Examples of fermenting organisms include fungal organisms, such as yeast. Preferred yeast includes strains of the genus *Saccharomyces*, in particular a strain of *Saccharomyces cerevisiae* or *Saccharomyces uvarum*; a strain of *Pichia*, in particular *Pichia stipitis* or *Pichia pastoris*; a strain of the genus *Candida*, in particular a strain of *Candida utilis*, *Candida arabinofementans*, *Candida diddensii*, *Candida sonorensis*, *Candida shehatae*, *Candida tropicalis*, or *Candida boidinii*. Other contemplated yeast includes strains of *Hansenula*, in particular *Hansenula polymorpha* or *Hansenula anomala*; strains of *Kluyveromyces*, in particular *Kluyveromyces marxianus* or *Kluyveromyces fragilis*, and strains of *Schizosaccharomyces*, in particular *Schizosaccharomyces pombe*.

Preferred bacterial fermenting organisms include strains of *Escherichia*, in particular *Escherichia coli*, strains of *Zymomonas*, in particular *Zymomonas mobilis*, strains of *Zymobacter*, in particular *Zymobacter palmae*, strains of *Klebsiella* in particular *Klebsiella oxytoca*, strains of *Leuconostoc*, in particular *Leuconostoc mesenteroides*, strains of *Clostridium*, in particular *Clostridium butyricum*, strains of *Enterobacter*, in particular *Enterobacter aerogenes* and strains of *Thermoanaerobacter*, in particular *Thermoanaerobacter* BG1L1 (*Appl. Microbiol. Biotech.* 77: 61-86) and *Thermoanaerobacter ethanolicus*, *Thermoanaerobacter thermosaccharolyticum*, or *Thermoanaerobacter mathranii*. Strains of *Lactobacillus* are also envisioned as are strains of *Corynebacterium glutamicum* R, *Bacillus thermoglucosidasius*, and *Geobacillus thermoglucosidasius*.

In an embodiment the fermenting organism is a C6 sugar (hexose) fermenting organism, such as a strain of, *e.g.*, *Saccharomyces cerevisiae*.

In connection with especially fermentation of lignocellulose derived materials, C5 sugar (pentose) fermenting organisms are also contemplated. Most C5 sugar fermenting organisms also ferment C6 sugars. Examples of C5 sugar fermenting organisms include strains of *Pichia*, such as of the species *Pichia stipitis*. C5 sugar fermenting bacteria are also known. Also some *Saccharomyces cerevisiae* strains ferment C5 (and C6) sugars. Examples are genetically modified strains of *Saccharomyces spp* that are capable of fermenting C5 sugars include the

12675-WO-PCT

ones concerned in, e.g., Ho et al., 1998, *Applied and Environmental Microbiology*, p. 1852-1859 and Karhumaa et al., 2006, *Microbial Cell Factories* 5:18.

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

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

According to the invention the fermenting organism capable of producing a desired fermentation product from fermentable sugars, including glucose, fructose maltose, xylose, mannose, and/or arabinose, is preferably grown under precise conditions at a particular growth
15 rate. When the fermenting organism is introduced into/added to the fermentation medium the inoculated fermenting organism pass through a number of stages. Initially growth does not occur. This period is referred to as the "lag phase" and may be considered a period of adaptation. During the next phase referred to as the "exponential phase" the growth rate gradually increases. After a period of maximum growth the rate ceases and the fermenting
20 organism enters "stationary phase". After a further period of time the fermenting organism enters the "death phase" where the number of viable cells declines.

In one embodiment lipoic acid, lipoate or lipoamide, or blends thereof, is(are) added to the fermentation medium when the fermenting organism is in the lag phase.

In one embodiment lipoic acid, lipoate or lipoamide, or blends thereof, is(are) added to
25 the fermentation medium when the fermenting organism is in exponential phase.

In one embodiment lipoic acid, lipoate or lipoamide, or blends thereof, is(are) added to the fermentation medium when the fermenting organism is in stationary phase.

Fermentation Products

30 The term "fermentation product" means a product produced in a process including a fermentation step using a fermenting organism. Fermentation products contemplated according to the invention include alcohols (e.g., ethanol, methanol, butanol); organic acids (e.g., citric acid, acetic acid, itaconic acid, lactic acid, 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. Preferred beer types comprise ales, stouts, porters, lagers, bitters, malt liquors, happoushu, high-alcohol beer, low-alcohol beer, low-calorie beer or light beer.

10 Fermentation

The sugars derived from plant material used in fermentation in a process of the invention may be derived from starch-containing material and/or lignocellulose-containing material. The fermentation conditions are determined based on, e.g., the kind of plant material, the fermentable sugars, the fermenting organism and/or the desired fermentation product. One skilled in the art can easily determine suitable fermentation conditions. The fermentation may according to the invention be carried out at conventionally used conditions. Preferred fermentation processes are anaerobic processes.

Fermentation of Sugars Derived from Starch-Containing Materials

As mentioned above different kinds of fermenting organisms may be used for fermenting sugars derived from starch-containing material. Fermentations are often carried out using yeast, such as *Saccharomyces cerevisiae*, as the fermenting organism. However, bacteria and filamentous fungi may also be used as fermenting organisms. Some bacteria have higher fermentation temperature optimum than, e.g., *Saccharomyces cerevisiae*. Therefore, fermentations may in such cases be carried out at temperatures as high as 75°C, e.g., between 40-70°C, such as between 50-60°C. However, bacteria with a significantly lower temperature optimum down to around room temperature (around 20°C) are also known. Examples of suitable fermenting organisms can be found in the "Fermenting Organisms"-section above.

For ethanol production using yeast, the fermentation may in one embodiment go on for 24 to 96 hours, in particular for 36 to 72 hours. In an embodiment the fermentation is carried out at a temperature between 20 to 40°C, preferably 28 to 36°C, in particular around 32°C. In an embodiment the pH is from pH 3 to 6, preferably around pH 4 to 5.

Especially contemplated is simultaneous hydrolysis/saccharification and fermentation (referred to as "SSF") where there is no separate holding stage for the hydrolysis/saccharification, meaning that the hydrolysing enzyme(s), the fermenting organism(s), and lipoic acid, lipoate or lipoamide, or blends thereof, may be added together. However, it should be understood that the lipoic acid, lipoate or lipoamide, or blends thereof, may also be added separately. When fermentation is performed simultaneous with saccharification (i.e., SSF) the temperature is preferably between 20 to 40°C, preferably 28 to 36°C, in particular around 32°C when the fermentation organism is a strain of *Saccharomyces cerevisiae* and the desired fermentation product is ethanol.

Other fermentation products may be fermented at temperatures known to the skilled person in the art to be suitable for the fermenting organism in question.

The process of the invention may be performed as a batch or as a continuous process. The fermentation process of the invention may be conducted in an ultrafiltration system where the retentate is held under recirculation in the presence of solids, water, and the fermenting organism, and where permeate is the desired fermentation product containing liquid. Equally contemplated are processes conducted in a continuous membrane reactor with ultrafiltration membranes and where the retentate is held under recirculation in presence of solids, water, the fermenting organism and where the permeate is the fermentation product containing liquid.

After fermentation the fermenting organism may be separated from the fermented slurry and recycled.

Fermentations are typically carried out at a pH in the range between 3 and 7, preferably from pH 3.5 to 6, such as around pH 5. Fermentations are typically ongoing for 24-96 hours.

Fermentation of Sugars Derived From Lignocellulose-Containing Materials

As mentioned above different kinds of fermenting organisms may be used for fermenting sugars derived from lignocellulose-containing materials. Fermentations are typically carried out by yeast, bacteria, or filamentous fungi, including the ones mentioned in the "Fermenting Organisms"-section above. If the aim is C6 fermentable sugars the conditions are usually similar to starch fermentations as described above. However, if the aim is to ferment C5 sugars (e.g., xylose) or a combination of C6 and C5 fermentable sugars the fermenting organism(s) and/or fermentation conditions may differ.

Bacteria fermentations may be carried out at higher temperatures, such as up to 75°C, e.g., between 40-70°C, such as between 50-60°C, than conventional yeast fermentations,

which are typically carried out at temperatures from 20-40°C. However, bacteria fermentations at temperature as low as 20°C are known. Fermentations are typically carried out at a pH in the range between 3 and 7, preferably from pH 3.5 to 6, such as around pH 5. Fermentations are typically ongoing for 24-96 hours.

5

Recovery

Subsequent to fermentation the fermentation product may be separated from the fermented slurry. The slurry may be distilled to extract the desired fermentation product or the desired fermentation product may be extracted from the fermented slurry by micro or membrane
10 filtration techniques. Alternatively the fermentation product may be recovered by stripping. Methods for recovery are well known in the art.

Production of Fermentation Products from Starch-Containing Materials

Processes for producing fermentation products from gelatinized starch-containing material

15 In this aspect the present invention relates to processes of producing fermentation products, especially ethanol, from starch-containing material, which processes includes a liquefaction step and sequentially or simultaneously performed saccharification and fermentation steps.

The invention relates to processes for producing fermentation products from starch-
20 containing material comprising the steps of:

- i) liquefying starch-containing material using an alpha-amylase;
- ii) saccharifying the liquefied material using a carbohydrate-source generating enzyme;
- iii) fermenting using a fermenting organism;

25 wherein fermentation is carried out in accordance with the fermentation process of the invention, i.e., wherein lipoic acid, lipoate or lipoamide, respectively, or a blends thereof, is(are) present in fermentation and/or added before and/or during fermentation.

The lipoic acid, lipoate and/or lipoamide, or a blends thereof, is(are) added before and/or during the fermentation step in an effective amount/concentration. This means that
30 lipoic acid, lipoate or lipoamide, or blends thereof, may be added in a separate saccharification step, such as pre-saccharification step, before fermentation. In an embodiment lipoic acid, lipoate and/or lipoamide, or a blends thereof, is(are) added directly to the fermentation medium or to the fermenting organism propagation medium. The lipoic acid, lipoate and/or lipoamide, or

12675-WO-PCT

blends thereof, concentration is according to the invention higher compared to when no such compound(s) is/are added in fermentation. When added in an effective amount lipoic acid, lipoate and/or lipoamide, or a blends thereof, has(have) a yield boosting effect when producing fermentation products, such as especially ethanol, from starch-containing material in a process including a fermentation step, such as a simultaneous saccharification and fermentation step (i.e., SSF step).

According to the invention effective amounts include embodiments where lipoic acid or lipoate, or blends thereof, is(are) present and/or dosed into fermentation in a concentration in the range from 0.001-1.8 mM/g mash, preferably 0.001- 1.5 mM/g mash, such as 0.01-1.2 mM/g mash, such as 0.02-1.0 mM/g mash, preferably 0.03-0.8 mM/g mash, such as 0.04-0.6 mM/g mash, such as around 0.4 mM/g mash.

According to the invention effective amounts include embodiments where lipoamide is present and/or dosed into fermentation in a concentration in the range from 0.001-10 mM/g mash, preferably 0.001- 8 mM/g mash, such as 0.01-6 mM/g mash, such as 0.02-4 mM/g mash, preferably 0.03-2 mM/g mash, such as 0.04-1 mM/g mash, such as around 0.4 mM/g mash.

According to the invention effective amounts include embodiments where lipoic acid and/or lipoate together with lipoamide are present and/or dosed/added into fermentation in a concentration in the range from 0.001-0.80 mM/g mash, preferably 0.001- 0.7 mM/g mash, such as 0.01-0.6 mM/g mash, such as 0.02-0.5 mM/g mash, preferably 0.03-0.4 mM/g mash, such as 0.04-0.3 mM/g mash, such as around 0.2 mM/g mash.

In an embodiment the ratio between lipoic acid and/or lipoate and lipoamide is between 1:9 and 9:1, such as between 1:5 and 5:1, such as around 1:1.

The fermentation product, such as especially ethanol, may optionally be recovered after fermentation, e.g., by distillation. Suitable starch-containing starting materials are listed in the section "Starch-Containing Materials"-section below. Contemplated enzymes are listed in the "Enzymes"-section below. The liquefaction is carried out in the presence of an alpha-amylase, preferably a bacterial alpha-amylase, especially *Bacillus* alpha-amylase. The fermenting organism is preferably yeast, preferably a strain of *Saccharomyces*, especially a strain of *Saccharomyces cerevisiae*. Suitable fermenting organisms are listed in the "Fermenting Organisms"-section above. In a preferred embodiment steps ii) and iii) are carried out sequentially or simultaneously (i.e., as SSF process).

12675-WO-PCT

In a particular embodiment, the process of the invention further comprises, prior to liquefaction step i), the steps of:

- x) reducing the particle size of the starch-containing material, preferably by milling;
- y) forming a slurry comprising the starch-containing material and water.

5 The aqueous slurry may contain from 10-55 wt.-% dry solids, preferably 25-45 wt.-% dry solids, more preferably 30-40 wt.-% dry solids of starch-containing material. The slurry is heated to above the initial gelatinization temperature. Alpha-amylase, preferably bacterial alpha-amylase, may be added to the slurry. In an embodiment the slurry is also jet-cooked to further gelatinize the slurry before being subjected to an alpha-amylase in liquefaction step i).

10 In an embodiment liquefaction is carried out as a three-step hot slurry process. The slurry is heated to between 60-95°C, preferably between 80-90°C, and alpha-amylase is added to initiate liquefaction (thinning). Then the slurry is jet-cooked at a temperature between 95-140°C, preferably 105-125°C, for 1-15 minutes, preferably for 3-10 minutes, especially around 5 minutes. The slurry is cooled to between 60-95°C, preferably between 80-90°C, such as
15 around 85°C, and more alpha-amylase is added to finalize hydrolysis (secondary liquefaction). The liquefaction process is usually carried out at pH 4.5-6.5, in particular at a pH between 5 and 6. Milled and liquefied starch is known as "mash".

The saccharification in step ii) may be carried out using conditions well known in the art. For instance, a full saccharification process may last up to from about 24 to about 72 hours. In
20 an embodiment a pre-saccharification step is done for 40-90 minutes at a temperature between 30-65°C, typically at about 60°C, followed by complete saccharification during fermentation in a simultaneous saccharification and fermentation step (i.e., SSF step). Saccharification is typically carried out at temperatures from 30-70°C, such as 55-65°C, typically around 60°C, and at a pH between 4 and 5, normally at about pH 4.5.

25 The most widely used process in fermentation product production, such as ethanol production, is simultaneous saccharification and fermentation (i.e., SSF process), in which there is no holding stage for the saccharification. This means that the fermenting organism, such as yeast, enzymes and lipoic acid, lipoate and/or lipoamide, or blends thereof, may be added together.

30 SSF may typically be carried out at a temperature between 25°C and 40°C, such as between 28°C and 36°C, such as between 30°C and 34°C, such as around 32°C, when the fermentation organism is yeast, such as a strain of *Saccharomyces cerevisiae*, and the desired fermentation product is ethanol.

12675-WO-PCT

Other fermentation products may be fermented at conditions and temperatures, well known to the skilled person in the art, suitable for the fermenting organism in question. According to the invention the temperature may be adjusted up or down during fermentation.

5 In an embodiment a protease is added during fermentation. Examples of proteases can be found in the "Proteases"-section below. Protease dosing can easily be determined by a person skilled in the art.

Processes for Producing Fermentation Products from Un-gelatinized Starch-containing Material

10 In this aspect the invention relates to processes for producing a fermentation product from starch-containing material without gelatinization of the starch-containing material (*i.e.*, uncooked starch-containing material). According to the invention the desired fermentation product, such as ethanol, can be produced without liquefying the aqueous slurry containing the starch-containing material. In one embodiment a process of the invention includes saccharifying (milled) starch-containing material, especially granular starch, below the initial gelatinization
15 temperature, preferably in the presence of a carbohydrate-source generating enzyme, preferably a glucoamylase, to produce sugars that can be fermented into the desired fermentation product by a suitable fermenting organism.

In this embodiment the desired fermentation product, especially ethanol, is produced from un-gelatinized (*i.e.*, uncooked) milled starch-containing material, especially granular
20 starch.

Accordingly, in this aspect the invention relates to processes of producing a fermentation product from starch-containing material, comprising the steps of:

(a) saccharifying starch-containing material at a temperature below the initial gelatinization temperature of said starch-containing material,

25 (b) fermenting using a fermenting organism,

wherein fermentation is carried out in accordance with the fermentation process of the invention, *i.e.*, wherein lipoic acid, lipoate and/or lipoamide, or blends thereof, is(are) present in fermentation and/or added before and/or during fermentation.

In a preferred embodiment steps (a) and (b) are carried out simultaneously (*i.e.*, one
30 step fermentation) or sequentially. Lipoic acid, lipoate and/or lipoamide, or blends thereof, may be added before and/or during the fermentation step, especially during simultaneous saccharification and fermentation in an effective amount/concentration. In an embodiment lipoic acid, lipoate and/or lipoamide, or blends thereof, is(are) added directly to the fermentation

12675-WO-PCT

medium or to the fermenting organism propagation medium. The lipoic acid, lipoate and/or lipoamide, or blends thereof, concentration is according to the invention higher compared to when no lipoic acid, lipoate and/or lipoamide, or blends thereof, is(are) added in fermentation. When added in an effective amount, lipoic acid, lipoate and/or lipoamide, or blends thereof, has(have) a yield boosting effect when producing fermentation products, such as especially ethanol, from starch-containing material, especially granular starch, in a process including a fermentation step, such as an one-step process of fermenting ungelatinized starch (i.e., no-cook process).

According to the invention effective amounts include embodiments where lipoic acid or lipoate, or blends thereof, is(are) present and/or dosed into fermentation in a concentration in the range from 0.001-1.8 mM/g mash, preferably 0.001- 1.5 mM/g mash, such as 0.01-1.2 mM/g mash, such as 0.02-1.0 mM/g mash, preferably 0.03-0.8 mM/g mash, such as 0.04-0.6 mM/g mash, such as around 0.4 mM/g mash.

According to the invention effective amounts include embodiments where lipoamide is present and/or dosed into fermentation in a concentration in the range from 0.001-10 mM/g mash, preferably 0.001- 8 mM/g mash, such as 0.01-6 mM/g mash, such as 0.02-4 mM/g mash, preferably 0.03-2 mM/g mash, such as 0.04-1 mM/g mash, such as around 0.4 mM/g mash.

According to the invention effective amounts include embodiments where lipoic acid and/or lipoate together with lipoamide are present and/or dosed/added into fermentation in a concentration in the range from 0.001-0.80 mM/g mash, preferably 0.001- 0.7 mM/g mash, such as 0.01-0.6 mM/g mash, such as 0.02-0.5 mM/g mash, preferably 0.03-0.4 mM/g mash, such as 0.04-0.3 mM/g mash, such as around 0.2 mM/g mash.

In an embodiment the ratio between lipoic acid or lipoate and lipoamide is between 1:9 and 9:1, such as between 1:5 and 5:1, such as around 1:1.

The fermentation product, such as especially ethanol, may optionally be recovered after fermentation, e.g., by distillation. Suitable starch-containing starting materials are listed in the section "Starch-Containing Materials"-section below. Contemplated enzymes are listed in the "Enzymes"-section below. A glucoamylase and/or an alpha-amylase may be present. Alpha-amylases used are preferably acidic, preferably acid fungal alpha-amylases. The fermenting organism is preferably yeast, preferably a strain of *Saccharomyces*. Suitable fermenting organisms are listed in the "Fermenting Organisms"-section above.

The term "below the initial gelatinization temperature" means below the lowest temperature where gelatinization of the starch commences. Starch heated in water typically begins to gelatinize between 50°C and 75°C; the exact temperature of gelatinization depends on the specific starch, and can readily be determined by the skilled artisan. Thus, the initial gelatinization temperature may vary according to the plant species, to the particular variety of the plant species as well as with the growth conditions. In the context of this invention the initial gelatinization temperature of a given starch-containing material is the temperature at which birefringence is lost in 5% of the starch granules using the method described by Gorinstein and Lii, 1992, *Starch/Stärke* 44 (12): 461-466.

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

The starch-containing material may be prepared by reducing the particle size, preferably by dry or wet milling, to 0.05 to 3.0 mm, preferably 0.1-0.5 mm. After being subjected to a process of the invention at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or preferably at least 99% of the dry solids of the starch-containing material is converted into a soluble starch hydrolyzate.

The process of the invention is conducted at a temperature below the initial gelatinization temperature. Preferably the temperature at which step (a) is carried out is between 30-75°C, preferably between 45-60°C.

In a preferred embodiment step (a) and step (b) are carried out as a simultaneous saccharification and fermentation process. In such preferred embodiment the process is typically carried at a temperature between 25°C and 40°C, such as between 28°C and 36°C, such as between 30°C and 34°C, such as around 32°C. According to the invention the temperature may be adjusted up or down during fermentation.

In an embodiment simultaneous saccharification and fermentation is carried out so that the sugar level, such as glucose level, is kept at a low level such as below 6 wt.-%, preferably below about 3 wt.-%, preferably below about 2 wt.-%, more preferred below about 1 wt.-%, even more preferred below about 0.5%, or even more preferred 0.25% wt.-%, such as below about 0.1 wt.-%. Such low levels of sugar can be accomplished by simply employing adjusted quantities of enzyme and fermenting organism. A skilled person in the art can easily determine which quantities of enzyme and fermenting organism to use. The employed quantities of enzyme and fermenting organism may also be selected to maintain low concentrations of maltose in the fermentation broth. For instance, the maltose level may be kept below about 0.5 wt.-% or below about 0.2 wt.-%.

The process of the invention may be carried out at a pH in the range between 3 and 7, preferably from pH 3.5 to 6, or more preferably from pH 4 to 5.

In an embodiment a protease is added during fermentation. Examples of proteases can be found in the "Proteases"-section below. Protease dosing can easily be determined by a person skilled in the art.

Starch-Containing Materials

According to the invention sugars may be derived from starch-containing materials. Any suitable starch-containing starting material, including granular starch, may be used according to the present invention. The starting material is generally selected based on the desired fermentation product. Examples of starch-containing starting materials, suitable for use in a process of present invention, include whole grains, corns, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, beans, and sweet potatoes, or mixtures thereof, or cereals, or sugar-containing raw materials, such as molasses, fruit materials, sugar cane or sugar beet, potatoes. Contemplated are both waxy and non-waxy types of corn and barley.

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

and fibers. The raw material, such as whole grain, is milled in order to open up the structure and allowing for further processing. Two milling processes are preferred according to the invention: wet and dry milling. In dry milling whole kernels are milled and used. Wet milling gives a good separation of germ and meal (starch granules and protein) and is often applied at
5 locations where the starch hydrolyzate is used in production of syrups. Both dry and wet milling is well known in the art of starch processing and is equally contemplated for the process of the invention.

The starch-containing material may be reduced in particle size, preferably by dry or wet milling, in order to expose more surface area. In an embodiment the particle size is between
10 0.05 to 3.0 mm, preferably 0.1-0.5 mm, or so that at least 30%, preferably at least 50%, more preferably at least 70%, even more preferably at least 90% of the starch-containing material fit through a sieve with a 0.05 to 3.0 mm screen, preferably 0.1-0.5 mm screen.

Production of Fermentation Products from Lignocellulose-Containing Material (Biomass)

15 In this aspect the invention relates to processes of producing desired fermentation products from lignocellulose-containing material. Conversion of lignocellulose-containing material into fermentation products, such as ethanol, has the advantages of the ready availability of large amounts of feedstock, including wood, agricultural residues, herbaceous crops, municipal solid wastes etc. Lignocellulose-containing materials primarily consist of
20 cellulose, hemicellulose, and lignin and are often referred to as "biomass".

The structure of lignocellulose is not directly accessible to enzymatic hydrolysis. Therefore, the lignocellulose-containing material has to be pre-treated, e.g., by acid hydrolysis under adequate conditions of pressure and temperature, in order to break the lignin seal and disrupt the crystalline structure of cellulose. This causes solubilization of the hemicellulose and
25 cellulose fractions. The cellulose and hemicelluloses can then be hydrolyzed enzymatically, e.g., by cellulolytic enzymes, to convert the carbohydrate polymers into fermentable sugars which may be fermented into a desired fermentation product, such as ethanol. Optionally the fermentation product may be recovered, e.g., by distillation.

In this aspect the invention relates to a process of producing a fermentation product
30 from lignocellulose-containing material, comprising the steps of:

- (a) pre-treating lignocellulose-containing material;
- (b) hydrolyzing the material;
- (c) fermenting using a fermenting organism;

wherein fermentation is carried out in accordance with the fermentation process of the invention, i.e., wherein lipoic acid, lipoate and/or lipoamide, or blends thereof, is(are) present in fermentation and/or added before and/or during fermentation.

Lipoic acid, lipoate and/or lipoamide, or blends thereof, may be added before and/or during fermentation. In a preferred embodiment lipoic acid, lipoate and/or lipoamide, or blends thereof, is(are) added to the fermentation medium. Fermentation step (c) may be carried out in accordance with the fermentation process of the invention. In preferred embodiments the steps are carried out as SSF, HHF, or SHF process steps which will be described further below.

Pre-treatment

The lignocellulose-containing material may according to the invention be pre-treated before being hydrolyzed and fermented. In a preferred embodiment the pre-treated material is hydrolyzed, preferably enzymatically, before and/or during fermentation. The goal of pre-treatment is to separate and/or release cellulose, hemicellulose and/or lignin and this way improve the rate of enzymatic hydrolysis.

According to the invention pre-treatment step (a) may be a conventional pre-treatment step known in the art. Pre-treatment may take place in aqueous slurry. The lignocellulose-containing material may during pre-treatment be present in an amount between 10-80 wt. %, preferably between 20-50 wt. %.

Chemical, Mechanical and/or Biological Pre-treatment

The lignocellulose-containing material may according to the invention be chemically, mechanically and/or biologically pre-treated before hydrolysis and/or fermentation. Mechanical treatment (often referred to as physical pre-treatment) may be used alone or in combination with subsequent or simultaneous hydrolysis, especially enzymatic hydrolysis, to promote the separation and/or release of cellulose, hemicellulose and/or lignin.

Preferably, chemical, mechanical and/or biological pre-treatment is(are) carried out prior to the hydrolysis and/or fermentation. Alternatively, the chemical, mechanical and/or biological pre-treatment is carried out simultaneously with hydrolysis, such as simultaneously with addition of one or more cellulolytic enzymes, or other enzyme activities mentioned below, to release fermentable sugars, such as glucose and/or maltose.

In an embodiment of the invention the pre-treated lignocellulose-containing material is washed and/or detoxified before or after hydrolysis step (b). This may improve the fermentability

of, *e.g.*, dilute-acid hydrolyzed lignocellulose-containing material, such as corn stover. Detoxification may be carried out in any suitable way, *e.g.*, by steam stripping, evaporation, ion exchange, resin or charcoal treatment of the liquid fraction or by washing the pre-treated material.

5 Chemical Pre-treatment

According to the present invention "chemical pre-treatment" refers to any chemical treatment which promotes the separation and/or release of cellulose, hemicellulose and/or lignin. Examples of suitable chemical pre-treatment steps include treatment with; for example, dilute acid, lime, alkaline, organic solvent, ammonia, sulphur dioxide, carbon dioxide. Further, wet
10 oxidation and pH-controlled hydrothermolysis are also contemplated chemical pre-treatments.

Preferably, the chemical pre-treatment is acid treatment, more preferably, a continuous dilute and/or mild acid treatment, such as, treatment with sulfuric acid, or another organic acid, such as acetic acid, citric acid, tartaric acid, succinic acid, or mixtures thereof. Other acids may also be used. Mild acid treatment means in the context of the present invention that the treatment
15 pH lies in the range from 1-5, preferably from pH 1-3. In a specific embodiment the acid concentration is in the range from 0.1 to 2.0 wt. % acid, preferably sulphuric acid. The acid may be mixed or contacted with the material to be fermented according to the invention and the mixture may be held at a temperature in the range of 160-220°C, such as 165-195°C, for periods ranging from minutes to seconds, *e.g.*, 1-60 minutes, such as 2-30 minutes or 3-12 minutes.
20 Addition of strong acids, such as sulphuric acid, may be applied to remove hemicellulose. This enhances the digestibility of cellulose.

Cellulose solvent treatment, also contemplated according to the invention, has been shown to convert about 90% of cellulose to glucose. It has also been shown that enzymatic hydrolysis could be greatly enhanced when the lignocellulosic structure is disrupted. Alkaline
25 H₂O₂, ozone, organosolv (uses Lewis acids, FeCl₃, (Al)₂SO₄ in aqueous alcohols), glycerol, dioxane, phenol, or ethylene glycol are among solvents known to disrupt cellulose structure and promote hydrolysis (Mosier et al., 2005, Bioresource Technology 96: 673-686).

Alkaline chemical pre-treatment with base, *e.g.*, NaOH, Na₂CO₃ and/or ammonia or the like, is also within the scope of the invention. Pre-treatment methods using ammonia are
30 described in, *e.g.*, WO 2006/110891, WO 2006/110899, WO 2006/110900, WO 2006/110901, which are hereby incorporated by reference.

Wet oxidation techniques involve use of oxidizing agents, such as: sulphite based oxidizing agents or the like. Examples of solvent pre-treatments include treatment with DMSO

(Dimethyl Sulfoxide) or the like. Chemical pre-treatment is generally carried out for 1 to 60 minutes, such as from 5 to 30 minutes, but may be carried out for shorter or longer periods of time dependent on the material to be pre-treated.

Other examples of suitable pre-treatment methods are described by Schell et al., 2003, Appl. Biochem and Biotechn. Vol. 105-108, p. 69-85, and Mosier et al., 2005, Bioresource Technology 96: 673-686, and US publication no. 2002/0164730, which references are hereby all incorporated by reference.

Mechanical Pre-treatment

As used in context of the present invention the term "mechanical pre-treatment" refers to any mechanical or physical pre-treatment which promotes the separation and/or release of cellulose, hemicellulose and/or lignin from lignocellulose-containing material. For example, mechanical pre-treatment includes various types of milling, irradiation, steaming/steam explosion, and hydrothermolysis.

Mechanical pre-treatment includes comminution (mechanical reduction of the particle size). Comminution includes dry milling, wet milling and vibratory ball milling. Mechanical pre-treatment may involve high pressure and/or high temperature (steam explosion). In an embodiment of the invention high pressure means pressure in the range from 300 to 600 psi, preferably 400 to 500 psi, such as around 450 psi. In an embodiment of the invention high temperature means temperatures in the range from about 100 to 300°C, preferably from about 140 to 235°C. In a preferred embodiment mechanical pre-treatment is a batch-process, steam gun hydrolyzer system which uses high pressure and high temperature as defined above. A Sunds Hydrolyzer (available from Sunds Defibrator AB (Sweden) may be used for this.

Combined Chemical and Mechanical Pre-treatment

In an embodiment of the invention both chemical and mechanical pre-treatments are carried out involving, for example, both dilute or mild acid pretreatment and high temperature and pressure treatment. The chemical and mechanical pretreatment may be carried out sequentially or simultaneously, as desired.

Accordingly, in a preferred embodiment, the lignocellulose-containing material is subjected to both chemical and mechanical pre-treatment to promote the separation and/or release of cellulose, hemicellulose and/or lignin.

In a preferred embodiment pre-treatment is carried out as a dilute or mild acid pre-treatment step. In another preferred embodiment pre-treatment is carried out as an ammonia fiber explosion step (or AFEX pre-treatment step).

5 Biological Pre-treatment

As used in the present invention the term "biological pre-treatment" refers to any biological pre-treatment which promotes the separation and/or release of cellulose, hemicellulose, and/or lignin from the lignocellulose-containing material. Biological pre-treatment techniques can involve applying lignin-solubilizing microorganisms (see, for example, Hsu, T.-A., 1996, Pretreatment of biomass, in *Handbook on Bioethanol: Production and Utilization*, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212; Ghosh, P., and Singh, A., 1993, Physicochemical and biological treatments for enzymatic/microbial conversion of lignocellulosic biomass, *Adv. Appl. Microbiol.* 39: 295-333; McMillan, J. D., 1994, Pretreating lignocellulosic biomass: a review, in *Enzymatic Conversion of Biomass for Fuels Production*, Himmel, M. E., Baker, J. O., and Overend, R. P., eds., ACS Symposium Series 566, American Chemical Society, Washington, DC, chapter 15; Gong, C. S., Cao, N. J., Du, J., and Tsao, G. T., 1999, Ethanol production from renewable resources, in *Advances in Biochemical Engineering/Biotechnology*, Scheper, T., ed., Springer-Verlag Berlin Heidelberg, Germany, 65: 207-241; Olsson, L., and Hahn-Hagerdal, B., 1996, Fermentation of lignocellulosic hydrolysates for ethanol production, *Enz. Microb. Tech.* 18: 312-331; and Vallander, L., and Eriksson, K.-E. L., 1990, Production of ethanol from lignocellulosic materials: State of the art, *Adv. Biochem. Eng./Biotechnol.* 42: 63-95).

Hydrolysis

Before and/or during fermentation the pre-treated lignocellulose-containing material may be hydrolyzed in order to break the lignin seal and disrupt the crystalline structure of cellulose. In a preferred embodiment hydrolysis is carried out enzymatically. According to the invention the pre-treated lignocellulose-containing material to be fermented may be hydrolyzed by one or more hydrolases (class E.C. 3 according to Enzyme Nomenclature), preferably one or more carbohydrases including cellulolytic enzymes and hemicellulolytic enzymes, or combinations thereof. Further, protease, alpha-amylase, glucoamylase and/or the like may also be present during hydrolysis and/or fermentation as the lignocellulose-containing material may include some, e.g., starchy and/or proteinaceous material.

The enzyme(s) used for hydrolysis may be capable of directly or indirectly converting carbohydrate polymers into fermentable sugars, such as glucose and/or maltose, which can be fermented into a desired fermentation product, such as ethanol.

5 In a preferred embodiment the carbohydrase(s) has(have) cellulolytic and/or hemicellulolytic enzyme activity.

In a preferred embodiment hydrolysis is carried out using a cellulolytic enzyme composition further comprising one or more polypeptides having cellulolytic enhancing activity.

10 In a preferred embodiment the polypeptide(s) having cellulolytic enhancing activity is(are) of family GH61 origin. Examples of suitable and preferred cellulolytic enzyme compositions and polypeptides having cellulolytic enhancing activity are described in the "Cellulases or Cellulolytic Enzymes"-section and "Cellulolytic Enhancing Polypeptides" sections below.

Suitable enzymes are described in the "Enzymes" section below.

15 Hemicellulose polymers can be broken down by hemicellulolytic enzymes and/or acid hydrolysis to release its five and six carbon sugar components. The six carbon sugars (hexoses), such as glucose, galactose, arabinose, and mannose, can readily be fermented to fermentation products such as ethanol, acetone, butanol, glycerol, citric acid, fumaric acid, etc. by suitable fermenting organisms including yeast.

20 Yeast is the preferred fermenting organism for ethanol fermentation. Preferred are strains of *Saccharomyces*, especially strains of the species *Saccharomyces cerevisiae*, preferably strains which are resistant towards high levels of ethanol, *i.e.*, up to, *e.g.*, about 10, 12, 15 or 20 vol. % or more ethanol.

25 Enzymatic hydrolysis is preferably carried out in a suitable aqueous environment under conditions which can readily be determined by one skilled in the art. In a preferred embodiment hydrolysis is carried out at suitable, preferably optimal, conditions for the enzyme(s) in question.

30 Suitable process time, temperature and pH conditions can readily be determined by one skilled in the art. Preferably, hydrolysis is carried out at a temperature between 25 and 70°C, preferably between 40 and 60°C, especially around 50°C. The step is preferably carried out at a pH in the range from 3-8, preferably pH 4-6. Hydrolysis is typically carried out for between 12 and 96 hours, preferable 16 to 72 hours, more preferably between 24 and 48 hours.

Fermentation of Lignocellulose derived Material

Fermentation of lignocellulose derived material is carried out in accordance with a fermentation process of the invention as described above, wherein lipoic acid, lipoate and/or lipoamide, or blends thereof, is(are) present in fermentation and/or added before and/or during
5 fermentation.

Lignocellulose-Containing Material (Biomass)

Any suitable lignocellulose-containing material is contemplated in context of the present invention. Lignocellulose-containing material may be any material containing lignocellulose. In
10 a preferred embodiment the lignocellulose-containing material contains at least 50 wt. %, preferably at least 70 wt. %, more preferably at least 90 wt. % lignocellulose. It is to be understood that the lignocellulose-containing material may also comprise other constituents such as cellulosic material, such as cellulose, hemicellulose and may also comprise constituents such as sugars, such as fermentable sugars and/or un-fermentable sugars.

15 Lignocellulose-containing material is generally found, for example, in the stems, leaves, hulls, husks, and cobs of plants or leaves, branches, and wood of trees. Lignocellulosic material can also be, but is not limited to, herbaceous material, agricultural residues, forestry residues, municipal solid wastes, waste paper, and pulp and paper mill residues. It is understood herein that lignocellulose-containing material may be in the form of plant cell wall
20 material containing lignin, cellulose, and hemi-cellulose in a mixed matrix.

In an embodiment the lignocellulose-containing material is corn fiber, rice straw, pine wood, wood chips, poplar, wheat straw, switchgrass, bagasse, paper and pulp processing waste.

Other more specific examples include corn stover, corn cobs, corn fiber, hardwood such
25 as poplar and birch, softwood, cereal straw such as wheat straw, switch grass, Miscanthus, rice hulls, municipal solid waste (MSW), industrial organic waste, office paper, or mixtures thereof.

In a preferred embodiment the lignocellulose-containing material is corn stover or corn cobs. In another preferred embodiment, the lignocellulose-containing material is corn fiber. In another preferred embodiment, the lignocellulose-containing material is switch grass. In another
30 preferred embodiment, the lignocellulose-containing material is bagasse.

SSF, HHF and SHF

In one embodiment of the present invention, hydrolysis and fermentation is carried out as a simultaneous hydrolysis and fermentation step (SSF). In general this means that combined/simultaneous hydrolysis and fermentation are carried out at conditions (e.g.,
5 temperature and/or pH) suitable, preferably optimal, for the fermenting organism(s) in question.

In another embodiment hydrolysis step and fermentation step are carried out as hybrid hydrolysis and fermentation (HHF). HHF typically begins with a separate partial hydrolysis step and ends with a simultaneous hydrolysis and fermentation step. The separate partial hydrolysis step is an enzymatic cellulose saccharification step typically carried out at conditions (e.g., at
10 higher temperatures) suitable, preferably optimal, for the hydrolyzing enzyme(s) in question. The subsequent simultaneous hydrolysis and fermentation step is typically carried out at conditions suitable for the fermenting organism(s) (often at lower temperatures than the separate hydrolysis step).

In another embodiment, the hydrolysis and fermentation steps may be carried out as
15 separate hydrolysis and fermentation, where the hydrolysis is taken to completion before initiation of fermentation. This is often referred to as "SHF".

Enzymes

Even if not specifically mentioned in context of a process of the invention, it is to be
20 understood that the enzyme(s) is(are) used in an "effective amount".

Alpha-Amylase

According to the invention an alpha-amylase may be used any alpha-amylase. In a preferred embodiment the alpha-amylase is an acid alpha-amylase, e.g., fungal acid alpha-
25 amylase or bacterial acid alpha-amylase. The term "acid alpha-amylase" means an alpha-amylase (E.C. 3.2.1.1) which added in an effective amount has activity optimum at a pH in the range of 3 to 7, preferably from 3.5 to 6, or more preferably from 4-5.

Bacterial Alpha-Amylase

The term "bacterial alpha-amylase" means any bacterial alpha-amylase classified under
30 EC 3.2.1.1. A bacterial alpha-amylase used according to the invention may, e.g., be derived from a strain of the genus *Bacillus*, which is sometimes also referred to as the genus *Geobacillus*. In an embodiment the *Bacillus* alpha-amylase is derived from a strain of *Bacillus*

amyloliquefaciens, *Bacillus licheniformis*, *Bacillus stearothermophilus*, *Bacillus* sp. TS-23, or *Bacillus subtilis*, but may also be derived from other *Bacillus* sp. Specific examples of bacterial alpha-amylases include the *Bacillus stearothermophilus* alpha-amylase of SEQ ID NO: 3 in WO 99/19467, the *Bacillus amyloliquefaciens* alpha-amylase of SEQ ID NO: 5 in WO 99/19467, and the *Bacillus licheniformis* alpha-amylase of SEQ ID NO: 4 in WO 99/19467 and the *Bacillus* sp. TS-23 alpha-amylase disclosed as SEQ ID NO: 1 in WO 2009/061380 (all sequences are hereby incorporated by reference). In an embodiment the bacterial alpha-amylase may be an enzyme having a degree of identity of at least 60%, e.g., at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% to any of the sequences shown in SEQ ID NOS: 3, 4 or 5, respectively, in WO 99/19467 and SEQ ID NO: 1 in WO 2009/061380. In an embodiment the alpha-amylase may be an enzyme having a degree of identity of at least 60%, e.g., at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% to any of the sequences shown in SEQ ID NO: 3 in WO 99/19467. In a preferred embodiment the alpha-amylase is derived from *Bacillus stearothermophilus*. The *Bacillus stearothermophilus* alpha-amylase may be a mature wild-type or a mature variant thereof. The mature *Bacillus stearothermophilus* alpha-amylases, or variant thereof, may be naturally truncated during recombinant production. For instance, the mature *Bacillus stearothermophilus* alpha-amylase may be a truncated so it has around 491 amino acids (compared to SEQ ID NO: 3 in WO 99/19467), such as from 480-495 amino acids. The *Bacillus* alpha-amylase may also be a variant and/or hybrid. Examples of such a variant can be found in any of WO 96/23873, WO 96/23874, WO 97/41213, WO 99/19467, WO 00/60059, WO 02/10355 and WO2009/061380 (all documents are hereby incorporated by reference). Specific alpha-amylase variants are disclosed in U.S. Patent Nos. 6,093,562, 6,187,576, 6,297,038, and 7,713,723 (hereby incorporated by reference) and include *Bacillus stearothermophilus* alpha-amylase (often referred to as BSG alpha-amylase) variants having a deletion of one or two amino acids at positions R179, G180, I181 and/or G182, preferably a double deletion disclosed in WO 96/23873 – see, e.g., page 20, lines 1-10 (hereby incorporated by reference), preferably corresponding to deletion of positions I181 and G182 compared to the amino acid sequence of *Bacillus stearothermophilus* alpha-amylase set forth in SEQ ID NO: 3 disclosed in WO 99/19467 or the deletion of amino acids R179 and G180 using SEQ ID NO: 3 in WO 99/19467 (which reference is hereby incorporated by reference). Even more preferred are *Bacillus* alpha-amylases, especially *Bacillus stearothermophilus* alpha-amylases, which have a double deletion

12675-WO-PCT

corresponding to a deletion of positions 181 and 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. The bacterial alpha-amylase may also have a substitution in a position corresponding to S239 in the *Bacillus licheniformis* alpha-amylase shown in SEQ ID NO: 4 in WO 99/19467, or a S242 variant of the *Bacillus stearothermophilus* alpha-amylase of SEQ ID NO: 3 in WO 99/19467. In an embodiment the variant is a S242A, E or Q variant, preferably a S242Q variant, of the *Bacillus stearothermophilus* alpha-amylase. In an embodiment the variant is a position E188 variant, preferably E188P variant of the *Bacillus stearothermophilus* alpha-amylase.

Other contemplated variant are *Bacillus* sp. TS-23 variant disclosed in WO2009/061380, especially variants defined in claim 1 of WO2009/061380.

Bacterial Hybrid Alpha-Amylases

The bacterial alpha-amylase may also be a hybrid bacterial alpha-amylase, e.g., an alpha-amylase comprising 445 C terminal amino acid residues of the *Bacillus licheniformis* alpha-amylase (shown in SEQ ID NO: 4 of WO 99/19467) and the 37 N terminal amino acid residues of the alpha-amylase derived from *Bacillus amyloliquefaciens* (shown in SEQ ID NO: 5 of WO 99/19467). In a preferred embodiment this hybrid has one or more, especially all, of the following substitutions:

G48A+T49I+G107A+H156Y+A181T+N190F+I201F+A209V+Q264S (using the *Bacillus licheniformis* numbering in SEQ ID NO: 4 of WO 99/19467). Also preferred are variants having one or more of the following mutations (or corresponding mutations in other *Bacillus* alpha-amylases): H154Y, A181T, N190F, A209V and Q264S and/or the deletion of two residues between positions 176 and 179, preferably the deletion of E178 and G179 (using SEQ ID NO: 5 of WO 99/19467 for position numbering).

In an embodiment the bacterial alpha-amylase is the mature part of the chimeric alpha-amylase disclosed in Richardson et al., 2002, The Journal of Biological Chemistry 277(29):. 267501-26507, referred to as BD5088 or a variant thereof. This alpha-amylase is the same as the one shown in SEQ ID NO: 2 in WO 2007134207. The mature enzyme sequence starts after the initial "Met" amino acid in position 1.

In an embodiment the bacterial alpha-amylase is dosed in an amount of 0.0005-5 KNU per g DS (dry solids), preferably 0.001-1 KNU per g DS, such as around 0.050 KNU per g DS.

Fungal Alpha-Amylase

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

5 A preferred acidic fungal alpha-amylase is a Fungamyl-like alpha-amylase which is derived from a strain of *Aspergillus oryzae*. According to the present invention, 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
10 to the mature part of the amino acid sequence shown in SEQ ID NO: 10 in WO 96/23874.

Another preferred acidic 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 WO 89/01969 (Example 3). A commercially available acid fungal alpha-
15 amylase derived from *Aspergillus niger* is SP288 (available from Novozymes A/S, Denmark).

Other contemplated wild-type alpha-amylases include those derived from a strain of the genera *Rhizomucor* and *Meripilus*, preferably a strain of *Rhizomucor pusillus* (WO 2004/055178 incorporated by reference) or *Meripilus giganteus*.

In a preferred embodiment the alpha-amylase is derived from *Aspergillus kawachii* and
20 disclosed by Kaneko et al., 1996, *J. Ferment. Bioeng.* 81: 292-298, "Molecular-cloning and determination of the nucleotide-sequence of a gene encoding an acid-stable alpha-amylase from *Aspergillus kawachii*"; and further as EMBL:#AB008370.

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

Fungal Hybrid Alpha-Amylase

In a preferred embodiment the fungal acid alpha-amylase is a hybrid alpha-amylase. Preferred examples of fungal hybrid alpha-amylases include the ones disclosed in WO
30 2005/003311 or U.S. application publication no. 2005/0054071 (Novozymes) or U.S. 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 U.S. application no. 60/638,614, including Fungamyl variant with catalytic domain JA118 and *Athelia rolfsii* SBD (SEQ ID NO:100 in US 60/638,614), *Rhizomucor pusillus* alpha-amylase with *Athelia rolfsii* AMG linker and SBD (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 U.S. application no. 11/316,535) or as V039 in Table 5 in WO 2006/069290, and *Meripilus giganteus* alpha-amylase with *Athelia rolfsii* glucoamylase linker and SBD (SEQ ID NO: 102 in U.S. application no. 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 U.S. application no. 11/316,535 and WO 2006/069290 (hereby incorporated by reference).

Other specific examples of contemplated hybrid alpha-amylases include those disclosed in U.S. application 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.

Contemplated are also alpha-amylases which exhibit a high identity to any of above mention alpha-amylases, *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 enzyme sequences.

An acid alpha-amylases may according to the invention be added in an amount of 0.1 to 10 AFAU/g DS, preferably 0.10 to 5 AFAU/g DS, especially 0.3 to 2 AFAU/g DS or 0.001 to 1 FAU-F/g DS, preferably 0.01 to 1 FAU-F/g DS.

Commercial Alpha-Amylase Products

Preferred commercial compositions comprising alpha-amylase include MYCOLASE from DSM (Gist Brocades), BAN™, TERMAMYL™ SC, FUNGAMYL™, LIQUOZYME™ X and SAN™ SUPER, SAN™ EXTRA L (Novozymes A/S) and CLARASE™ L-40,000, DEX-LO™, SPEZYME™ FRED, SPEZYME™ AA, SPEZYME™ DELTA AA, SPEZYME XTRA™ (Genencor Int., USA), FUELZYME™ (from Verenum Corp, USA) and the acid fungal alpha-amylase sold under the trade name SP288 (available from Novozymes A/S, Denmark).

Carbohydrate-Source Generating Enzyme

The term "carbohydrate-source generating enzyme" includes glucoamylase (being glucose generators), beta-amylase and maltogenic amylase (being maltose generators). A carbohydrate-source generating enzyme is capable of producing a carbohydrate that can be used as an energy-source by the fermenting organism(s) in question, for instance, when used in a process of the invention for producing a fermentation product, such as ethanol. The generated carbohydrate may be converted directly or indirectly to the desired fermentation product, preferably ethanol. According to the invention a mixture of carbohydrate-source generating enzymes may be used. Especially contemplated mixtures are mixtures of at least a glucoamylase and an alpha-amylase, especially an acid amylase, even more preferred an acid fungal alpha-amylase. The ratio between acid fungal alpha-amylase activity (FAU) per glucoamylase activity (AGU) (FAU per AGU) may in an embodiment of the invention be at least 0.1, or at least 0.16, such as in the range from 0.12 to 0.50 or more.

The ratio between acid fungal alpha-amylase activity (FAU-F) and glucoamylase activity (AGU) (*i.e.*, FAU-F per AGU) may in an embodiment of the invention be between 0.1 and 100, in particular between 2 and 50, such as in the range from 10-40.

Glucoamylase

A glucoamylase used according to the invention may be derived from any suitable source, *e.g.*, derived from a microorganism or a plant. Preferred glucoamylases are of fungal or bacterial origin, selected from the group consisting of *Aspergillus* glucoamylases, in particular *A. niger* G1 or G2 glucoamylase (Boel et al., 1984, *EMBO J.* 3 (5): 1097-1102), or variants thereof, such as those disclosed in WO 92/00381, WO 00/04136 and WO 01/04273 (from Novozymes, Denmark); the *A. awamori* glucoamylase disclosed in WO 84/02921, *A. oryzae* glucoamylase (*Agric. Biol. Chem.*, 1991, 55 (4): 941-949), or variants or fragments thereof. Other *Aspergillus* glucoamylase variants include variants with enhanced thermal stability: G137A and G139A (Chen et al., 1996, *Prot. Eng.* 9: 499-505); D257E and D293E/Q (Chen et al., 1995, *Prot. Eng.* 8: 575-582); N182 (Chen et al., 1994, *Biochem. J.* 301: 275-281); disulphide bonds, A246C (Fierobe et al., 1996, *Biochemistry* 35: 8698-8704; and introduction of Pro residues in position A435 and S436 (Li et al., 1997, *Protein Eng.* 10: 1199-1204).

Other glucoamylases include *Athelia rolfsii* (previously denoted *Corticium rolfsii*) glucoamylase (see U.S. Patent No. 4,727,026 and Nagasaka et al., 1998, "Purification and properties of the raw-starch-degrading glucoamylases from *Corticium rolfsii*, *Appl Microbiol*

12675-WO-PCT

Biotechnol 50:323-330), *Talaromyces* glucoamylases, in particular derived from *Talaromyces emersonii* (WO 99/28448), *Talaromyces leycettanus* (U.S. Patent No. Re. 32,153), *Talaromyces duponti*, and *Talaromyces thermophilus* (U.S. Patent No. 4,587,215).

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

Contemplated fungal glucoamylases include *Trametes cingulata*, *Pachykytospora papyracea*; and *Leucopaxillus giganteus* all disclosed in WO 2006/069289; and *Peniophora rufomarginata* disclosed in WO2007/124285; or a mixture thereof. Also hybrid glucoamylase
10 are contemplated according to the invention. Examples include the hybrid glucoamylases disclosed in WO 2005/045018. Specific examples include the hybrid glucoamylase disclosed in Table 1 and 4 of Example 1 (which hybrids are hereby incorporated by reference).

In an embodiment the glucoamylase is derived from a strain of the genus *Pycnoporus*, in particular a strain of *Pycnoporus* as described in WO 2011/066576 (SEQ ID NOs 2, 4 or 6),
15 or from a strain of the genus *Gloeophyllum*, in particular a strain of *Gloeophyllum* as described in WO 2011/068803 (SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16) or a strain of the genus *Nigrofomes*, in particular a strain of *Nigrofomes* sp. disclosed in WO 2012/064351 (SEQ ID NO: 2) (all references hereby incorporated by reference). Contemplated are also glucoamylases which exhibit a high identity to any of the above-mentioned glucoamylases, i.e., at least 70%, at least
20 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, such as 100% identity to any one of the mature parts of the enzyme sequences mentioned above.

Commercially available compositions comprising glucoamylase include AMG 200L; AMG 300 L; SAN™ SUPER, SAN™ EXTRA L, SPIRIZYME™ PLUS, SPIRIZYME™ FUEL,
25 SPIRIZYME™ B4U, SPIRIZYME ULTRA™ and AMG™ E (from Novozymes A/S, Denmark); OPTIDEX™ 300, GC480™ and GC147™ (from Genencor Int., USA); 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 0.02-20 AGU/g DS,
30 preferably 0.1-10 AGU/g DS, especially between 1-5 AGU/g DS, such as 0.1-2 AGU/g DS, such as 0.5 AGU/g DS or in an amount of 0.0001-20 AGU/g DS, preferably 0.001-10 AGU/g DS, especially between 0.01-5 AGU/g DS, such as 0.1-2 AGU/g DS.

Beta-Amylase

At least according to the invention the a beta-amylase (E.C 3.2.1.2) is the name traditionally given to exo-acting maltogenic amylases, which catalyze the hydrolysis of 1,4-alpha-glucosidic linkages in amylose, amylopectin and related glucose polymers. Maltose units
5 are successively removed from the non-reducing chain ends in a step-wise manner until the molecule is degraded or, in the case of amylopectin, until a branch point is reached. The maltose released has the beta anomeric configuration, hence the name beta-amylase.

Beta-amylases have been isolated from various plants and microorganisms (W.M. Fogarty and C.T. Kelly, 1979, *Progress in Industrial Microbiology* 15: 112-115). These beta-
10 amylases are characterized by having optimum temperatures in the range from 40°C to 65°C and optimum pH in the range from 4.5 to 7. A commercially available beta-amylase from barley is NOVOZYM™ WBA from Novozymes A/S, Denmark and SPEZYME™ BBA 1500 from Genencor Int., USA.

Maltogenic amylase

The amylase may also be a maltogenic alpha-amylase. A "maltogenic alpha-amylase" (glucan 1,4-alpha-maltohydrolase, E.C. 3.2.1.133) is able to hydrolyze amylose and amylopectin to maltose in the alpha-configuration. A maltogenic amylase from *Bacillus*
20 *stearothermophilus* strain NCIB 11837 is commercially available from Novozymes A/S. Maltogenic alpha-amylases are described in U.S. Patent Nos. 4,598,048, 4,604,355 and 6,162,628, which are hereby incorporated by reference.

The maltogenic amylase may in a preferred embodiment be added in an amount of 0.05- 5 mg total protein/gram DS or 0.05- 5 MANU/g DS.

Proteases

A protease used in accordance with the present invention may be any protease, such as of microbial or plant origin. In a preferred embodiment the protease is an acid protease of microbial origin, preferably of fungal or bacterial origin.

Suitable proteases include microbial proteases, such as fungal and bacterial proteases.
30 Preferred proteases are acidic proteases, *i.e.*, proteases characterized by the ability to hydrolyze proteins under acidic conditions below pH 7.

Contemplated acid fungal proteases include fungal proteases derived from *Aspergillus*, *Mucor*, *Rhizopus*, *Candida*, *Coriolus*, *Endothia*, *Enthomophtra*, *Irpex*, *Penicillium*, *Sclerotium* and

Torulopsis. Especially contemplated are proteases derived from *Aspergillus niger* (see, e.g., Koaze et al., 1964, *Agr. Biol. Chem. Japan* 28: 216), *Aspergillus saitoi* (see, e.g., Yoshida, 1954, *J. Agr. Chem. Soc. Japan* 28: 66), *Aspergillus awamori* (Hayashida et al., 1977, *Agric. Biol. Chem.* 42(5): 927-933, *Aspergillus aculeatus* (WO 95/02044), or *Aspergillus oryzae*, such as the pepA protease; and acidic proteases from *Mucor pusillus* or *Mucor miehei*.

Contemplated are also neutral or alkaline proteases, such as a protease derived from a strain of *Bacillus*. A particular protease contemplated for the invention is derived from *Bacillus amyloliquefaciens* and has the sequence obtainable at Swissprot as Accession No. P06832.

Also contemplated are the proteases having at least 90% identity to amino acid sequence obtainable at Swissprot as Accession No. P06832 such as at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or particularly at least 99% identity.

Further contemplated are the proteases having at least 90% identity to amino acid sequence disclosed as SEQ.ID.NO: 1 in the WO 2003/048353 such as at 92%, at least 95%, at least 96%, at least 97%, at least 98%, or particularly at least 99% identity.

Also contemplated are papain-like proteases such as proteases within E.C. 3.4.22.* (cysteine protease), such as EC 3.4.22.2 (papain), EC 3.4.22.6 (chymopapain), EC 3.4.22.7 (asclepain), EC 3.4.22.14 (actinidain), EC 3.4.22.15 (cathepsin L), EC 3.4.22.25 (glycyl endopeptidase) and EC 3.4.22.30 (caricain).

In an embodiment the protease is a protease preparation derived from a strain of *Aspergillus*, such as *Aspergillus oryzae*. In another embodiment the protease is derived from a strain of *Rhizomucor*, preferably *Rhizomucor miehei*. In another contemplated embodiment the protease is a protease preparation, preferably a mixture of a proteolytic preparation derived from a strain of *Aspergillus*, such as *Aspergillus oryzae*, and a protease derived from a strain of *Rhizomucor*, preferably *Rhizomucor mehei*.

Aspartic acid proteases are described in, for example, Handbook of Proteolytic Enzymes, Edited by Barrett, Rawlings and Woessner, Academic Press, San Diego, 1998, Chapter 270). Suitable examples of aspartic acid protease include, e.g., those disclosed in Berka et al., 1990, *Gene* 96: 313; Berka et al., 1993, *Gene* 125: 195-198; and Gomi et al., 1993, *Biosci. Biotech. Biochem.* 57: 1095-1100, which are hereby incorporated by reference.

Commercially available products include ALCALASE®, ESPERASE™, FLAVOURZYME™, PROMIX™, NEUTRASE®, RENNILASE®, NOVOZYM™ FM 2.0L, and NOVOZYM™ 50006 (available from Novozymes A/S, Denmark) and GC106™ and SPEZYME™ FAN from Genencor Int., Inc., USA.

The protease may be present in an amount of 0.0001-1 mg enzyme protein per g DS, preferably 0.001 to 0.1 mg enzyme protein per g DS. Alternatively, the protease may be present in an amount of 0.0001 to 1 LAPU/g DS, preferably 0.001 to 0.1 LAPU/g DS and/or 0.0001 to 1 mAU-RH/g DS, preferably 0.001 to 0.1 mAU-RH/g DS or 0.1-1000 AU/kg DM (dry matter), preferably 1-100 AU/kg DS and most preferably 5-25 AU/kg DS.

Cellulases or Cellulolytic Enzymes

The terms "cellulases" or "cellulolytic enzymes" as used herein are understood as comprising the cellobiohydrolases (EC 3.2.1.91), *e.g.*, cellobiohydrolase I and cellobiohydrolase II, as well as the endo-glucanases (EC 3.2.1.4) and beta-glucosidases (EC 3.2.1.21). See relevant sections below with further description of such enzymes.

In order to be efficient, the digestion of cellulose may require several types of enzymes acting cooperatively. At least three categories of enzymes are often needed to convert cellulose into glucose: endoglucanases (EC 3.2.1.4) that cut the cellulose chains at random; cellobiohydrolases (EC 3.2.1.91) which cleave cellobiosyl units from the cellulose chain ends and beta-glucosidases (EC 3.2.1.21) that convert cellobiose and soluble cellodextrins into glucose. Among these three categories of enzymes involved in the biodegradation of cellulose, cellobiohydrolases are the key enzymes for the degradation of native crystalline cellulose. The term "cellobiohydrolase I" is defined herein as a cellulose 1,4-beta-cellobiosidase (also referred to as Exo-glucanase, Exo-cellobiohydrolase or 1,4-beta-cellobiohydrolase) activity, as defined in the enzyme class EC 3.2.1.91, which catalyzes the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose and cellotetraose, by the release of cellobiose from the non-reducing ends of the chains. The definition of the term "cellobiohydrolase II activity" is identical, except that cellobiohydrolase II attacks from the reducing ends of the chains.

The cellulases may comprise a carbohydrate-binding module (CBM) which enhances the binding of the enzyme to a cellulose-containing fiber and increases the efficacy of the catalytic active part of the enzyme. A CBM is defined as contiguous amino acid sequence within a carbohydrate-active enzyme with a discreet fold having carbohydrate-binding activity. For further information of CBMs see the CAZy internet server (Supra) or Tomme et al. (1995) in *Enzymatic Degradation of Insoluble Polysaccharides* (Saddler and Penner, eds.), Cellulose-binding domains: classification and properties. pp. 142-163, American Chemical Society, Washington.

In a preferred embodiment the cellulases or cellulolytic enzymes may be a cellulolytic preparation or cellulolytic enzymes composition as defined in WO 2008/151079 or WO 2013/028928, which are both hereby incorporated by reference. In a preferred embodiment the cellulolytic preparation comprising a polypeptide having cellulolytic enhancing activity (GH61),
5 preferably a GH61A polypeptide disclosed in WO 2005/074656. The cellulolytic preparation may further comprise a beta-glucosidase, such as a beta-glucosidase derived from a strain of the genus *Trichoderma*, *Aspergillus*, or *Penicillium*, including the fusion protein having beta-glucosidase activity disclosed in WO 2008/057637 (Novozymes). In an embodiment the cellulolytic preparation may also comprises a CBH II, preferably *Thielavia terrestris*
10 cellobiohydrolase II (CEL6A). In an embodiment the cellulolytic preparation also comprises a cellulase enzymes preparation, preferably the one derived from *Trichoderma reesei*.

The cellulolytic activity may, in a preferred embodiment, be derived from a fungal source, such as a strain of the genus *Trichoderma*, preferably a strain of *Trichoderma reesei*; or a strain of the genus *Humicola*, such as a strain of *Humicola insolens*.

15 In an embodiment the cellulolytic enzyme preparation comprises a polypeptide having cellulolytic enhancing activity (GH61A) disclosed in WO 2005/074656; a cellobiohydrolase, such as *Thielavia terrestris* cellobiohydrolase II (CEL6A), a beta-glucosidase (e.g., the fusion protein disclosed in WO 2008/057637 and cellulolytic enzymes, e.g., derived from *Trichoderma reesei*.

20 In an embodiment the cellulolytic preparation comprises a polypeptide having cellulolytic enhancing activity, e.g., a GH61A polypeptide disclosed in WO 2005/074656; a beta-glucosidase, e.g., a fusion protein disclosed in WO 2008/057637 and cellulolytic enzymes, e.g., derived from *Trichoderma reesei*.

25 In an embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition, further comprising *Thermoascus aurantiacus* GH61A polypeptide having cellulolytic enhancing activity (SEQ ID NO: 2 in WO 2005/074656) and *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 2 of WO 2005/047499).

30 In an embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition further comprising *Penicillium emersonii* GH61A polypeptide disclosed as SEQ ID NO: 2 in WO 2011/041397, and *Aspergillus fumigatus* beta-glucosidase disclosed as SEQ ID NO: 2 in WO 2005/047499, or a variant thereof, which variant has one of, preferably all of, the following substitutions: F100D, S283G, N456E, F512Y. In an embodiment the cellulolytic enzyme composition further comprises a cellobiohydrolase I and cellobiohydrolase II derived from *Aspergillus fumigatus*. In a preferred embodiment the

cellulolytic enzyme composition comprises the *Aspergillus fumigatus* Cel7A CBH I disclosed as SEQ ID NO: 6 in WO2011/057140 and the *Aspergillus fumigatus* CBH II disclosed as SEQ ID NO: 18 in WO 2011/057140.

The cellulolytic enzyme composition may also comprise some hemicellulase, such as up to 20% hemicellulase, such as up to 15%, such as up to 10%, such as xylanase and/or xylosidase, such as beta-xylosidase. In an embodiment the cellulolytic enzyme composition comprises *Aspergillus fumigatus* GH10 xylanase, such as the one disclosed as SEQ ID NO: 6 (Xyl III) in WO 2006/078256, and *Aspergillus fumigatus* beta-xylosidase, such as the one disclosed in WO 2013/028928 (Example 16 and 17).

In an embodiment the cellulolytic enzyme is the commercially available product CELLUCLAST® 1.5L, CELLUZYME™, CELLIC CTEC™, CELLIC CTEC2™, CELLIC CTEC3™ (all available from Novozymes A/S, Denmark), ACCELLERASE 1000™, ACCELLERASE 1500™, ACCELLERASE DUET™, ACCELLERASE TRIO™ (all available from DuPont, USA).

Dosing of cellulases or cellulolytic enzymes can easily be determined by the skilled person in the art. Cellulases or cellulolytic enzymes may typically be dosed in the range from 0.1-100 FPU per gram total solids (TS), preferably 0.5-50 FPU per gram TS, especially 1-20 FPU per gram TS.

Endoglucanase (EG)

Endoglucanases (EC No. 3.2.1.4) catalyses endo hydrolysis of 1,4-beta-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxy methyl cellulose and hydroxy ethyl cellulose), lichenin, beta-1,4 bonds in mixed beta-1,3 glucans such as cereal beta-D-glucans or xyloglucans and other plant material containing cellulosic parts. The authorized name is endo-1,4-beta-D-glucan 4-glucano hydrolase, but the abbreviated term endoglucanase is used in the present specification. Endoglucanase activity may be determined using carboxymethyl cellulose (CMC) hydrolysis according to the procedure of Ghose, 1987, *Pure and Appl. Chem.* 59: 257-268.

In a preferred embodiment endoglucanases may be derived from a strain of the genus *Trichoderma*, preferably a strain of *Trichoderma reesei*; a strain of the genus *Humicola*, such as a strain of *Humicola insolens*; or a strain of *Chrysosporium*, preferably a strain of *Chrysosporium lucknowense*.

Cellobiohydrolase (CBH)

The term “cellobiohydrolase” means a 1,4-beta-D-glucan cellobiohydrolase (E.C. 3.2.1.91), which catalyzes the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, cellobiosaccharides, or any beta-1,4-linked glucose containing polymer, releasing cellobiose
5 from the reducing or non-reducing ends of the chain.

Examples of cellobiohydrolases are mentioned above including CBH I and CBH II from *Trichoderma reesei*; *Humicola insolens*, *Aspergillus fumigatus*, and CBH II from *Thielavia terrestris* cellobiohydrolase (CELL6A). In a preferred embodiment the cellulolytic enzyme composition comprises the *Aspergillus fumigatus* Cel7A CBH I disclosed as SEQ ID NO: 6 in
10 WO2011/057140 and the *Aspergillus fumigatus* CBH II disclosed as SEQ ID NO: 18 in WO 2011/057140.

Cellobiohydrolase activity may be determined according to the procedures described by Lever *et al.*, 1972, *Anal. Biochem.* 47: 273-279 and by van Tilbeurgh *et al.*, 1982, *FEBS Letters* 149: 152-156; van Tilbeurgh and Claeysens, 1985, *FEBS Letters* 187: 283-288. The Lever *et al.* method is suitable for assessing hydrolysis of cellulose in corn stover and the method of van
15 Tilbeurgh *et al.* is suitable for determining the cellobiohydrolase activity on a fluorescent disaccharide derivative.

Beta-glucosidase

20 One or more beta-glucosidases (often referred to as “cellobiases”) may be present during hydrolysis.

The term “beta-glucosidase” means a beta-D-glucoside glucohydrolase (E.C. 3.2.1.21), which catalyzes the hydrolysis of terminal non-reducing beta-D-glucose residues with the release of beta-D-glucose. For purposes of the present invention, beta-glucosidase activity is
25 determined according to the basic procedure described by Venturi *et al.*, 2002, *J. Basic Microbiol.* 42: 55-66, except different conditions were employed as described herein. One unit of beta-glucosidase activity is defined as 1.0 μ mole of p-nitrophenol produced per minute at 50°C, pH 5 from 4 mM p-nitrophenyl-beta-D-glucopyranoside as substrate in 100 mM sodium citrate, 0.01% TWEEN® 20.

30 In an embodiment the beta-glucosidase is of fungal origin, such as a strain of the genus *Trichoderma*, *Aspergillus* or *Penicillium*. In a preferred embodiment the beta-glucosidase is derived from *Trichoderma reesei*, such as the beta-glucosidase encoded by the *bgl1* gene (see Fig. 1 of EP 562,003). In another preferred embodiment the beta-glucosidase is derived from

Aspergillus oryzae (recombinantly produced in *Aspergillus oryzae* according to WO 02/095014).

In a preferred embodiment the beta-glucosidase is derived from *Aspergillus fumigatus* such as the one disclosed as SEQ ID NO: 2 in WO 2005/047499, or a variant thereof, which variant has one of, preferably all of, the following substitutions: F100D, S283G, N456E, F512Y.

5

Cellulolytic Enhancing Polypeptides

The term "cellulolytic enhancing activity" is defined herein as a biological activity that enhances the hydrolysis of a lignocellulose derived material by proteins having cellulolytic activity. For purposes of the present invention, cellulolytic enhancing activity is determined by measuring the increase in reducing sugars or in the increase of the total of cellobiose and glucose from the hydrolysis of a lignocellulose derived material, e.g., pre-treated lignocellulose-containing material by cellulolytic protein under the following conditions: 1-50 mg of total protein/g of cellulose in PCS (pre-treated corn stover), wherein total protein is comprised of 80-99.5% w/w cellulolytic protein/g of cellulose in PCS and 0.5-20% w/w protein of cellulolytic enhancing activity for 1-7 day at 50°C compared to a control hydrolysis with equal total protein loading without cellulolytic enhancing activity (1-50 mg of cellulolytic protein/g of cellulose in PCS).

The polypeptides having cellulolytic enhancing activity enhance the hydrolysis of a lignocellulose derived material catalyzed by proteins having cellulolytic activity by reducing the amount of cellulolytic enzyme required to reach the same degree of hydrolysis preferably at least 0.1-fold, more at least 0.2-fold, more preferably at least 0.3-fold, more preferably at least 0.4-fold, more preferably at least 0.5-fold, more preferably at least 1-fold, more preferably at least 3-fold, more preferably at least 4-fold, more preferably at least 5-fold, more preferably at least 10-fold, more preferably at least 20-fold, even more preferably at least 30-fold, most preferably at least 50-fold, and even most preferably at least 100-fold.

In an embodiment the hydrolysis and/or fermentation is carried out in the presence of a cellulolytic enzyme in combination with a polypeptide having enhancing activity. In a preferred embodiment the polypeptide having enhancing activity is a family GH61A polypeptide. WO 2005/074647 discloses isolated polypeptides having cellulolytic enhancing activity and polynucleotides thereof from *Thielavia terrestris*. WO 2005/074656 discloses an isolated polypeptide having cellulolytic enhancing activity and a polynucleotide thereof from *Thermoascus aurantiacus*. U.S. Application Publication No. 2007/0077630 discloses an

isolated polypeptide having cellulolytic enhancing activity and a polynucleotide thereof from *Trichoderma reesei*.

In an embodiment the cellulolytic enzyme composition comprising *Thermoascus aurantiacus* GH61A polypeptide having cellulolytic enhancing activity (SEQ ID NO: 2 in WO 2005/074656). In another preferred embodiment the cellulolytic enzyme composition comprising *Penicillium emersonii* GH61A polypeptide disclosed as SEQ ID NO: 2 in WO 2011/041397.

Hemicellulolytic enzymes

Hemicellulose can be broken down by hemicellulases and/or acid hydrolysis to release its five and six carbon sugar components.

In an embodiment of the invention the lignocellulose derived material may be treated with one or more hemicellulases.

Any hemicellulase suitable for use in hydrolyzing hemicellulose, preferably into xylose, may be used. Preferred hemicellulases include xylanases, beta-xylosidase, arabinofuranosidases, acetyl xylan esterase, feruloyl esterase, glucuronidases, galactanase, endo-galactanase, mannases, endo or exo arabinases, exo-galactanases, pectinase, xyloglucanase, or mixtures of two or more thereof. Preferably, the hemicellulase for use in the present invention is an exo-acting hemicellulase, and more preferably, the hemicellulase is an exo-acting hemicellulase which has the ability to hydrolyze hemicellulose under acidic conditions of below pH 7, preferably pH 3-7.

In an embodiment the hemicellulase is a xylanase. In an embodiment the xylanase is of microbial origin, such as of fungal origin (e.g., *Trichoderma*, *Meripilus*, *Humicola*, *Aspergillus*, *Fusarium*) or from a bacterium (e.g., *Bacillus*). In a preferred embodiment the xylanase is derived from a filamentous fungus, preferably derived from a strain of *Aspergillus*, such as *Aspergillus aculeatus* or *Aspergillus fumigatus*; or a strain of *Humicola*, preferably *Humicola lanuginosa*. The xylanase may preferably be an endo-1,4-beta-xylanase, more preferably an endo-1,4-beta-xylanase of GH10 or GH11. In an embodiment the xylanase is an *Aspergillus aculeatus* GH10 xylanase, such as the one disclosed as SEQ ID NO: 2 (Xyl II) in WO WO9421785.

In an embodiment the xylanase is an *Aspergillus fumigatus* GH10 xylanase, such as the one disclosed as SEQ ID NO: 6 (Xyl III) in WO 2006/078256.

In an embodiment the hemicellulase is a beta-xylosidase. In an embodiment the beta-xylosidase is an *Aspergillus fumigatus* beta-xylosidase, such as the one disclosed in WO

12675-WO-PCT

2013/028928 (Example 16 and 17). Examples of commercial xylanases include SHEARZYME™ and CELLIC HTEC™, CELLIC HTEC2™ CELLIC HTEC3™ from Novozymes A/S, Denmark.

Arabinofuranosidase (EC 3.2.1.55) catalyzes the hydrolysis of terminal non-reducing alpha-L-arabinofuranoside residues in alpha-L-arabinosides.

5 Galactanase (EC 3.2.1.89), arabinogalactan endo-1,4-beta-galactosidase, catalyses the endohydrolysis of 1,4-D-galactosidic linkages in arabinogalactans.

Pectinase (EC 3.2.1.15) catalyzes the hydrolysis of 1,4-alpha-D-galactosiduronic linkages in pectate and other galacturonans.

Xyloglucanase catalyzes the hydrolysis of xyloglucan.

10 The hemicellulase may be added in an amount effective to hydrolyze hemicellulose, such as, in amounts from about 0.001 to 0.5 wt.-% of total solids (TS), more preferably from about 0.05 to 0.5 wt.-% of TS.

Xylanases may be added in amounts of 0.001-1.0 g/kg DM (Dry Matter) substrate, preferably in the amounts of 0.005-0.5 g/kg DM substrate, and most preferably from 0.05-0.10
15 g/kg DM substrate.

Composition

In this aspect the invention relates to a composition comprising lipoic acid, lipoate and/or lipoamide, or blends thereof, and further one or more enzymes and/or one or more fermenting
20 organisms.

In an embodiment the ratio between lipoic acid and/or lipoate and lipoamide is between 1:9 and 1:9, such as between 1:5 to 5:1, such as around 1:1.

The composition of the invention may comprise an enzyme from the class of hydrolases (class EC 3 according to Enzyme Nomenclature). In an embodiment the enzyme is selected
25 from the group consisting of cellulase, hemicellulase, protease, alpha-amylase, and glucoamylase, or a mixture thereof.

In a preferred embodiment the enzyme is a glucoamylase, such as one listed in the "Glucoamylase"-section above.

The composition may also comprise a fermenting organism, such as yeast or another
30 fermenting organism mentioned in the "Fermenting Organism"-section above.

12675-WO-PCT

Use

In this aspect the invention relates to the use of lipoic acid, lipoate and/or lipoamide, or blends thereof, for propagating fermenting organisms, such as yeast, especially yeast derived from a strain of *Saccharomyces*, such as *Saccharomyces cerevisiae*.

- 5 In invention also relates to the use of lipoic acid, lipoate and/or lipoamide, or blends thereof, in a fermentation process or a process of the invention.

The present invention is further described in the following numbered paragraphs:

- 10 1. A process of fermenting sugars derived from plant material into a fermentation product using a fermenting organism, wherein lipoic acid, lipoate and/or lipoamide, or blends thereof is(are) present or added before and/or during fermentation.
2. The process of paragraph 1, wherein lipoic acid and/or lipoate is added in fermentation.
- 15 3. The process of paragraph 1, wherein lipoamide is added in fermentation.
4. The process of any of paragraphs 1-3, wherein a blend of lipoic acid, lipoate and lipoamide, such as lipoic acid and lipoamide, lipoate and lipoamide, or lipoic acid, lipoate and lipoamide are added in fermentation.
- 20 5. The process of any of paragraphs 1-4, wherein lipoic acid, lipoate and/or lipoamide is(are) added before fermentation, such as in a pre-saccharification step.
6. The process of any of paragraphs 1-5, wherein the fermenting organism is yeast, filamentous fungus and/or a bacterium.
- 25 7. The process of any of paragraphs 1-6, wherein the fermenting organism is derived from a strain of *Saccharomyces*, such as *Saccharomyces cerevisiae*.
- 30 8. The process of any of paragraphs 1-7, wherein the sugars derived from plant material are derived from starch-containing material or lignocellulose-containing material, or a mixture thereof.

12675-WO-PCT

9. The process of any of paragraphs 1-8, wherein lipoic acid and/or lipoate is(are) present and/or dosed into fermentation in a concentration in the range from 0.001-1.8 mM/g mash, preferably 0.001- 1.5 mM/g mash, such as 0.01-1.2 mM/g mash, such as 0.02-1.0 mM/g mash, preferably 0.03-0.8 mM/g mash, such as 0.04-0.6 mM/g mash, such as around 0.4 mM/g mash.

5

10. The process of any of paragraphs 1-8, wherein lipoamide is present and/or dosed into fermentation in a concentration in the range from 0.001-10 mM/g mash, preferably 0.001- 8 mM/g mash, such as 0.01-6 mM/g mash, such as 0.02-4 mM/g mash, preferably 0.03-2 mM/g mash, such as 0.04-1 mM/g mash, such as around 0.4 mM/g mash.

10

11. The process of any of paragraphs 1-8, wherein lipoic acid and/or lipoate together with lipoamide are present and/or dosed into fermentation in a concentration in the range from 0.001-0.80 mM/g mash, preferably 0.001- 0.7 mM/g mash, such as 0.01-0.6 mM/g mash, such as 0.02-0.5 mM/g mash, preferably 0.03-0.4 mM/g mash, such as 0.04-0.3 mM/g mash, such as around 0.2 mM/g mash.

15

12. The process of any of paragraphs 1-11, wherein the ratio between lipoic acid and/or lipoate and ii) lipoamide is between 1:9 and 9:1, such as between 1:5 and 5:1, such as around 1:1.

20

13. The process of any of paragraphs 1-11, wherein the fermentation product is an alcohol, preferably ethanol, especially fuel ethanol, potable ethanol and/or industrial ethanol.

14. The process of any of paragraphs 1-13, wherein lipoic acid/lipoate and/or lipoamide boosts the fermentation yield compared to a corresponding fermentation where no lipoic acid/lipoate and lipoamide is present and/or added.

25

15. A process of producing a fermentation product from starch-containing material comprising the steps of:

i) liquefying starch-containing material using an alpha-amylase;

30

ii) saccharifying the liquefied material using a carbohydrate-source generating enzyme;

iii) fermenting using a fermenting organism; wherein fermentation is carried out as defined in any of paragraphs 1-14.

12675-WO-PCT

16. The process of paragraph 15, further comprises, prior to the liquefaction step i), the steps of:

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

17. The process of paragraphs 15 or 16, wherein the temperature during liquefaction is above the initial gelatinization temperature of starch

18. The process of any of paragraphs 15-17, wherein in the range from 70-100°C, such as between 70-95°C, such as between 75-90°C, preferably between 80-90°C, such as around 85°C.

19. The process of any of paragraphs 15-18, wherein a jet-cooking step is carried out prior to liquefaction in step i).

20. The process of paragraph 19, wherein the jet-cooking is carried out at a temperature between 95-140°C, preferably 105-125°C, for 1-15 minutes, preferably for 3-10 minutes, especially around 5 minutes, such as between 110-145°C, preferably 120-140°C, such as 125-135°C, preferably around 130°C for about 1-15 minutes, preferably for about 3-10 minutes, especially around about 5 minutes.

21. The process of any of paragraphs 15-20, wherein the pH during liquefaction is between 4.0-6.5, such as 4.5-6.2, such as above 4.8-6.0, such as between 5.0-5.8.

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

23. The process of any of paragraphs 15-22, wherein the alpha-amylase is of bacterial origin.

24. The process of paragraph 23, wherein the alpha-amylase is from the genus *Bacillus*, such as a strain of *Bacillus stearothermophilus*, in particular a variant of a *Bacillus stearothermophilus* alpha-amylase, such as the one shown in SEQ ID NO: 3 in WO 99/019467.

12675-WO-PCT

25. The process of paragraph 25, wherein the *Bacillus stearothermophilus* alpha-amylase or variant thereof is truncated, preferably to have around 491 amino acids, such as from 480-495 amino acids.

5

26. The process of any of paragraphs 15-25, wherein the liquefied material, in step ii), is saccharified using a carbohydrate-source generating enzyme.

27. The process of any of paragraphs 15-26, wherein the saccharification step ii) is carried out for from about 24 to about 72 hours.

10

28. The process of any of paragraphs 15-27, wherein the carbohydrate-source generating enzyme is added during pre-saccharification carried out before saccharification step ii) or simultaneous saccharification and fermentation (SSF).

15

29. The process of paragraphs 28, wherein pre-saccharification is done for 40-90 minutes at a temperature between 30-65°C, typically about 60°C.

30. The process of paragraph 29, wherein pre-saccharification is followed by saccharification during fermentation in simultaneous saccharification and fermentation ("SSF").

20

31. The process of paragraph 30, wherein saccharification is carried out at temperatures from 20-75°C, preferably from 40-70°C, typically around 60°C, and at a pH between 4 and 5, such as around pH 4.5.

25

32. The process of paragraph 30, wherein fermentation or SSF is carried out at a temperature from 25°C to 40°C, such as from 28°C to 36°C, such as from 30°C to 34°C, preferably around about 32°C.

33. The process of any of paragraphs 15-32, wherein fermentation is ongoing for 6 to 120 hours, in particular 24 to 96 hours.

30

12675-WO-PCT

34. The process of any of paragraphs 15-33, wherein the pH is between 3.5-5, in particular between 3.8 and 4.3.

35. The process of any of paragraphs 15-34, wherein the carbohydrate-source generating enzyme, preferably a glucoamylase, is present or added in presaccharification before
5 saccharification step ii), in saccharification step ii), or simultaneous saccharification and fermentation steps ii) and iii).

36. The process of any of paragraphs 15-35, wherein the carbohydrate-source generating
10 enzyme is a glucoamylase of fungal origin, preferably from a stain of *Aspergillus*, preferably *Aspergillus niger*, *Aspergillus awamori*, or *Aspergillus oryzae*; or a strain of *Trichoderma*, preferably *T. reesei*; or a strain of *Talaromyces*, preferably *T. emersonii*, or a strain of *Pycnoporus*, or a strain of *Gloeophyllum*, or a strain of the *Nigrofomes*.

37. The process of paragraph 36, wherein the glucoamylase is derived from a strain of in particular derived from a strain of *Talaromyces emersonii* disclosed as SEQ ID NO: 34 in WO
15 99/28448.

38. The process of paragraph 37, wherein the glucoamylase is derived from a strain of
20 *Gloeophyllum*, such as a strain of *Gloeophyllum sepiarium* or *Gloeophyllum trabeum*, such as one disclosed in WO 2011/068803 as any of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14 or 16, preferably SEQ ID NO: 2 in WO 2011/068803 in WO 2011/068803.

39. The process of any of paragraphs 15-38, wherein the fermenting organism is yeast,
25 filamentous fungus and/or a bacterium.

40. The process of any of paragraphs 15-39, wherein the fermenting organism is derived from a strain of *Saccharomyces*, such as *Saccharomyces cerevisiae*.

41. The process of producing a fermentation product from starch-containing material, comprising the steps of:

(a) saccharifying starch-containing material at a temperature below the initial gelatinization temperature of said starch-containing material,

12675-WO-PCT

(b) fermenting using a fermenting organism,
wherein fermentation is carried out as defined in any of paragraphs 1-14.

42. The process of paragraph 41, wherein steps (a) and (b) are carried out simultaneously
5 or sequentially.

43. The process of paragraph 41 or 42, wherein the saccharification or simultaneous
saccharification and fermentation are carried out in using a carbohydrate-source generating
enzyme selected from the group consisting of glucoamylase, beta-amylase, and maltogenic
10 amylase, or a mixture thereof.

44. The process of any of paragraphs 41-43, wherein an alpha-amylase is present during
saccharification and/or fermentation, preferably simultaneous saccharification and fermentation.

15 45. The process of any of paragraphs 41-44, wherein the starch-containing material is
granular starch.

46. The process of producing a fermentation product from lignocellulose-containing
material, comprising the steps of:

- 20 (a) pre-treating lignocellulose-containing material;
(b) hydrolyzing the material;
(c) fermenting using a fermenting organism;

wherein fermentation is carried out as defined in any of paragraphs 1-14.

25 47. The process of paragraph 46, wherein the lignocellulose-containing material is
chemically, mechanically and/or biologically pre-treated in step (a).

48. The process of paragraph 46 or 47, wherein hydrolysis in step (b) and fermentation in
step (c) is carried out as a SSF, HHF or SHF process.

30 49. The process of any of paragraphs 46-48, wherein the fermenting organism is yeast,
preferably a strain of the genus *Saccharomyces* or *Pichia*.

12675-WO-PCT

50. A composition comprising lipoic acid, lipoate and/or lipoamide, or blends thereof, and one or more enzymes and/or one or more fermenting organisms.

51. The composition of paragraph 50, wherein the enzyme(s) is(are) one or more
5 hydrolases (class EC 3 according to Enzyme Nomenclature) selected from the group consisting of cellulases, hemicellulases, proteases, alpha-amylases, glucoamylases, or a mixture thereof.

52. The composition of paragraph 50 or 51, wherein the fermenting organism is selected from the group of yeast, filamentous fungus and/or a bacteria.

10 53. The composition of any of paragraphs 50-52, wherein the ratio between lipoic acid and/or lipoate and lipoamide in the composition is between 1:9 and 9:1, such as between 1:5 and 5:1, such as around 1:1.

15 54. Use of lipoic acid, lipoate, and/or lipoamide, or blends thereof for propagating fermenting organisms.

55. Use of lipoic acid, lipoate, and/or lipoamide, or blends thereof, in a fermentation process.

20
The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the
25 scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure, including definitions will be controlling.

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

12675-WO-PCT

MATERIALS & METHODS**Materials:**

Lipoic acid: Alpha-lipoic acid sourced from Sigma Product# T5625-5G

5 Lipoamide: Alpha-lipoamide sourced from Sigma Product# T5875-5G

Glucoamylase E: Enzyme blend comprising *Talaromyces emersonii* glucoamylase disclosed as SEQ ID NO: 34 in WO99/28448, *Trametes cingulata* glucoamylase disclosed as SEQ ID NO: 2 in WO 06/69289, and *Rhizomucor pusillus* alpha-amylase with *Aspergillus niger* glucoamylase linker and SBD disclosed as V039 in Table 5 in WO 2006/069290 (ratio about 75:20:5).

10

Yeast:

- RED STAR™ available from Red Star/Lesaffre, USA

15 **Methods:**

Identity

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

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

20

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

25

30

12675-WO-PCT

Glucoamylase activity (AGU)

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

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

<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

10

<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.

15 **Alpha-amylase activity (KNU)**

The alpha-amylase activity may be determined using potato starch as substrate. This method is based on the break-down of modified potato starch by the enzyme, and the reaction

12675-WO-PCT

is followed by mixing samples of the starch/enzyme solution with an iodine solution. Initially, a blackish-blue color is formed, but during the break-down of the starch the blue color gets weaker and gradually turns into a reddish-brown, which is compared to a colored glass standard.

5 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 upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

10

Acid alpha-amylase activity (AFAU)

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

15

Determination of FAU-F

FAU-F Fungal Alpha-Amylase Unts (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

20 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.

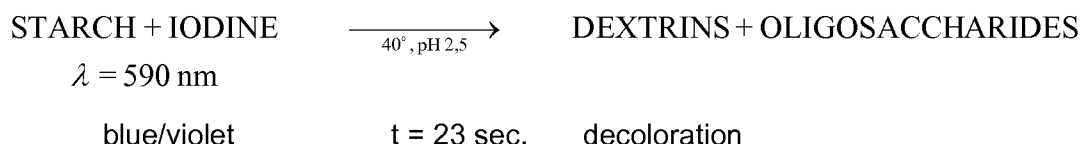
Acid alpha-amylase activity (AFAU)

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

12675-WO-PCT

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

ALPHA - AMYLASE



Standard conditions/reaction conditions:

Substrate:	Soluble 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:	590nm
Enzyme concentration:	0.025 AFAU/mL
Enzyme working range:	0.01-0.04 AFAU/mL

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

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

1. Source of Method

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

12675-WO-PCT

2. Procedure

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

5 2.2 Enzyme Assay Tubes:

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

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

10 2.2.3 The tubes containing filter paper and buffer are incubated 5 min. at 50°C ($\pm 0.1^\circ\text{C}$) in a circulating water bath.

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

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

15 2.2.6 After vortexing, the tubes are incubated for 60 mins. at 50°C ($\pm 0.1^\circ\text{C}$) in a circulating water bath.

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

20 2.3 Blank and Controls

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

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

25 2.3.3 Enzyme controls are prepared for each enzyme dilution by mixing 1.0 mL of citrate buffer with 0.5 mL of the appropriate enzyme dilution.

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

2.4 Glucose Standards

30 2.4.1 A 100 mL stock solution of glucose (10.0 mg/mL) is prepared, and 5 mL aliquots are frozen. Prior to use, aliquots are thawed and vortexed to mix.

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

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

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

12675-WO-PCT

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

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

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

5 2.4.4 The glucose standard tubes are assayed in the same manner as the enzyme assay tubes, and done along with them.

2.5 Color Development

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

10 2.5.2 After boiling, they are immediately cooled in an ice/water bath.

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

2.6 Calculations (examples are given in the NREL document)

15 2.6.1 A glucose standard curve is prepared by graphing glucose concentration (mg/0.5 mL) for the four standards (G1-G4) vs. A₅₄₀. This is fitted using a linear regression (Prism Software), and the equation for the line is used to determine the glucose produced for each of the enzyme assay tubes.

20 2.6.2 A plot of glucose produced (mg/0.5 mL) vs. total enzyme dilution is prepared, with the Y-axis (enzyme dilution) being on a log scale.

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

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

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

Protease Assay method - AU(RH)

30 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.

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

Proteolytic Activity (AU)

- 10 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.

- 15 One Anson Unit (AU) is defined as the amount of enzyme which under standard conditions (*i.e.*, 25°C, pH 7.5 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.

- 20 A folder AF 4/5 describing the analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Protease assay method (LAPU)

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

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

Determination of Maltogenic Amylase activity (MANU)

- 30 One MANU (Maltogenic Amylase Novo Unit) may be defined as the amount of enzyme required to release one micro mole of maltose per minute at a concentration of 10 mg of maltotriose (Sigma M 8378) substrate per ml of 0.1 M citrate buffer, pH 5.0 at 37°C for 30 minutes.

EXAMPLES**Example 1**Effect of Using of Lipoic acid and/or Lipoamide in SSF

5 Trials to gauge the effectiveness of lipoic acid and lipoamide during fermentation were performed.

Industrial liquefied corn mash from Corn LP was used in SSF (≈5g mash). The liquefied mash (≈ 35.7% DS (Dry Solids)) was pH adjusted to 5.0 using 40% v/v H₂SO₄. Urea and
 10 penicillin was added at 500 ppm and 3 ppm, respectively, prior to the mash being aliquoted into 15 ml conical centrifuge tubes. Enzymes and compounds were dosed using the equation below to calculate the dosage. Glucoamylase E was dosed at a rate of 0.6 AGU/g solids. Finally, H₂O was added as a volume correction to make the ethanol concentration of each tube comparable. A target of ≈30 million cells/g mash of hydrated RED STAR™ yeast was added to each tube
 15 before being incubated in a 32°C water bath for 54 hours. After a 54 hour fermentation time tubes were centrifuged and the liquid fraction was syringe filtered for HPLC analysis of ethanol and glucose.

$$\text{Enz. dose (ml)} = \frac{\text{Final enz. dose (mg/g DS)} \times \text{Mash weight (g)} \times \text{Solid content (\%DS)}}{(\text{Conc. enzyme mg/ml})}$$

20

As shown in Fig. 1 the ethanol yield was increased when

- 2 mM (i.e., 2 mmol) lipoic acid (LA);
- 2 mM or 10 mM lipoamide (LAM); or
- 1 mM LA + LAM (ratio 1:1);

25 was added to 5 g mash.

A dose of 10 mM lipoic acid/tube caused a marked reduction in ethanol production. When lipoic acid and lipoamide were combined at 5mM a strong reduction in ethanol production was noted. Fig. 2 shows that glycerol production is lowered or unchanged despite having increased amounts of ethanol thus indicating that carbohydrates are being utilized more
 30 efficiently. Increased levels of glucose in addition to higher ethanol titer indicate a more efficient fermentation is taking place (See Fig. 3).

CLAIMS

1. A process of fermenting sugars derived from plant material into a fermentation product using a fermenting organism, wherein lipoic acid, lipoate and/or lipoamide, or blends thereof is(are)
5 present or added before and/or during fermentation.
2. The process of claim 1, wherein the fermenting organism is derived from a strain of *Saccharomyces*, such as *Saccharomyces cerevisiae*.
- 10 3. The process of any of claim 1 or 2, wherein lipoic acid and/or lipoate is(are) present and/or dosed/added into fermentation in a concentration in the range from 0.001-1.8 mM/g mash, preferably 0.001- 1.5 mM/g mash, such as 0.01-1.2 mM/g mash, such as 0.02-1.0 mM/g mash, preferably 0.03-0.8 mM/g mash, such as 0.04-0.6 mM/g mash, such as around 0.4 mM/g mash.
- 15 4. The process of any of claims 1-3, wherein lipoamide is present and/or dosed into fermentation in a concentration in the range from 0.001-10 mM/g mash, preferably 0.001- 8 mM/g mash, such as 0.01-6 mM/g mash, such as 0.02-4 mM/g mash, preferably 0.03-2 mM/g mash, such as 0.04-1 mM/g mash, such as around 0.4 mM/g mash.
- 20 5. The process of any of claims 1-4, wherein lipoic acid and/or lipoate together with lipoamide are present and/or dosed into fermentation in a concentration in the range from 0.001-0.80 mM/g mash, preferably 0.001- 0.7 mM/g mash, such as 0.01-0.6 mM/g mash, such as 0.02-0.5 mM/g mash, preferably 0.03-0.4 mM/g mash, such as 0.04-0.3 mM/g mash, such
25 as around 0.2 mM/g mash.
6. The process of any of claims 1-5, wherein the ratio between lipoic acid and/or lipoate and ii) lipoamide is between 1:9 and 9:1, such as between 1:5 and 5:1, such as around 1:1.
- 30 7. The process of any of claims 1-6, wherein the fermentation product is an alcohol, preferably ethanol, especially fuel ethanol, potable ethanol and/or industrial ethanol.

12675-WO-PCT

8. The process of any of claims 1-7, wherein lipoic acid/lipoate and/or lipoamide boosts the fermentation yield compared to a corresponding fermentation where no lipoic acid/lipoate and lipoamide is present and/or added.

- 5 9. A process of producing a fermentation product from starch-containing material comprising the steps of:
- i) liquefying starch-containing material using an alpha-amylase;
 - ii) saccharifying the liquefied material using a carbohydrate-source generating enzyme;
 - 10 iii) fermenting using a fermenting organism; wherein fermentation is carried out as defined in any of claims 1-8.

10. The process of producing a fermentation product from starch-containing material, comprising the steps of:

- 15 (a) saccharifying starch-containing material at a temperature below the initial gelatinization temperature of said starch-containing material,
- (b) fermenting using a fermenting organism,
- wherein fermentation is carried out as defined in any of claims 1-8.

- 20 11. The process of claim 10, wherein steps (a) and (b) are carried out simultaneously or sequentially.

12. The process of claim 10 or 11, wherein the starch-containing material is granular starch.

- 25 13. The process of producing a fermentation product from lignocellulose-containing material, comprising the steps of:

- (a) pre-treating lignocellulose-containing material;
- (b) hydrolyzing the material;
- (c) fermenting using a fermenting organism;

30 wherein fermentation is carried out as defined in any of claims 1-8.

14. A composition comprising lipoic acid, lipoate and/or lipoamide, or blends thereof, and one or more enzymes and/or one or more fermenting organisms.

12675-WO-PCT

15. The composition of claim 14, wherein the enzyme(s) is(are) one or more hydrolases (class EC 3 according to Enzyme Nomenclature) selected from the group consisting of cellulases, hemicellulases, proteases, alpha-amylases, glucoamylases, or a mixture thereof.

5

16. The composition of claim 14 or 15, wherein the fermenting organism is selected from the group of yeast, filamentous fungus and/or a bacteria.

10 17. The composition of any of claims 14-16, wherein the ratio between lipoic acid and/or lipoate and lipoamide in the composition is between 1:9 and 9:1, such as between 1:5 and 5:1, such as around 1:1.

18. Use of lipoic acid, lipoate, and/or lipoamide, or blends thereof for propagating fermenting organisms.

15

19. Use of lipoic acid, lipoate, and/or lipoamide, or blends thereof, in a fermentation process.

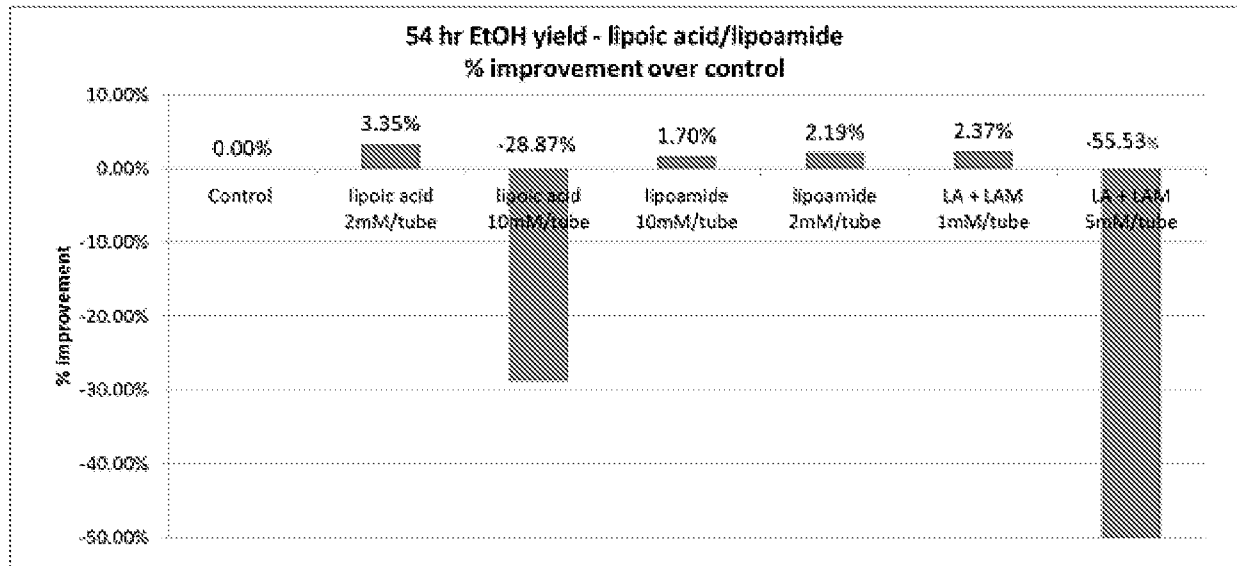


Fig. 1

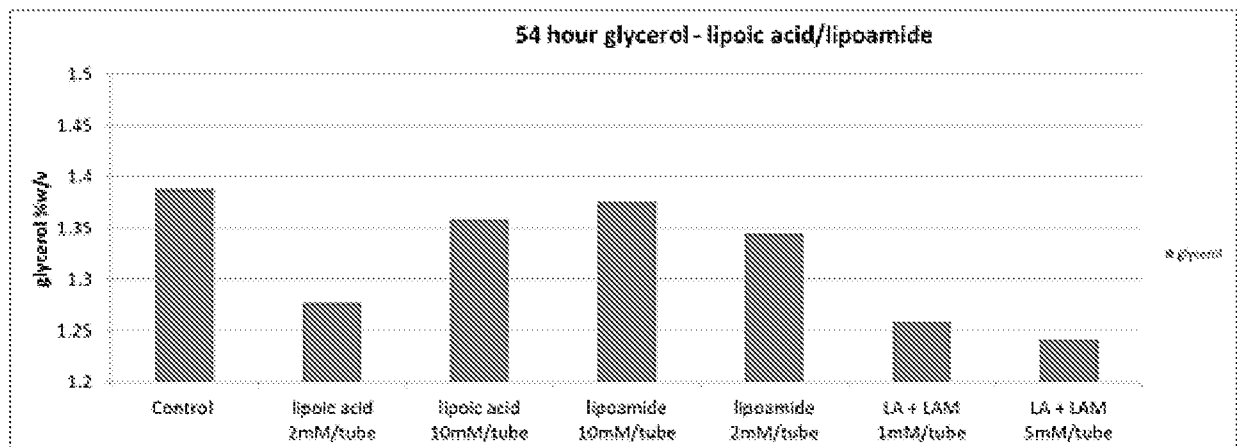


Fig. 2

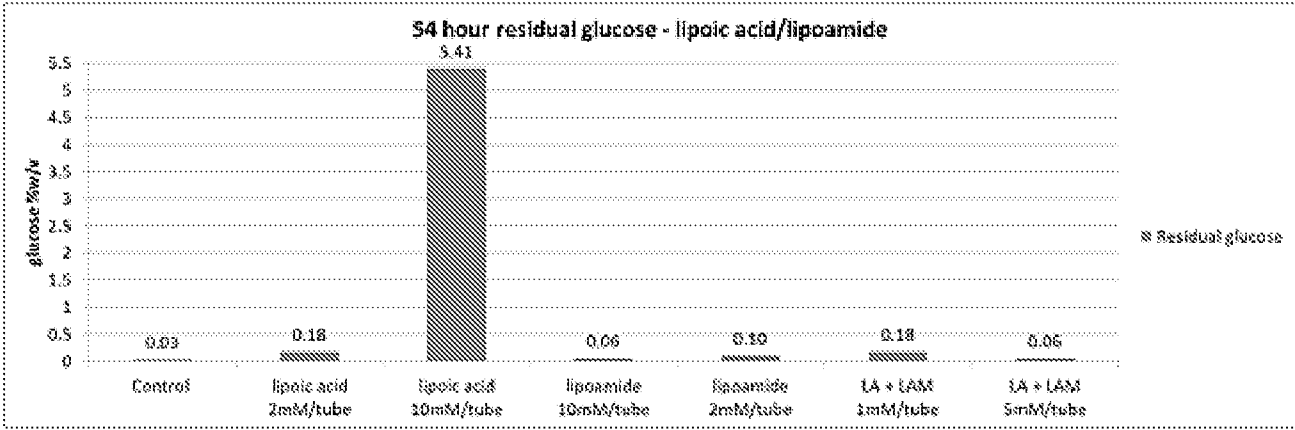


Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/057997**A. CLASSIFICATION OF SUBJECT MATTER****C12P 1/00(2006.01)i, C12N 1/14(2006.01)i, C12N 1/16(2006.01)i, C12N 1/20(2006.01)i, C12R 1/865(2006.01)n**

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 1/00; C12P 7/08; A61K 031/385; A61K 33/42; C12P 7/14; C12N 15/74; C12S 1/00; C12P 17/00; A61K 031/202; C12N 1/14; C12N 1/16; C12N 1/20; C12R 1/865

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: lipoic acid, lipoate, lipoamide, fermentation, plant, propagation, fermentation organism

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2002-0192774 A1 (AHRING, BIRGITTE KIAER et al.) 19 December 2002 See abstract; claims 1-26; and paragraphs [0112]-[0186].	1-3, 14-16, 18, 19
A	US 2003-0190742 A1 (WHITEMAN, G. ROBERT) 09 October 2003 See abstract and paragraphs [0065]-[0066].	1-3, 14-16, 18, 19
A	US 2011-0262976 A1 (KANDULA, MAHESH et al.) 27 October 2011 See abstract and claim 34.	1-3, 14-16, 18, 19
A	LAI, YUAN-SHU et al., 'Antiplatlet activity of α -lipoic acid', Journal of Agricultural and Food Chemistry, 2010, Vol. 58, No. 15, pp. 8596-8603 See abstract and pages 8596, 8600-8602.	1-3, 14-16, 18, 19
A	US 2009-0142410 A1 (HEUER, MARVIN A. et al.) 04 June 2009 See abstract and claims 1-6.	1-3, 14-16, 18, 19
A	US 2003-0187058 A1 (HASSELWANDER, OLIVER et al.) 02 October 2003 See abstract and claims 1-13.	1-3, 14-16, 18, 19



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

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

"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

12 January 2015 (12.01.2015)

Date of mailing of the international search report

12 January 2015 (12.01.2015)

Name and mailing address of the ISA/KR

International Application Division
Korean Intellectual Property Office
189 Cheongsu-ro, Seo-gu, Daejeon Metropolitan City, 302-701,
Republic of Korea

Facsimile No. ++82 42 472 3473

Authorized officer

HEO, Joo Hyung

Telephone No. +82-42-481-8150



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/057997

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GURVITZ, ANER et al. `Heterologous expression of Mycobacterial proteins in Saccharomyces cerevisiae reveals two physiologically functional 3-hydroxyacyl-thioester dehydratases, HtdX and HtdY, in addition to HadABC and HtdZ`, Jouanal of Bacteriology, 2009, Vol. 191, No. 8, pp.2683-2690 See the whole document.	1-3,14-16,18,19
A	BRODY, STUART et al. `Mitochondrial acyl carrier protein is involved in lipoic acid synthesis in Saccharomyces cerevisiae`, FEBS Letters, 1997, Vol. 408, pp. 217-220 See the whole document.	1-3,14-16,18,19
A	MARVIN, MARCUS E. et al. `The isolation and characterisation of a Saccharomyces cerevisiae gene (LIP2) involved in the attachment of lipoic acid groups to mitochondrial enzymes`, FEMS Microbiology Letters, 2001, Vol. 199, pp. 131-136 See the whole document.	1-3,14-16,18,19
A	HERMES, FATEMAH A. et al. `The role of the Saccharomyces cerevisiae lipoate protein ligase homologue, Lip3, in lipoic acid synthesis`, Yeast, 2 September 2013, Vol. 30, pp.415-427 See the whole document.	1-3,14-16,18,19

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US2014/057997**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.: 11
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 11 is unclear since it refers to a multiple dependent claim which does not comply with PCT Rule 6.4(a) (PCT Article 6).
3. ☒ Claims Nos.: 4-10, 12-13, 17
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/US2014/057997

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2002-0192774 A1	19/12/2002	EP 1259466 A1 EP 1259466 B1 US 6555350 B2 WO 01-60752 A1	27/11/2002 22/10/2008 29/04/2003 23/08/2001
US 2003-0190742 A1	09/10/2003	US 2002-0117445 A1 US 2008-0210610 A1 US 2008-0210630 A1 US 2008-0277338 A1 US 2010-0089824 A1 US 2010-0089826 A1 US 2010-0096323 A1 US 2010-0193430 A1 US 7879593 B2 WO 01-44119 A1	29/08/2002 04/09/2008 04/09/2008 13/11/2008 15/04/2010 15/04/2010 22/04/2010 05/08/2010 01/02/2011 21/06/2001
US 2011-0262976 A1	27/10/2011	WO 2009-091582 A1	23/07/2009
US 2009-0142410 A1	04/06/2009	EP 1871183 A1 EP 1991241 A1 EP 1991241 A4 EP 1996199 A1 EP 1996199 A4 JP 2009-527504 A US 2007-0196348 A1 US 2007-0196470 A1 US 2007-0196508 A1 US 2007-0202166 A1 US 2007-0264337 A1 US 2008-0105946 A1 US 2009-0011015 A1 US 7476405 B2 US 7749547 B2 US 7794749 B2 US 7906154 B2 US 7914826 B2 WO 2006-108295 A1 WO 2007-053943 A1 WO 2007-095716 A1 WO 2007-095717 A1 WO 2007-095718 A1 WO 2007-095733 A1 WO 2007-095734 A1 WO 2008-051675 A1	02/01/2008 19/11/2008 08/07/2009 03/12/2008 15/07/2009 30/07/2009 23/08/2007 23/08/2007 23/08/2007 30/08/2007 15/11/2007 08/05/2008 08/01/2009 13/01/2009 06/07/2010 14/09/2010 15/03/2011 29/03/2011 19/10/2006 18/05/2007 30/08/2007 30/08/2007 30/08/2007 30/08/2007 30/08/2007 02/05/2008
US 2003-0187058 A1	02/10/2003	EP 1317264 A2 EP 1317264 B1 JP 2004-508399 A	11/06/2003 28/07/2004 18/03/2004