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(54) Title: METHODS FOR PRODUCING FERMENTATION PRODUCTS

(57) Abstract: The invention relates to processes for producing a fermentation product from wood-containing material, wherein the process comprises the steps of i) pre-treating wood-containing material; ii) hydrolysing by subjecting said pre-treated wood-containing material to one or more cellulolytic enzymes; iii) fermenting using a fermenting organism; wherein the wood-containing material is subjected to one or more esterases before and/or during pre-treatment in step i) and/or hydrolysis in step ii) and/or fermentation in step iii).



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METHODS FOR PRODUCING FERMENTATION PRODUCTS

TECHNICAL FIELD

The present invention relates to methods for producing fermentation products from wood-
5 containing material using one or more fermenting organisms.

BACKGROUND OF THE INVENTION

Due to the limited reserves of fossil fuels and worries about emission of greenhouse gasses there is an increasing focus on using renewable energy sources such as plant material and wood.

10 Production of fermentation products from wood-containing material is known in the art and generally includes the steps of pre-treating, hydrolysing, and fermenting.

WO 2005/118828 concerns a process of producing ethanol from wood-containing materials by operating hydrolysis and fermentation at high substrate concentration and by avoiding the inhibitory effects of the hemicellulose filtrate to reduce the amount of enzyme needed.

15 However, even though converting wood-containing material into (bio)fuel (e.g. ethanol) is possible, it is still more expensive than making ethanol from, e.g., starchy materials.

Consequently, there is a need for providing further methods and processes for producing fermentation products from wood-containing-materials.

20 SUMMARY OF THE INVENTION

The present invention relates to processes for producing fermentation products from wood-containing material using fermenting organisms.

In the first aspect the invention relates to processes of producing fermentation products comprising the steps of:

- 25 i) pre-treating wood-containing material;
ii) hydrolysing by subjecting said pre-treated wood-containing material to one or more cellulolytic enzymes;
iii) fermenting using a fermenting organism;

wherein the wood-containing material is subjected to one or more esterases before and/or during
30 pre-treatment in step i) and/or hydrolysis in step ii) and/or fermentation in step iii).

In a preferred embodiment the wood-containing material is softwood-containing material.

DETAILED DESCRIPTION OF THE INVENTION

Wood-containing material that includes softwood and/or hardwood residues may, according to the invention be used for producing fermentation products, such as ethanol.

Wood-Containing Material

5 The feedstock used in a process of the invention is wood-containing material. The wood-containing material may contain softwood and/or hardwood, or a combination therefore. However, the wood-containing material may comprise other constituents. In a preferred embodiment a significant portion of the feedstock is wood. In preferred embodiments at least 30 wt.-%, preferably at least 50 wt.-%, more preferably at least 70 wt.-%, even more preferably at least 90 wt.-% of the
10 wood-containing material is wood.

Especially contemplated is soft wood-containing material. The terms "soft wood" and "hard wood" are used herein as they are used in the art. The difference between softwood and hardwood has to do with plant reproduction. All trees reproduce by producing seeds, but the seed structures vary. Hardwood trees are angiosperms, which mean plants that produce seeds with some sort of
15 covering. It might be a fruit, such as an apple, or a hard shell, such as an acorn or hickory nut. Softwood trees are gymnosperms (conifers) with "naked" seed. Softwood trees are usually evergreen, bear cones, and have needles or scale-like leaves.

Examples of softwoods include softwood derived from pine, spruce, fir, hemlock, larch, redwood, and/or cedar tree. For instance, the fir may be Douglas fir, the pine may be Ponderosa
20 pine or Loblolly pine; the spruce may be Sitka spruce, the hemlock may be Eastern and/or Western hemlock.

Examples of hardwoods include hardwood derived from ash, aspen, beech, basswood, birch, black cherry, black walnut/butternut, buckeye, American chestnut, cottonwood, dogwood, elm, hackberry, hickory, holly, locust, magnolia, maple, oak, poplar, red alder, redbud, royal,
25 paulownia, sassafras, sweetgum, sycamore, tupelo, willow, and/or yellow-poplar, or a combination thereof.

The generic chemical composition of hardwood and softwood are as follows:

Hardwood:

- Lignin (20-25%) – coniferyl & sinapyl alcohol
- 30 • Cellulose (40-45%)
- Hemicellulose (25-35%)
 - Glucuronoxylan (15-30%)
 - Glucomannan (2-5%)

- Extractives (1-5%)

Softwood:

- Lignin (25-35%) – coniferyl & p-coumaryl alcohol
 - Cellulose (40-45%)
 - 5 • Hemicellulose (25-30%)
 - Galactoglucomannan (5-8%)
 - Glucomannan (10-15%)
 - Arabinoglucuronoxylan (7-10%)
 - High concentration of localized esterified pectin
 - 10 • Extractives (1-5%)
- Extractives comprise a significant fraction of raw wood (1-5%).

“Extractives” is a collective term that includes: terpenoids (e.g. resin acids), phenolics (e.g. lignans, Tannins, flavonoids, etc.), waxes, fats, fatty acids, steryl esters, and sterols.

15 Processes of the invention

The invention relates to methods for producing fermentation products such as ethanol from wood-containing material using one or more fermenting organisms. Before the wood-containing material can be fermented into the desired fermentation product, such as ethanol, it has to be converted into fermentable sugars.

- 20 Firstly, the wood-containing material is pre-treated. Mechanical, chemical and/or physical pre-treatments are generally carried out to reduce the particle size, to disrupt fiber walls and to expose carbohydrates. This way the susceptibility of the wood material to enzymatic hydrolysis is increased. However, it also exposes, alters and redistributes extractives. This redeposition of certain extractives such as waxes, fats, steryl esters and sterols onto the
- 25 newly available surface area can effectively shield the underlying carbohydrates from enzymatic hydrolysis. Therefore, these extractives make wood-containing material (e.g., wood chip) a difficult feedstock to use in fermentation product production processes. The inventors have found a way to increase the hydrolysis rate of wood-containing feedstock. Without being bound by any theory it is believed that esterase treatment improves the accessibility of hydrolysing enzymes to the substrate
- 30 (cellulose and hemicellulose). This is more pronounced for softwood-containing material than for hardwood-containing material.

Consequently, in the first aspect the invention relates to processes for producing fermentation products, especially ethanol, from wood-containing materials, wherein the processes comprise the steps of:

- i) pre-treating wood-containing material;
- 5 ii) hydrolysing by subjecting said pre-treated wood-containing material to one or more cellulolytic enzymes;
- iii) fermenting using a fermenting organism;

wherein the wood-containing material is subjected to one or more esterases before and/or during pre-treatment in step i) and/or hydrolysis in step ii) and/or fermentation in step iii).

10 In an embodiment the wood-containing material is subjected to esterase before and/or during step i) and/or ii) and/or iii). Esterase treatment may also take place in a separate step before step i); or in a separate step between pre-treatment step i) and hydrolysis step ii).

Hydrolysis step ii) and fermentation step iii) may be carried out separately or simultaneously. In preferred embodiments the hydrolysis and fermentation steps are carried out as
15 HHF (hybrid hydrolysis and fermentation) or SHF (simultaneous hydrolysis and fermentation).

The pH during esterase treatment, especially when carried out as a separate step, is preferably in the range from pH 4.5-8.5. The temperature during esterase treatment, especially when carried out as a separate step, is preferably in the range from 40-90°C.

In a preferred embodiment the esterase is dosed in the range from 7.5-25 LU per dry
20 gram of substrate. In a preferred embodiment the esterase is a lipolytic enzyme, preferably lipase; a cutinase; a phospholipase, or a combination of two or more thereof. Examples of enzymes are described in the "Enzymes"-section below.

Pre-treatment

25 As mentioned above the structure of wood is not directly accessible to enzymatic hydrolysis and therefore has to be pre-treated. The wood-containing material may according to the invention be pre-treated in any suitable way.

Pre-treatment methods including wet-oxidation and alkaline pre-treatment, target lignin, while dilute acid pre-treatment and auto-hydrolysis targets hemicellulose. Steam explosion is an
30 example of pre-treatment that targets cellulose.

According to the invention the pre-treatment step may be a conventional pre-treatment step using techniques well known in the art. In a preferred embodiment pre-treatment takes place in aqueous slurry. In preferred embodiments the wood-containing material are present during pre-

treatment in amounts between 10-80 wt.-%, preferably between 20-70 wt.-%, especially between 30-60 wt.-%, such as around 50 wt.-%.

Chemical, Mechanical and/or Biological Pre-treatment

5 The wood-containing material may according to the invention be chemically, mechanically and/or biologically pre-treated before hydrolysis and/or fermentation. Mechanical pre-treatment (often referred to as "physical"-pre-treatment) may be used alone or in combination with subsequent and/or simultaneous hydrolysis, especially enzymatic hydrolysis.

Preferably chemical, mechanical and/or biological pre-treatment is carried out prior to the
10 hydrolysis. Alternatively, the chemical, mechanical and/or biological pre-treatment may be carried out simultaneously with hydrolysis, such as simultaneously with treatment of the wood-containing material to one or more cellulolytic enzymes, or other enzyme activities, to release, e.g., fermentable sugars, such as glucose and/or maltose.

In an embodiment of the invention the pre-treated wood-containing material may be washed
15 or detoxified in another way.

Chemical Pre-treatment

The term "chemical treatment" refers to any chemical pre-treatment which promotes the separation and/or release of cellulose, hemicellulose and/or lignin. Examples of suitable chemical
20 pre-treatment methods include treatment with; for example, dilute acid, lime, alkaline, organic solvent, ammonia, sulfur dioxide, carbon dioxide. Further, wet oxidation and pH-controlled hydrothermolysis are also considered chemical pre-treatment.

In a preferred embodiment the chemical pre-treatment is carried out as acid treatment, more preferably, a continuous dilute and/or mild acid treatment, such as, treatment with sulfuric acid, or
25 another organic acid, such as acetic acid, citric acid, tartaric acid, succinic acid, hydrogen chloride or mixtures thereof. Other acids may also be used. Mild acid treatment means that the treatment pH lies in the range from 1-5, preferably 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 contacted with the wood-containing material and the mixture may be held at a temperature in the range of 160-220°C,
30 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. Addition of strong acids, such as sulphuric acid, may be applied to remove hemicellulose. This enhances the digestibility of cellulose.

Other techniques are also contemplated. Cellulose solvent treatment 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 wood structure is disrupted. Alkaline H_2O_2 , ozone, organosolv (uses Lewis acids, $FeCl_3$, $(Al)_2SO_4$ in aqueous alcohols), glycerol, dioxane, phenol, or ethylene glycol are among solvents known to disrupt cellulose structure and promote hydrolysis (Mosier et al. Bioresource Technology 96 (2005), p. 673-686).

Alkaline chemical pre-treatment with base, e.g., NaOH, Na_2CO_3 and/or ammonia or the like, is also contemplated according to the invention. Pre-treatment methods using ammonia are described in, e.g., WO 2006/110891, WO 2006/11899, WO 2006/11900, WO 2006/110901, which are hereby incorporated by reference.

Wet oxidation techniques involve the 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 depending 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. Bioresource Technology 96 (2005) 673-686, and US publication no. 2002/0164730, which references are hereby all incorporated by reference.

Mechanical Pre-treatment

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 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

5 In a preferred embodiment the wood-containing material is pre-treated both chemically and mechanically. For instance, the pre-treatment step may involve dilute or mild acid treatment and high temperature and/or pressure treatment. The chemical and mechanical pre-treatments may be carried out sequentially or simultaneously, as desired.

Accordingly, in a preferred embodiment, the wood-containing material is subjected to both
10 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 and/or mild acid steam explosion step. In another preferred embodiment pre-treatment is carried out as an ammonia fiber explosion step (or AFEX pre-treatment step).

15

Biological Pre-treatment

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
20 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*
25 *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
30 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 the pre-treated wood-containing material is fermented it may be hydrolyzed to break down cellulose and hemicellulose. As mentioned above esterase treatment may be carried out as a separate step between the pre-treatment step i) and the hydrolysis step ii), but may also be carried out during hydrolysis with cellulolytic enzymes.

The dry solids content during hydrolysis may be in the range from 5-50 wt.-%, preferably 10-40 wt.-%, preferably 20-30 wt.-%. Hydrolysis may in a preferred embodiment be carried out as a fed batch process where the pre-treated wood-containing material (substrate) is fed gradually to, e.g., an enzyme containing hydrolysis solution.

10 In a preferred embodiment hydrolysis is carried out enzymatically. According to the invention the pre-treated wood-containing material may be hydrolyzed by one or more cellulolytic enzymes, such as one or more cellulases. One or more hemicellulolytic enzymes, such as hemicellulases may also be present during hydrolysis.

In a preferred embodiment hydrolysis is carried out using a cellulolytic enzyme preparation comprising one or more polypeptides having cellulolytic enhancing activity. In a preferred embodiment the polypeptide(s) having cellulolytic enhancing activity is(are) of family GH61A origin. Examples of suitable and preferred cellulolytic enzyme preparations and polypeptides having cellulolytic enhancing activity are described in the "Cellulolytic Enzymes"-section and "Cellulolytic Enhancing polypeptides"-section below.

20 As the wood-containing material contain other constituents than lignin, cellulose, hemicellulose and extractives hydrolysis and/or fermentation in steps ii) and iii) may be carried out in the presence of additional enzyme activities such as: protease activity, amylase activity, carbohydrate-generating enzyme activity; and pectinase activity.

Suitable enzyme activities are described in the "Enzymes"-section below.

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.

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, especially around pH 5. Hydrolysis is typically carried out for between 12 and 96 hours, preferable 16 to 72 hours, more preferably between 24 and 48 hours.

Fermentation

According to the invention fermentable sugars from pre-treated and hydrolyzed wood-containing material may be fermented by one or more fermenting organisms capable of fermenting sugars, such as glucose, xylose, mannose, and galactose directly or indirectly into a desired
5 fermentation product. The fermentation conditions depend on the desired fermentation product and fermenting organism and can easily be determined by one of ordinary skill in the art.

Especially in the case of ethanol fermentation the fermentation may be ongoing for between 1-48 hours, preferably 1-24 hours. In an embodiment the fermentation is carried out at a temperature between 20 to 40°C, preferably 26 to 34°C, in particular around 32°C. In an
10 embodiment the pH is from pH 3-7, preferably 4-6. However, some, e.g., bacterial fermenting organisms have higher fermentation temperature optima. Therefore, in an embodiment the fermentation is carried out at temperature between 40-60°C, such as 50-60°C. The skilled person in the art can easily determine suitable fermentation conditions.

15 SHF and HHF

In a preferred embodiment hydrolysis and fermentation is carried out as simultaneous hydrolysis and fermentation (SHF). In general this means that combined hydrolysis and fermentation is carried out at conditions (e.g. temperature and/or pH) suitable for the fermenting organism in question.

20 In another preferred embodiment hydrolysis steps ii) and fermentation step iii) is carried out as hybrid hydrolysis and fermentation (HHF). HHF begins with a separate hydrolysis step and ends with a simultaneous hydrolysis and fermentation step. The separate hydrolysis step is an enzymatic cellulose saccharification step typically carried out at conditions (e.g., at higher temperatures) suitable, preferably optimal, for the hydrolysing enzyme(s) in question. The
25 following simultaneous hydrolysis and fermentation step is typically carried out at conditions suitable for the fermenting organism (often at lower temperatures than the separate hydrolysis step).

Recovery

30 Subsequent to fermentation, the fermentation product may optionally be separated from the fermentation medium in any suitable way. For instance, the medium may be distilled to extract the fermentation product or the fermentation product may be extracted from the fermentation medium

by micro or membrane filtration techniques. Alternatively the fermentation product may be recovered by stripping. Recovery methods are well known in the art.

Fermentation Products

5 The present invention may be used for producing any fermentation product. Preferred fermentation products include alcohols (e.g., ethanol, methanol, butanol); organic acids (e.g., citric acid, acetic acid, itaconic acid, lactic acid, gluconic acid, succinic acid, fumaric 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.

10 Other products include consumable alcohol industry products, e.g., beer and wine; dairy industry products, e.g., fermented dairy products; leather industry products and tobacco industry products. In a preferred embodiment the fermentation product is an alcohol, especially ethanol. The fermentation product, such as ethanol, obtained according to the invention, may preferably be used as fuel alcohol/ethanol. However, in the case of ethanol it may also be used as potable ethanol.

15

Fermenting Organism

 The term "fermenting organism" refers to any organism, including bacterial and fungal organisms, suitable for producing a desired fermentation product. The fermenting organism may be C₆ and/or C₅ fermenting organisms, or a combination thereof. Both C₆ and C₅ fermenting organisms
20 are well known in the art.

 Suitable fermenting organisms are able to ferment, i.e., convert, fermentable sugars, such as glucose, xylulose, and maltose, directly or indirectly into the desired fermentation product.

 Examples of bacterial and fungal fermenting organisms producing ethanol can be found in Lin et al (2006), Appl. Microbiol. Biotechnol 69:627-642, see especially pages 630-632, Table 1 and
25 2 (which is hereby incorporated by reference).

 Examples of fermenting organisms that can ferment C₆ sugars include fungal and bacterial organisms, especially yeast. Preferred yeast includes strains of *Saccharomyces*, in particular strains of *Saccharomyces cerevisiae* or *Saccharomyces uvarum*.

 Examples of fermenting organisms that can ferment C₅ sugars include fungal or bacterial
30 organism. In a preferably embodiment the C₅ fermenting organism is yeast, preferably yeast from a strain of *Pichia*, preferably *Pichia stipitis*, such as *Pichia stipitis* CBS 5773; a strain of *Candida*, in particular a strain of *Candida utilis*, *Candida diddensii*, or *Candida boidinii*.

Other fermenting organisms include strains of *Zymomonas*, such as *Zymomonas mobilis*; *Hansenula*, in particular *Hansenula anomala*; *Kluyveromyces*, in particular *K. fragilis*; and *Schizosaccharomyces*, in particular *S. pombe*.

Commercially available yeast suitable for ethanol production includes, e.g., ETHANOL 5 RED™ yeast (available from Fermentis/Lesaffre, USA), FALI™ (available from Fleischmann's Yeast, USA), SUPERSTART and THERMOSACC™ fresh yeast (available from Ethanol Technology, WI, USA), BIOFERM AFT and XR (available from NABC - North American Bioproducts Corporation, GA, USA), GERT STRAND (available from Gert Strand AB, Sweden), and FERMIOL (available from DSM Specialties).

10

Enzymes

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

15 Esterases

An "esterase", also referred to as a carboxylic ester hydrolases, refers to enzymes acting on ester bonds, and includes enzymes classified in EC 3.1.1 Carboxylic Ester Hydrolases according to Enzyme Nomenclature (available at <http://www.chem.qmw.ac.uk/iubmb/enzyme> or from Enzyme Nomenclature 1992, Academic Press, San Diego, California, with Supplement 1 (1993), 20 Supplement 2 (1994), Supplement 3 (1995), Supplement 4 (1997) and Supplement 5, in Eur. J. Biochem. 1994, 223, 1-5; Eur. J. Biochem. 1995, 232, 1-6; Eur. J. Biochem. 1996, 237, 1-5; Eur. J. Biochem. 1997, 250, 1-6, and Eur. J. Biochem. 1999, 264, 610-650; respectively). Non-limiting examples of esterases include arylesterase, triacylglycerol lipase, acylesterase, acetylcholinesterase, cholinesterase, tropinesterase, pectinesterase, sterol esterase, 25 chlorophyllase, L-arabinonolactonase, gluconolactonase, uronolactonase, tannase, retinyl-palmitate esterase, hydroxybutyrate-dimer hydrolase, acylglycerol lipase, 3-oxoadipate enol-lactonase, 1,4-lactonase, galactolipase, 4-pyridoxolactonase, acylcarnitine hydrolase, aminoacyl-tRNA hydrolase, D-arabinonolactonase, 6-phosphogluconolactonase, phospholipase A1, 6-acetylglucose deacetylase, lipoprotein lipase, dihydrocoumarin lipase, limonin-D-ring-lactonase, steroid-lactonase, 30 triacetate-lactonase, actinomycin lactonase, orsellinate-depside hydrolase, cephalosporin-C deacetylase, chlorogenate hydrolase, alpha-amino-acid esterase, 4-methyloxaloacetate esterase, carboxymethylenebutenolidase, deoxylimonate A-ring-lactonase, 2-acetyl-1-alkylglycerophosphocholine esterase, fusarinine-C ornithinesterase, sinapine esterase, wax-ester

hydrolase, phorbol diester hydrolase, phosphatidylinositol deacylase, sialate O-acetyl esterase, acetoxybutyryl bithiophene deacetylase, acetylsalicylate deacetylase, methylumbelliferyl-acetate deacetylase, 2-pyrone-4,6-dicarboxylate lactonase, N-acetylgalactosaminoglycan deacetylase, juvenile-hormone esterase, bis(2-ethylhexyl)phthalate esterase, protein-glutamate methyl esterase, 11-cis-retinyl-palmitate hydrolase, all-trans-retinyl-palmitate hydrolase, L-rhamnono-1,4-lactonase, 5-(3,4-diacetoxybut-1-ynyl)-2,2'-bithiophene deacetylase, fatty-acyl ethyl-ester synthase, xylono-1,4-lactonase, N-acetylglucosaminylphosphatidylinositol deacetylase, cetaxate benzyl esterase, acetylalkylglycerol acetylhydrolase, and acetylxylan esterase.

Preferred esterases for use in the present invention are lipolytic enzymes, such as, lipases (as classified by EC 3.1.1.3, EC 3.1.1.23 and/or EC 3.1.1.26), phospholipases (as classified by EC 3.1.1.4 and/or EC 3.1.1.32, including lysophospholipases as classified by EC 3.1.1.5), and cutinases classified as EC 3.1.1.74.

An esterase enzyme may in an embodiment of the invention be dosed in the range from 7.5-25 LU per dry gram of substrate.

15

Lipolytic Enzymes

The lipolytic enzyme is preferably of microbial origin, in particular of bacterial, fungal or yeast origin. The lipolytic enzyme used may be derived from any source, including, for example, a strain of *Absidia*, in particular *Absidia blakesleena* and *Absidia corymbifera*, a strain of *Achromobacter*, in particular *Achromobacter iophagus*, a strain of *Aeromonas*, a strain of *Alternaria*, in particular *Alternaria brassiciola*, a strain of *Aspergillus*, in particular *Aspergillus niger* and *Aspergillus flavus*, a strain of *Achromobacter*, in particular *Achromobacter iophagus*, a strain of *Aureobasidium*, in particular *Aureobasidium pullulans*, a strain of *Bacillus*, in particular *Bacillus pumilus*, *Bacillus stearothermophilus* and *Bacillus subtilis*, a strain of *Beauveria*, a strain of *Brochothrix*, in particular *Brochothrix thermosphacta*, a strain of *Candida*, in particular *Candida cylindracea* (*Candida rugosa*), *Candida parapsilosis*, and *Candida antarctica*, a strain of *Chromobacter*, in particular *Chromobacter viscosum*, a strain of *Coprinus*, in particular *Coprinus cinereus*, a strain of *Fusarium*, in particular *Fusarium oxysporum*, *Fusarium solani*, *Fusarium solani pisi*, and *Fusarium roseum culmorum*, a strain of *Geotrichum*, in particular *Geotrichum penicillatum*, a strain of *Hansenula*, in particular *Hansenula anomala*, a strain of *Humicola*, in particular *Humicola brevispora*, *Humicola brevis* var. *thermoidea*, *Humicola insolens*, and *Humicola lanuginosa* (also known as *Thermomyces lanuginosus*), a strain of *Hypozyma*, a strain of *Lactobacillus*, in particular *Lactobacillus curvatus*, a strain of *Metarhizium*, a strain of *Mucor*, a strain of *Paecilomyces*, a strain

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of *Penicillium*, in particular *Penicillium cyclopium*, *Penicillium crustosum* and *Penicillium expansum*, a strain of *Pseudomonas* in particular *Pseudomonas aeruginosa*, *Pseudomonas alcaligenes*, *Pseudomonas cepacia* (syn. *Burkholderia cepacia*), *Pseudomonas fluorescens*, *Pseudomonas fragi*, *Pseudomonas maltophilia*, *Pseudomonas mendocina*, *Pseudomonas mephitica* lipolytica, 5 *Pseudomonas alcaligenes*, *Pseudomonas plantari*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas putida*, *Pseudomonas stutzeri*, and *Pseudomonas wisconsinensis*, a strain of *Rhizoctonia*, in particular *Rhizoctonia solani*, a strain of *Rhizomucor*, in particular *Rhizomucor miehei*, a strain of *Rhizopus*, in particular *Rhizopus japonicus*, *Rhizopus microsporus* and *Rhizopus nodosus*, a strain of *Rhodospiridium*, in particular *Rhodospiridium toruloides*, a strain of 10 *Rhodotorula*, in particular *Rhodotorula glutinis*, a strain of *Sporobolomyces*, in particular *Sporobolomyces shibatanus*, a strain of *Thermomyces*, in particular *Thermomyces lanuginosus* (also know as *Humicola lanuginosa*), a strain of *Thiarosporella*, in particular *Thiarosporella phaseolina*, a strain of *Trichoderma*, in particular *Trichoderma harzianum*, and *Trichoderma reesei*, and/or a strain of *Verticillium*. In a preferred embodiment, the lipolytic enzyme used according to 15 the invention is derived from a strain of *Aspergillus*, a strain of *Achromobacter*, a strain of *Bacillus*, a strain of *Candida*, a strain of *Chromobacter*, a strain of *Fusarium*, a strain of *Humicola*, a strain of *Hyphozyma*, a strain of *Pseudomonas*, a strain of *Rhizomucor*, a strain of *Rhizopus*, or a strain of *Thermomyces*.

20 Lipases

In a preferred embodiment the lipolytic enzymes is a lipase.

Examples of suitable lipases include lipases from *Humicola* (synonym *Thermomyces*), e.g. from *H. lanuginosa* (*T. lanuginosus*) as described in EP 258 068 and EP 305 216 or from *H. insolens* as described in WO 96/13580, a *Pseudomonas* lipase, e.g. from *P. alcaligenes* or *P. pseudoalcaligenes* (EP 218 272), *P. cepacia* (EP 331 376), *P. stutzeri* (GB 1,372,034), *P. fluorescens*, *Pseudomonas* sp. strain SD 705 (WO 95/06720 and WO 96/27002), *P. wisconsinensis* (WO 96/12012), a *Bacillus* lipase, e.g. from *B. subtilis* (Dartois et al. (1993), Biochemica et Biophysica Acta, 1131, 253-360), *B. stearothermophilus* (JP 64/744992) or *B. pumilus* (WO 91/16422), *Candida antarctica* lipases, such as *Candida antarctica* lipase A, 25 *Candida antarctica* lipase (lipase B), *Candida cylindracea* lipase, and *Penicillium camembertii* lipase. 30

In an embodiment the lipase is a variant of *Humicola lanuginosa*. Examples of specifically contemplated lipase variant are described in WO 1992/005249; WO 1992/019726; WO

1995/022615; WO 1997/007202; WO 2000/060063; and WO 2002/055679 (which references are all incorporated by reference).

In a preferred embodiment the lipase is a variant of *Thermomyces lanuginosus* (previously *Humicola lanuginosa*) with one or more or all of the following mutations:

D111G + S216P
D27N
L227F
S224I + G225W + T226N + L227P + V228C
L227F + V228I
G225P
W221C + G246C
D27R + D111G + S216P
D27N + D111A
D27R + D111G + S216P + L227G + P256T
D27R + D111G + S216P + L227F + P256T
D27R + D111G + S216P + L227G
D27S + D111G + S216P
D27R + D111A + S216P + L227G + P256T
D27R + D111G + S216P + G225P + L227G + P256T
D27R + T37S + D111G + S216P + L227G + P256T
D27R + N39F + D111G + S216P + L227G + P256T
D27R + G38C + D111G + S216P + L227G + P256T
D27R + D111G + S216P + L227G + T244I + P256T
D27R + G91A + D111G + S216P + L227G + P256T
N25I + D27R + D111A + S216P + L227G + P256T
N25L + D27R + D111A + S216P + L227G + P256T
N26D + D27R + D111A + S216P + L227G + P256T
D27R + K46R + D111A + S216P + L227G + P256T
D27R + V60N + D111A + S216P + L227G + P256T
D27R + D111A + P136A + S216P + L227G + P256T
D27R + D111A + S216P + L227G + P256T + I265F
D27R + S58Y + D111A + S216P + L227G + P256T +

N26D +D27R +E56Q +D111A + S216P + L227G + P256T
D27R +G91A +D96E +L97Q +D111A +S216P + L227G + P256T
D27R +G91A +D111A + S216P + L227G + P256T +
D27R + G91T +N94S +D111A +S216P + L227G + P256T
D27R +G91S +D111A + S216P + L227G + P256T +
D27R +G91N +D111A + S216P + L227G + P256T
D27R +D96E +D111A + S216P + L227G + P256T
D27R +I90L +G91A +N94K +D111A + S216P + L227G + P256T
D27R +G91S +F95V +D111A + S216P + L227G + P256T
D27R +I90V +G91S +D111A + S216P + L227G + P256T
D27R +G91N +N94R +D111A + S216P + L227G + P256T
D27R +I90L +L93F +D96N +D111A + S216P + L227G + P256T
D27R +I90L +G91A +D96E +D111A + S216P + L227G + P256T
D27R +G91S +L93F +D111A + S216P + L227G + P256T
D27R +G91T +N94K +D111A + S216P + L227G + P256T
D27R +G91T +D111A + S216P + L227G + P256T
D27R +L93F +D111A +D137N + S216P + L227G + P256T
D27R +G91S +D96N +D111A + S216P + L227G + P256T
D27R +G91W +D111A + S216P + L227G + P256T
D27R +I90L +G91T +D111A + S216P + L227G + P256T
D27R +G91S +L93F +N94R +D96G +D111A +S216P + L227G + P256T
D27R +G91T +D96N +D111A +S216P + L227G + P256T
D27R +I90V +G91T +L93F +N94K +D111A +S216P + L227G + P256T
D27R +L93V +D111A +S216P + L227G + P256T
D27R +G91S +N94K +D111A +S216P + L227G + P256T
D27R +I90L +G91T +D111A +S216P + L227G + P256T
D27R +G91S +L93F +F95I +D96N +D111A +S216P + L227G + P256T

D27R + D111A + V187I + S216P + L227G + P256T
D27R + D111A + F211Y + S216P + L227G + P256T
D27R + R118M + D111A + A131V + S216P + L227G + P256T
D27R + P29S + R84C + D111A + H135Y + S216P + L227G + P256T
D27R + T32S + D111A + H135Y + S216P + L227G + P256T
D27R + G91R + D111A + I238V + S216P + L227G + P256T
D27R + F51I + I76V + N101D + D111A + N162R + S216P + L227G + P256T
D27R + F51L + D111A + S216P + L227G + P256T

Commercial lipases include LIPOLASE™, LIPOLASE ULTRA™, LIPEX™, RESINASE™ A2x, RESINASE™ HT (available from Novozymes A/S) and G AMANO 50 (available from Amano).

5 Cutinase

The cutinase used according to the invention may be of any origin. Preferably the cutinase is of microbial origin, in particular of bacterial, of fungal or of yeast origin.

In a preferred embodiment, the cutinase is derived from a strain of *Aspergillus*, in particular *Aspergillus oryzae*, a strain of *Alternaria*, in particular *Alternaria brassiciola*, a strain of *Fusarium*, in particular *Fusarium solani*, *Fusarium solani pisi*, *Fusarium roseum culmorum*, or *Fusarium roseum sambucium*, a strain of *Helminthosporium*, in particular *Helminthosporium sativum*, a strain of *Humicola*, in particular *Humicola insolens*, a strain of *Pseudomonas*, in particular *Pseudomonas mendocina*, or *Pseudomonas putida*, a strain of *Rhizoctonia*, in particular *Rhizoctonia solani*, a strain of *Streptomyces*, in particular *Streptomyces scabies*, or a strain of *Ulocladium*, in particular *Ulocladium consortiale*. In a most preferred embodiment the cutinase is derived from a strain of *Humicola insolens*, in particular the strain *Humicola insolens* DSM 1800. *Humicola insolens* cutinase is described in WO 96/13580 which is hereby incorporated by reference. The cutinase may be a variant, such as one of the variants disclosed in WO 00/34450 and WO 01/92502, which are hereby incorporated by reference. Preferred cutinase variants include variants listed in Example 2 of WO 01/92502, which is hereby specifically incorporated by reference.

Preferred commercial cutinases include NOVOZYM™ 51032 (available from Novozymes A/S, Denmark).

Phospholipases

As used herein, the term phospholipase is an enzyme which has activity towards phospholipids. Phospholipids, such as lecithin or phosphatidylcholine, consist of glycerol
 5 esterified with two fatty acids in an outer (sn-1) and the middle (sn-2) positions and esterified with phosphoric acid in the third position; the phosphoric acid, in turn, may be esterified to an amino-alcohol. Phospholipases are enzymes which participate in the hydrolysis of phospholipids. Several types of phospholipase activity can be distinguished, including phospholipases A₁ and A₂ which hydrolyze one fatty acyl group (in the sn-1 and sn-2 position,
 10 respectively) to form lysophospholipid; and lysophospholipase (or phospholipase B) which can hydrolyze the remaining fatty acyl group in lysophospholipid. Phospholipase C and phospholipase D (phosphodiesterases) release diacyl glycerol or phosphatidic acid respectively.

The term phospholipase includes enzymes with phospholipase activity, e.g., phospholipase A (A₁ or A₂), phospholipase B activity, phospholipase C activity or phospholipase
 15 D activity. The term "phospholipase A" used herein in connection with an enzyme of the invention is intended to cover an enzyme with Phospholipase A₁ and/or Phospholipase A₂ activity. The phospholipase activity may be provided by enzymes having other activities as well, such as, e.g., a lipase with phospholipase activity. The phospholipase activity may, e.g., be from a lipase with phospholipase side activity. In other embodiments of the invention the
 20 phospholipase enzyme activity is provided by an enzyme having essentially only phospholipase activity and wherein the phospholipase enzyme activity is not a side activity.

The phospholipase may be of any origin, e.g., of animal origin (such as, e.g., mammalian), e.g. from pancreas (e.g., bovine or porcine pancreas), or snake venom or bee venom. Preferably the phospholipase may be of microbial origin, e.g., from filamentous fungi,
 25 yeast or bacteria, such as the genus or species *Aspergillus*, e.g., *A. niger*, *Dictyostelium*, e.g., *D. discoideum*; *Mucor*, e.g. *M. javanicus*, *M. mucedo*, *M. subtilissimus*; *Neurospora*, e.g. *N. crassa*; *Rhizomucor*, e.g., *R. pusillus*; *Rhizopus*, e.g. *R. arrhizus*, *R. japonicus*, *R. stolonifer*; *Sclerotinia*, e.g., *S. libertiana*; *Trichophyton*, e.g. *T. rubrum*; *Whetzelinia*, e.g., *W. sclerotiorum*; *Bacillus*, e.g., *B. megaterium*, *B. subtilis*; *Citrobacter*, e.g., *C. freundii*; *Enterobacter*, e.g., *E.*
 30 *aerogenes*, *E. cloacae* *Edwardsiella*, *E. tarda*; *Erwinia*, e.g., *E. herbicola*; *Escherichia*, e.g., *E. coli*; *Klebsiella*, e.g., *K. pneumoniae*; *Proteus*, e.g., *P. vulgaris*; *Providencia*, e.g., *P. stuartii*; *Salmonella*, e.g. *S. typhimurium*; *Serratia*, e.g., *S. liquefaciens*, *S. marcescens*; *Shigella*, e.g., *S. flexneri*; *Streptomyces*, e.g., *S. violeceoruber*; *Yersinia*, e.g., *Y. enterocolitica*. Thus, the

phospholipase may be fungal, e.g., from the class *Pyrenomycetes*, such as the genus *Fusarium*, such as a strain of *F. culmorum*, *F. heterosporum*, *F. solani*, or a strain of *F. oxysporum*. The phospholipase may also be from a filamentous fungus strain within the genus *Aspergillus*, such as a strain of *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*,
5 *Aspergillus niger* or *Aspergillus oryzae*.

Preferred phospholipases are derived from a strain of *Humicola*, especially *Humicola lanuginosa*. The phospholipase may be a variant, such as one of the variants disclosed in WO 00/32758, which are hereby incorporated by reference. Preferred phospholipase variants include variants listed in Example 5 of WO 00/32758, which is hereby specifically incorporated by
10 reference. In another preferred embodiment the phospholipase is one described in WO 04/111216, especially the variants listed in the table in Example 1.

In another preferred embodiment the phospholipase is derived from a strain of *Fusarium*, especially *Fusarium oxysporum*. The phospholipase may be the one concerned in WO 98/026057 displayed in SEQ ID NO: 2 derived from *Fusarium oxysporum* DSM 2672, or variants
15 thereof.

Examples of commercial phospholipases include LECITASE™ and LECITASE™ ULTRA, YIELSMAX, or LIPOPAN F (available from Novozymes A/S, Denmark).

Cellulolytic Activity

20 The term "cellulolytic activity" as used herein are understood as comprising enzymes having cellobiohydrolase activity (EC 3.2.1.91), e.g., cellobiohydrolase I and cellobiohydrolase II, as well as endo-glucanase activity (EC 3.2.1.4) and beta-glucosidase activity (EC 3.2.1.21).

At least three categories of enzymes are important for converting cellulose into fermentable sugars: endo-glucanases (EC 3.2.1.4) that cut the cellulose chains at random; cellobiohydrolases
25 (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 seems to be the key enzymes for degrading native crystalline cellulose.

The cellulolytic activity may, in a preferred embodiment, be in the form of a preparation of
30 enzymes of fungal origin, such as 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*.

In preferred embodiment the cellulolytic enzyme preparation contains one or more of the following activities: cellulase, hemicellulase, cellulolytic enzyme enhancing activity, beta-glucosidase activity, endoglucanase, cellobiohydrolase, or xylose-isomerase.

In a preferred embodiment cellulolytic enzyme preparation is a composition concerned in
5 co-pending application US application # 60/941,251, which is hereby incorporated by reference.
In a preferred embodiment the cellulolytic enzyme preparation comprising a polypeptide having cellulolytic enhancing activity, preferably a family GH61A polypeptide, preferably the one disclosed in WO 2005/074656 (Novozymes). The cellulolytic enzyme preparation may further comprise a beta-glucosidase, such as a beta-glucosidase derived from a strain of the genus
10 *Trichoderma*, *Aspergillus* or *Penicillium*, including the fusion protein having beta-glucosidase activity disclosed in co-pending application US 60/832,511 (Novozymes). In a preferred embodiment the cellulolytic enzyme preparation may also comprises a CBH II enzyme, preferably *Thielavia terrestris* cellobiohydrolase II CEL6A. In another preferred embodiment the cellulolytic enzyme preparation may also comprises cellulolytic enzymes, preferably one derived
15 from *Trichoderma reesei* or *Humicola insolens*.

The cellulolytic enzyme preparation may also comprising a polypeptide having cellulolytic enhancing activity (GH61A) disclosed in WO 2005/074656; a beta-glucosidase (fusion protein disclosed in US 60/832,511) and cellulolytic enzymes derived from *Trichoderma reesei*.

20 In an embodiment the cellulolytic enzyme composition is the commercially available product CELLUCLAST™ 1.5L or CELLUZYME™ (Novozymes A/S, Denmark).

The cellulolytic activity may 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.

25 Endoglucanase (EG)

The term "endoglucanase" means an endo-1,4-(1,3;1,4)-beta-D-glucan 4-glucanohydrolase (E.C. No. 3.2.1.4), which catalyses endo-hydrolysis of 1,4-beta-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxymethyl cellulose and hydroxyethyl cellulose), lichenin, beta-1,4 bonds in mixed beta-1,3 glucans such as cereal beta-D-glucans or
30 xyloglucans, and other plant material containing cellulosic components. 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*.

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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, celooligosaccharides, or any beta-1,4-linked glucose containing polymer, releasing cellobiose
10 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* and CBH II from *Thielavia terrestris* cellobiohydrolase (CELL6A)

Cellobiohydrolase activity may be determined according to the procedures described by
15 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 Tilbeurgh *et al.* is suitable for determining the cellobiohydrolase activity on a fluorescent disaccharide derivative.

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Beta-glucosidase

One or more beta-glucosidases 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
25 release of beta-D-glucose. For purposes of the present invention, beta-glucosidase activity is 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
30 citrate, 0.01% TWEEN® 20.

In a preferred 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 562003). In another preferred embodiment the beta-glucosidase is derived from *Aspergillus oryzae* (recombinantly produced in *Aspergillus oryzae* according to WO 02/095014), *Aspergillus fumigatus* (recombinantly produced in *Aspergillus oryzae* according to Example 22 of WO 02/095014) or *Aspergillus niger* (1981, J. Appl. Vol 3, pp 157-163).

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Hemicellulolytic enzymes

According to the invention the pre-treated lignocellulose-containing material may further be subjected to one or more hemicellulolytic enzymes, e.g., one or more hemicellulases.

Hemicellulose can be broken down by hemicellulases and/or acid hydrolysis to release its
10 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, arabinofuranosidases, acetyl xylan esterase,
15 feruloyl esterase, glucuronidases, endo-galactanase, mannases, endo or exo arabinases, exo-galactanases, and 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. An example of hemicellulase suitable for use in the present
20 invention includes VISCOZYME™ (available from Novozymes A/S, Denmark).

In an embodiment the hemicellulase is a xylanase. In an embodiment the xylanase may preferably be 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
25 *Aspergillus aculeatus*; 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. Examples of commercial xylanases include SHEARZYME™ and BIOFEED WHEAT™ from Novozymes A/S, Denmark.

The hemicellulase may be added in an amount effective to hydrolyze hemicellulose, such
30 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 g/kg DM substrate.

5 Cellulolytic Enhancing Activity

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
10 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
15 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
20 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.

25 In a preferred 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
30 isolated polypeptide having cellulolytic enhancing activity and a polynucleotide thereof from *Thermoascus aurantiacus*. U.S. Published Application Serial No. 2007/0077630 discloses an isolated polypeptide having cellulolytic enhancing activity and a polynucleotide thereof from *Trichoderma reesei*.

Proteases

A protease may be added during hydrolysis in step ii), fermentation in step iii) or simultaneous hydrolysis and fermentation. The protease may be any protease. In a preferred embodiment the protease is an acid protease of microbial origin, preferably of fungal or bacterial origin. An acid fungal protease is preferred, but also other proteases can be used.

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

10 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 20 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 25 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 (asclapain), 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).

30 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 mehei*. 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, Hand-book of Proteolytic Enzymes, Edited by A.J. Barrett, N.D. Rawlings and J.F. Woessner, Academic Press, San Diego, 1998, Chapter 270). Suitable examples of aspartic acid protease include, e.g., those disclosed in R.M. Berka et al. Gene, 96, 313 (1990)); (R.M. Berka et al. Gene, 125, 195-198 (1993)); and Gomi et al. Biosci. Biotech. Biochem. 57, 1095-1100 (1993), which are hereby incorporated by reference.

Commercially available products include ALCALASE®, ESPERASE™, 10 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 15 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.

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

25 Various references are cited herein, the disclosures of which are incorporated by reference in their entireties. The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

MATERIALS & METHODS

30 Methods

Determination of identity

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

The degree of identity between two amino acid sequences may be 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

5 Ktuple=1, gap penalty=3, windows=5, and diagonals=5.

The degree of identity between two nucleotide sequences may be 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

10 gap length penalty of 10. Pairwise alignment parameters are Ktuple=3, gap penalty=3, and windows=20.

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

1. Source of Method

15 1.1 The method is disclosed in a document entitled "Measurement of Cellulase Activities" by Adney, B. and Baker, J. 1996. Laboratory Analytical Procedure, LAP-006, National Renewable Energy Laboratory (NREL). It is based on the IUPAC method for measuring cellulase activity (Ghose, T.K., Measurement of Cellulase Activities, *Pure & Appl. Chem.* 59, pp. 257-268, 1987.

20 2. Procedure

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

2.2 Enzyme Assay Tubes:

25 2.2.1 A rolled filter paper strip (#1 Whatman; 1 X 6 cm; 50 mg) is added to the bottom of a test tube (13 X 100 mm).

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

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

30 2.2.4 Following incubation, 0.5 mL of enzyme dilution in citrate buffer is added to the tube. Enzyme dilutions are designed to produce values slightly above and below the target value of 2.0 mg glucose.

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

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

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

2.3 Blank and Controls

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

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

10 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

15 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

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

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

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

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

2.5 Color Development

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

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

30 2.5.3 When cool, the tubes are briefly vortexed, and the pulp is allowed to settle. Then each tube is diluted by adding 50 microL from the tube to 200 microL of ddH₂O in a 96-well plate. Each well is mixed, and the absorbance is read at 540 nm.

2.6 Calculations (examples are given in the NREL document)

- 2.6.1 A glucose standard curve is prepared by graphing glucose concentration (mg/0.5 mL) for the four standards (G1-G4) vs. A_{540} . This is fitted using a linear regression (Prism Software), and the equation for the line is used to determine the glucose produced for each of the enzyme assay tubes.
- 5 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.
- 10 2.6.4 The Filter Paper Units/mL (FPU/mL) are calculated as follows:
FPU/mL = $0.37 / \text{enzyme dilution producing 2.0 mg glucose}$

Lipase activity (LU)

The esterase activity is determined using tributyrine as substrate. This method is based
15 on the hydrolysis of tributyrin by the enzyme, and the alkali consumption is registered as a function of time.

One Lipase Unit (LU) is defined as the amount of enzyme which, under standard conditions (i.e., at 30°C; pH 7.0; with Gum Arabic as emulsifier and tributyrine as substrate) liberates 1 micro-mol titrable butyric acid per minute. Folders AF 95/5 or EB-SM-0095-02-D
20 which describes this analytical method in more detail is available upon request to Novozymes A/S, Denmark. The folders are hereby included by reference.

CLAIMS

1. A process for producing a fermentation product from wood-containing material, wherein the process comprises the steps of:
- 5 i) pre-treating wood-containing material;
- ii) hydrolysing by subjecting said pre-treated wood-containing material to one or more cellulolytic enzymes;
- iii) fermenting using a fermenting organism;
- wherein the wood-containing material is subjected to one or more esterases before and/or during
- 10 pre-treatment in step i) and/or hydrolysis in step ii) and/or fermentation in step iii).
2. The process of claim 1, wherein the wood-containing material contains softwood and/or hardwood.
- 15 3. The process of claim 2, wherein the softwood is derived from pine, spruce, fir, hemlock, larch, redwood, and/or cedar tree or a combination thereof.
4. The process of claim 3, wherein the fir is a Douglas fir; the pine is a Ponderosa pine or Loblolly pine; the spruce is a Sitka spruce, the hemlock is an Eastern or Western hemlock.
- 20 5. The process of claim 2, wherein the hardwood is derived from ash, aspen, beech, basswood, birch, black cherry, black walnut/butternut, buckeye, American chestnut, cottonwood, dogwood, elm, hackberry, hickory, holly, locust, magnolia, maple, oak, poplar, red alder, redbud, royal, paulownia, sassafras, sweetgum, sycamore, tupelo, willow, and/or yellow-poplar, or a combination
- 25 thereof.
6. The process of claims 1-5, wherein the pH during esterase treatment is in the range from pH 4.5-8.5.
- 30 7. The process of claims 1-6, wherein the temperature during esterase treatment is in the range from 40-90°C.

8. The process of any of claims 1-7, wherein the esterase dose used is in the range from 7.5-25 LU per dry gram of substrate.

9. The process of any of claims 1-8, wherein the esterase is a lipolytic enzyme, preferably lipase; a cutinase; a phospholipase, or a combination of two or more thereof.

10 The process of any of claims 1-9, wherein the lipase is derived from *Thermomyces*, preferably a strain of *Thermomyces lanuginosus* (previously *Humicola lanuginosa*) or a variant thereof.

10

11. The process of any of claims 1-10, wherein hydrolysis step ii) and fermentation step iii) are carried out separately or as HHF (hybrid hydrolysis and fermentation) or SHF (simultaneous hydrolysis and fermentation).

15 12. The process of any of claims 1-11, wherein said wood-containing material is subjected to esterase:

- before and/or during step i) and/or ii) and/or iii); or
- in a separate step before step i); or
- in a separate step between pre-treatment step i) and hydrolysis step ii).

20

13. The process of any of claims 1-12 wherein the wood-containing material is detoxified before fermentation and/or hydrolysis

14. The process of any of claims 1-13, wherein the wood-containing material is un-detoxified.

25

15. The process of any of claims 1-14, wherein the wood-containing material is chemically, mechanical and/or biologically pre-treated in step i).

16. The process of any of claims 1-15, wherein the cellulolytic enzymes used for hydrolysis of the pretreated material includes one or more cellulases.

30

17. The process of any of claims 1-16, further comprising subjecting the pre-treated material to one or more hemicellulolytic enzymes.

18. The process of any of claims 1-17, wherein the pretreated material is subjected to a pectinase before and/or during step i) and/or step ii) and/or step iii).

5 19. The process of any of claims 1-17, further wherein one or more polypeptides having cellulolytic enhancing activity is(are) present during hydrolysis and/or fermentation or HHF or SHF.

20. The method of claim 19, wherein the polypeptide having cellulolytic enhancing activity is a
10 family GH61A polypeptide, such as a family GH61A polypeptide derived from a strain of *Thermoascus*, preferably the *Thermoascus aurantiacus* GH61A polypeptide, especially the *Thermoascus aurantiacus* GH61A polypeptide disclosed in WO 2005/074656; derived from a strain of *Thielavia*, preferably *Thielavia terrestris*, more preferably the polypeptide disclosed in WO 2005/074656; a strain derived from *Trichoderma*, preferably a strain of *Trichoderma reesei*,
15 more preferably the polypeptide disclosed in U.S. Published Application Serial No. 2007/0077630.

21. The method of claim 1-20, further wherein one or more beta-glucosidases is(are) present during hydrolysis or fermentation or HHF (hybrid hydrolysis and fermentation) or SHF
20 (simultaneous hydrolysis and fermentation).

22. The method of claim 21, wherein the beta-glucosidase is derived from a strain of *Aspergillus*, preferably *Aspergillus oryzae*, or a strain of *Trichoderma*, preferably a strain of *Trichoderma reesei*.

25

23. The method of any of claims 1-22, wherein the cellulolytic enzymes used for hydrolysis is a cellulolytic preparation derived from a strain of *Trichoderma*, preferably a strain of *Trichoderma reesei*.

30 24. The method of any of claims 1-23, wherein the cellulolytic enzymes used for hydrolysis is a cellulolytic composition comprising a polypeptide having cellulolytic enhancing activity (GH61A) disclosed in WO 2005/074656; a beta-glucosidase (fusion protein disclosed in US 60/832,511) and cellulolytic enzymes preparation derived from *Trichoderma reesei*.

25. The method of any of claims 1-24, wherein the fermentation product is an alcohol, such as ethanol or butanol) or an organic acid, such as acetic acid or succinic acid.

5 26. The method of any of claims 1-25, wherein the fermentation is carried out using one or more C₆ and/or C₅ fermenting organisms.

27. The method of any of claims 1-26, wherein the fermenting organism is yeast, preferably a strain of the genus *Saccharomyces*, preferably *Saccharomyces cerevisiae*.

10

28. The method of any of claims 1-27, wherein the fermenting organism is yeast, preferably a strain of *Pichia*, preferably *Pichia stipitis*.

29. The method of any of claims 1-28, wherein the fermentation or SHF is carried out at a
15 temperature between 25°C and 40°C, such as between 29°C and 35°C, such as between 30°C and 34°C, such as around 32°C.

30. The method of any of claims 1-29, wherein the pH during fermentation is between 3 and 7, preferably between 4 and 6.

20

31. The method of any of claims 1-30, wherein fermentation is carried out for 1-96 hours, preferably 1-48 hours.

32. The process of any of claims 1-31, wherein the fermentation product is recovered after
25 fermentation.