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(71) Applicant: **DSM IP ASSETS B.V.** [NL/NL]; Het Overloon 1, 6411 TE HEERLEN (NL).

(72) Inventors: **NOORDAM, Bertus**; P.O.Box 4, 6100 AA ECHT (NL). **BOOGERS, Ilco Adrianus Lambertus Antonius**; P.O. Box 4, 6100 AA ECHT (NL). **PEL, Herman Jan**; P.O. Box 4, 6100 AA ECHT (NL).

(74) Agent: **DSM INTELLECTUAL PROPERTY**; P.O. Box 4, 6100 AA ECHT (NL).

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(54) Title: PROCESS FOR ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSIC MATERIAL AND FERMENTATION OF SUGARS

(57) Abstract: The invention relates to a process for the preparation of a sugar and/or fermentation product from lignocellulosic material.

PROCESS FOR ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSIC MATERIAL AND FERMENTATION OF SUGARS

Field

The application relates to a process for preparing a sugar product from lignocellulosic material by enzymatic hydrolysis and a process for preparing a fermentation product by fermentation of sugars.

Background

Lignocellulosic material is primarily composed of cellulose, hemicellulose and lignin and provides an attractive platform for generating alternative energy sources to fossil fuels. The material is available in large amounts and can be converted into valuable products *e.g.* sugars or biofuel, such as bioethanol.

Producing fermentation products from lignocellulosic material is known in the art and generally includes the steps of pretreatment, hydrolysis, fermentation, and optionally recovery of the fermentation products.

During the hydrolysis, which may comprise the steps of liquefaction, pre-saccharification and/or saccharification, cellulose and hemicellulose present in the lignocellulosic material are partly (typically 30 to 95 %, dependable on enzyme activity and hydrolysis conditions) converted into sugars by cellulolytic enzymes. The hydrolysis typically takes place during a process lasting 6 to 168 hours (see Kumar, S., Chem. Eng. Technol. 32 (2009), 517-526) under elevated temperatures of 45 to 50°C and non-sterile conditions.

Commonly, the sugars are then converted into valuable fermentation products such as ethanol by microorganisms such as yeast. The fermentation takes place in a separate, preferably anaerobic, process step, either in the same or in a different vessel. The temperature during fermentation is adjusted to 30 to 33°C to accommodate growth and ethanol production by microorganisms, commonly yeasts. During the fermentation process, the remaining cellulosic and/or hemicellulosic material is converted into sugars by the enzymes already present from the hydrolysis step, while microbial biomass and ethanol are produced. The fermentation is finished once the cellulosic and/or hemicellulosic material is converted into fermentable sugars and all fermentable sugars are converted into ethanol, carbon dioxide and microbial biomass. This may take up to 6 days. In general, the overall process time of hydrolysis and fermentation may amount up to 13 days.

In general, cost of enzyme production is a major cost factor in the overall production process of fermentation products from lignocellulosic material (see Kumar, S., Chem. Eng. Technol. 32 (2009), 517-526). Thus far, reduction of enzyme production costs is achieved by applying enzyme products from a single or from multiple microbial sources (see WO 2008/008793) with broader and/or higher (specific) hydrolytic activity. This leads to a lower enzyme need, faster conversion rates and/or higher conversion yields and thus to lower overall production costs.

Next to the optimization of enzymes, optimization of process design is a crucial tool to reduce overall costs of the production of sugar products and fermentation products.

For economic reasons, it is therefore desirable to include new and innovative process configurations aimed at reducing overall production costs in the process involving hydrolysis and fermentation of lignocellulosic material.

Summary

An object of the application is to provide an improved process for the preparation of a sugar product and/or a fermentation product from lignocellulosic material. The process is improved by removing volatiles during the process.

Detailed description

Throughout the present specification and the accompanying claims, the words "comprise" and "include" and variations such as "comprises", "comprising", "includes" and "including" are to be interpreted inclusively. That is, these words are intended to convey the possible inclusion of other elements or integers not specifically recited, where the context allows. The articles “a” and “an” are used herein to refer to one or to more than one (*i.e.* to one or at least one) of the grammatical object of the article. By way of example, “an element” may mean one element or more than one element.

It is to be understood that all features and embodiments described in relation to the processes can also be applied to the apparatuses and all features and embodiment described in relation to the apparatuses can also be applied to the processes.

The present application relates to a process for the preparation of a sugar product from lignocellulosic material, comprising the steps of (a) enzymatically hydrolysing lignocellulosic material in a hydrolysis reactor, (b) adding gas to the lignocellulosic material in the hydrolysis reactor during the enzymatic hydrolysis, wherein at least part of the gas ends up in the headspace of the hydrolysis reactor, and (c) removing volatiles from the gas that ends up in the headspace.

The present application also relates to a process for the preparation of a fermentation product from lignocellulosic material, comprising the steps of (a) performing a process for the preparation of a sugar product from lignocellulosic material as described herein, (b) fermenting the

hydrolysed lignocellulosic material to produce the fermentation product, and (c) optionally, recovering the fermentation product.

In an embodiment the processes as described herein further comprise the step of recycling at least part of the gas in the headspace to step (b). In other words, in an embodiment the processes as described herein further comprise a recycling step wherein at least part of the gas added in step (b) originates from the headspace of the hydrolysis reactor. In an embodiment the step of removing volatiles from the gas that ends up in the headspace may be done before, during and/or after the step of recycling at least part of the gas in the headspace to step (b). In a preferred embodiment the volatiles are at least partially removed from the gas during the recycling step.

In an embodiment the volatiles are removed by means of *e.g.* a filter containing an adsorbant (such as a filter containing active carbon, bentonite or a synthetic adsorbant), a condenser, a membrane, a scrubber.

"Volatiles" as used herein means volatile compounds, *i.e.* compounds that inhibit enzymatic hydrolysis of lignocellulosic material and/or fermentation of hydrolysed lignocellulosic material (e.g. fermentation of sugars to alcohol). Inhibition can be a results of enzyme inactivation, competitive inhibition, non-competitive inhibition, to name just a few. Volatiles can be formed during enzymatic hydrolysis of lignocellulosic material or before, for example, during pretreatment of lignocellulosic material. Volatiles include, but are not limited to, methanol; acetic acid; furoic acid; propionic acid; 4-hydroxyphenolic acid; vanillic acid; syringic acid; gluconic acid; glucaric acid; xylonic acid; 2-furoic acid; salicylic acid; adipic acid; fumaric acid; itaconic acid; sinapinic acid; syringic acid; succinic acid; gentisic acid; isoferulic acid; levulinic acid; homovanillic acid; dihydroxybenzoic acids; aldehydes such as 2-furaldehyde (i.e. furfural), 5-hydroxymethyl-2-furaldehyde (HMF), protocatechuic aldehyde, formaldehyde, acetaldehyde, acetone, acrolein, propionaldehyde, crotonaldehyde, butyraldehyde, benzaldehyde, isovaleraldehyde, valeraldehyde, o-tolualdehyde, m-tolualdehyde, p-toluialdehyde, hexaldehyde, 2,5-dimethylbenzaldehyde, syringaldehyde, coniferyl aldehyde; parabenes such as ethylparaben, methylparaben; phenolic compounds such as 4-hydroxybenzoic acid, acetosyringone, catechol, 4-hydroxybenzaldehyde, vanillin, apocynin, dihydroconiferyl alcohol, and Hibbert's ketones, p-coumaric acid, pyrogallol, gallic acid and ferulic acid; non-phenolic aromatic compounds such as benzoic acid, benzyl alcohol, cinnamic acid, cinnamaldehyde, 3,4-dimethoxy-cinnamic acid, and para- and ortho-toluic acid; *p*-benzoquinones.

As shown in the examples, volatiles are removed to a concentration that no longer hampers yeast propagation and/or ethanol production by yeast. In an embodiment furfural is removed to a concentration of below 0.88 g/l in the hydrolysate to improve yeast propagation, preferably to a concentration of below 0.85 g/l in the hydrolysate to improve yeast propagation, even more preferably to a concentration of below 0.80 g/l in the hydrolysate to improve yeast propagation, yet even more preferably to a concentration of below 0.75 g/l in the hydrolysate to improve yeast propagation, most preferably to a concentration of below 0.70 g/l in the hydrolysate to improve yeast propagation, in particular to a concentration of below 0.60 g/l in the hydrolysate to improve

yeast propagation. In an embodiment furfural is removed to a concentration of below 2.70 g/l in the hydrolysate to improve ethanol production, preferably to a concentration of below 2.60 g/l in the hydrolysate to improve ethanol production, even more preferably to a concentration of below 2.50 g/l in the hydrolysate to improve ethanol production, most preferably to a concentration of below 2.50 g/l in the hydrolysate to improve ethanol production, most preferably to a concentration of below 2.40 g/l in the hydrolysate to improve ethanol production and in particular to a concentration of below 2.30 g/l in the hydrolysate to improve ethanol production.

The application also relates to an apparatus which comprises: (a) a reactor of at least 10 m^3, (b) a gas introducing means for addition of gas into the reactor, (c) a gas pump for introducing gas into the reactor, (d) a means for recycling gas from the headspace of the reactor into the reactor, (e) an exhaust for removing gas from the reactor, (f) a gas inlet for introducing fresh gas into the reactor, (g) a means for controlling the ratio between recycled gas and fresh gas, and (h) a means for removing volatiles from the gas in the headspace.

In an embodiment the gas introducing means for addition of gas into the reactor (i.e. element (b)) is a gas introducing means for addition of gas into the content of the reactor. In an embodiment the gas introducing means for addition of gas into the reactor (i.e. element (b)) is a gas introducing means for addition of gas into the lignocellulosic material present in the reactor.

In an embodiment the gas inlet for introducing fresh gas into the reactor (i.e. element (f)) is connected directly to the reactor. In another embodiment the gas inlet for introducing fresh gas into the reactor is connected to the means for recycling gas from the headspace of the reactor into the reactor (i.e. element (d)). In other words, fresh gas may be added outside the reactor into the recycle stream. In a preferred embodiment fresh gas is added outside the reactor into the recycle stream after the volatiles have been removed from the gas in the headspace. In an embodiment the apparatus as described herein comprises - instead of element (f) as described above (i.e. a gas inlet for introducing fresh gas into the reactor) - the following element (f): a means to add fresh gas to the means for recycling gas.

In an embodiment the apparatus as described herein comprises instead of element (h) as described above (i.e. a means for removing volatiles from the gas in the headspace) the following element (h): a means for removing volatiles from the gas originating from the headspace.

In an embodiment of the apparatus as described herein the means for removing volatiles from the gas is connected to the means for recycling gas. “Connected” as used herein means that the means for removing volatiles from the gas is incorporated in and/or linked to the means for recycling gas.

In an embodiment the apparatus as described herein comprises an exhaust for removing gas from the reactor. The exhaust may also comprise and/or may be connected to a means for removing volatiles. Consequently, volatiles are removed from the gas before the gas is removed from the reactor.

In an embodiment the apparatus as described herein further comprises a stirring means for stirring the content of the reactor. The stirring means may comprise an impeller. In an embodiment the impeller is mounted at a distal end of a shaft. By rotating the shaft, the impeller can be used to stir the content of the reactor. The impeller may comprise one blade, two blades, three blades, four blades, five blades, six blades or even more blades. In an embodiment the apparatus comprises more than one stirring means, for example two, three, four or even more stirring means.

In the processes as described herein, lignocellulosic material may be added to the hydrolysis reactor. In an embodiment the enzymatic hydrolysis is done with an enzyme composition. In an embodiment the enzyme composition is already present in the hydrolysis reactor before the lignocellulosic material is added. In another embodiment the enzyme composition may be added to the hydrolysis reactor. In an embodiment the lignocellulosic material is already present in the hydrolysis reactor before the enzyme composition is added. In an embodiment both the lignocellulosic material and the enzyme composition are added simultaneously to the hydrolysis reactor. The enzyme composition may be an aqueous composition.

In an embodiment the process for the preparation of a sugar product from lignocellulosic material (i.e. an enzymatic hydrolysis process) comprises at least a liquefaction step wherein the lignocellulosic material is enzymatically hydrolysed in a first hydrolysis reactor, and a saccharification step wherein the liquefied lignocellulosic material is hydrolysed in the first hydrolysis reactor and/or in a second hydrolysis reactor. Saccharification can be done in the same hydrolysis reactor as the liquefaction (*i.e.* the first hydrolysis reactor). It can also be done in a separate hydrolysis reactor (*i.e.* the second hydrolysis reactor). In the enzymatic hydrolysis process liquefaction and saccharification may be separate steps. Alternatively, the liquefaction and saccharification may be combined. Liquefaction and saccharification may be performed at different temperatures, but may also be performed at a single temperature. In an embodiment the temperature of the liquefaction is higher than the temperature of the saccharification. Liquefaction is preferably carried out at a temperature of 60 - 75 ºC and saccharification is preferably carried out at a temperature of 45 - 65 ºC.

In an embodiment the enzyme composition used in the liquefaction is different from the enzyme compostion in the saccharification. In another embodiment the enzyme composition used in the liquefaction is the same as the enzyme compostion in the saccharification. In an embodiment additional enzyme composition is added during and/or after liquefaction. In an embodiment additional enzyme composition is added during and/or after liquefaction, but before saccharification. In an embodiment additional enzyme composition is added before and/or during and/or after saccharification.

In an embodiment the liquefaction can be batch, fed-batch or continuous. In an embodiment the total liquefaction time is 1 to 48 hours, 1 to 36 hours, preferably 1 to 24 hours, more preferably 1 to 12 hours, most preferably 1 to 8 hours.

In an embodiment the total saccharification time is 1 to 300 hours, 1 to 200 hours, preferably 1 to 120 hours, more preferably 1 to 72 hours, most preferably 1 to 48 hours.

In an embodiment the total enzymatic hydrolysis time is 10 hours or more, 12 hours or more, 14 hours or more, 16 hours or more, 18 hours or more, 20 hours or more, 30 hours or more, 40 hours or more, 50 hours or more, 60 hours or more, 70 hours or more, 80 hours or more, 90 hours or more, 100 hours or more, 110 hours or more, 120 hours or more, 130 hours or more, 140 hours or more, 150 hours or more, 160 hours or more, 170 hours or more, 180 hours or more, 190 hours or more, 200 hours or more.

In an embodiment the total enzymatic hydrolysis time is 10 to 300 hours, 16 to 275 hours, preferably 20 to 250 hours, more preferably 30 to 200 hours, most preferably 40 to 150 hours.

In an embodiment gas is added during the process for the preparation of a sugar product from lignocellulosic material as described herein. In an embodiment the gas added to the lignocellulosic material in the hydrolysis reactor is non-oxygen-containing gas. In an embodiment the gas added to the lignocellulosic material in the hydrolysis reactor is oxygen-containing gas. In an embodiment oxygen is added during at least a part of the process (*i.e.* during part of the enzymatic hydrolysis). Gas can be added during the liquefaction and/or during the saccharification step. Gas can also be added before liquefaction and before saccharification (but after liquefaction). Gas can be added continuously or discontinuously during the enzymatic hydrolysis. In an embodiment gas is added one or more times during the process for the preparation of a sugar product from lignocellulosic material as described herein. In an embodiment gas may be added before the process for the preparation of a sugar product from lignocellulosic material as described herein, during the addition of lignocellulosic material to a hydrolysis reactor used for the process for the preparation of a sugar product from lignocellulosic material as described herein, during the addition of enzyme to a hydrolysis reactor used for the process for the preparation of a sugar product from lignocellulosic material as described herein, during a part of the process for the preparation of a sugar product from lignocellulosic material as described herein, during the whole process for the preparation of a sugar product from lignocellulosic material as described herein, or any combination thereof. Gas is added to the hydrolysis reactors used in the enzymatic hydrolysis. Gas can be added to the content (e.g. lignocellulosic material) present in the hydrolysis reactor or it can be added to the headspace of the hydrolysis reactor. Gas can be added during the liquefaction step of the process for the preparation of a sugar product from lignocellulosic material as described herein. Gas can be added during the saccharifiction step of the process for the preparation of a sugar product from lignocellulosic material as described herein. Gas can be added during the liquefaction step and the saccharification step of the process for the preparation of a sugar product from lignocellulosic material as described herein.

In an embodiment gas is added during the saccharification step.

In an embodiment gas addition starts 24 hours after start of the enzymatic hydrolysis, preferably 36 hours after start of the enzymatic hydrolysis and more preferably 48 hours after start of the enzymatic hydrolysis.

In an embodiment the total gas addition period is at least 12 hours, more preferably at least 24 hours and even more preferably at least 36 hours.

In an embodiment the oxygen concentration in the gas is between 1 and 99%, preferably between 1 and 90%, more preferably between 1 and 80%, even more preferably between 1 and 70%, yet even more preferably between 1 and 60%, most preferably between 1 and 50% and in particular between 1 and 40%. In an embodiment the oxygen concentration in the gas is between 1 and 21%, more preferably between 3 and 21%, even more preferably between 5 and 21% and most preferably between 7 and 21%.

The gas added to the lignocellulosic material in the hydrolysis reactor can be oxygen-containing gas, oxygen-enriched gas, such as oxygen-enriched air, air or inert gas. In a preferred embodiment the gas contains oxygen.

In a preferred embodiment the gas added to the lignocellulosic material in the hydrolysis reactor is added in the form of bubbles. This can for example be done by sparging. Sparging can be done by means of a sparger, a porous tube, diffuser, to name just a few. In an embodiment the gas is added at the lower part of the hydrolysis reactor.

In an embodiment at least part of the gas added to the lignocellulosic material in the hydrolysis reactor is gas originating from the headspace of the hydrolysis reactor. In other words, in an embodiment the processes as described herein further comprise a recycling step wherein at least part of the gas added in step (b) originates from the headspace of the hydrolysis reactor. When gas is added to the lignocellulosic material in the hydrolysis reactor, part of the gas may be absorbed/used/converted by the lignocellulosic material, the enzymes or other content of the hydrolysis reactor. The part of the gas that is not absorbed/used/converted may end up in headspace of the hydrolysis reactor. The gas that ends up in the headspace of the hydrolysis reactor or at least a part of that gas can be recycled, *i.e.* added again to the lignocellulosic material in the hydrolysis reactor. In other words, the gas that ends up in the headspace of the hydrolysis reactor or at least a part of that gas can be recycled to step (b) of the processes for the preparation of a sugar product from lignocellulosic material as described herein. The recycled gas can be combined with another gas before it is added to the lignocellulosic material in the hydrolysis reactor. Another gas can for example be oxygen-containing gas, oxygen-enriched gas, such as oxygen-enriched air, air or an inert gas. By combining the recycled gas with the other gas, the oxygen level of the gas added to the lignocellulosic material in the hydrolysis reactor can be controlled, varied and/or steered.

Recycling at least part of the gas in the headspace to the hydrolysis reactor can be done by means of a pipe, tube, pipeline, tubing or any other suitable means for transporting gas.

In an embodiment volatiles are removed from the gas that ends up in the headspace. The gas that ends up in the headspace has moved through the lignocellulosic material in the hydrolysis reactor during the enzymatic hydrolysis and may have taken up volatiles that have formed during the enzymatic hydrolysis and/or that were already present in the lignocellulosic material before enzymatic hydrolysis (for example as a result of the pretereatment of the lignocellulosic materia)l. Removal of the volatiles can be done by means of a filter containing an adsorbant (such as a filter containing active carbon, bentonite or a synthetic adsorbant), a condenser, a membrane, a scrubber, to name just a few.

In an embodiment the volatiles are at least partially removed from the gas during the recycling step. The means for recycling the gas may be connected to the means for removing volatiles from the gas. For example, the pipe used to recycle the gas may comprise or may be linked to the means for removing volatiles from the gas, *e.g.* a filter or condensor.

In an embodiment the hydrolysis reactor(s) as described herein have a volume of at least 1 m^3. Preferably, the bioreactors have a volume of at least 1 m^3, at least 2 m^3, at least 3 m^3, at least 4 m^3, at least 5 m^3, at least 6 m^3, at least 7 m^3, at least 8 m^3, at least 9 m^3, at least 10 m^3, at least 15 m^3, at least 20 m^3, at least 25 m^3, at least 30 m^3, at least 35 m^3, at least 40 m^3, at least 45 m^3, at least 50 m^3, at least 60 m^3, at least 70 m^3, at least 75 m^3, at least 80 m^3, at least 90 m^3, at least 100 m^3, at least 200 m^3, at least 300 m^3, at least 400 m^3, at least 500 m^3, at least 600 m^3, at least 700 m^3, at least 800 m^3, at least 900 m^3, at least 1000 m^3, at least 1500 m^3, at least 2000 m^3, at least 2500 m^3. In general, the bioreactor(s) will be smaller than 3000 m^3 or 5000 m^3. In an embodiment the size of the bioreactor(s) is from 50 m^3 to 5000 m^3. In case multiple hydrolysis reactors are used, they may have the same volume, but also may have a different volume.

In an embodiment the enzyme composition used in the enzymatic hydrolysis as described herein is from a fungus, preferably a filamentous fungus. In an embodiment the enzymes in the enzyme composition are derived from a fungus, preferably a filamentous fungus or the enzymes comprise a fungal enzyme, preferably a filamentous fungal enzyme. The enzymes used in the enzymatic hydrolysis as described herein are derived from a fungus or the enzymes used in the enzymatic hydrolysis as described herein comprise a fungal enzyme. "Filamentous fungi" include all filamentous forms of the subdivision *Eumycota* and *Oomycota* (as defined by Hawksworth *et al.*, In, Ainsworth and Bisby's Dictionary of The Fungi, 8th edition, 1995, CAB International, University Press, Cambridge, UK). Filamentous fungi include, but are not limited to *Acremonium, Agaricus, Aspergillus, Aureobasidium, Beauvaria, Cephalosporium, Ceriporiopsis, Chaetomium paecilomyces, Chrysosporium, Claviceps, Cochiobolus, Coprinus, Cryptococcus, Cyathus, Emericella, Endothia, Endothia mucor, Filibasidium, Fusarium, Geosmithia, Gilocladium, Humicola, Magnaporthe, Mucor, Myceliophthora, Myrothecium, Neocallimastix, Neurospora, Paecilomyces, Penicillium, Piromyces, Panerochaete, Pleurotus, Podospora, Pyricularia, Rasamsonia, Rhizomucor, Rhizopus, Scylatidium, Schizophyllum, Stagonospora, Talaromyces, Thermoascus, Thermomyces, Thielavia, Tolypocladium, Trametes pleurotus, Trichoderma* and *Trichophyton*. In a

preferred embodiment the fungus is *Rasamsonia*, with *Rasamsonia emersonii* being most preferred. *Ergo*, the processes as described herein are advantageously applied in combination with enzymes derived from a microorganism of the genus *Rasamsonia* or the enzymes used in the processes as described herein comprise a *Rasamsonia* enzyme.

Several strains of filamentous fungi are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

The enzymatic hydrolysis as described herein is preferably conducted at a temperature of 45ºC or more, for example at 45 - 90ºC. Preferably, the processes as described herein are done with thermostable enzymes. “Thermostable” enzyme as used herein means that the enzyme has a temperature optimum of 50ºC or higher, 60ºC or higher, 70ºC or higher, 75ºC or higher, 80ºC or higher, or even 85ºC or higher. They may for example be isolated from thermophilic microorganisms or may be designed by the skilled person and artificially synthesized. In an embodiment the polynucleotides encoding the thermostable enzymes may be isolated or obtained from thermophilic or thermotolerant filamentous fungi or isolated from non-thermophilic or non-thermotolerant fungi, but are found to be thermostable. By “thermophilic fungus” is meant a fungus that grows at a temperature of 50°C or higher. By “themotolerant” fungus is meant a fungus that grows at a temperature of 45°C or higher, having a maximum near 50°C.

Suitable thermophilic or thermotolerant fungal cells may be *Humicola*, *Rhizomucor*, *Myceliophthora*, *Rasamsonia, Talaromyces*, *Thermomyces, Thermoascus* or *Thielavia* cells, preferably *Rasamsonia* cells. Preferred thermophilic or thermotolerant fungi are *Humicola grisea var. thermoidea, Humicola lanuginosa, Myceliophthora thermophila, Papulaspora thermophilia, Rasamsonia byssochlamydoides, Rasamsonia emersonii, Rasamsonia argillacea, Rasamsonia eburnean, Rasamsonia brevistipitata, Rasamsonia cylindrospora, Rhizomucor pusillus, Rhizomucor miehei, Talaromyces bacillisporus, Talaromyces leycettanus, Talaromyces thermophilus, Thermomyces lenuginosus, Thermoascus crustaceus, Thermoascus thermophilus Thermoascus aurantiacus* and *Thielavia terrestris*.

Rasamsonia is a new genus comprising thermotolerant and thermophilic *Talaromyces* and *Geosmithia* species. Based on phenotypic, physiological and molecular data, the species *Talaromyces emersonii, Talaromyces byssochlamydoides, Talaromyces eburneus, Geosmithia argillacea* and *Geosmithia cylindrospora* were transferred to *Rasamsonia* gen. nov. *Talaromyces emersonii, Penicillium geosmithia emersonii* and *Rasamsonia emersonii* are used interchangeably herein.

In the processes as described herein enzyme compositions are used. Preferably, the compositions are stable. “Stable enzyme compositions” as used herein means that the enzyme compositions retain activity after 30 hours of hydrolysis reaction time, preferably at least 10%, 20%,

30%, 40%, 50%, 60%, 65%, 70%, 75%, 80% 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% of its initial activity after 30 hours of hydrolysis reaction time. Preferably, the enzyme composition retains activity after 40, 50, 60, 70, 80, 90 100, 150, 200, 250, 300, 350, 400, 450, 500 hours of hydrolysis reaction time.

The enzymes may be prepared by fermentation of a suitable substrate with a suitable microorganism, *e.g. Rasamsonia emersonii* or *Aspergillus niger*, wherein the enzymes are produced by the microorganism. The microorganism may be altered to improve or to make the enzymes. For example, the microorganism may be mutated by classical strain improvement procedures or by recombinant DNA techniques. Therefore, the microorganisms mentioned herein can be used as such to produce the enzymes or may be altered to increase the production or to produce altered enzymes which might include heterologous enzymes, *e.g.* cellulases, thus enzymes that are not originally produced by that microorganism. Preferably, a fungus, more preferably a filamentous fungus is used to produce the enzymes. Advantageously, a thermophilic or thermotolerant microorganism is used. Optionally, a substrate is used that induces the expression of the enzymes by the enzyme producing microorganism.

The enzymes are used to liquefy the lignocellulosic material and/or release sugars from lignocellulosic material that comprises polysaccharides. The major polysaccharides are cellulose (glucans), hemicelluloses (xylans, heteroxylans and xyloglucans). In addition, some hemicellulose may be present as glucomannans, for example in wood-derived lignocellulosic material. The enzymatic hydrolysis of these polysaccharides to soluble sugars, including both monomers and multimers, for example glucose, cellobiose, xylose, arabinose, galactose, fructose, mannose, rhamnose, ribose, galacturonic acid, glucoronic acid, gluconic acid and other hexoses and pentoses occurs under the action of different enzymes acting in concert. By sugar product is meant the enzymatic hydrolysis product of the lignocellulosic material and/or the hydrolysed lignocellulosic material. The sugar product comprises soluble sugars, including both monomers and multimers. Preferably, it comprises glucose. Examples of other sugars are cellobiose, xylose, arabinose, galactose, fructose, mannose, rhamnose, ribose, galacturonic acid, glucoronic acid and other hexoses and pentoses. The sugar product may be used as such or may be further processed for example recovered and/or purified.

In addition, pectins and other pectic substances such as arabinans may make up considerably proportion of the dry mass of typically cell walls from non-woody plant tissues (about a quarter to half of dry mass may be pectins). Furthermore, the lignocellulosic material may comprise lignin.

Enzymes that may be used in the processes as described herein are described in more detail below.

Lytic polysaccharide monooxygenases, endoglucanases (EG) and exo-cellobiohydrolases (CBH) catalyze the hydrolysis of insoluble cellulose to products such as cellooligosaccharides, while β-glucosidases (BG) convert the oligosaccharides, mainly cellobiose and cellotriose, to

glucose.

Xylanases together with other accessory enzymes, for example α-L- arabinofuranosidases, feruloyl and acetylxylan esterases, glucuronidases, and β-xylosidases catalyze the hydrolysis of hemicellulose.

An enzyme composition for use in the processes as described herein may comprise at least two activities, although typically a composition will comprise more than two activities, for example, three, four, five, six, seven, eight, nine or even more activities. Typically, an enzyme composition for use in the processes as described herein comprises at least two cellulases. The at least two cellulases may contain the same or different activities. The enzyme composition for use in the processes as described herein may also comprises at least one enzyme other than a cellulase. Preferably, the at least one other enzyme has an auxiliary enzyme activity, *i.e.* an additional activity which, either directly or indirectly leads to lignocellulose degradation. Examples of such auxiliary activities are mentioned herein and include, but are not limited, to hemicellulases.

In an embodiment the enzyme composition used in the liquefaction step differs from the enzyme composition used in the saccharification step. In another embodiment the enzyme compositions are similar or even identical.

In an embodiment an enzyme composition for use in the processes as described herein comprises at least a lytic polysaccharide monooxygenase. It may comprise a lytic polysaccharide monooxygenase, an endoglucanase, a cellobiohydrolase and/or a beta-glucosidase. An enzyme composition may comprise more than one enzyme activity per activity class. For example, a composition may comprise two endoglucanases, for example an endoglucanase having endo-1,3(1,4)-β glucanase activity and an endoglucanase having endo-β-1,4-glucanase activity.

A composition for use in the processes as described herein may be derived from a fungus, such as a filamentous fungus, such as *Rasamsonia*, such as *Rasamsonia emersonii*. In an embodiment a core set of enzymes may be derived from *Rasamsonia emersonii*. If needed, the set of enzymes can be supplemented with additional enzymes from other sources. Such additional enzymes may be derived from classical sources and/or produced by genetically modified organisms.

In addition, enzymes in the enzyme compositions for use in the processes as described herein may be able to work at low pH. For the purposes of this invention, low pH indicates a pH of 5.5 or lower, 5 or lower, 4.9 or lower, 4.8 or lower, 4.7 or lower, 4.6 or lower, 4.5 or lower, 4.4 or lower, 4.3 or lower, 4.2 or lower, 4.1 or lower, 4.0 or lower 3.9 or lower, 3.8 or lower, 3.7 or lower, 3.6 or lower, 3.5 or lower.

The enzyme composition for use in the processes as described herein may comprise a cellulase and/or a hemicellulase and/or a pectinase from *Rasamsonia*. They may also comprise a cellulase and/or a hemicellulase and/or a pectinase from a source other than *Rasamsonia*. They may be used together with one or more *Rasamsonia* enzymes or they may be used without additional *Rasamsonia* enzymes being present.

An enzyme composition for use in the processes as described herein may comprise a lytic polysaccharide monooxygenas (LPMO), an endoglucanase (EG), one or two cellobiohydrolases (CBH) and/or a beta-glucosidase (BG).

An enzyme composition for use in the processes as described herein may comprise one type of cellulase activity and/or hemicellulase activity and/or pectinase activity provided by a composition as described herein and a second type of cellulase activity and/or hemicellulase activity and/or pectinase activity provided by an additional cellulase/hemicellulase/pectinase.

As used herein, a cellulase is any polypeptide which is capable of degrading or modifying cellulose. A polypeptide which is capable of degrading cellulose is one which is capable of catalyzing the process of breaking down cellulose into smaller units, either partially, for example into cellodextrins, or completely into glucose monomers. A cellulase as described herein may give rise to a mixed population of cellodextrins and glucose monomers. Such degradation will typically take place by way of a hydrolysis reaction.

As used herein, a hemicellulase is any polypeptide which is capable of degrading or modifying hemicellulose. That is to say, a hemicellulase may be capable of degrading or modifying one or more of xylan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan. A polypeptide which is capable of degrading a hemicellulose is one which is capable of catalyzing the process of breaking down the hemicellulose into smaller polysaccharides, either partially, for example into oligosaccharides, or completely into sugar monomers, for example hexose or pentose sugar monomers. A hemicellulase as described herein may give rise to a mixed population of oligosaccharides and sugar monomers. Such degradation will typically take place by way of a hydrolysis reaction.

As used herein, a pectinase is any polypeptide which is capable of degrading or modifying pectin. A polypeptide which is capable of degrading pectin is one which is capable of catalyzing the process of breaking down pectin into smaller units, either partially, for example into oligosaccharides, or completely into sugar monomers. A pectinase as described herein may give rise to a mixed population of oligosacchardies and sugar monomers. Such degradation will typically take place by way of a hydrolysis reaction.

Accordingly, an enzyme composition for use in the processes as described herein may comprise one or more of the following enzymes, a lytic polysaccharide monooxygenase (*e.g.* GH61), a cellobiohydrolase, an endo-β-1,4-glucanase, a beta-glucosidase, and a β-(1,3)(1,4)-glucanase. A composition for use in the processes as described herein may also comprise one or more hemicellulases, for example, an endoxylanase, a β-xylosidase, a α-L-arabionofuranosidase, an α-D-glucuronidase, an acetyl xylan esterase, a feruloyl esterase, a coumaroyl esterase, an α-galactosidase, a β-galactosidase, a β-mannanase and/or a β-mannosidase. A composition for use in the processes as described herein may also comprise one or more pectinases, for example, an endo polygalacturonase, a pectin methyl esterase, an endo-galactanase, a beta galactosidase, a pectin acetyl esterase, an endo-pectin lyase, pectate lyase, alpha rhamnosidase, an exo-

galacturonase, an expolygalacturonate lyase, a rhamnogalacturonan hydrolase, a rhamnogalacturonan lyase, a rhamnogalacturonan acetyl esterase, a rhamnogalacturonan galacturonohydrolase, and/or a xylogalacturonase. In addition, one or more of the following enzymes, an amylase, a protease, a lipase, a ligninase, a hexosyltransferase, a glucuronidase, an expansin, a cellulose induced protein or a cellulose integrating protein or like protein may be present in a composition for use in the processes of the current invention (these are referred to as auxiliary activities above).

As used herein, lytic polysaccharide monooxygenases are enzymes that have recently been classified by CAZy in family AA9 (Auxiliary Activity Family 9) or family AA10 (Auxiliary Activity Family 10). *Ergo*, there exist AA9 lytic polysaccharide monooxygenases and AA10 lytic polysaccharide monooxygenases. Lytic polysaccharide monooxygenases are able to open a crystalline glucan structure and enhance the action of cellulases on lignocellulose substrates. They are enzymes having cellulolytic enhancing activity. Lytic polysaccharide monooxygenases may also affect cello-oligosaccharides. According to the latest literature, (see Isaksen et al., Journal of Biological Chemistry, vol. 289, no. 5, p. 2632-2642), proteins named GH61 (glycoside hydrolase family 61 or sometimes referred to EGIV) are lytic polysaccharide monooxygenases. GH61 was originally classified as endoglucanase based on measurement of very weak endo-1,4-β-d-glucanase activity in one family member, but have recently been reclassified by CAZy in family AA9. CBM33 (family 33 carbohydrate-binding module) is also a lytic polysaccharide monooxygenase (see Isaksen et al, Journal of Biological Chemistry, vol. 289, no. 5, pp. 2632-2642). CAZy has recently reclassified CBM33 in the AA10 family.

In an embodiment the lytic polysaccharide monooxygenase comprises an AA9 lytic polysaccharide monooxygenase. This means that at least one of the lytic polysaccharide monooxygenases in the enzyme composition is an AA9 lytic polysaccharide monooxygenase. In an embodiment all lytic polysaccharide monooxygenases in the enzyme composition are AA9 lytic polysaccharide monooxygenase.

In an embodiment the enzyme composition comprises a lytic polysaccharide monooxygenase from *Thermoascus*, such as *Thermoascus aurantiacus*, such as the one described in WO 2005/074656 as SEQ ID NO:2 and SEQ ID NO:1 in WO2014/130812 and in WO 2010/065830; or from *Thielavia*, such as *Thielavia terrestris*, such as the one described in WO 2005/074647 as SEQ ID NO: 8 or SEQ ID NO:4 in WO2014/130812 and in WO 2008/148131, and WO 2011/035027; or from *Aspergillus*, such as *Aspergillus fumigatus*, such as the one described in WO 2010/138754 as SEQ ID NO:2 or SEQ ID NO: 3 in WO2014/130812; or from *Penicillium*, such as *Penicillium emersonii*, such as the one disclosed as SEQ ID NO:2 in WO 2011/041397 or SEQ ID NO:2 in WO2014/130812. Other suitable lytic polysaccharide monooxygenases include, but are not limited to, *Trichoderma reesei* (see WO 2007/089290), *Myceliophthora thermophila* (see WO 2009/085935, WO 2009/085859, WO 2009/085864, WO 2009/085868), *Penicillium pinophilum* (see WO 2011/005867), *Thermoascus sp.* (see WO 2011/039319), and *Thermoascus crustaceous*

(see WO 2011/041504). Other cellulolytic enzymes that may be comprised in the enzyme composition are described in WO 98/13465, WO 98/015619, WO 98/015633, WO 99/06574, WO 99/10481, WO 99/025847, WO 99/031255, WO 2002/101078, WO 2003/027306, WO 2003/052054, WO 2003/052055, WO 2003/052056, WO 2003/052057, WO 2003/052118, WO 2004/016760, WO 2004/043980, WO 2004/048592, WO 2005/001065, WO 2005/028636, WO 2005/093050, WO 2005/093073, WO 2006/074005, WO 2006/117432, WO 2007/071818, WO 2007/071820, WO 2008/008070, WO 2008/008793, US 5,457,046, US 5,648,263, and US 5,686,593, to name just a few. In an embodiment, the lytic polysaccharide monooxygenase is from *Rasamsonia, e.g. Rasamsonia emersonii* (see WO 2012/000892).

As used herein, endoglucanases are enzymes which are capable of catalyzing the endohydrolysis of 1,4-β-D-glucosidic linkages in cellulose, lichenin or cereal β-D-glucans. They belong to EC 3.2.1.4 and may also be capable of hydrolyzing 1,4-linkages in β-D-glucans also containing 1,3-linkages. Endoglucanases may also be referred to as cellulases, avicelases, β-1,4-endoglucan hydrolases, β-1,4-glucanases, carboxymethyl cellulases, celludextrinases, endo-1,4-β-D-glucanases, endo-1,4-β-D-glucanohydrolases or endo-1,4-β-glucanases.

In an embodiment the endoglucanase comprises a GH5 endoglucanase and/or a GH7 endoglucanase. This means that at least one of the endoglucanases in the enzyme composition is a GH5 endoglucanase or a GH7 endoglucanase. In case there are more endoglucanases in the enzyme composition, these endoglucanases can be GH5 endoglucanases, GH7 endoglucanases or a combination of GH5 endoglucanases and GH7 endoglucanases. In a preferred embodiment the endoglucanase comprises a GH5 endoglucanase.

In an embodiment the enzyme composition comprises an endoglucanase from *Trichoderma*, such as *Trichoderma reesei*; from *Humicola*, such as a strain of *Humicola insolens*; from Aspergillus, such as *Aspergillus aculeatus* or *Aspergillus kawachii*; from *Erwinia*, such as *Erwinia carotovara*; from *Fusarium*, such as *Fusarium oxysporum*; from *Thielavia*, such as *Thielavia terrestris*; from *Humicola*, such as *Humicola grisea var. thermoidea* or *Humicola insolens*; from *Melanocarpus*, such as *Melanocarpus albomyces*; from *Neurospora*, such as *Neurospora crassa*; from *Myceliophthora*, such as *Myceliophthora thermophila*; from *Cladorrhinum*, such as *Cladorrhinum foecundissimum*; and/or from *Chrysosporium*, such as a strain of *Chrysosporium lucknowense*. In an embodiment the endoglucanase is from *Rasamsonia*, such as a strain of *Rasamsonia emersonii* (see WO 01/70998). In an embodiment even a bacterial endoglucanase can be used including, but are not limited to, *Acidothermus cellulolyticus* endoglucanase (see WO 91/05039; WO 93/15186; US 5,275,944; WO 96/02551; US 5,536,655, WO 00/70031, WO 05/093050); *Thermobifida fusca* endoglucanase III (see WO 05/093050); and *Thermobifida fusca* endoglucanase V (see WO 05/093050).

As used herein, beta-xylosidases (EC 3.2.1.37) are polypeptides which are capable of catalysing the hydrolysis of 1,4-β-D-xylans, to remove successive D-xylose residues from the non-reducing termini. Beta-xylosidases may also hydrolyze xylobiose. Beta-xylosidase may also be

referred to as xylan 1,4-β-xylosidase, 1,4-β-D-xylan xylohydrolase, exo-1,4-β-xylosidase or xylobiase.

In an embodiment the beta-xylosidase comprises a GH3 beta-xylosidase. This means that at least one of the beta-xylosidases in the enzyme composition is a GH3 beta-xylosidase. In an embodiment all beta-xylosidases in the enzyme composition are GH3 beta-xylosidases.

In an embodiment the enzyme composition comprises a beta-xylosidase from *Neurospora crassa, Aspergillus fumigatus* or *Trichoderma reesei*. In an embodiment the enzyme composition comprises a beta-xylosidase from *Rasamsonia*, such as *Rasamsonia emersonii* (see WO 2014/118360).

As used herein, an endoxylanase (EC 3.2.1.8) is any polypeptide which is capable of catalysing the endohydrolysis of 1,4-β-D-xylosidic linkages in xylans. This enzyme may also be referred to as endo-1,4-β-xylanase or 1,4-β-D-xylan xylanohydrolase. An alternative is EC 3.2.1.136, a glucuronoarabinoxylan endoxylanase, an enzyme that is able to hydrolyze 1,4 xylosidic linkages in glucuronoarabinoxylans.

In an embodiment the endoxylanase comprises a GH10 xylanase. This means that at least one of the endoxylanases in the enzyme composition is a GH10 xylanase. In an embodiment all endoxylanases in the enzyme composition are GH10 xylanases.

In an embodiment the enzyme composition comprises an endoxylanase from *Aspergillus aculeatus* (see WO 94/21785), *Aspergillus fumigatus* (see WO 2006/078256), *Penicillium pinophilum* (see WO 2011/041405), *Penicillium sp.* (see WO 2010/126772), *Thielavia terrestris NRRL 8126* (see WO 2009/079210), *Talaromyces leycettanus*, *Thermobifida fusca,* or *Trichophaea saccata GH10* (see WO 2011/057083). In an embodiment the enzyme composition comprises an endoxylanase from *Rasamsonia*, such as *Rasamsonia emersonii* (see WO 02/24926).

As used herein, a beta-glucosidase (EC 3.2.1.21) is any polypeptide which is capable of catalysing the hydrolysis of terminal, non-reducing β-D-glucose residues with release of β-D-glucose. Such a polypeptide may have a wide specificity for β-D-glucosides and may also hydrolyze one or more of the following: a β-D-galactoside, an α-L-arabinoside, a β-D-xyloside or a β-D-fucoside. This enzyme may also be referred to as amygdalase, β-D-glucoside glucohydrolase, cellobiase or gentobiase.

In an embodiment the enzyme composition comprises a beta-glucosidase from *Aspergillus*, such as *Aspergillus oryzae*, such as the one disclosed in WO 02/095014 or the fusion protein having beta-glucosidase activity disclosed in WO 2008/057637, or *Aspergillus fumigatus*, such as the one disclosed as SEQ ID NO:2 in WO 2005/047499 or SEQ ID NO:5 in WO 2014/130812 or an *Aspergillus fumigatus* beta-glucosidase variant, such as one disclosed in WO 2012/044915, such as one with the following substitutions: F100D, S283G, N456E, F512Y (using SEQ ID NO: 5 in WO 2014/130812 for numbering), or *Aspergillus aculeatus, Aspergillus niger* or *Aspergillus kawachi*. In another embodiment the beta-glucosidase is derived from *Penicillium*, such as *Penicillium brasilianum* disclosed as SEQ ID NO:2 in WO 2007/019442, or from *Trichoderma*, such

as *Trichoderma reesei*, such as ones described in US 6,022,725, US 6,982,159, US 7,045,332, US 7,005,289, US 2006/0258554 US 2004/0102619. In an embodiment even a bacterial beta-glucosidase can be used. In another embodiment the beta-glucosidase is derived from *Thielavia terrestris* (WO 2011/035029) or *Trichophaea saccata* (WO 2007/019442). In an embodiment the enzyme composition comprises a beta-glucosidase from Rasamsonia, such as Rasamsonia emersonii (see WO 2012/000886).

As used herein, a cellobiohydrolase (EC 3.2.1.91) is any polypeptide which is capable of catalyzing the hydrolysis of 1,4-β-D-glucosidic linkages in cellulose or cellotetraose, releasing cellobiose from the ends of the chains. This enzyme may also be referred to as cellulase 1,4-β-cellobiosidase, 1,4-β-cellobiohydrolase, 1,4-β-D-glucan cellobiohydrolase, avicelase, exo-1,4-β-D-glucanase, exocellobiohydrolase or exoglucanase.

In an embodiment the enzyme composition comprises a cellobiohydrolase I from *Aspergillus*, such as *Aspergillus fumigatus*, such as the Cel7A CBH I disclosed in SEQ ID NO:6 in WO 2011/057140 or SEQ ID NO:6 in WO 2014/130812; from *Trichoderma*, such as *Trichoderma reesei*; from *Chaetomium*, such as *Chaetomium thermophilum*; from *Talaromyces*, such as *Talaromyces leycettanus* or from *Penicillium,* such as *Penicillium emersonii.* In an embodiment the enzyme composition comprises a cellobiohydrolase I from *Rasamsonia*, such as *Rasamsonia emersonii* (see WO 2010/122141).

In an embodiment the enzyme composition comprises a cellobiohydrolase II from *Aspergillus*, such as *Aspergillus fumigatus*, such as the one in SEQ ID NO:7 in WO 2014/130812 or from *Trichoderma*, such as *Trichoderma reesei*, or from *Talaromyces*, such as *Talaromyces leycettanus*, or from *Thielavia*, such as *Thielavia terrestris*, such as cellobiohydrolase II CEL6A from *Thielavia terrestris*. In an embodiment the enzyme composition comprises a cellobiohydrolase II from *Rasamsonia*, such as *Rasamsonia emersonii* (see WO 2011/098580).

In an embodiment the enzyme composition comprises at least two cellulases. As used herein, a cellulase is any polypeptide which is capable of degrading or modifying cellulose. The at least two cellulases may contain the same or different activities. The enzyme composition may also comprise at least one enzyme other than a cellulase, *e.g.* a hemicellulase or a pectinase. As used herein, a hemicellulase is any polypeptide which is capable of degrading or modifying hemicellulose. As used herein, a pectinase is any polypeptide which is capable of degrading or modifying pectin. The at least one other enzyme may have an auxiliary enzyme activity, *i.e.* an additional activity which, either directly or indirectly leads to lignocellulose degradation. Examples of such auxiliary activities are mentioned herein.

In an embodiment the enzyme composition as described herein comprises one, two, three, four classes or more of cellulase, for example one, two, three or four or all of a lytic polysaccharide monooxygenase (LPMO), an endoglucanase (EG), one or two cellobiohydrolases (CBH) and a beta-glucosidase (BG).

In an embodiment the enzyme composition as described herein comprises a lytic

polysaccharide monooxygenase, an endoglucanase, a cellobiohydrolase I, a cellobiohydrolase II, a beta-glucosidase, a beta-xylosidase and an endoxylanase.

In an embodiment the enzyme composition also comprises one or more of the below mentioned enzymes.

As used herein, a β-(1,3)(1,4)-glucanase (EC 3.2.1.73) is any polypeptide which is capable of catalysing the hydrolysis of 1,4-β-D-glucosidic linkages in β-D-glucans containing 1,3- and 1,4-bonds. Such a polypeptide may act on lichenin and cereal β-D-glucans, but not on β-D-glucans containing only 1,3- or 1,4-bonds. This enzyme may also be referred to as licheninase, 1,3-1,4-β-D-glucan 4-glucanohydrolase, β-glucanase, endo-β-1,3-1,4 glucanase, lichenase or mixed linkage β-glucanase. An alternative for this type of enzyme is EC 3.2.1.6, which is described as endo-1,3(4)-beta-glucanase. This type of enzyme hydrolyses 1,3- or 1,4-linkages in beta-D-glucanse when the glucose residue whose reducing group is involved in the linkage to be hydrolysed is itself substituted at C-3. Alternative names include endo-1,3-beta-glucanase, laminarinase, 1,3-(1,3;1,4)-beta-D-glucan 3 (4) glucanohydrolase. Substrates include laminarin, lichenin and cereal beta-D-glucans.

As used herein, an α-L-arabinofuranosidase (EC 3.2.1.55) is any polypeptide which is capable of acting on α-L-arabinofuranosides, α-L-arabinans containing (1,2) and/or (1,3)- and/or (1,5)-linkages, arabinoxylans and arabinogalactans. This enzyme may also be referred to as α-N-arabinofuranosidase, arabinofuranosidase or arabinosidase. Examples of arabinofuranosidases that may be comprised in the enzyme composition include, but are not limited to, arabinofuranosidases from *Aspergillus niger, Humicola insolens* DSM 1800 (see WO 2006/114094 and WO 2009/073383) and *M. giganteus* (see WO 2006/114094).

As used herein, an α-D-glucuronidase (EC 3.2.1.139) is any polypeptide which is capable of catalysing a reaction of the following form: alpha-D-glucuronoside + H(2)O = an alcohol + D-glucuronate. This enzyme may also be referred to as alpha-glucuronidase or alpha-glucosiduronase. These enzymes may also hydrolyse 4-O-methylated glucoronic acid, which can also be present as a substituent in xylans. An alternative is EC 3.2.1.131: xylan alpha-1,2-glucuronosidase, which catalyses the hydrolysis of alpha-1,2-(4-O-methyl)glucuronosyl links. Examples of alpha-glucuronidases that may be comprised in the enzyme composition include, but are not limited to, alpha-glucuronidases from *Aspergillus clavatus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus terreus*, *Humicola insolens* (see WO 2010/014706), *Penicillium aurantiogriseum* (see WO 2009/068565) and *Trichoderma reesei*.

As used herein, an acetyl xylan esterase (EC 3.1.1.72) is any polypeptide which is capable of catalysing the deacetylation of xylans and xylo-oligosaccharides. Such a polypeptide may catalyze the hydrolysis of acetyl groups from polymeric xylan, acetylated xylose, acetylated glucose, alpha-napthyl acetate or p-nitrophenyl acetate but, typically, not from triacetylglycerol. Such a polypeptide typically does not act on acetylated mannan or pectin. Examples of acetylxylan esterases that may be comprised in the enzyme composition include, but are not limited to,

acetylxylan esterases from *Aspergillus aculeatus* (see WO 2010/108918), *Chaetomium globosum*, *Chaetomium gracile*, *Humicola insolens DSM 1800* (see WO 2009/073709), *Hypocrea jecorina* (see WO 2005/001036), *Myceliophtera thermophila* (see WO 2010/014880), *Neurospora crassa*, *Phaeosphaeria nodorum* and *Thielavia terrestris* NRRL 8126 (see WO 2009/042846). In an embodiment the enzyme composition comprises an acetyl xylan esterase from *Rasamsonia*, such as *Rasamsonia emersonii* (see WO 2010/000888)

As used herein, a feruloyl esterase (EC 3.1.1.73) is any polypeptide which is capable of catalysing a reaction of the form: feruloyl-saccharide + H_2O = ferulate + saccharide. The saccharide may be, for example, an oligosaccharide or a polysaccharide. It may typically catalyse the hydrolysis of the 4-hydroxy-3-methoxycinnamoyl (feruloyl) group from an esterified sugar, which is usually arabinose in 'natural' substrates. p-nitrophenol acetate and methyl ferulate are typically poorer substrates. This enzyme may also be referred to as cinnamoyl ester hydrolase, ferulic acid esterase or hydroxycinnamoyl esterase. It may also be referred to as a hemicellulase accessory enzyme, since it may help xylanases and pectinases to break down plant cell wall hemicellulose and pectin. Examples of feruloyl esterases (ferulic acid esterases) that may be comprised in the enzyme composition include, but are not limited to, feruloyl esterases form *Humicola insolens* DSM 1800 (see WO 2009/076122), *Neosartorya fischeri*, *Neurospora crassa*, *Penicillium aurantiogriseum* (see WO 2009/127729), and *Thielavia terrestris* (see WO 2010/053838 and WO 2010/065448).

As used herein, a coumaroyl esterase (EC 3.1.1.73) is any polypeptide which is capable of catalysing a reaction of the form: coumaroyl-saccharide + H(2)O = coumarate + saccharide. The saccharide may be, for example, an oligosaccharide or a polysaccharide. This enzyme may also be referred to as trans-4-coumaroyl esterase, trans-p-coumaroyl esterase, p-coumaroyl esterase or p-coumaric acid esterase. This enzyme also falls within EC 3.1.1.73 so may also be referred to as a feruloyl esterase.

As used herein, an α-galactosidase (EC 3.2.1.22) is any polypeptide which is capable of catalysing the hydrolysis of terminal, non-reducing α-D-galactose residues in α-D-galactosides, including galactose oligosaccharides, galactomannans, galactans and arabinogalactans. Such a polypeptide may also be capable of hydrolyzing α-D-fucosides. This enzyme may also be referred to as melibiase.

As used herein, a β-galactosidase (EC 3.2.1.23) is any polypeptide which is capable of catalysing the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides. Such a polypeptide may also be capable of hydrolyzing α-L-arabinosides. This enzyme may also be referred to as exo-(1->4)-β-D-galactanase or lactase.

As used herein, a β-mannanase (EC 3.2.1.78) is any polypeptide which is capable of catalysing the random hydrolysis of 1,4-β-D-mannosidic linkages in mannans, galactomannans and glucomannans. This enzyme may also be referred to as mannan endo-1,4-β-mannosidase or endo-1,4-mannanase.

As used herein, a β-mannosidase (EC 3.2.1.25) is any polypeptide which is capable of catalysing the hydrolysis of terminal, non-reducing β-D-mannose residues in β-D-mannosides. This enzyme may also be referred to as mannanase or mannase.

As used herein, an endo-polygalacturonase (EC 3.2.1.15) is any polypeptide which is capable of catalysing the random hydrolysis of 1,4-α-D-galactosiduronic linkages in pectate and other galacturonans. This enzyme may also be referred to as polygalacturonase pectin depolymerase, pectinase, endopolygalacturonase, pectolase, pectin hydrolase, pectin polygalacturonase, poly-α-1,4-galacturonide glycanohydrolase, endogalacturonase; endo-D-galacturonase or poly(1,4-α-D-galacturonide) glycanohydrolase.

As used herein, a pectin methyl esterase (EC 3.1.1.11) is any enzyme which is capable of catalysing the reaction: pectin + n H_2O = n methanol + pectate. The enzyme may also have been known as pectinesterase, pectin demethoxylase, pectin methoxylase, pectin methylesterase, pectase, pectinoesterase or pectin pectylhydrolase.

As used herein, an endo-galactanase (EC 3.2.1.89) is any enzyme capable of catalysing the endohydrolysis of 1,4-β-D-galactosidic linkages in arabinogalactans. The enzyme may also be known as arabinogalactan endo-1,4-β-galactosidase, endo-1,4-β-galactanase, galactanase, arabinogalactanase or arabinogalactan 4-β-D-galactanohydrolase.

As used herein, a pectin acetyl esterase is defined herein as any enzyme which has an acetyl esterase activity which catalyses the deacetylation of the acetyl groups at the hydroxyl groups of GalUA residues of pectin.

As used herein, an endo-pectin lyase (EC 4.2.2.10) is any enzyme capable of catalysing the eliminative cleavage of (1→4)-α-D-galacturonan methyl ester to give oligosaccharides with 4-deoxy-6-*O*-methyl-α-D-galact-4-enuronosyl groups at their non-reducing ends. The enzyme may also be known as pectin lyase, pectin *trans*-eliminase; endo-pectin lyase, polymethylgalacturonic transeliminase, pectin methyltranseliminase, pectolyase, PL, PNL or PMGL or (1→4)-6-*O*-methyl-α-D-galacturonan lyase.

As used herein, a pectate lyase (EC 4.2.2.2) is any enzyme capable of catalysing the eliminative cleavage of (1→4)-α-D-galacturonan to give oligosaccharides with 4-deoxy-α-D-galact-4-enuronosyl groups at their non-reducing ends. The enzyme may also be known polygalacturonic transeliminase, pectic acid transeliminase, polygalacturonate lyase, endopectin methyltranseliminase, pectate transeliminase, endogalacturonate transeliminase, pectic acid lyase, pectic lyase, α-1,4-D-endopolygalacturonic acid lyase, PGA lyase, PPase-N, endo-α-1,4-polygalacturonic acid lyase, polygalacturonic acid lyase, pectin *trans*-eliminase, polygalacturonic acid *trans*-eliminase or (1→4)-α-D-galacturonan lyase.

As used herein, an alpha rhamnosidase (EC 3.2.1.40) is any polypeptide which is capable of catalysing the hydrolysis of terminal non-reducing α-L-rhamnose residues in α-L-rhamnosides or alternatively in rhamnogalacturonan. This enzyme may also be known as α-L-rhamnosidase T, α-L-rhamnosidase N or α-L-rhamnoside rhamnohydrolase.

As used herein, exo-galacturonase (EC 3.2.1.82) is any polypeptide capable of hydrolysing pectic acid from the non-reducing end, releasing digalacturonate. The enzyme may also be known as exo-poly-α-galacturonosidase, exopolygalacturonosidase or exopolygalacturanosidase.

As used herein, exo-galacturonase (EC 3.2.1.67) is any polypeptide capable of catalysing: (1,4-α-D-galacturonide)$_n$ + H_2O = (1,4-α-D-galacturonide)$_{n-1}$ + D-galacturonate. The enzyme may also be known as galacturan 1,4-α-galacturonidase, exopolygalacturonase, poly(galacturonate) hydrolase, exo-D-galacturonase, exo-D-galacturonanase, exopoly-D-galacturonase or poly(1,4-α-D-galacturonide) galacturonohydrolase.

As used herein, exopolygalacturonate lyase (EC 4.2.2.9) is any polypeptide capable of catalysing eliminative cleavage of 4-(4-deoxy-α-D-galact-4-enuronosyl)-D-galacturonate from the reducing end of pectate, i.e. de-esterified pectin. This enzyme may be known as pectate disaccharide-lyase, pectate exo-lyase, exopectic acid transeliminase, exopectate lyase, exopolygalacturonic acid-*trans*-eliminase, PATE, exo-PATE, exo-PGL or (1→4)-α-D-galacturonan reducing-end-disaccharide-lyase.

As used herein, rhamnogalacturonan hydrolase is any polypeptide which is capable of hydrolyzing the linkage between galactosyluronic acid and rhamnopyranosyl in an endo-fashion in strictly alternating rhamnogalacturonan structures, consisting of the disaccharide [(1,2-alpha-L-rhamnoyl-(1,4)-alpha-galactosyluronic acid].

As used herein, rhamnogalacturonan lyase is any polypeptide which is any polypeptide which is capable of cleaving α-L-Rha*p*-(1→4)-α-D-Gal*p*A linkages in an endo-fashion in rhamnogalacturonan by beta-elimination.

As used herein, rhamnogalacturonan acetyl esterase is any polypeptide which catalyzes the deacetylation of the backbone of alternating rhamnose and galacturonic acid residues in rhamnogalacturonan.

As used herein, rhamnogalacturonan galacturonohydrolase is any polypeptide which is capable of hydrolyzing galacturonic acid from the non-reducing end of strictly alternating rhamnogalacturonan structures in an exo-fashion.

As used herein, xylogalacturonase is any polypeptide which acts on xylogalacturonan by cleaving the β-xylose substituted galacturonic acid backbone in an *endo*-manner. This enzyme may also be known as xylogalacturonan hydrolase.

As used herein, an α-L-arabinofuranosidase (EC 3.2.1.55) is any polypeptide which is capable of acting on α-L-arabinofuranosides, α-L-arabinans containing (1,2) and/or (1,3)- and/or (1,5)-linkages, arabinoxylans and arabinogalactans. This enzyme may also be referred to as α-N-arabinofuranosidase, arabinofuranosidase or arabinosidase.

As used herein, endo-arabinanase (EC 3.2.1.99) is any polypeptide which is capable of catalysing endohydrolysis of 1,5-α-arabinofuranosidic linkages in 1,5-arabinans. The enzyme may also be known as endo-arabinase, arabinan endo-1,5-α-L-arabinosidase, endo-1,5-α-L-arabinanase, endo-α-1,5-arabanase; endo-arabanase or 1,5-α-L-arabinan 1,5-α-L-

arabinanohydrolase.

"Protease" includes enzymes that hydrolyze peptide bonds (peptidases), as well as enzymes that hydrolyze bonds between peptides and other moieties, such as sugars (glycopeptidases). Many proteases are characterized under EC 3.4 and are suitable for use in the processes as described herein. Some specific types of proteases include, cysteine proteases including pepsin, papain and serine proteases including chymotrypsins, carboxypeptidases and metalloendopeptidases.

"Lipase" includes enzymes that hydrolyze lipids, fatty acids, and acylglycerides, including phospoglycerides, lipoproteins, diacylglycerols, and the like. In plants, lipids are used as structural components to limit water loss and pathogen infection. These lipids include waxes derived from fatty acids, as well as cutin and suberin.

"Ligninase" includes enzymes that can hydrolyze or break down the structure of lignin polymers. Enzymes that can break down lignin include lignin peroxidases, manganese peroxidases, laccases and feruloyl esterases, and other enzymes described in the art known to depolymerize or otherwise break lignin polymers. Also included are enzymes capable of hydrolyzing bonds formed between hemicellulosic sugars (notably arabinose) and lignin. Ligninases include but are not limited to the following group of enzymes: lignin peroxidases (EC 1.11.1.14), manganese peroxidases (EC 1.11.1.13), laccases (EC 1.10.3.2) and feruloyl esterases (EC 3.1.1.73).

“Hexosyltransferase” (2.4.1-) includes enzymes which are capable of catalysing a transferase reaction, but which can also catalyze a hydrolysis reaction, for example of cellulose and/or cellulose degradation products. An example of a hexosyltransferase which may be used is a ß-glucanosyltransferase. Such an enzyme may be able to catalyze degradation of (1,3)(1,4)glucan and/or cellulose and/or a cellulose degradation product.

"Glucuronidase" includes enzymes that catalyze the hydrolysis of a glucuronoside, for example β-glucuronoside to yield an alcohol. Many glucuronidases have been characterized and may be suitable for use, for example β-glucuronidase (EC 3.2.1.31), hyalurono-glucuronidase (EC 3.2.1.36), glucuronosyl-disulfoglucosamine glucuronidase (3.2.1.56), glycyrrhizinate β-glucuronidase (3.2.1.128) or α-D-glucuronidase (EC 3.2.1.139).

Expansins are implicated in loosening of the cell wall structure during plant cell growth. Expansins have been proposed to disrupt hydrogen bonding between cellulose and other cell wall polysaccharides without having hydrolytic activity. In this way, they are thought to allow the sliding of cellulose fibers and enlargement of the cell wall. Swollenin, an expansin-like protein contains an N-terminal Carbohydrate Binding Module Family 1 domain (CBD) and a C-terminal expansin-like domain. As described herein, an expansin-like protein or swollenin-like protein may comprise one or both of such domains and/or may disrupt the structure of cell walls (such as disrupting cellulose structure), optionally without producing detectable amounts of reducing sugars.

A cellulose induced protein, for example the polypeptide product of the *cip1* or *cip2* gene

or similar genes (see Foreman *et al.*, J. Biol. Chem. 278(34), 31988-31997, 2003), a cellulose/cellulosome integrating protein, for example the polypeptide product of the cipA or cipC gene, or a scaffoldin or a scaffoldin-like protein. Scaffoldins and cellulose integrating proteins are multi-functional integrating subunits which may organize cellulolytic subunits into a multi-enzyme complex. This is accomplished by the interaction of two complementary classes of domain, *i.e.* a cohesion domain on scaffoldin and a dockerin domain on each enzymatic unit. The scaffoldin subunit also bears a cellulose-binding module (CBM) that mediates attachment of the cellulosome to its substrate. A scaffoldin or cellulose integrating protein may comprise one or both of such domains.

A catalase; the term "catalase" means a hydrogen-peroxide: hydrogen-peroxide oxidoreductase (EC 1.11.1.6 or EC 1.11.1.21) that catalyzes the conversion of two hydrogen peroxides to oxygen and two waters. Catalase activity can be determined by monitoring the degradation of hydrogen peroxide at 240 nm based on the following reaction: $2H_2O_2 \rightarrow 2H_2O + O_2$. The reaction is conducted in 50 mM phosphate pH 7.0 at 25°C with 10.3 mM substrate (H_20_2) and approximately 100 units of enzyme per ml. Absorbance is monitored spectrophotometrically within 16-24 seconds, which should correspond to an absorbance reduction from 0.45 to 0.4. One catalase activity unit can be expressed as one micromole of H_20_2 degraded per minute at pH 7.0 and 25°C.

The term “amylase” as used herein means enzymes that hydrolyze alpha-1,4-glucosidic linkages in starch, both in amylose and amylopectin, such as alpha-amylase (EC 3.2.1.1), beta-amylase (EC 3.2.1.2), glucan 1,4-alpha-glucosidase (EC 3.2.1.3), glucan 1,4-alpha-maltotetraohydrolase (EC 3.2.1.60), glucan 1,4-alpha-maltohexaosidase (EC 3.2.1.98), glucan 1,4-alpha-maltotriohydrolase (EC 3.2.1.116) and glucan 1,4-alpha-maltohydrolase (EC 3.2.1.133), and enzymes that hydrolyze alpha-1,6-glucosidic linkages, being the branch-points in amylopectin, such as pullulanase (EC 3.2.1.41) and limit dextinase (EC 3.2.1.142).

A composition for use in the processes as described herein may be composed of enzymes from (1) commercial suppliers; (2) cloned genes expressing enzymes; (3) broth (such as that resulting from growth of a microbial strain in media, wherein the strains secrete proteins and enzymes into the media; (4) cell lysates of strains grown as in (3); and/or (5) plant material expressing enzymes. Different enzymes in a composition of the invention may be obtained from different sources.

The enzymes can be produced either exogenously in microorganisms, yeasts, fungi, bacteria or plants, then isolated and added, for example, to lignocellulosic material. Alternatively, the enzyme may be produced in a fermentation that uses (pretreated) lignocellulosic material (such as corn stover or wheat straw) to provide nutrition to an organism that produces an enzyme(s). In this manner, plants that produce the enzymes may themselves serve as a lignocellulosic material and be added into lignocellulosic material.

In the uses and processes described herein, the components of the compositions

described above may be provided concomitantly (*i.e.* as a single composition *per se*) or separately or sequentially.

In an embodiment the enzyme composition is a whole fermentation broth of a fungus, preferably *Rasamsonia*. The whole fermentation broth can be prepared from fermentation of non-recombinant and/or recombinant filamentous fungi. In an embodiment the filamentous fungus is a recombinant filamentous fungus comprising one or more genes which can be homologous or heterologous to the filamentous fungus. In an embodiment, the filamentous fungus is a recombinant filamentous fungus comprising one or more genes which can be homologous or heterologous to the filamentous fungus wherein the one or more genes encode enzymes that can degrade a cellulosic substrate. The whole fermentation broth may comprise any of the polypeptides described above or any combination thereof.

Preferably, the enzyme composition is a whole fermentation broth wherein the cells are killed. The whole fermentation broth may contain organic acid(s) (used for killing the cells), killed cells and/or cell debris, and culture medium.

Generally, the filamentous fungi are cultivated in a cell culture medium suitable for production of enzymes capable of hydrolyzing a cellulosic substrate. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable culture media, temperature ranges and other conditions suitable for growth and cellulase and/or hemicellulase and/or pectinase production are known in the art. The whole fermentation broth can be prepared by growing the filamentous fungi to stationary phase and maintaining the filamentous fungi under limiting carbon conditions for a period of time sufficient to express the one or more cellulases and/or hemicellulases and/or pectinases. Once enzymes, such as cellulases and/or hemicellulases and/or pectinases, are secreted by the filamentous fungi into the fermentation medium, the whole fermentation broth can be used. The whole fermentation broth of the present invention may comprise filamentous fungi. In some embodiments, the whole fermentation broth comprises the unfractionated contents of the fermentation materials derived at the end of the fermentation. Typically, the whole fermentation broth comprises the spent culture medium and cell debris present after the filamentous fungi is grown to saturation, incubated under carbon-limiting conditions to allow protein synthesis (particularly, expression of cellulases and/or hemicellulases and/or pectinases). In some embodiments, the whole fermentation broth comprises the spent cell culture medium, extracellular enzymes and filamentous fungi. In some embodiments, the filamentous fungi present in whole fermentation broth can be lysed, permeabilized, or killed using methods known in the art to produce a cell-killed whole fermentation broth. In an embodiment, the whole fermentation broth is a cell-killed whole fermentation broth, wherein the whole fermentation broth containing the filamentous fungi cells are lysed or killed. In some embodiments, the cells are killed by lysing the filamentous fungi by chemical and/or pH treatment to generate the cell-killed whole broth of a fermentation of the filamentous fungi. In some embodiments, the cells are killed by lysing the filamentous fungi by

chemical and/or pH treatment and adjusting the pH of the cell-killed fermentation mix to a suitable pH. In an embodiment, the whole fermentation broth comprises a first organic acid component comprising at least one 1-5 carbon organic acid and/or a salt thereof and a second organic acid component comprising at least 6 or more carbon organic acid and/or a salt thereof. In an embodiment, the first organic acid component is acetic acid, formic acid, propionic acid, a salt thereof, or any combination thereof and the second organic acid component is benzoic acid, cyclohexanecarboxylic acid, 4-methylvaleric acid, phenylacetic acid, a salt thereof, or any combination thereof.

The term "whole fermentation broth" as used herein refers to a preparation produced by cellular fermentation that undergoes no or minimal recovery and/or purification. For example, whole fermentation broths are produced when microbial cultures are grown to saturation, incubated under carbon-limiting conditions to allow protein synthesis (*e.g.*, expression of enzymes by host cells) and secretion into cell culture medium. Typically, the whole fermentation broth is unfractionated and comprises spent cell culture medium, extracellular enzymes, and microbial, preferably non-viable, cells.

If needed, the whole fermentation broth can be fractionated and the one or more of the fractionated contents can be used. For instance, the killed cells and/or cell debris can be removed from a whole fermentation broth to provide a composition that is free of these components.

The whole fermentation broth may further comprise a preservative and/or anti-microbial agent. Such preservatives and/or agents are known in the art.

The whole fermentation broth as described herein is typically a liquid, but may contain insoluble components, such as killed cells, cell debris, culture media components, and/or insoluble enzyme(s). In some embodiments, insoluble components may be removed to provide a clarified whole fermentation broth.

In an embodiment, the whole fermentation broth may be supplemented with one or more enzyme activities that are not expressed endogenously, or expressed at relatively low level by the filamentous fungi, to improve the degradation of the cellulosic substrate, for example, to fermentable sugars such as glucose or xylose. The supplemental enzyme(s) can be added as a supplement to the whole fermentation broth and the enzymes may be a component of a separate whole fermentation broth, or may be purified, or minimally recovered and/or purified.

In an embodiment, the whole fermentation broth comprises a whole fermentation broth of a fermentation of recombinant filamentous fungi overexpressing one or more enzymes to improve the degradation of the cellulosic substrate. Alternatively, the whole fermentation broth can comprise a mixture of a whole fermentation broth of a fermentation of a non-recombinant filamentous fungus and a recombinant filamentous fungus overexpressing one or more enzymes to improve the degradation of the cellulosic substrate. In an embodiment, the whole fermentation broth comprises a whole fermentation broth of a fermentation of filamentous fungi overexpressing beta-glucosidase. Alternatively, the whole fermentation broth for use in the present processes can comprise a mixture

of a whole fermentation broth of a fermentation of a non-recombinant filamentous fungus and a whole fermentation broth of a fermentation of recombinant filamentous fungi overexpressing a beta-glucosidase.

Lignocellulosic material as used herein includes any lignocellulosic and/or hemicellulosic material. Lignocellulosic material suitable for use in the processes as described herein includes biomass, *e.g.* virgin biomass and/or non-virgin biomass such as agricultural biomass, commercial organics, construction and demolition debris, municipal solid waste, waste paper and yard waste. Common forms of biomass include trees, shrubs and grasses, wheat, wheat straw, sugar cane, cane straw, sugar cane bagasse, switch grass, miscanthus, energy cane, corn, corn stover, corn husks, corn cobs, corn fiber, corn kernels, canola stems, soybean stems, sweet sorghum, products and by-products from milling of grains such as corn, wheat and barley (including wet milling and dry milling) often called “bran or fibre”, distillers dried grains, as well as municipal solid waste, waste paper and yard waste. The biomass 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. "Agricultural biomass" includes branches, bushes, canes, corn and corn husks, energy crops, forests, fruits, flowers, grains, grasses, herbaceous crops, leaves, bark, needles, logs, roots, saplings, short rotation woody crops, shrubs, switch grasses, trees, vegetables, fruit peels, vines, sugar beet pulp, wheat midlings, oat hulls, and hard and soft woods (not including woods with deleterious materials). In addition, agricultural biomass includes organic waste materials generated from agricultural processes including farming and forestry activities, specifically including forestry wood waste. Agricultural biomass may be any of the afore-mentioned singularly or in any combination or mixture thereof.

In an embodiment the lignocellulosic material is pretreated before and/or during the enzymatic hydrolysis. In an embodiment the lignocellulosic material is pretreated before and/or during step (a) of the processes as described in the present application. Pretreatment methods are known in the art and include, but are not limited to, heat, mechanical, chemical modification, biological modification and any combination thereof. Pretreatment is typically performed in order to enhance the accessibility of the lignocellulosic material to enzymatic hydrolysis and/or hydrolyse the hemicellulose and/or solubilize the hemicellulose and/or cellulose and/or lignin, in the lignocellulosic material. In an embodiment, the pretreatment comprises treating the lignocellulosic material with steam explosion, hot water treatment or treatment with dilute acid or dilute base. Examples of pretreatment methods include, but are not limited to, steam treatment (*e.g.* treatment at 100-260ºC, at a pressure of 7-45 bar, at neutral pH, for 1-10 minutes), dilute acid treatment (*e.g.* treatment with 0.1 – 5% H_2SO_4 and/or SO_2 and/or HNO_3 and/or HCl, in presence or absence of steam, at 120-200ºC, at a pressure of 2-15 bar, at acidic pH, for 2-30 minutes), organosolv treatment (*e.g.* treatment with 1 – 1.5% H_2SO_4 in presence of organic solvent and steam, at 160-200ºC, at a pressure of 7-30 bar, at acidic pH, for 30-60 minutes), lime treatment (*e.g.* treatment with 0.1 - 2% NaOH/$Ca(OH)_2$ in the presence of water/steam at 60-160ºC, at a pressure of 1-10

bar, at alkaline pH, for 60-4800 minutes), ARP treatment (*e.g.* treatment with 5 - 15% NH_3, at 150-180ºC, at a pressure of 9-17 bar, at alkaline pH, for 10-90 minutes), AFEX treatment (*e.g.* treatment with > 15% NH_3, at 60-140ºC, at a pressure of 8-20 bar, at alkaline pH, for 5-30 minutes).

The lignocellulosic material may be washed. In an embodiment the lignocellulosic material may be washed after the pretreatment. The washing step may be used to remove water soluble compounds that may act as inhibitors for the fermentation and/or hydrolysis step. The washing step may be conducted in manner known to the skilled person. Next to washing, other detoxification methods do exist. The lignocellulosic material may also be detoxified by any (or any combination) of these methods which include, but are not limited to, solid/liquid separation, vacuum evaporation, extraction, adsorption, neutralization, overliming, addition of reducing agents, addition of detoxifying enzymes such as laccases or peroxidases, addition of microorganisms capable of detoxification of hydrolysates.

In an embodiment the enzymatically hydrolysed lignocellulosic material is washed and/or detoxified. In an embodiment the solid fraction and/or the liquid fraction obtained after solid/liquid separation of the enzymatically hydrolysed lignocellulosic material is washed and/or detoxified. In an embodiment the liquid fraction obtained after solid/liquid separation of the enzymatically hydrolysed lignocellulosic material is subjected to a detoxification step and/or a concentration step. The detoxification step can be done by any of the methods as described above. The concentration step can be done by methods well known to a person skilled in the art including, but not limited to, centrifugation.

The enzyme composition used in the process as described herein can extremely effectively hydrolyze lignocellulosic material, for example corn stover, wheat straw, cane straw, and/or sugar cane bagasse, which can then be further converted into a product, such as ethanol, biogas, butanol, a plastic, an organic acid, a solvent, an animal feed supplement, a pharmaceutical, a vitamin, an amino acid, an enzyme or a chemical feedstock. Additionally, intermediate products from a process following the hydrolysis, for example lactic acid as intermediate in biogas production, can be used as building block for other materials.

In an embodiment the amount of enzyme added (herein also called enzyme dosage or enzyme load) is low. In an embodiment the amount of enzyme is 10 mg protein / g dry matter weight or lower, 9 mg protein / g dry matter weight or lower, 8 mg protein / g dry matter weight or lower, 7 mg protein / g dry matter weight or lower, 6 mg protein / g dry matter weight or lower, 5 mg protein / g dry matter or lower, 4 mg protein / g dry matter or lower, 3 mg protein / g dry matter or lower, 2 mg protein / g dry matter or lower, or 1 mg protein / g dry matter or lower (expressed as protein in mg protein / g dry matter). In an embodiment, the amount of enzyme is 5 mg enzyme / g dry matter weight or lower, 4 mg enzyme / g dry matter weight or lower, 3 mg enzyme / g dry matter weight or lower, 2 mg enzyme / g dry matter weight or lower, 1 mg enzyme / g dry matter weight or lower, 0.5 mg enzyme / g dry matter weight or lower, 0.4 mg enzyme composition / g dry matter weight or lower, 0.3 mg enzyme / g dry matter weight or lower, 0.25 mg enzyme / g dry matter weight or

lower, 0.20 mg enzyme / g dry matter weight or lower, 0.18 mg enzyme / g dry matter weight or lower, 0.15 mg enzyme / g dry matter weight or lower or 0.10 mg enzyme / g dry matter weight or lower (expressed as total of cellulase enzymes in mg enzyme / g dry matter). A low enzyme dosage is possible, because of the activity and stability of the enzymes. When the enzymatic hydrolysis comprises a separate liquefaction step and a saccharification step, enzyme may be added before and/or during only one of the steps or before and/or during both steps. The amount given above are amounts as measured by Bradford protein assay.

The pH during the enzymatic hydrolysis may be chosen by the skilled person. In an embodiment the pH during the hydrolysis may be 3.0 to 6.4. The stable enzymes of the invention may have a broad pH range of up to 2 pH units, up to 3 pH units, up to 5 pH units. The optimum pH may lie within the limits of pH 2.0 to 8.0, 2.5 to 7.5, 3.0 to 7.0, 3.5 to 6.5, 4.0 to 5.0, 4.0 to 4.5 or is about 4.2. The pH used in the liquefaction step of the enzymatic hydrolysis and the saccharification step of the enzymatic hydrolysis may differ or may be the same. In case different enzymes are used during the liquefaction step and the saccharification step, the optimum pH of said enzymes may differ or may be the same.

In an embodiment the hydrolysis step is conducted until 70% or more, 80% or more, 85% or more, 90% or more, 92% or more, 95% or more of available sugar in the lignocellulosic material is released.

Significantly, the processes as described herein may be carried out using high levels of dry matter (of the lignocellulosic material) in the hydrolysis reaction. In an embodiment the dry matter content at the end of the enzymatic hydrolysis is 5 wt% or higher, 6 wt% or higher, 7 wt% or higher, 8 wt% or higher, 9 wt% or higher, 10 wt% or higher, 11 wt% or higher, 12 wt% or higher, 13 wt% or higher, 14 wt% or higher, 15 wt% or higher, 16 wt% or higher, 17 wt% or higher, 18 wt% or higher, 19 wt% or higher, 20 wt% or higher, 21 wt% or higher, 22 wt% or higher, 23 wt% or higher, 24 wt% or higher, 25 wt% or higher, 26 wt% or higher, 27 wt% or higher, 28 wt% or higher, 29 wt% or higher, 30 wt% or higher, 31 wt% or higher, 32 wt% or higher, 33 wt% or higher, 34 wt% or higher, 35 wt% or higher, 36 wt% or higher, 37 wt% or higher, 38 wt% or higher or 39 wt% or higher. In an embodiment the dry matter content at the end of the enzymatic hydrolysis is between 5 wt% - 40 wt%, 6 wt% - 40 wt%, 7 wt% - 40 wt%, 8 wt% - 40 wt%, 9 wt% - 40 wt%, 10 wt% - 40 wt%, 11 wt% - 40 wt%, 12 wt% - 40 wt%, 13 wt% - 40 wt%, 14 wt% - 40 wt%, 15 wt% - 40 wt%, 16 wt% - 40 wt%, 17 wt% - 40 wt%, 18 wt% - 40 wt%, 19 wt% - 40 wt%, 20 wt% - 40 wt%, 21 wt% - 40 wt%, 22 wt% - 40 wt%, 23 wt% - 40 wt%, 24 wt% - 40 wt%, 25 wt% - 40 wt%, 26 wt% - 40 wt%, 27 wt% - 40 wt%, 28 wt% - 40 wt%, 29 wt% - 40 wt%, 30 wt% - 40 wt%, 31 wt% - 40 wt%, 32 wt% - 40 wt%, 33 wt% - 40 wt%, 34 wt% - 40 wt%, 35 wt% - 40 wt%, 36 wt% - 40 wt%, 37 wt% - 40 wt%, 38 wt% - 40 wt%, 39 wt% - 40 wt%.

In an embodiment the dry matter content at the end of the liquefaction step of the enzymatic hydrolysis is 5 wt% or higher, 6 wt% or higher, 7 wt% or higher, 8 wt% or higher, 9 wt% or higher, 10 wt% or higher, 11 wt% or higher, 12 wt% or higher, 13 wt% or higher, 14 wt% or

higher, 15 wt% or higher, 16 wt% or higher, 17 wt% or higher, 18 wt% or higher, 19 wt% or higher, 20 wt% or higher, 21 wt% or higher, 22 wt% or higher, 23 wt% or higher, 24 wt% or higher, 25 wt% or higher, 26 wt% or higher, 27 wt% or higher, 28 wt% or higher, 29 wt% or higher, 30 wt% or higher, 31 wt% or higher, 32 wt% or higher, 33 wt% or higher, 34 wt% or higher, 35 wt% or higher, 36 wt% or higher, 37 wt% or higher, 38 wt% or higher or 39 wt% or higher. In an embodiment the dry matter content at the end of the liquefaction step of the enzymatic hydrolysis is between 5 wt% - 40 wt%, 6 wt% - 40 wt%, 7 wt% - 40 wt%, 8 wt% - 40 wt%, 9 wt% - 40 wt%, 10 wt% - 40 wt%, 11 wt% - 40 wt%, 12 wt% - 40 wt%, 13 wt% - 40 wt%, 14 wt% - 40 wt%, 15 wt% - 40 wt%, 16 wt% - 40 wt%, 17 wt% - 40 wt%, 18 wt% - 40 wt%, 19 wt% - 40 wt%, 20 wt% - 40 wt%, 21 wt% - 40 wt%, 22 wt% - 40 wt%, 23 wt% - 40 wt%, 24 wt% - 40 wt%, 25 wt% - 40 wt%, 26 wt% - 40 wt%, 27 wt% - 40 wt%, 28 wt% - 40 wt%, 29 wt% - 40 wt%, 30 wt% - 40 wt%, 31 wt% - 40 wt%, 32 wt% - 40 wt%, 33 wt% - 40 wt%, 34 wt% - 40 wt%, 35 wt% - 40 wt%, 36 wt% - 40 wt%, 37 wt% - 40 wt%, 38 wt% - 40 wt%, 39 wt% - 40 wt%.

In an embodiment the dry matter content at the end of the saccharification step of the enzymatic hydrolysis is 5 wt% or higher, 6 wt% or higher, 7 wt% or higher, 8 wt% or higher, 9 wt% or higher, 10 wt% or higher, 11 wt% or higher, 12 wt% or higher, 13 wt% or higher, 14 wt% or higher, 15 wt% or higher, 16 wt% or higher, 17 wt% or higher, 18 wt% or higher, 19 wt% or higher, 20 wt% or higher, 21 wt% or higher, 22 wt% or higher, 23 wt% or higher, 24 wt% or higher, 25 wt% or higher, 26 wt% or higher, 27 wt% or higher, 28 wt% or higher, 29 wt% or higher, 30 wt% or higher, 31 wt% or higher, 32 wt% or higher, 33 wt% or higher, 34 wt% or higher, 35 wt% or higher, 36 wt% or higher, 37 wt% or higher, 38 wt% or higher or 39 wt% or higher. In an embodiment the dry matter content at the end of the saccharification step of the enzymatic hydrolysis is between 5 wt% - 40 wt%, 6 wt% - 40 wt%, 7 wt% - 40 wt%, 8 wt% - 40 wt%, 9 wt% - 40 wt%, 10 wt% - 40 wt%, 11 wt% - 40 wt%, 12 wt% - 40 wt%, 13 wt% - 40 wt%, 14 wt% - 40 wt%, 15 wt% - 40 wt%, 16 wt% - 40 wt%, 17 wt% - 40 wt%, 18 wt% - 40 wt%, 19 wt% - 40 wt%, 20 wt% - 40 wt%, 21 wt% - 40 wt%, 22 wt% - 40 wt%, 23 wt% - 40 wt%, 24 wt% - 40 wt%, 25 wt% - 40 wt%, 26 wt% - 40 wt%, 27 wt% - 40 wt%, 28 wt% - 40 wt%, 29 wt% - 40 wt%, 30 wt% - 40 wt%, 31 wt% - 40 wt%, 32 wt% - 40 wt%, 33 wt% - 40 wt%, 34 wt% - 40 wt%, 35 wt% - 40 wt%, 36 wt% - 40 wt%, 37 wt% - 40 wt%, 38 wt% - 40 wt%, 39 wt% - 40 wt%.

As described above, the present invention also relates to a process for the preparation of a fermentation product from lignocellulosic material, comprising the steps of (a) performing a process for the preparation of a sugar product from lignocellulosic material as described above, (b) fermenting the hydrolysed lignocellulosic material to produce the fermentation product; and (c) optionally, recovering the fermentation product.

In an embodiment the fermentation is performed in one or more bioreacors. In an embodiment the fermentation is done by an alcohol producing microorganism to produce alcohol. The fermentation can be done in the same bioreactor wherein the liquefaction and/or saccharification is performed. Alternatively, the fermentation can be performed in one or more

separate bioreactors.

In an embodiment the fermentation is done by a yeast. In an embodiment the alcohol producing microorganism is a yeast. In an embodiment the alcohol producing microorganism is able to ferment at least a C5 sugar and at least a C6 sugar.

In a further aspect, the invention thus includes fermentation processes in which a microorganism is used for the fermentation of a carbon source comprising sugar(s), *e.g.* glucose, L-arabinose and/or xylose. The carbon source may include any carbohydrate oligo- or polymer comprising L-arabinose, xylose or glucose units, such as *e.g.* lignocellulose, xylans, cellulose, starch, arabinan and the like. For release of xylose or glucose units from such carbohydrates, appropriate carbohydrases (such as xylanases, glucanases, amylases and the like) may be added to the fermentation medium or may be produced by the modified host cell. In the latter case, the modified host cell may be genetically engineered to produce and excrete such carbohydrases. An additional advantage of using oligo- or polymeric sources of glucose is that it enables to maintain a low(er) concentration of free glucose during the fermentation, *e.g.* by using rate-limiting amounts of the carbohydrases. This, in turn, will prevent repression of systems required for metabolism and transport of non-glucose sugars such as xylose. In a preferred process the modified host cell ferments both the L-arabinose (optionally xylose) and glucose, preferably simultaneously in which case preferably a modified host cell is used which is insensitive to glucose repression to prevent diauxic growth. In addition to a source of L-arabinose, optionally xylose (and glucose) as carbon source, the fermentation medium will further comprise the appropriate ingredient required for growth of the modified host cell. Compositions of fermentation media for growth of microorganisms such as yeasts or filamentous fungi are well known in the art.

The fermentation process may be an aerobic or an anaerobic fermentation process. An anaerobic fermentation process is herein defined as a fermentation process run in the absence of oxygen or in which substantially no oxygen is consumed, preferably less than 5, 2.5 or 1 mmol/L/h, more preferably 0 mmol/L/h is consumed (*i.e.* oxygen consumption is not detectable), and wherein organic molecules serve as both electron donor and electron acceptors. In the absence of oxygen, NADH produced in glycolysis and biomass formation, cannot be oxidised by oxidative phosphorylation. To solve this problem many micro-organisms use pyruvate or one of its derivatives as an electron and hydrogen acceptor thereby regenerating NAD^+. Thus, in a preferred anaerobic fermentation process pyruvate is used as an electron (and hydrogen acceptor) and is reduced to fermentation products such as ethanol, lactic acid, 3-hydroxy-propionic acid, acrylic acid, acetic acid, succinic acid, citric acid, malic acid, fumaric acid, an amino acid, 1,3-propane-diol, ethylene, glycerol, butanol, a β-lactam antibiotic and a cephalosporin. In a preferred embodiment, the fermentation process is anaerobic. An anaerobic process is advantageous, since it is cheaper than aerobic processes: less special equipment is needed. Furthermore, anaerobic processes are expected to give a higher product yield than aerobic processes. Under aerobic conditions, usually the biomass yield is higher than under anaerobic conditions. As a consequence, usually under

aerobic conditions, the expected product yield is lower than under anaerobic conditions.

In another embodiment, the fermentation process is under oxygen-limited conditions. More preferably, the fermentation process is aerobic and under oxygen-limited conditions. An oxygen-limited fermentation process is a process in which the oxygen consumption is limited by the oxygen transfer from the gas to the liquid. The degree of oxygen limitation is determined by the amount and composition of the ingoing gas flow as well as the actual mixing/mass transfer properties of the fermentation equipment used. Preferably, in a process under oxygen-limited conditions, the rate of oxygen consumption is at least 5.5, more preferably at least 6 and even more preferably at least 7 mmol/L/h. In an embodiment the alcohol fermentation process is anaerobic.

The fermentation process is preferably run at a temperature that is optimal for the microorganism used. Thus, for most yeasts or fungal cells, the fermentation process is performed at a temperature which is less than 42ºC, preferably 38ºC or lower. For yeast or filamentous fungal host cells, the fermentation process is preferably performed at a temperature which is lower than 35, 33, 30 or 28ºC and at a temperature which is higher than 20, 22, or 25ºC. In an embodiment the alcohol fermentation step is performed between 25ºC and 35ºC.

In an embodiment the fermentations are conducted with a fermenting microorganism. In an embodiment of the invention, the alcohol (*e.g.* ethanol) fermentations of C5 sugars are conducted with a C5 fermenting microorganism. In an embodiment of the invention, the alcohol (*e.g.* ethanol) fermentations of C6 sugars are conducted with a C5 fermenting microorganism or a commercial C6 fermenting microorganism. Commercially available yeast suitable for ethanol production include, but are not limited to, BIOFERM™ AFT and XR (NABC—North American Bioproducts Corporation, GA, USA), ETHANOL RED™ yeast (Fermentis/Lesaffre, USA), FALI™ (Fleischmann's Yeast, USA), FERMIOL™ (DSM Specialties), GERT STRAND™ (Gert Strand AB, Sweden), and SUPERSTART™ and THERMOSACC™ fresh yeast (Ethanol Technology, WI, USA).

In an embodiment the alcohol producing microorganism is a microorganism that is able to ferment at least one C5 sugar. Preferably, it also is able to ferment at least one C6 sugar. In an embodiment the application describes a process for the preparation of ethanol from lignocellulosic material, comprising the steps of (a) performing a process for the preparation of a sugar product from lignocellulosic material as described above, (b) fermentation of the sugar product to produce ethanol; and (c) optionally, recovery of the ethanol. The fermentation can be done with a microorganism that is able to ferment at least one C5 sugar.

The alcohol producing microorganisms may be a prokaryotic or eukaryotic organism. The microorganism used in the process may be a genetically engineered microorganism. Examples of suitable alcohol producing organisms are yeasts, for instance *Saccharomyces, e.g. Saccharomyces cerevisiae, Saccharomyces pastorianus or Saccharomyces uvarum, Hansenula, Issatchenkia*, *e.g. Issatchenkia orientalis, Pichia*, *e.g. Pichia stipites* or *Pichia pastoris, Kluyveromyces, e.g. Kluyveromyces fagilis*, *Candida, e.g. Candida pseudotropicalis* or *Candida*

acidothermophilum, Pachysolen, e.g. Pachysolen tannophilus or bacteria, for instance *Lactobacillus, e.g. Lactobacillus lactis, Geobacillus, Zymomonas, e.g. Zymomonas mobilis, Clostridium, e.g. Clostridium phytofermentans, Escherichia, e.g. E. coli, Klebsiella, e.g. Klebsiella oxytoca*. In an embodiment the microorganism that is able to ferment at least one C5 sugar is a yeast. In an embodiment, the yeast is belongs to the genus *Saccharomyces*, preferably of the species *Saccharomyces cerevisiae*. The yeast, *e.g. Saccharomyces cerevisiae,* used in the processes according to the present invention is capable of converting hexose (C6) sugars and pentose (C5) sugars. The yeast, *e.g. Saccharomyces cerevisiae,* used in the processes according to the present invention can anaerobically ferment at least one C6 sugar and at least one C5 sugar. For example, the yeast is capable of using L-arabinose and xylose in addition to glucose anaerobically. In an embodiment, the yeast is capable of converting L-arabinose into L-ribulose and/or xylulose 5-phosphate and/or into a desired fermentation product, for example into ethanol. Organisms, for example *Saccharomyces cerevisiae* strains, able to produce ethanol from L-arabinose may be produced by modifying a host yeast introducing the *araA* (L-arabinose isomerase), *araB* (L-ribuloglyoxalate) and *araD* (L-ribulose-5-P4-epimerase) genes from a suitable source. Such genes may be introduced into a host cell in order that it is capable of using arabinose. Such an approach is given is described in WO 2003/095627. *araA*, *araB* and *araD* genes from *Lactobacillus plantarum* may be used and are disclosed in WO 2008/041840. The *araA* gene from *Bacillus subtilis* and the *araB* and *araD* genes from *Escherichia coli* may be used and are disclosed in EP1499708. In another embodiment, *araA*, *araB* and *araD* genes may derived from of at least one of the genus *Clavibacter*, *Arthrobacter* and/or *Gramella*, in particular one of *Clavibacter michiganensis, Arthrobacter aurescens,* and/or *Gramella forsetii,* as disclosed in WO 2009/011591. In an embodiment, the yeast may also comprise one or more copies of xylose isomerase gene and/or one or more copies of xylose reductase and/or xylitol dehydrogenase.

The yeast may comprise one or more genetic modifications to allow the yeast to ferment xylose. Examples of genetic modifications are introduction of one or more *xylA*-gene, *XYL1* gene and *XYL2* gene and/or *XKS1*-gene; deletion of the aldose reductase (*GRE3*) gene; overexpression of PPP-genes *TAL1*, *TKL1*, *RPE1* and *RKI1* to allow the increase of the flux through the pentose phosphate pathway in the cell. Examples of genetically engineered yeast are described in EP1468093 and/or WO2006/009434.

An example of a suitable commercial yeast is RN1016 that is a xylose and glucose fermenting *Saccharomyces cerevisiae* strain from DSM, the Netherlands.

In an embodiment, the fermentation process for the production of ethanol is anaerobic. Anaerobic has already been defined earlier herein. In another preferred embodiment, the fermentation process for the production of ethanol is aerobic. In another preferred embodiment, the fermentation process for the production of ethanol is under oxygen-limited conditions, more preferably aerobic and under oxygen-limited conditions. Oxygen-limited conditions have already been defined earlier herein.

Alternatively, to the fermentation processes described above, at least two distinct cells may be used, this means this process is a co-fermentation process. All preferred embodiments of the fermentation processes as described above are also preferred embodiments of this co-fermentation process: identity of the fermentation product, identity of source of L-arabinose and source of xylose, conditions of fermentation (aerobic or anaerobic conditions, oxygen-limited conditions, temperature at which the process is being carried out, productivity of ethanol, yield of ethanol).

Fermentation products that may be produced by the processes of the invention can be any substance derived from fermentation. They include, but are not limited to, alcohol (such as arabinitol, butanol, ethanol, glycerol, methanol, 1,3-propanediol, sorbitol, and xylitol); organic acid (such as acetic acid, acetonic acid, adipic acid, ascorbic acid, acrylic acid, citric acid, 2,5-diketo-D-gluconic acid, formic acid, fumaric acid, glucaric acid, gluconic acid, glucuronic acid, glutaric acid, 3- hydroxypropionic acid, itaconic acid, lactic acid, maleic acid, malic acid, malonic acid, oxalic acid, oxaloacetic acid, propionic acid, succinic acid, and xylonic acid); ketones (such as acetone); amino acids (such as aspartic acid, glutamic acid, glycine, lysine, serine, tryptophan, and threonine); alkanes (such as pentane, hexane, heptane, octane, nonane, decane, undecane, and dodecane), cycloalkanes (such as cyclopentane, cyclohexane, cycloheptane, and cyclooctane), alkenes (such as pentene, hexene, heptene, and octene); and gases (such as methane, hydrogen (H_2), carbon dioxide ($C0_2$), and carbon monoxide (CO)). The fermentation product can also be a protein, a vitamin, a pharmaceutical, an animal feed supplement, a specialty chemical, a chemical feedstock, a plastic, a solvent, ethylene, an enzyme, such as a protease, a cellulase, an amylase, a glucanase, a lactase, a lipase, a lyase, an oxidoreductase, a transferase or a xylanase. In a preferred embodiment an alcohol is prepared in the fermentation processes as described herein. In a preferred embodiment ethanol is prepared in the fermentation processes as described herein.

The processes as described herein may comprise recovery of all kinds of products made during the processes including fermentation products such as ethanol. A fermentation product may be separated from the fermentation broth in manner know to the skilled person. Examples of techniques for recovery include, but are not limited to, chromatography, electrophoretic procedures, differential solubility, distillation, or extraction. For each fermentation product, the skilled person will thus be able to select a proper separation technique. For instance, ethanol may be separated from a yeast fermentation broth by distillation, for instance steam distillation/vacuum distillation in conventional way.

EXAMPLES

Example 1

The influence of process conditions on volatiles removal during the enzymatic hydrolysis of lignocellulosic feedstock

The enzymatic hydrolysis was performed using acid pretreated cornstover (aCS) feedstock at a concentration of 17 % (w/w) dry matter (DM). The feedstock solution was prepared by the dilution of concentrated feedstock slurry with water. The pH was adjusted to pH 4.5 with a 10% (w/w) NH_4OH-solution. The enzymatic hydrolysis was performed at 1 kg scale using a 1.5 liter reactor. The pH was controlled at 4.5 and the temperature was controlled at 62°C. The dissolved oxygen during the process was controlled by headspace gas recycling and additional fresh air (containing 20 - 21% oxygen).

Prior to enzyme addition, headspace gas was recylced at a gas flow of 3 l/hour using a peristaltic pump and a sparger. Due to the fact that the feedstock consumes oxygen through a chemical reaction, the DO level reached a level of 0% DO within one hour resulting in an anaerobic feedstock and a headspace which was completely depleted from oxygen. The resulting inert headspace gas (oxygen free) was used throughout the entire hydrolysis as carrier gas for the introduced fresh air.

Next, the *Talaromyces emersonii* cellulose enzyme cocktail TEC-210 was added to the feedstock at a dosage of 3.75 mg (TCA/Biuret protein)/g dm. TEC-210 was produced according to the inoculation and fermentation procedures described in WO 2011/000949. The total hydrolysis time was 120 hours.

Fresh air was introduced into the recycle loop of inert headspace gas during the entire hydrolysis process at a fresh air flow of 60 ml per kg reaction mixture per hour, starting directly after enzyme addition.

The following enzymatic hydrolysis processes were conducted:

a) Headspeace gas recycle at a speed of 3 l/hour during the entire enzymatic hydrolysis process in conbination with a fresh air flow introduced in the gas recycle stream at a speed of 60 ml/hour.
b) As experiment (a) but having an active carbon filter (Whatman™ 6722-1001 VACU-GUARD™ 150 Vacuum Protection In-Line Filter Capsule with 0.2µm PTFE Membrane & Activated Carbon Trap, 150mm) in the gas recycle stream to reduce volatiles in the recycled gas stream.
c) A control experiment with neither gas recycle nor fresh air introduction.

Samples were drawn at the start and end of each enzymatic hydrolysis process and directly centrifuged for 10 minutes at 5000xg for analysis of volatiles (e.g. furfural and hydroxymethylfurfural (HMF)) in the supernatant by means of HPLC.

Furfural and HMF concentrations were measured as follows: After centrifugation, the supernatant was subsequently filtered (0.2 µm filter, Whatman PN UN203NPENYL mini uniprep) and the filtrates were analysed for sugars, organic caids, furfural and HMF. Furfural and HMF concentrations of the diluted samples were measured using an HPLC equipped with an Aminex HPX-87H column (Biorad) by elution with 0.005 M sulphuric acid at 65°C at a flow rate of 0.55 ml per minute and quantified by integration of the signals from refractive index detection (R.I.) calibrated with furfural and HMF standard solutions.

The results are presented in Table 1. The results clearly indicate a considerable furfural and HMF reduction in case the gas recyle was used. They further show an even more efficient furfural and HMF reduction in case means for removing volatiles from the gas in the headspace were used.

Example 2

The effect of the furfural concentration in hydrolysed lignocellulosic materials on yeast propagation

Hydrolysed lignocellulosic material is produced as described in Example 1, experiment (b). The hydrolysate is centrifuged to remove insoluble solids.

The resulting supernatant is enriched with furfural up to concentrations of 0.6 – 1.0 – 1.5 – 2.0 – 2.5 – 3.0 – 4.0 – 5.0 g/l. The pH of each mixture is adjusted to pH 7.0 using KOH. The hydrolysates containing different concentrations of furfural are used for yeast propagation according to the following procedure.

Yeast propagation procedure**:** First, 900 ml solution is prepared which contains 25 g of Bacto Yeast Extract (DIFCO) and 50 g of Bacto or Phytone Peptone (DIFCO) in demineralized water. The pH is corrected to 7.0 using KOH or H_2SO_4. Next, demineralized water is added to a total weight of 1000 g. Finally, the solution is sterilised for 15 minutes at 110 ºC and cooled down to room temperature. Next, 40 g of the above described mixture is added to 60 g of each hydrolysate containing different concentrations of furfural (prepared as described above).These mixtures are called inoculation media. Inoculation of the media (containing different concentrations of furfural) is done under aseptic conditions (flow cabinet or flame) by addition of 0.1 ml of yeast culture from glycerol stock. The cultures are incubated for 24 hours at 30ºC in a shaking incubator at 200 rpm. Finally, the pH, OD_{600nm} and the dry weight are measured for every incubation. For a good propagation, the final pH should be 4.5 – 5.0, the OD_{600} should be 18 +/- 3 and the dry weight should be 4 – 6 g/kg.

The results indicate a reduced yeast propagation performance at furfural levels of > 1 g/l in the hydrolysate, *i.e.* a reduced OD_{600} and a reduced dry weight which both are indicative for yeast growth.

Furthermore, the following experiment was performed. Hydrolysed lignocellulosic material was produced as described in Example 1, experiment (b). The produced hydrolysate contained 0.46 g/l furfural at the start of this experiment. The hydrolysate was centrifuged to remove insoluble solids.

The resulting supernatant was enriched with furfural (99% pure, from Acros Organics) up to concentrations of 0.61 – 0.88 – 1.34 – 1.82 – 2.27 – 2.70 – 3.70 – 4.60 g/l. The pH of each mixture was adjusted to pH 7.0 using KOH. The hydrolysates containing different concentrations of furfural were used for yeast propagation according to the following procedure.

Yeast propagation procedure**:** First, 900 ml solution was prepared which contains 25 g of Bacto Yeast Extract (DIFCO) and 50 g of Bacto or Phytone Peptone (DIFCO) in demineralized

water. The pH was corrected to 7.0 using KOH or H_2SO_4. Next, demineralized water was added to a total weight of 1000 g. Finally, the solution was sterilised by filtration on a 0.45 µm filter. Next, 40 g of the above described mixture was added to 60 g of each hydrolysate containing different concentrations of furfural (prepared as described above).These mixtures were called inoculation media. Inoculation of the media (containing different concentrations of furfural) was done under aseptic conditions (flow cabinet or flame) by addition of 0.1 ml of yeast culture from glycerol stock (strain RN1016). The cultures were incubated for 24 hours at 30ºC in a shaking incubator at 200 rpm. Finally, the pH, OD_{600nm} and the dry weight were measured for every incubation.

The results are presented in Table 2. The results indicate a reduced yeast propagation performance at furfural levels of 0.88 g/l and above in the hydrolysate, *i.e.* a reduced OD_{600} and a reduced dry weight which both are indicative for yeast growth. So, volatiles removal as described herein is beneficial for yeast propagation.

Example 3

The effect of the furfural concentration in hydrolysed lignocellulosic materials on fermentation of sugars in these hydrolysed feedstocks

The following procedures are done.

Feedstock preparation: Hydrolysed lignocellulosic material is produced as described in Example 1, experiment (b). The hydrolysate is enriched with furfural up to concentrations of 0.6 – 1.0 – 1.5 – 2.0 – 2.5 – 3.0 – 4.0 – 5.0 g/l. The pH of each mixture is adjusted to pH 5.5 using KOH. The hydrolysates containing different concentrations of furfural are used as substrate in ethanol fermentations of sugars according to the procedure described below.

Yeast propagation procedure: First, 450 ml solution is prepared which contains 5 g of Bacto Yeast Extract (DIFCO) and 10 g of Bacto or Phytone Peptone (DIFCO) in demineralized water. The pH is corrected to 7.0 using KOH or H_2SO_4. Next, demineralized water is added to a total weight of 500 g. Finally, the solution is sterilised for 15 minutes at 110ºC and cooled down to room temperature. Next, 30 ml of a solution containing 50% w/v glucose.1aq is added to prepare the growth medium. Inoculation of the growth medium is done under aseptic conditions (flow cabinet or flame) by addition of 0.1 ml of yeast culture from glycerol stock. The culture is incubated for 24 hours at 30ºC in a shaking incubator at 200 rpm.

Cream yeast preparation: After 24 hours of incubation, the yeast is concentrated by centrifugation at 4000 rpm for 10 minutes until a solid pellet is formed. Then, the supernatant is carefully decanted and the biomass pellet is washed by suspension in sterile water. The amount of water used for the re-suspension is roughly the same as the supernatant that is discarded. Finally, a second centrifugation step follows at 4000 rpm for 10 min and again the supernatant is carefully decanted. This time the washed pellet is re-suspended in sterile water (total weight is 30 g) creating the cream yeast. The dry weight of the cream yeast is determined using an infrared balance. The

dry matter of the cream yeast is used for dosing the correct amount of yeast at the start of the fermentation.

Ethanol fermentation: Ethanol fermentations are conducted in 250 ml erlenmeyer flasks containing 100 g of hydrolysed lignocellulosic feedstock containing different amounts of furfural as described above under feedstock preparation. All flasks are inoculated with cream yeast as prepared above in a concentration of 0.5 g of dry weight yeast per kg of hydrolysed lignocellulosic material. All flasks are closed using a rubber stopper equiped with a water lock. Incubation is done for 72 hours in a shaking incubator at 150 rpm at 32°C. Samples are taken after 0 – 6 – 24 – 48 – 72 hours of fermentation for analysis by HPLC (ethanol, glucose and xylose measurement).

After centrifugation, the diluted supernatant was subsequently filtered (0.2 µm filter, Whatman PN UN203NPENYL mini uniprep) and the filtrates were analysed for glucose, xylose, organic acids, furfural and HMF. Furfural and HMF concentrations of the diluted samples were measured using an HPLC equipped with an Aminex HPX-87H column (Biorad) by elution with 0.005 M sulphuric acid at 65°C at a flow rate of 0.55 ml per minute and quantified by integration of the signals from refractive index detection (R.I.) calibrated with glucose, xylose, ethanol, furfural and HMF standard solutions.

The fermentation results indicate a slower ethanol fermentation in case the furfural concentration in the hydrolysed lignocellulosic material is >1.5 g/l.

Moreover, the following experiment was done.

Feedstock preparation: Hydrolysed lignocellulosic material was produced as described in Example 1, experiment (b). The hydrolysate contained 0.46 g/l furfural. The furfural concentration was increased with furfural (99 % pure, obtained from Acros Organics, Cas 98-01-1, lot nr. A03387741) up to concentrations of 0.61 – 0.88 – 1.34 – 1.82 – 2.27 – 2.70 – 3.70 – 4.60 g/l. The pH of each mixture was adjusted to pH 5.5 using KOH. The hydrolysates containing different concentrations of furfural were used as substrate in ethanol fermentations of sugars according to the procedure described below.

Yeast propagation procedure: First, 450 ml solution was prepared which contains 5 g of Bacto Yeast Extract (DIFCO) and 10 g of Bacto or Phytone Peptone (DIFCO) in demineralized water. The pH was corrected to 7.0 using KOH or H_2SO_4. Next, demineralized water was added to a total weight of 500 g. Finally, the solution was sterilised for 15 minutes at 110°C and cooled down to room temperature. Next, 30 ml of a solution containing 50% w/v glucose.1aq was added to prepare the growth medium. Inoculation of the growth medium was done under aseptic conditions (flow cabinet or flame) by addition of 0.1 ml of yeast culture from glycerol stock (strain RN1016). The culture was incubated for 24 hours at 30°C in a shaking incubator at 200 rpm.

Cream yeast preparation: After 24 hours of incubation, the yeast was concentrated by centrifugation at 4000 rpm for 10 minutes until a solid pellet was formed. Then, the supernatant was carefully decanted and the biomass pellet was washed by suspension in sterile water. The amount of water used for the re-suspension was roughly the same as the supernatant that was discarded.

Finally, a second centrifugation step followed at 4000 rpm for 10 min and again the supernatant was carefully decanted. This time the washed pellet was re-suspended in sterile water (total weight was 26.9 g) creating the cream yeast. The dry weight of the cream yeast was determined using an infrared balance. The dry matter was 18.9 % (w/w). The dry matter of the cream yeast was used for dosing the correct amount of yeast at the start of the fermentation.

Ethanol fermentation: Ethanol fermentations were conducted in 125 ml erlenmeyer flasks containing 50 g of hydrolysed lignocellulosic feedstock containing different amounts of furfural as described above under feedstock preparation. All flasks were inoculated with cream yeast as prepared above in a concentration of 0.5 g of dry weight yeast per kg of hydrolysed lignocellulosic material. All flasks were closed using a rubber stopper equiped with a water lock. Incubation was done for 48 hours in a shaking incubator at 150 rpm at 32°C. Samples were taken after 0 – 6 – 24 – 48 hours of fermentation for analysis by HPLC (ethanol, glucose and xylose measurement).

After centrifugation, the diluted supernatant was subsequently filtered (0.2 µm filter, Whatman PN UN203NPENYL mini uniprep) and the filtrates were analysed for glucose, xylose, ethanol using an HPLC equipped with an Aminex HPX-87H column (Biorad) by elution with 0.005 M sulphuric acid at 65°C at a flow rate of 0.55 ml per minute and quantified by integration of the signals from refractive index detection (R.I.) calibrated with glucose, xylose and ethanol standard solutions.

The results of are presented in Table 3. The results show a slower ethanol fermentation with increasing furfural concentrations in the hydrolysed lignocellulosic material. So, volatiles removal as described herein is beneficial for ethanol fermentation.

Table 1: The effect of process conditions on volatiles removal

Experiment	Furfural at start of hydrolysis (g/l*)	HMF at start of hydrolysis (g/l*)	Furfural at end of hydrolysis (g/l*)	HMF at end of hydrolysis (g/l*)
A	2.3	0.63	1.2	0.40
B	2.3	0.63	0.6	0.38
C	2.3	0.63	1.9	0.48

* Measured in the supernatant

Table 2: The effect of the furfural concentration on yeast growth during propagation

Furfural content (g/l)	OD (600 nm)	Dry matter (g/l)
0.61	1.86	3.0
0.88	0.0	> 0.3
1.34	0.0	> 0.3
1.82	0.0	> 0.3

2.27	0.0	> 0.3
2.70	0.0	> 0.3
3.70	0.0	> 0.3
4.60	0.0	> 0.3

Table 3: The effect of furfural on ethanol fermentation.

Furfural (g/l)	Glucose + xylose (g/l)				Ethanol (g/l)			
	0h	6h	24h	48h	0h	6h	24h	48h
0.61	81.0	49.3	17.0	11.4	0.1	13.3	24.8	26.5
0.88	80.6	51.1	18.5	13.1	0.1	12.6	25.9	27.9
1.34	80.5	54.2	18.8	14.6	0.1	11.0	25.9	27.7
1.82	80.7	58.7	20.2	15.2	0.1	9.1	25.9	28.0
2.27	80.7	60.6	21.3	17.4	0.1	8.1	25.6	26.8
2.70	80.7	62.6	22.8	19.5	0.1	7.2	24.3	25.0
3.70	80.4	65.7	24.0	20.5	0.1	5.8	24.0	24.0
4.60	80.4	67.6	26.3	21.3	0.1	4.7	23.1	25.7

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CLAIMS

1. A process for the preparation of a sugar product from lignocellulosic material, comprising the steps of:
   a) enzymatically hydrolysing lignocellulosic material in a hydrolysis reactor,
   b) adding gas to the lignocellulosic material in the hydrolysis reactor during the enzymatic hydrolysis, wherein at least part of the gas ends up in the headspace of the hydrolysis reactor, and
   c) removing volatiles from the gas that ends up in the headspace.

2. A process for the preparation of a fermentation product from lignocellulosic material, comprising the steps of:
   a) enzymatically hydrolysing lignocellulosic material in a hydrolysis reactor,
   b) adding gas to the lignocellulosic material in the hydrolysis reactor during the enzymatic hydrolysis, wherein at least part of the gas ends up in the headspace of the hydrolysis reactor,
   c) removing volatiles from the gas that ends up in the headspace,
   d) fermenting the hydrolysed lignocellulosic material to produce the fermentation product, and
   e) optionally, recovering the fermentation product.

3. A process according to claim 1 or 2, further comprising the step of recycling at least part of the gas in the headspace to step (b).

4. A process according to claim 3, wherein the volatiles are at least partially removed from the gas during the recycling step.

5. A process according to any of the claims 1 to 4, wherein the volatiles are removed by means of a filter containing an adsorbant, a condenser, a membrane, a scrubber.

6. A process according to any of the claims 1 to 5, wherein the gas added in step (b) is oxygen-containing gas.

7. A process according to any of the claims 1 to 6, wherein the gas added in step (b) is added in the form of bubbles.

8. A process according to any of the claims 1 to 7, wherein the enzymatic hydrolysis is done with an enzyme composition that comprises at least a lytic polysaccharide monooxygenase.

9. A process according to any of the claims 1 to 8, wherein the hydrolysis reactor has a volume of 10 m^3 or more.

10. A process according to any of the claims 1 to 9, wherein the hydrolysis is conducted at a temperature of 45°C or more.

11. A process according to any of the claims 1 to 10, wherein the dry matter content in the hydrolysis step is 10% (w/w) or more.

12. A process according to any of the claims 2 to 11, wherein the fermentation is conducted with a microorganism that is able to ferment at least one C5 sugar.

13. An apparatus which comprises:
    a) a reactor of at least 10 m^3,
    b) a gas introducing means for addition of gas into the reactor,
    c) a gas pump for introducing gas into the reactor,
    d) a means for recycling gas from the headspace of the reactor into the reactor,
    e) an exhaust for removing gas from the reactor,
    f) a gas inlet for introducing fresh gas into the reactor,
    g) a means for controlling the ratio between recycled gas and fresh gas, and
    h) a means for removing volatiles from the gas in the headspace.

14. An apparatus according to claim 13, wherein the means for removing volatiles from the gas is connected to the means for recycling gas.

15. An apparatus according to claim 13 or 14, further comprising a stirring means for stirring the content of the reactor.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/058427

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P19/02 C12P7/10 C12P19/14 C12M1/00
ADD.

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 C12M

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2015/075277 A1 (DSM IP ASSETS BV [NL]) 28 May 2015 (2015-05-28) claims 1-15	1-15
A	WO 2009/046538 A1 (SUNOPTA BIOPROCESS INC [CA]; BURKE MURRAY J [CA]; SAVILLE BRADLEY [CA]) 16 April 2009 (2009-04-16) the whole document	1-15

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 May 2018	07/06/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Siatou, Evangelia

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KARA K PODKAMINER ET AL: "Ethanol and anaerobic conditions reversibly inhibit commercial cellulase activity in thermophilic simultaneous saccharification and fermentation (tSSF)", BIOTECHNOLOGY FOR BIOFUELS, vol. 5, no. 1, 1 January 2012 (2012-01-01) , page 43, XP055058045, ISSN: 1754-6834, DOI: 10.1186/1754-6834-5-43 the whole document -----	1-12

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2018/058427

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