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

(57) Abstract: A process for the preparation of a sugar and/or fermentation product from lignocellulosic material is disclosed comprising enzymatic hydrolysis of lignocellulosic material, lowering the pH by addition of an acid, and solid/liquid separation.

PROCESS FOR ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSIC MATERIAL AND FERMENTATION OF SUGARS

Field of the invention

The invention 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 of the invention

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 present in the lignocellulosic material is 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 like 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 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 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 of the invention

An object of the invention 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 lowering the pH of the lignocellulosic material after enzymatic hydrolysis. By lowering the pH of the enzymatically hydrolysed lignocellulosic material, the material can more easily be clarified which reduces costs, especially in large scale facilities. Benefits are lower sugar losses due to sugars present in the solid fraction and a lower solid fraction volume. Furthermore, the presence of particles in the liquid fraction may give clogging problems in valves, pumps and tubes. To remove the particles additional filtration may be necessary, which is time consuming and expensive. The present invention has as a further advantage that less particles are present in the liquid fraction.

Detailed description of the invention

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.

The present invention relates to a process for the preparation of a sugar product from lignocellulosic material, comprising the steps of (a) enzymatically hydrolysing the lignocellulosic material in one or more containers using an enzyme composition to obtain enzymatically hydrolysed lignocellulosic material comprising the sugar product, (b) lowering the pH of the enzymatically hydrolysed lignocellulosic material by adding an acid to the enzymatically hydrolysed lignocellulosic material, (c) subjecting the enzymatically hydrolysed lignocellulosic material to a solid/liquid separation to obtain a solid fraction and a liquid fraction, and (d) optionally, recovering the solid fraction and/or the liquid fraction. The sugar product is present in the solid and/or liquid fraction.

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 herein, (b) fermenting the solid fraction and/or liquid fraction to produce a fermentation product; and (c) optionally, recovering of the fermentation product.

In an embodiment the pH is lowered to 3.0 or lower. So, the pH of the enzymatically hydrolysed lignocellulosic material is lowered to 3.0 or lower. In an embodiment the pH is lowered to 2.9 or lower, 2.8 or lower, 2.7 or lower, 2.6 or lower, 2.5 or lower, 2.4 or lower, 2.3 or lower, 2.2 or lower, 2.1 or lower, 2.0 or lower, 1.9 or lower, 1.8 or lower, 1.7 or lower, 1.6 or lower, 1.5 or lower, 1.4 or lower, 1.3 or lower, 1.2 or lower, 1.1 or lower, 1.0 or lower. In an embodiment the pH is lowered to between 0.5 and 3.0, between 0.5 and 2.9, between 0.5 and 2.8, between 0.5 and 2.7, between 0.5 and 2.6, between 0.5 and 2.5, between 0.5 and 2.4, between 0.5 and 2.3, between 0.5 and 2.2, between 0.5 and 2.1, between 0.5 and 2.0, between 0.5 and 1.9, between 0.5 and 1.8, between 0.5 and 1.7, between 0.5 and 1.6, between 0.5 and 1.5, between 0.5 and 1.4, between 0.5 and 1.3, between 0.5 and 1.2, between 0.5 and 1.1, between 0.5 and 1.0.

All types of acids can be used to lower the pH. Examples include, but are not limited to, hydrochloric acid and sulfuric acid. Sulfuric acid is normally preferred on an industrial scale to minimise corrosion of equipment and to minimise waste treatment costs. The acid can be added directly to the hydrolysate or it can be added to a circulation loop. The pH is typically measured inline in a container or in a withdrawal line. Alternatively, it can be measured offline in samples taken from the lignocellulosic material.

The methods and conditions of solid/liquid separation will depend on the type of lignocellulosic material used and are well within the scope of the skilled artisan. Examples include, but are not limited to, centrifugation, cyclonic separation, filtration, decantation, sieving and sedimentation. In a preferred embodiment the solid/liquid separation is performed by centrifugation or sedimentation. During solid/liquid separation, means and/or aids for improving the separation may be used.

In an embodiment the lignocellulosic material is subjected to a pretreatment step before the enzymatic hydrolysis. In an embodiment the lignocellulosic material is subjected to a washing step before the enzymatic hydrolysis. In an embodiment the lignocellulosic material is subjected to at least one solid/liquid separation before the enzymatic hydrolysis. So, before subjecting the lignocellulosic material to enzymatic hydrolysis, it can be subjected to at least one solid/liquid separation. The solid/liquid separation may be done before and/or after the pretreatment step. Suitable methods and conditions for a solid/liquid separation have been described above.

In an embodiment the invention relates to a process wherein the enzymatically hydrolysed lignocellulosic material is subjected to an additional solid/liquid separation step followed by a detoxification step and/or a concentration step before lowering the pH of the enzymatically hydrolysed lignocellulosic material.

In another embodiment the invention relates to a process wherein the enzymatically hydrolysed lignocellulosic material is subjected to an additional solid/liquid separation step followed by a detoxification step before lowering the pH of the enzymatically hydrolysed lignocellulosic material. After lowering the pH of the enzymatically hydrolysed lignocellulosic material and before subjecting the material to a solid/liquid separation step, the material may be subjected to a concentration step.

The solid/liquid separation leads to at least a solid fraction and at least a liquid fraction. In an embodiment the solid fraction comprises between 1 and 80 wt% C6 sugars and/or the liquid fraction comprises between 1 and 80 wt% C6 sugars. In an embodiment the solid fraction comprises between 5 and 75 wt% C6 sugars, between 10 and 70 wt% C6 sugars, between 15 and 65 wt% C6 sugars, between 20 and 60 wt% C6 sugars and/or the liquid fraction comprises between 2 and 70 wt% C6 sugars, between 3 and 65 wt% C6 sugars, between 4 and 60 wt% C6 sugars, between 5 and 55 wt% C6 sugars, between 6 and 50 wt% C6 sugars, between 7 and 45 wt% C6 sugars, between 8 and 40 wt% C6 sugars.

In the processes according to the present invention lignocellulosic material may be added to the one or more containers. In an embodiment the enzyme composition is already present in the one or more containers before the lignocellulosic material is added. In another embodiment the enzyme composition may be added to the one or more containers. In an embodiment the lignocellulosic material is already present in the one or more containers before the enzyme composition is added. In an embodiment both the lignocellulosic material and the enzyme composition are added simultaneously to the one or more containers. The enzyme composition present in the one or more containers may be an aqueous composition.

In an embodiment the enzymatic hydrolysis comprises at least a liquefaction step wherein the lignocellulosic material is hydrolysed in at least a first container, and a saccharification step wherein the liquefied lignocellulosic material is hydrolysed in the at least first container and/or in at least a second container. Saccharification can be done in the same container as the liquefaction (*i.e.* the at least first container), it can also be done in a separate container (*i.e.* the at least second container). So, in the enzymatic hydrolysis of the processes according to the present invention liquefaction and saccharification may be combined. Alternatively, the liquefaction and saccharification may be separate steps. 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 °C and saccharification is preferably carried out at a temperature of 50 - 65 °C.

The enzymatic hydrolysis can be performed in one or more containers, but can also be performed in one or more tubes or any other continuous system. This also holds true when the enzymatic hydrolysis comprises a liquefaction step and a saccharification step. The liquefaction step can be performed in one or more containers, but can also be performed in one or more

tubes or any other continuous system and/or the saccharification step can be performed in one or more containers, but can also be performed in one or more tubes or any other continuous system. Examples of containers to be used in the present invention include, but are not limited to, fed-batch stirred containers, batch stirred containers, continuous flow stirred containers with ultrafiltration, and continuous plug-flow column reactors. Stirring can be done by one or more impellers, pumps and/or static mixers.

The enzymes used in the enzymatic hydrolysis may be added before and/or during the enzymatic hydrolysis. As indicated above, when the lignocellulosic material is subjected to a solid/liquid separation before enzymatic hydrolysis, the enzymes used in the enzymatic hydrolysis may be added before the solid/liquid separation. Alternatively, they may also be added after solid/liquid separation or before and after solid/liquid separation. The enzymes may also be added during the enzymatic hydrolysis. In case the enzymatic hydrolysis comprises a liquefaction step and saccharification step, additional enzymes may be added during and/or after the liquefaction step. The additional enzymes may be added before and/or during the saccharification step. Additional enzymes may also be added after the saccharification step.

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.

The viscosity of the lignocellulosic material in the one or more containers used for the enzymatic hydrolysis is kept between 10 and 4000 cP, between 10 and 2000 cP, preferably between 10 and 1000 cP.

In case the process comprises an enzymatic hydrolysis comprising a liquefaction step and a saccharification step, the viscosity of the lignocellulosic material in the liquefaction step is kept between 10 and 4000 cP, between 10 and 2000 cP, preferably between 10 and 1000 cP and/or the viscosity of the lignocellulosic material in the saccharification step is kept between 10 and 1000 cP, between 10 and 900 cP, preferably between 10 and 800 cP.

The viscosity can be determined with a Brookfield DV III Rheometer at the temperature used for the hydrolysis.

In an embodiment oxygen is added during the enzymatic hydrolysis. In an embodiment oxygen is added during at least a part of the enzymatic hydrolysis. Oxygen can be added continuously or discontinuously during the enzymatic hydrolysis. In an embodiment oxygen is added one or more times during the enzymatic hydrolysis. In an embodiment oxygen may be

added before the enzymatic hydrolysis, during the addition of lignocellulosic material to a container used of enzymatic hydrolysis, during the addition of enzyme to a container used of enzymatic hydrolysis, during a part of the enzymatic hydrolysis, during the whole enzymatic hydrolysis or any combination thereof. Oxygen is added to the one or more containers used in the enzymatic hydrolysis.

Oxygen can be added in several forms. For example, oxygen can be added as oxygen gas, oxygen-enriched gas, such as oxygen-enriched air, or air. Oxygen may also be added by means of *in situ* oxygen generation. For example, oxygen may be generated by electrolysis, oxygen may be produced enzymatically, *e.g.* by the addition of peroxide, or oxygen may be produced chemically, *e.g.* by an oxygen generating system such as $KHSO_5$. For example, oxygen is produced from peroxide by catalase. The peroxide can be added in the form of dissolved peroxide or generated by an enzymatic or chemical reaction. In case catalase is used as enzyme to produce oxygen, catalase present in the enzyme composition for the hydrolysis can be used or catalase can be added for this purpose.

Examples how to add oxygen include, but are not limited to, addition of oxygen by means of sparging, electrolysis, chemical addition of oxygen, filling the one or more containers used in the enzymatic hydrolysis from the top (plunging the hydrolysate into the tank and consequently introducing oxygen into the hydrolysate) and addition of oxygen to the headspace of said one or more containers. When oxygen is added to the headspace of the container(s), sufficient oxygen necessary for the hydrolysis reaction may be supplied. In general, the amount of oxygen added to the container(s) can be controlled and/or varied. Restriction of the oxygen supplied is possible by adding only oxygen during part of the hydrolysis time in said container(s). Another option is adding oxygen at a low concentration, for example by using an mixture of air and recycled air (air leaving the container) or by “diluting” air with an inert gas. Increasing the amount of oxygen added can be achieved by addition of oxygen during longer periods of the hydrolysis time, by adding the oxygen at a higher concentration or by adding more air. Another way to control the oxygen concentration is to add an oxygen consumer and/or an oxygen generator. Oxygen can be introduced, for example blown, into the liquid hydrolysis container contents of lignocellulosic material. It can also be blown into the headspace of the container.

In an embodiment oxygen is added to the one or more containers used in the enzymatic hydrolysis before and/or during and/or after the addition of the lignocellulosic material to said one or more containers. The oxygen may be introduced together with the lignocellulosic material that enters the hydrolysis container(s). The oxygen may be introduced into the material stream that will enter the container(s) or with part of the container(s) contents that passes an external loop of the container(s).

In an embodiment the container(s) used in the enzymatic hydrolysis and/or the fermentation have a volume of at least 1 m^3. Preferably, the containers 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 container(s) will be smaller than 3000 m^3 or 5000 m^3. In case several containers are used in the enzymatic hydrolysis of the processes of the present invention, they may have the same volume, but also may have a different volume. In case the enzymatic hydrolysis of the processes of the present invention comprises a separate liquefaction step and saccharification step the container(s) used for the liquefaction step and the container(s) used for the saccharification step may have the same volume, but also may have a different volume.

In an embodiment the enzyme composition 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. In a preferred embodiment the fungus is *Rasamsonia*, with *Rasamsonia emersonii* being most preferred. The enzymes used in the enzymatic hydrolysis of the processes of the present invention are derived from a fungus or the enzymes used in the enzymatic hydrolysis of the processes of the present invention 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). The filamentous fungi are characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligatory aerobic. Filamentous fungal strains include, but are not limited to, strains of *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*.

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). Examples of such strains include *Aspergillus niger* CBS 513.88, *Aspergillus oryzae* ATCC 20423, IFO 4177, ATCC 1011, ATCC 9576, ATCC14488-14491, ATCC 11601,

ATCC12892, *P. chrysogenum* CBS 455.95, P*enicillium citrinum* ATCC 38065, *Penicillium chrysogenum* P2, *Talaromyces emersonii* CBS 393.64, *Acremonium chrysogenum* ATCC 36225 or ATCC 48272, *Trichoderma reesei* ATCC 26921 or ATCC 56765 or ATCC 26921, *Aspergillus sojae* ATCC11906, *Chrysosporium lucknowense* C1, Garg 27K, VKM F-3500-D, ATCC44006 and derivatives thereof.

The enzymatic hydrolysis of the processes of the present invention are advantageously applied in combination with enzymes derived from a microorganism of the genus *Rasamsonia* or the enzymes used in the enzymatic hydrolysis of the processes of the present invention comprise a *Rasamsonia* enzyme.

The enzymatic hydrolysis is preferably done at 50–90ºC. In this step thermostable cellulolytic enzymes are preferred. A “thermostable” enzyme as used herein means that the enzyme has a temperature optimum of 50°C or higher, 60°C or higher, 70°C or higher, 75°C or higher, 80°C or higher, 85°C or higher. They may for example be isolated from thermophilic microorganisms or may be designed by the skilled person and artificially synthesized. In one embodiment the polynucleotides 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 a *Humicola*, *Rhizomucor*, *Myceliophthora*, *Rasamsonia, Talaromyces*, *Thermomyces, Thermoascus* or *Thielavia* cell, preferably a *Rasamsonia* cell. 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.*

Thermophilic fungi are not restricted to a specific taxonomic order and occur all over the fungal tree of life. Examples are *Rhizomucor* in the Mucorales, *Myceliophthora* in Sordariales and *Talaromyces*, *Thermomyces* and *Thermoascus* in the Eurotiales (see Mouchacca, 1997). The majority of *Talaromyces* species are mesophiles, but exceptions are species within sections *Emersonii* and *Thermophila*. Section *Emersonii* includes *Talaromyces emersonii*, *Talaromyces byssochlamydoides, Talaromyces bacillisporus and Talaromyces leycettanus,* all of which grow well at 40°C. *Talaromyces bacillisporus* is thermotolerant, *Talaromyces leycettanus* is thermotolerant to thermophilic, and *Talaromyces emersonii* and *Talaromyces byssochlamydoides* are truly thermophilic (see Stolk and Samson, 1972). The sole member of *Talaromyces* section

Thermophila, Talaromyces thermophilus, grows rapidly at 50°C (see Stolk and Samson, 1972). The current classification of these thermophilic *Talaromyces* species is mainly based on phenotypic and physiological characters, such as their ability to grow above 40ºC, ascospore color, the structure of ascornatal covering and the formation of a certain type of anamorph. Stolk and Samson (1972) stated that the members of the section *Emersonii* have anamorphs of either *Paecilomyces* (*Talaromyces byssochlamydoides* and *Talaromyces leycettanus*) or *Penicillium cylindrosporum* series (*Talaromyces emersonii* and *Talaromyces bacillisporus*). Later, Pitt (1979) transferred the species belonging to the *Penicillium cylindrosporum* series to the genus *Geosmithia*, based on various characters such as the formation of conidia from terminal pores instead of on collula (necks), a character of *Penicillium* and *Paecilomyces*. Within the genus *Geosmithia*, only *Geosmithia argillacea* is thermotolerant, and Stolk et al. (1969) and Evans (1971) proposed a connection with members of *Talaromyces* sect. *Emersonii*. The phylogenetic relationship of the themophilic *Talaromyces* species within *Talaromyces* and the Trichocomaceae is unknown. (see J. Houbraken, Antonie van Leeuwenhoek 2012 Feb; 101(2): 403-21).

Rasamsonia is a new genus comprising thermotolerant and thermophilic *Talaromyces* and *Geosmithia* species (J. Houbraken et al., *vida supra*). Based on phenotypic, physiological and molecular data, Houbraken et al. proposed to transfer the species *Talaromyces emersonii, Talaromyces byssochlamydoides, Talaromyces eburneus, Geosmithia argillacea* and *Geosmithia cylindrospora* to *Rasamsonia* gen. nov.

Preferred thermophilic fungi are *Rasamsonia byssochlamydoides, Rasamsonia emersonii, Thermomyces lenuginosus, Talaromyces thermophilus, Thermoascus crustaceus, Thermoascus thermophilus and Thermoascus aurantiacus,* with *Rasamsonia emersonii* being most preferred. *Talaromyces emersonii, Penicillium geosmithia emersonii* and *Rasamsonia emersonii* are used interchangeably herein.

In a common process for converting lignocellulosic material into fermentation products, process steps are preferably done under septic conditions to lower the operational costs. Contamination and growth of contaminating microorganisms can therefore occur and result in undesirable side effects, such as lactic acid, formic acid and acetic acid production, yield losses of fermentation product on substrate, production of toxins and extracellular polysaccharides. These effects may affect production costs significantly. A high process temperature and/or a short process time limits the risk on contamination during hydrolysis and fermentation. Thermostable enzymes, like those of *Rasamsonia,* are capable of hydrolysing lignocellulosic material at temperatures of higher than 60°C. At these temperatures, the risk that a contaminating microorganism will cause undesired side effects is little to almost zero.

During the fermentation step, in which for example ethanol is produced, temperatures are typically between 30 to 38°C and are preferably not raised because of production losses. By applying short fermentation process times, the risks and effects of contamination and/or growth of contaminants are reduced as much as possible. With stable enzymes, like those of *Rasamsonia,*

a short fermentation time can be applied and thus risks of contamination and/or growth of contaminants are reduced as much as possible. The cost reduction achieved with applying thermostable cellulolytic enzymes of *Rasamsonia* in this way, results in a lower risk of process failures due to contamination.

The first step after thermal pretreatment is to cool the pretreated material to temperatures wherein the enzymes have an optimal activity. On large scale, this is typically done by adding (cooled) water, which, besides decreasing the temperature, reduces the dry matter content. By using thermostable enzymes, like those of *Rasamsonia*, cost reduction can be achieved, because (i) less cooling of the pretreated material is required since higher temperatures are allowed during hydrolysis, and (ii) less water is added, which increases the dry matter content during hydrolysis and fermentation and thus increase the ethanol production capacity (amount produced per time unit per volume) of an ethanol plant. By using thermostable enzymes, like those of *Rasamsonia,* cost reduction may also be achieved by using cooling water having a higher temperature than the water that is used in a process with non-thermostable enzyme.

At the end of the hydrolysis, enzyme activities appear to be low, since little reducing sugars are released once almost all cellulose is converted. The amount of enzymatic activity present, however, has decreased only a little, assumingly mainly due to absorption of the enzymes to the substrate. By applying solid-liquid separation after hydrolysis, such as centrifugation, filtration, cantation, sedimentation, 60% or more (*e.g.* 70%) of the enzyme activity in solution can be recovered and re-used for hydrolysis of a new pretreated lignocellulosic material during the next hydrolysis.

Moreover, after solid/liquid separation the enzyme in solution can be separated from the solution containing reducing sugars and other hydrolysis products from the enzymatic actions. This separation can be done by techniques including, but not limited to, ultra- and microfiltration, centrifugation, cantation, sedimentation, with or without first adsorption of the enzyme to a carrier of any kind. For example, after hydrolysis of pretreated material with 0.175 ml/g material dry matter enzyme load for 20h, 50% of the theoretical maximum amount of reducing sugars is liberated and after the same hydrolysis for 72h, 90% of the theoretical maximum amount of reducing sugars is liberated. By centrifugation and ultrafiltration, 60-70% of the enzyme activity was recovered in the retentate, while the filtrate contained more than 80% of the liberated reducing sugars. By re-using the retentate, either as it is or after further purification and/or concentration, enzyme dosage during the next hydrolysis step can be reduced with 60 to 70%. The cost reduction achieved by using stable cellulolytic enzymes, such as those of *Rasamsonia*, in this way is the consequence of a lower enzyme dosage.

The process including enzyme recycling after hydrolysis, as described above, can be combined with recycling of the microorganism producing the fermentation product after fermentation and with the use of the reducing sugars containing filtrate as a substrate (purified and/or concentrated or diluted) in the propagation and cultivation of the microorganism producing

the fermentation product and the microorganism producing the enzymes for hydrolysis. The reducing sugars containing filtrate can also be used as a substrate (purified and/or concentrated or diluted) in the production of the fermentation product by the fermentation product producing microorganism and the production of the enzymes for hydrolysis by the enzyme producing microorganism.

The thermostability of enzymes, like those from *Rasamsonia,* causes remaining cellulolytic activity after hydrolysis, fermentation and vacuum distillation in the thin stillage. The total activity of the enzyme is reduced during the three successive process steps. The thin stillage obtained after vacuum distillation can thus be re-used as a source of enzyme for a newly started hydrolysis–fermentation–distillation process cycle of pretreated material conversion into for example ethanol. The thin stillage can be used either in concentrated or (un)diluted form and/or purified and with or without additional enzyme supplementation.

In an optimal process, an amount of enzyme is supplemented into the thin stillage, before its re-use in a new process cycle, equal to the amount of activity lost during the three successive process steps of the previous process cycle. In this way over dosage of enzyme is avoided and thus most efficient use of enzyme is obtained. Moreover, by providing high enzyme dosage in the first process cycle, and supplementing enzyme equal to the amount of activity lost during the three successive process steps in the following process cycles, highest possible hydrolysis rates can be obtained in each process cycle resulting in short hydrolysis times of less than 48h in combination with most efficient use of enzymes.

By applying mixing during hydrolysis, enzymes come more often in contact with substrates, which results in a more efficient use of the catalytic activity. This will result in a lower enzyme dosages and thus in lower costs, unless the mixing has a negative effect on the enzymes. Stable enzymes, like the thermostable enzymes from *Rasamsonia*, are robust and can resist circumstances of (locally) high shear and temperatures, which is the case during intensive mixing of slurries. The use of them in mixed systems is therefore beneficial and will lead to dosage and thus costs reduction.

An advantage of expression and production of the enzymes (for example at least two, three or four different cellulases) in a suitable microorganism may be a high enzyme composition yield which can be used in the processes of the present invention.

In the processes of the present invention 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 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 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. 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).

Enzymes that may be used in the invention 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 (cellobiose as a main product), 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 process of the current invention comprises preferably 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 of the current invention 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 of the current invention 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.

Thus, a composition for use in the processes of the current invention may comprise lytic polysaccharide monooxygenase activity, endoglucanase activity and/or cellobiohydrolase activity and/or beta-glucosidase activity. A composition for use in the invention may comprise more than one enzyme activity per activity class. For example, a composition for use in the invention may comprise two endoglucanase activities, for example, endo-1,3(1,4)-β glucanase activity and endo-β-1,4-glucanase activity.

A composition for use in the processes of the current invention 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 (lignocellulose degrading) enzyme activities may be derived from *Rasamsonia emersonii*. *Rasamsonia emersonii* can provide a highly effective set of activities as demonstrated herein for the hydrolysis of lignocellulosic material. If needed, the set of activities can be supplemented with additional enzyme activities from other sources. Such additional activities may be derived from classical sources and/or produced by genetically modified organisms.

The activities in a composition for use in the processes of the current invention may be thermostable. Herein, this means that the activity has a temperature optimum of 60°C or higher, 70°C or higher, 75°C or higher, 80°C or higher, 85°C or higher. Activities in a composition for use in the processes of the current invention will typically not have the same temperature optima, but preferably will, nevertheless, be thermostable.

In addition, enzyme activities in a composition for use in the processes of the current invention 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.

Activities in a composition for use in the processes of the current invention may be defined by a combination of any of the above temperature optima and pH values.

The enzyme composition for use in the processes of the current invention may 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.

For example, enzymes for use in the processes of the current invention may comprise a beta-glucosidase (BG) 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).

For example, enzymes for use in the processes of the current invention may comprise an endoglucanase (EG) 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 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).

For example, enzymes for use in the processes of the current invention may comprise 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, or from *Trichoderma*, such as *Trichoderma reesei*.

For example, enzymes for use in the processes of the current invention may comprise 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 *Thielavia*, such as *Thielavia terrestris*, such as cellobiohydrolase II CEL6A from *Thielavia terrestris*.

For example, enzymes for use in the processes of the current invention may comprise a GH61 polypeptide (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 GH61 polypeptides 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). In one aspect, the GH61 polypeptide is used in the presence of a soluble activating divalent metal cation according to WO 2008/151043, *e.g.* manganese sulfate. In one aspect, the GH61 polypeptide is used in the presence of a dioxy compound, a bicylic compound, a heterocyclic compound, a nitrogen-containing compound, a quinone compound, a sulfur-containing compound, or a liquor obtained from a pretreated cellulosic material such as pretreated corn stover.

Other cellulolytic enzymes that may be used in the processes of the present invention 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 addition, examples of xylanases useful in the processes of the present invention include, but are not limited to, xylanases 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), and *Trichophaea saccata GH10* (see WO 2011/057083). Examples of beta-xylosidases useful in the processes of the present invention include, but are not limited to, beta-xylosidases from *Neurospora crassa* and *Trichoderma reesei*. Examples of acetylxylan esterases useful in the processes of the present invention 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). Examples of feruloyl esterases (ferulic acid esterases) useful in the processes of the present invention 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). Examples of arabinofuranosidases useful in the processes of the present invention 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). Examples of alpha-glucuronidases useful in the processes of the present invention 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*.

An enzyme composition for use in the processes of the current invention may comprise one, two, three, four classes or more of cellulase, for example one, two, three or four or all of a lytic polysaccharide monooxygenas (LPMO) , an endoglucanase (EG), one or two exo-cellobiohydrolase (CBH) and a beta-glucosidase(BG). A composition for use in the processes of the current invention may comprise two or more of any of these classes of cellulase.

An enzyme composition for use in the processes of the current invention 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 according to the invention may give rise to a mixed population of cellodextrins and glucose monomers. Such degradation will typically take place by way of a hydrolysis reaction.

Lytic polysaccharide monooxygenases (LPMO) are recently classified by CAZy in family AA9 (Auxiliary Activity Family 9) or family AA10 (Auxiliary Activity Family 10). As mentioned above, lytic polysaccharide monooxygenases are able to open a crystalline glucan structure. Lytic polysaccharide monooxygenases may also affect cello-oligosaccharides. GH61 (glycoside hydrolase family 61 or sometimes referred to EGIV) proteins are (lytic) oxygen-dependent polysaccharide monooxygenases (PMO's/LPMO's) according to the latest literature (see Isaksen et al., Journal of Biological Chemistry, vol. 289, no. 5, pp. 2632-2642). PMO and LPMO are used herein interchangeably. Often in literature these proteins are mentioned to enhance the action of cellulases on lignocellulose substrates. GH61 was originally classified as endoglucanase based on measurement of very weak endo-1,4-β-d-glucanase activity in one family member. The term "GH61" as used herein, is to be understood as a family of enzymes, which share common conserved sequence portions and folding to be classified in family 61 of the well-established CAZy GH classification system (http://www.cazy.org/GH61.html). The glycoside hydrolase family 61 is a member of the family of glycoside hydrolases EC 3.2.1. GH61 are recently now reclassified by CAZy in family AA9 (Auxiliary Activity Family 9). GH61 is used herein as being part of the cellulases.

CBM33 (family 33 carbohydrate-binding module) is 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 AA10 (Auxiliary Activity Family 10).

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 according to the invention 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 according to the invention 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 of the current invention may comprise any cellulase, for example, a lytic polysaccharide monooxygenase (*e.g.* GH61), a cellobiohydrolase, an endo-β-1,4-glucanase, a beta-glucosidaseor a β-(1,3)(1,4)-glucanase.

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.

As used herein, an endo-β-1,4-glucanase (EC 3.2.1.4) is any polypeptide which is capable of catalyzing the endohydrolysis of 1,4-β-D-glucosidic linkages in cellulose, lichenin or cereal β-D-glucans. Such a polypeptide may also be capable of hydrolyzing 1,4-linkages in β-D-glucans also containing 1,3-linkages. This enzyme may also be referred to as cellulase, avicelase, β-1,4-endoglucan hydrolase, β-1,4-glucanase, carboxymethyl cellulase, celludextrinase, endo-1,4-β-D-glucanase, endo-1,4-β-D-glucanohydrolase, endo-1,4-β-glucanase or endoglucanase.

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.

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.

A composition for use in the processes of the current invention may comprise any hemicellulase, 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 or a β-mannosidase.

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.

As used herein, a β-xylosidase (EC 3.2.1.37) is any polypeptide which is capable of catalysing the hydrolysis of 1,4-β-D-xylans, to remove successive D-xylose residues from the non-reducing termini. Such enzymes may also hydrolyze xylobiose. This enzyme may also be referred to as xylan 1,4-β-xylosidase, 1,4-β-D-xylan xylohydrolase, exo-1,4-β-xylosidase or xylobiase.

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, 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.

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.

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.

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.

A composition for use in the processes of the current invention may comprise any pectinase, 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, a xylogalacturonase.

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 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 hydrolysis of 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: $(1,4\text{-}\alpha\text{-D-galacturonide})_n + H_2O = (1,4\text{-}\alpha\text{-D-galacturonide})_{n-1} + \text{D-galacturonate}$. 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.

An enzyme composition for use in the processes of the current invention will typically

comprise at least two cellulases and optionally at least one hemicellulase and optionally at least one pectinase. A composition for use in the processes of the current invention may comprise a lytic polysaccharide monooxygenases (such as GH61), a cellobiohydrolase, an endoglucanase and/or a beta-glucosidase. Such a composition may also comprise one or more hemicellulases and/or one or more pectinases.

In addition, one or more (for example two, three, four or all) of 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).

"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 of the current invention. 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 in the invention 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 glucoronoside, for example β-glucuronoside to yield an alcohol. Many glucuronidases have been characterized and may be suitable for use in the invention, 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).

A composition for use in the processes of the current invention may comprise an

expansin or expansin-like protein, such as a swollenin (see Salheimo *et al.*, Eur. J. Biochem. 269, 4202-4211, 2002) or a swollenin-like protein.

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. For the purposes of this invention, 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 composition for use in the processes of the current invention may comprise 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 for the purposes of this invention may comprise one or both of such domains.

A composition for use in the processes of the current invention may also comprise 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.

A composition for use in the processes of the current invention may be composed of a member of each of the classes of enzymes mentioned above, several members of one enzyme class, or any combination of these enzymes classes or helper proteins (*i.e.* those proteins mentioned herein which do not have enzymatic activity *per se*, but do nevertheless assist in lignocellulosic degradation).

A composition for use in the processes of the current invention 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 is 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 a 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 a filamentous fungi overexpressing beta-glucosidase. Alternatively, the whole fermentation broth for use in the present methods and reactive compositions 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 a recombinant filamentous fungi overexpressing a beta-glucosidase.

Enzymes are present in the liquefaction step and in the saccharification step of the enzymatic hydrolysis. These enzymes may be the same or may be different. Furthermore, as described above, additional enzymes are added during the liquefaction step and the saccharification step of the integrated processes according to the present invention. The enzymes added may be enzymes that are already present in the liquefaction step and in the saccharification step. Alternatively, they may be different enzymes. Moreover, the additional enzymes added during the liquefaction step may differ or may be the same as the additional enzymes added during the saccharification step of the integrated processes according to the present invention.

Lignocellulosic material as used herein includes any lignocellulosic and/or hemicellulosic material. Lignocellulosic material suitable for use in the processes of the current invention 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, canola stems, soybean stems, sweet sorghum, corn kernel including fiber from kernels, 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” 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 aforementioned singularly or in any combination or mixture thereof.

Cellulose is an organic compound with the formula $(C_6H_{10}O_5)_n$, a polysaccharide consisting of a linear chain of several hundred to over ten thousand β(1→4) linked D-glucose units. A glucan molecule is a polysaccharide of D-glucose monomers linked by glycosidic bonds. Herein glucan and cellulose are used interchangeably for a polysaccharide of D-glucose monomers linked by glycosidic bonds. Methods for the quantitative analysis of glucan or polysaccharide compositions are well-known and described in the art and are for example summarized in Carvalho de Souza et al., Carbohydrate Polymers 95 (2013) 657-663. In general, 50 to 70% of the glucan is crystalline cellulose, the remainder is amorphous cellulose.

In an embodiment the lignocellulosic material is pretreated before and/or during the enzymatic hydrolysis. 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 of the invention 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 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, a process of the invention 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) fermentation of the solid fraction and/or liquid fraction to produce a fermentation product; and (c) optionally, recovery of the fermentation product.

In an embodiment the fermentation (*i.e.* step b) is performed in one or more containers. In an embodiment the fermentation of the solid fraction and/or the liquid fraction is done by an alcohol producing microorganism to produce alcohol. In a preferred embodiment the fermentation of the solid fraction is done by an alcohol producing microorganism to produce alcohol. In an embodiment the fermentation of the solid fraction and/or the liquid fraction is done by an organic acid producing microorganism to produce an organic acid. In a preferred embodiment the fermentation of the liquid fraction is done by an organic acid producing microorganism to produce an organic acid. The fermentation of the solid fraction and/or the liquid fraction by an alcohol producing microorganism to produce alcohol can be done in the same container(s) wherein the enzymatic hydrolysis is performed. Alternatively, the fermentation of the solid fraction and/or the liquid fraction by an alcohol producing microorganism to produce alcohol and the fermentation of the liquid fraction and/or the solid fraction by an organic acid producing microorganism to produce an organic acid can be performed in one or more separate containers, but may also be done in one or more of the same containers.

In an embodiment the fermentation is done by a yeast. In an embodiment the alcohol producing microorganism and/or the organic acid 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 an embodiment the organic acid producing microorganism is able to ferment at least a C6 sugar. In an embodiment the alcohol producing microorganism and the organic acid producing microorganism are different microorganisms. In another embodiment the alcohol producing microorganism and the organic acid producing microorganism are the same microorganism, *i.e.* the alcohol producing microorganism is also able to produce organic acid such as succinic acid.

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 time may be shorter than in conventional fermentation at the same conditions, wherein part of the enzymatic hydrolysis still has to take part during fermentation. In one embodiment, the fermentation time is 100 hours or less, 90 hours or less, 80 hours or less, 70 hours or less, or 60 hours or less, for a sugar composition of 50 g/l glucose and corresponding other sugars from the lignocellulosic material (*e.g.* 50 g/l xylose, 35 g/l L-arabinose and 10 g/l galactose). For more dilute sugar compositions, the fermentation time may correspondingly be reduced. In an embodiment the fermentation time of the ethanol production step is between 10 and 50 hours for ethanol made out of C6 sugars and between 20 and 100 hours for ethanol made out of C5 sugars. In an embodiment the fermentation time of the succinic acid production step is between 20 and 70 hours.

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 microorganisms 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 antibiotics 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, while the organic acid fermentation process is aerobic, but done under oxygen-limited conditions.

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 and the organic acid fermentation step are performed between 25ºC and 35ºC.

In an embodiment of the invention, 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 propagation of the alcohol producing microorganism and/or the organic acid producing microorganism is performed in one or more propagation containers. The propagation can be done by fermentation of the liquid fraction and/or the solid fraction. After propagation, the alcohol producing microorganism and/or the organic acid producing microorganism may be added to one or more fermentation containers. Alternatively, the propagation of the alcohol producing microorganism and/or the organic acid producing microorganism is combined with the fermentation of the liquid fraction and/or the solid fraction by the alcohol producing microorganism and/or the organic acid producing microorganism to produce alcohol and/or organic acid, respectively.

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 invention relates to 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 solid fraction and/or liquid fraction 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.

In an embodiment the organic acid producing microorganism is a microorganism that is able to ferment at least one C6 sugar. In an embodiment the invention relates to a process for the preparation of succinic acid 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 solid fraction and/or liquid fraction to produce succinic acid; and (c) optionally, recovery of the succinic acid. The fermentation can be done with a microorganism that is able to ferment at least one C6 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 WO2003/095627. *araA*, *araB* and *araD* genes from *Lactobacillus plantarum* may be used and are disclosed in WO2008/041840. 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 2009011591. 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.

The volumetric ethanol productivity is preferably at least 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0 or

10.0 g ethanol per litre per hour. The ethanol yield on L-arabinose and optionally xylose and/or glucose in the process preferably is at least 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 95 or 98%. The ethanol yield is herein defined as a percentage of the theoretical maximum yield, which, for glucose and L-arabinose and optionally xylose is 0.51 g ethanol per g glucose or xylose.

In one aspect, the fermentation process leading to the production of ethanol, has several advantages by comparison to known ethanol fermentations processes: anaerobic processes are possible; oxygen limited conditions are possible; higher ethanol yields and ethanol production rates can be obtained; the strain used may be able to use L-arabinose and optionally xylose.

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).

The organic acid producing microorganisms may be a prokaryotic or eukaryotic organism. The microorganism used in the process may be a genetically engineered microorganism. Examples of suitable organic acid producing organisms are yeasts, for instance *Saccharomyces, e.g. Saccharomyces* cerevisiae; fungi for instance *Aspergillus* strains, such as *Aspergillus niger* and *Aspergillus fumigatus, Byssochlamys nivea*, *Lentinus degener*, *Paecilomyces varioti* and *Penicillium viniferum*; and bacteria, for instance *Anaerobiospirillum succiniciproducens, Actinobacillus succinogenes, Mannhei succiniciproducers* MBEL 55E, *Escherichia coli, Propionibacterium* species, *Pectinatus sp.*, *Bacteroides sp.*, such as *Bacteroides amylophilus, Ruminococcus flavefaciens*, *Prevotella ruminicola*, *Succcinimonas amylolytica*, *Succinivibrio dextrinisolvens*, *Wolinella succinogenes*, and *Cytophaga succinicans*. In an embodiment the organic acid producing microorganism that is able to ferment at least one C6 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 production processes of organic acid according to the present invention is capable of converting hexose (C6) sugars. The yeast, *e.g. Saccharomyces cerevisiae,* used in the processes according to the present invention can anaerobically ferment at least one C6 sugar.

The overall reaction time (or the reaction time of hydrolysis step and fermentation step together) may be reduced. In one embodiment, the overall reaction time is 300 hours or less, 200 hours or less, 150 hours or less, 140 hours or less, 130 or less, 120 hours or less, 110 hours or less, 100 hours of less, 90 hours or less, 80 hours or less, 75 hours or less, or about 72 hours at 90% glucose yield. Correspondingly, lower overall reaction times may be reached at lower glucose yield.

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 organic acid and/or an alcohol is prepared in the fermentation processes of the present invention. In a preferred embodiment succinic acid and/or ethanol is prepared in the fermentation processes of the present invention.

In an embodiment the alcohol, the organic acid, the enzymes, the enzyme producing microorganism, the alcohol producing microorganism and/or the organic acid producing microorganism are recovered. The processes according to the invention may comprise recovery of all kinds of products made during the processes including fermentation products such as ethanol and succinic acid. 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.

In an embodiment the processes of the invention also produce energy, heat, electricity and/or steam.

In an embodiment the solid fraction obtained after solid/liquid separation of the enzymatically hydrolysed lignocellulosic material and the waste obtained after purification/recovery of the fermentation product can be used in the production of electricity. Electricity can be made by incineration of any one of the above-mentioned materials. The electricicty can be used in any one of the steps of the processes according to the present invention.

The beneficial effects of the present invention are found for several lignocellulosic materials and therefore believed to be present for the hydrolysis of all kind of lignocellulosic

materials. This beneficial effects of the present invention are found for several enzymes and therefore believed to be present for all kind of hydrolysing enzyme compositions.

In an embodiment a part of the enzymatically hydrolysed lignocellulosic material is used in the propagation of the enzyme producing microorganism and/or the production of enzymes by the enzyme producing microorganism. In an embodiment the part of the enzymatically hydrolysed lignocellulosic material that is used in the propagation of the enzyme producing microorganism and/or the production of enzymes by the enzyme producing microorganism is the liquid fraction obtained after solid/liquid separation of the enzymatically hydrolysed lignocellulosic material. In an embodiment a part of the enzymatically hydrolysed lignocellulosic material and a part of the lignocellulosic material is used in the propagation of the enzyme producing microorganism and/or the production of enzymes by the enzyme producing microorganism. This means that a part of the enzymatically hydrolysed lignocellulosic material and/or a part of the lignocellulosic material is added to the enzyme producing microorganism before and/or during propagation and/or before and/or during production of enzymes by the enzyme producing microorganism. Of course, the enzyme producing microorganism can also be added to the part of the enzymatically hydrolysed lignocellulosic material and/or the part of the lignocellulosic material and/or the pretreated lignocellulosic material. The lignocellulosic material and/or the pretreated lignocellulosic material used in the propagation of the enzyme producing microorganism and/or the production of enzymes by the enzyme producing microorganism has not undergone enzymatic hydrolysis. In an embodiment the part of the lignocellulosic material and/or the pretreated lignocellulosic material that is used in the propagation of the enzyme producing microorganism and/or the production of enzymes by the enzyme producing microorganism has not been subjected to a solid/liquid separation. In another embodiment the part of the lignocellulosic material and/or the pretreated lignocellulosic material that is used in the propagation of the enzyme producing microorganism and/or the production of enzymes by the enzyme producing microorganism has been subjected to a solid/liquid separation. In the latter case, the solid fraction obtained after solid/liquid separation of the lignocellulosic material and/or the pretreated lignocellulosic material is used in the propagation of the enzyme producing microorganism and/or the production of enzymes by the enzyme producing microorganism.

In a preferred embodiment the enzymes produced by the enzyme producing microorganism are used in the enzymatic hydrolysis of the lignocellulosic material and/or the pretreated lignocellulosic material to obtain enzymatically hydrolysed lignocellulosic material.

In an embodiment the propagation of the enzyme producing microorganism and the production of enzymes by the enzyme producing microorganism are a single step, meaning that during propagation of the enzyme producing microorganism enzymes are already produced by the microorganism.

The enzymatically hydrolysed lignocellulosic material that is added to the enzyme producing microorganism before and/or during propagation of the enzyme producing

microorganism and/or before and/or during production of enzymes by the enzyme producing microorganism can be concentrated before addition. In an embodiment the part of the enzymatically hydrolysed lignocellulosic material that is used in the propagation of the enzyme producing microorganism and/or the production of enzymes by the enzyme producing microorganism has been subjected to a solid/liquid separation. The liquid fraction obtained after solid/liquid separation of the enzymatically hydrolysed lignocellulosic material may be used in the propagation of the enzyme producing microorganism and/or the production of enzymes by the enzyme producing microorganism. In an embodiment that liquid fraction may be subjected to a concentration step before it is used in the propagation of the enzyme producing microorganism and/or the production of enzymes by the enzyme producing microorganism.

The lignocellulosic material and/or the pretreated lignocellulosic material that is added to the enzyme producing microorganism before and/or during propagation of the enzyme producing microorganism and/or before and/or during production of enzymes by the enzyme producing microorganism can be washed before addition.

In an embodiment the solid fraction obtained after solid/liquid separation of the enzymatically hydrolysed lignocellulosic material is recycled back to the enzymatic hydrolysis container and subjected to another round of enzymatic hydrolysis.

EXAMPLES

Example 1

Lowering the pH of the enzymatically hydrolysed lignocellulosic material with H_2SO_4 and solid/liquid separation by means of sedimentation

Enzymatic hydrolysis was started by adding an enzyme composition (100 mg eWB-CE enriched in BG/g DM) to the hydrolysis container. TEC-210 cellulase-containing composition was produced as described in WO 2011/000949. The whole broth of the TEC-210 cellulase-containing composition (eWB-CE) comprised 44 mg protein/g whole broth. The hydrolysis was performed with pretreated sugar cane straw (pSCS) at a final concentration of 20% w/w total solids. The hydrolysis was performed at 62°C at pH 4.5 for 120 hours.

The pH of the obtained enzymatically hydrolysed lignocellulosic material was 4.54. Next, H_2SO_4 was added to a portion (17 gram) of the obtained enzymatically hydrolysed lignocellulosic material to lower the pH the respective portion to pH 2.20. H_2SO_4 was added at room temperature under constant stirring.

After pH addition, the stirring was stopped and the enzymatically hydrolysed lignocellulosic material was subjected to a solid/liquid separation by centrifugation for 10 minutes at a relative centrifugal force (RCF) of 2880 to obtain a liquid fraction and a solid fraction.

The solid/liquid separation was quantified as follows:

- Excellent solid/liquid separation (clear distinction between solid and liquid fraction and clear liquid fraction;) = 5;
- Good solid/liquid separation (clear distinction between solid and liquid fraction, but slightly hazy liquid fraction) = 3;
- No solid/liquid separation (distinction between solid and liquid fraction) = 1.

The results are shown in Table 1. Table 1 clearly demonstrates that by lowering the pH of the enzymatically hydrolysed lignocellulosic material to 3.0 or lower with H_2SO_4, there is a good separation between the liquid and solid fraction after 10 minutes of centrifugation, while at pH conditions of more than 3.0 there is no solid/liquid separation of the enzymatically hydrolysed lignocellulosic material.

Table 1: Solid/liquid separation after lowering pH with H_2SO_4 after 10 minutes of centrifugation.

pH	Solid/liquid separation
4.54	1
2.20	5

CLAIMS

1. Process for the preparation of a sugar product from lignocellulosic material, comprising the steps of:
   a) enzymatically hydrolysing the lignocellulosic material in one or more containers using an enzyme composition to obtain enzymatically hydrolysed lignocellulosic material comprising the sugar product,
   b) lowering the pH of the enzymatically hydrolysed lignocellulosic material by adding an acid to the enzymatically hydrolysed lignocellulosic material,
   c) subjecting the enzymatically hydrolysed lignocellulosic material to a solid/liquid separation to obtain a solid fraction and a liquid fraction, and
   d) optionally, recovering of the solid fraction and/or the liquid fraction.

2. Process for the preparation of a fermentation product from lignocellulosic material, comprising the steps of:
   a) performing a process according to claim 1,
   b) fermentation of the solid fraction and/or liquid fraction to produce a fermentation product, and
   c) optionally, recovering of the fermentation product.

3. Process according to claim 1 or 2, wherein the pH is lowered to 3.0 or lower.

4. Process according to any of the claims 1 to 3, wherein the solid/liquid separation is performed by centrifugation or sedimentation.

5. Process according to any of the claims 1 to 4, wherein
   a) the solid fraction comprises between 1 and 80 wt% C6 sugars, and/or
   b) the liquid fraction comprises between 1 and 80 wt% C6 sugars.

6. Process according to any of the claims 1 to 5, wherein the enzymatic hydrolysis comprises at least:
   - a liquefaction step wherein the lignocellulosic material is hydrolysed in at least a first container, and
   - a saccharification step wherein the liquefied lignocellulosic material is hydrolysed in the at least first container and/or in at least a second container.

7. Process according to any of the claims 1 to 6, wherein the lignocellulosic material is subjected to a pretreatment step before the enzymatic hydrolysis.

8. Process according to any of the claims 1 to 7, wherein oxygen is added during the enzymatic hydrolysis.

9. Process according to any of the claims 1 to 8, wherein the enzyme composition is a whole fermentation broth of a fungus.

10. Process according to claim 9, wherein the fungus is *Rasamsonia*.

11. Process according to any of the claims 1 to 10, wherein the liquid fraction is subjected to a detoxification step and/or a concentration step.

12. Process according to any of the claims 2 to 11, wherein the fermentation is done by a yeast.

13. Process according to any of the claims 2 to 12, wherein the fermentation product is an organic acid or an alcohol.

14. Process according to any of the claims 1 to 13, wherein the one or more containers have a volume of at least 1 m^3.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/064277

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P19/00 C12P19/02 C12P19/12 C12P19/14 C12P7/06
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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X Y	EP 2 860 258 A1 (TORAY INDUSTRIES [JP]) 15 April 2015 (2015-04-15) paragraphs [0019] - [0024], [0035], [0049] - [0055]; claims; examples -----	1-14 1-14
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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

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"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
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A	EP 2 548 965 A1 (TORAY INDUSTRIES [JP]) 23 January 2013 (2013-01-23) claims; figure 1	1-14
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