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(54) Title: FRACTIONATION OF LIGNOCELLULOSIC MATTER

(57) Abstract: The invention relates to methods for the fractionation of lignocellulosic matter. More specifically, the invention relates to methods for separating sugar-containing components of lignocellulosic matter from lignin.

FRACTIONATION OF LIGNOCELLULOSIC MATTER

Technical Field

The invention relates to methods for the fractionation of lignocellulosic matter. More specifically, the invention relates to methods for fractionating sugar-containing components of lignocellulosic matter from lignin.

Background

With the continuing high price of oil and its increasing importation costs in many countries, new strategies for the fractionation of lignocellulosic biomass into its core components (sugars and lignin) are attracting increasing interest. Following fractionation, these sugars and lignin-based compounds can then be converted into a range of products including fuel ethanol and higher value chemicals, thereby providing an alternative and renewable feedstock to the depleting sources of hydrocarbon-based raw materials. In the US, the Department of Energy is providing strong financial support for the development of 'biorefineries' based on the use of these biomass-based raw materials with funding of up to $385m over 4 years for the period 2007-2011 (see www.energy.gov/news/4827.htm).

Lignocellulosic matter consists of carbohydrate polymers (celluloses and hemicelluloses) and the phenolic polymer lignin. Cellulose and hemicellulose polymers are tightly bound to lignin by hydrogen and covalent bonds making separation difficult. Lignin bound to cellulose and hemicellulose hinders the hydrolysis of these components into simple sugars suitable for fermentation to bioethanol and other target products. By decreasing lignin content, the remaining carbohydrate components can be more readily accessed by hydrolytic enzymes and more efficient production of fermentable sugars can thus be achieved. Lignin makes up a significant proportion of lignocellulosic matter and offers another utilisable resource in addition to the cellulosic and hemicellulosic components. However, many current methods for the production of biofuels from lignocellulosic matter do not effectively utilise the lignin component, which instead goes to waste.

A need exists for methods capable of efficiently separating lignin from the cellulose and/or hemicellulose components of lignocellulosic biomass during biofuel production processes. A need also exists for methods that better utilise the energy producing

potential of lignin during the production of fermentable sugars from lignocellulosic biomass.

Summary of the invention

In a first aspect, the invention provides a method of fractionating lignocellulosic matter, the method comprising the steps of:

(a) fractionating hemicellulose from the lignocellulosic matter using a solvent

(b) removing the solvated hemicellulose component

(c) fractionating lignin from the remaining solid matter using a supercritical solvent; and optionally

(d) removing the solvated lignin component.

In a second aspect, the invention provides a method of producing an alkylated lignin product from lignocellulosic matter, the method comprising the steps of:

(a) fractionating hemicellulose from the lignocellulosic matter using a solvent

(b) removing the solvated hemicellulose component

(c) fractionating lignin from the remaining solid matter using a supercritical solvent; and optionally

(d) removing the solvated lignin component,

wherein said supercritical solvent is an alkylating agent.

In a third aspect, the invention provides a method of fractionating lignocellulosic matter, the method comprising the steps of:

(a) fractionating hemicellulose from the lignocellulosic matter using a solvent

(b) removing the solvated hemicellulose component

(c) fractionating lignin from the remaining solid matter using a supercritical solvent

(d) removing the solvated lignin component

(e) fractionating cellulose from the remaining solid matter using a solvent; and optionally

(f) removing the solvated cellulose component

In a fourth aspect, the invention provides a method of producing fermentable saccharides from lignocellulosic matter, the method comprising the steps of:

(a) fractionating hemicellulose from the lignocellulosic matter using a solvent

(b) removing the solvated hemicellulose component

(c) fractionating lignin from the remaining solid matter using a supercritical solvent

(d) removing the solvated lignin component

(e) fractionating cellulose from the remaining solid matter using a solvent; and optionally

(f) saccharification of either or both of:

(i) the solvated hemicellulose component of step (b)

(ii) the solvated cellulose component of step (e)

to produce fermentable saccharides.

In a fifth aspect, the invention provides a method of producing a fermented sugar product from lignocellulosic matter, the method comprising:

(a) fractionating hemicellulose from the lignocellulosic matter using a solvent

(b) removing the solvated hemicellulose component

(c) fractionating lignin from the remaining solid matter using a supercritical solvent

(d) removing the solvated lignin component

(e) fractionating cellulose from the remaining solid matter using a solvent; and optionally

(f) saccharification of either or both of:

(i) the solvated hemicellulose component of step (b)

(ii) the solvated cellulose component of step (e)

to produce fermentable saccharides; and

(g) fermenting the saccharides.

In one embodiment of the fifth aspect, the fermented sugar product is an alcohol or an organic acid. The alcohol may be selected from the group consisting of ethanol, butanol, xylitol, mannitol, and arabinol. The organic acid may be glutamic acid or succinic acid.

In one embodiment of the fifth aspect, step (g) of fermenting the sugars is performed using bacteria, yeast or fungi. The bacteria, yeast or fungi may be recombinant bacteria, recombinant yeast or recombinant fungi.

In another embodiment of the fifth aspect, the saccharification and fermenting are performed simultaneously.

In one embodiment of the fourth or fifth aspect, the fermentable saccharides are selected from the group consisting of polysaccharides, oligosaccharides, disaccharides,

monosaccharides and mixtures thereof. The fermentable saccharides may be 5 carbon saccharides.

In one embodiment of the fourth or fifth aspect, the saccharification of step (f) is performed using pyrolysis.

In one embodiment of the fourth or fifth aspect, the saccharification of step (f) is substituted with hydrothermal upergrading in sub-supercritical water.

In one embodiment of the fourth or fifth aspect, the saccharification of step (f) is performed using one or more hydrolytic enzymes. The hydrolytic enzyme may be a hydrolase. The hydrolase may be a glycosylase. The glycosylase may be selected from the group consisting of xylanases, glucosidases, cellulases, xylosidases, mannanases, arabinosidases, glycoside hydrolases, dextranases, and endoglucanases. The hydrolytic enzyme may be a thermophilic hydrolytic enzyme. The thermophilic enzyme may be derived from one or more microorganisms selected from the group consisting of thermophilic bacteria, thermophilic fungi, thermophilic yeast and combinations thereof.

In one embodiment of the third, fourth or fifth aspect, step (e) of fractionating cellulose from the remaining solid matter using a solvent is performed at a temperature of between 80°C and 400°C.

In another embodiment of the third, fourth or fifth aspect, step (e) of fractionating cellulose from the remaining solid matter using a solvent is performed at a temperature of 340°C.

In one embodiment of the third, fourth or fifth aspect, the solvent of step (e) is selected from the group consisting of a subcritical solvent, a critical solvent and a supercritical solvent.

In another embodiment of the third, fourth or fifth aspect, step (e) of fractionating cellulose from the remaining solid matter using a solvent is performed with a reaction pH of between about 3.5 and about 10.5.

In a further embodiment of the third, fourth or fifth aspect, the solvent of step (e) is water.

In one embodiment of the first, second, third, fourth or fifth aspect, step (d) of removing the solvated lignin component is performed using a cyclone apparatus.

In another embodiment of the first, second, third, fourth or fifth aspect, step (b) of removing the solvated hemicellulose component is performed using a hydrocyclone apparatus.

In another embodiment of the first, second, third, fourth or fifth aspect, step (a) of fractionating hemicellulose from the lignocellulosic matter using a solvent is performed at a temperature of between about 120°C and about 250°C.

In a further embodiment of the first, second, third, fourth or fifth aspect, step (a) of fractionating hemicellulose from the lignocellulosic matter using a solvent is performed at a temperature of about 210°C.

In another embodiment of the first, second, third, fourth or fifth aspect, step (a) of fractionating hemicellulose from the lignocellulosic matter using a solvent is performed with a reaction pH of between about 3.5 and about 10.5.

In one embodiment of the first, second, third, fourth or fifth aspect, the supercritical solvent of step (c) is an alkylating agent is selected from the group consisting of an alkylhalide, an alkylsulfate, an olefin, an alkylphosphate, and an alcohol. The alcohol may be selected from the group consisting of methanol, ethanol, isopropyl alcohol, isobutyl alcohol, pentyl alcohol, hexanol and iso-hexanol. The alcohol may be ethanol.

In one embodiment of the first, second, third, fourth or fifth aspect, step (c) of fractionating lignin from the remaining solid matter using a supercritical solvent is perfomred at a temperature of about 514 Kelvin.

In another embodiment of the first, second, third, fourth or fifth aspect, step (c) of fractionating lignin from the remaining solid matter using a supercritical solvent is performed at a pressure of about 6.14 MPa.

In a sixth aspect the invention provides a product obtained in accordance with the method of any one of the first, second, third, fourth and fifth aspects.

In a seventh aspect, the invention provides a method of fractionating hemicellulose from lignocellulosic matter, said method comprising treating the lignocellulosic matter with a solvent at a reaction temperature of between about 100 and about 250 degrees Celsius, and a reaction pressure of between about 2 and about 50 atmospheres.

In one embodiment of the seventh aspect, the reaction pressure is between about 10 and about 40 atmospheres.

In one embodiment of the seventh aspect, the lignocellulosic matter comprises at least about 10% lignin, at least about 15% cellulose, and at least about 10% hemicellulose.

In one embodiment of the seventh aspect, the lignocellulosic matter comprises at least about 20% lignin, at least about 25% cellulose, and at least about 20% hemicellulose.

In one embodiment of the seventh aspect, the solvent is an aqueous acid and the treatment is performed at a pH of below about 6.5.

In one embodiment of the seventh aspect, the solvent is an aqueous base and the treatment is performed at a pH of above about 7.5.

In one embodiment of the seventh aspect, the solvent is water and the treatment is performed at a pH of between about 6.5 and about 7.5.

In one embodiment of the seventh aspect, the method further comprises pre-treating the lignocellulosic matter prior to fractionating said hemicellulose. The pre-treating may comprise producing a slurry comprising a mixture of the solvent and particles derived from the lignocellulosic matter.

In one embodiment of the seventh aspect, the particles are between about 10 microns and about 10,000 microns in size.

In another embodiment of the seventh aspect, between about 100 and about 400 microns in size.

In one embodiment of the seventh aspect, the particles may be between about 10 microns and about 10,000 microns in size and more preferably between 100 and 400 microns in size.

In one embodiment of the seventh aspect, the slurry comprises between about 5% and about 20% lignocellulosic matter.

In an eighth aspect, the invention provides a method of fractionating lignocellulosic matter, the method comprising the steps of:

(a) solvating hemicellulose from said lignocellulosic matter using the method according to the seventh aspect,

(b) removing solvated hemicellulose from solid matter remaining after step (a),

(c) solvating lignin from the solid matter remaining after step (a) using a supercritical solvent.

In one embodiment of the eighth aspect, the supercritical solvent is ethanol and the solvating in step (c) is performed at a temperature of above about 230 degrees Celsius and a pressure of above about 55 atmospheres.

In one embodiment of the eighth aspect, the supercritical solvent is ethanol and the solvating in step (c) is performed at a pH of between about 6.5 and 7.5.

In one embodiment of the eighth aspect, the method comprises the further step of removing solvated lignin from solid matter remaining after step (c).

In one embodiment of the eighth aspect, the method comprises the further step of solvating cellulose from said solid matter remaining after step (c) to produce solvated cellulose.

In one embodiment of the eighth aspect, the method comprises the step of saccharifying the solvated hemicellulose to produce a fermentable saccharide.

In one embodiment of the eighth aspect, the method comprises the step of saccharifying either or both of:

(a) the solvated hemicellulose

(b) the solvated cellulose,

to produce a fermentable saccharide.

In one embodiment of the eighth aspect the saccharide is fermented to produce a fermented sugar product.

In one embodiment of the eighth aspect, the saccharifying and fermenting are performed simultaneously.

In one embodiment of the eighth aspect, the fermented sugar product is an alcohol or an organic acid. The alcohol may be selected from the group consisting of ethanol, butanol, xylitol, mannitol, and arabinol. The organic acid may be glutamic acid or succinic acid.

In a ninth aspect, the invention provides a method of producing an alkylated lignin product from lignocellulosic matter, the method comprising fractionating lignocellulosic matter in accordance with the method of the eighth aspect, wherein an alkylating agent is used as the supercritical solvent, and wherein said agent alkylates the lignin of step (c).

In one embodiment of the ninth aspect, the supercritical solvent is ethanol and the alkylated lignin product is ethylated lignin.

In a tenth aspect, the invention provides a product obtained by the process of the seventh, eighth or ninth aspect.

Brief Description of the Drawings

A preferred embodiment of the present invention will now be described, by way of an example only, with reference to the accompanying drawings wherein:

Figure 1 is a graph showing the results of a dinitrosalicyclic acid (DNS) assay conducted on hemicellulose liquor samples subjected to saccharification using hydrolytic enzymes. Absorbance readings (I_{540}, in mOD) from substrate only controls and enzyme

only controls were subtracted from readings obtained from enzyme-substrate samples. Sample numbers are shown on x-axis. Y-axis shows amounts of reducing sugars present in each sample.

Figure 2 shows high performance liquid chromatography (HPLC) traces of standard mixtures subjected to acidic hydrolysis. Top panel: 50μM; Centre panel: standard sample (1/50) with 15mM NaOH; Lower panel: standard sample (1/50) with water. Peak numbering: 1 (fucose), 2 (deOH-glucose); 3 (galactosamine); 4 (arabinose); 5 (galactose); 6 (glucose); 7 (lyxose); 8 (xylose); 9 (mannose), 10 (fructose); 11 (ribose).

Figure 3 shows high performance liquid chromatography (HPLC) traces of standard mixtures subjected to acidic hydrolysis. Peak numbering: 1 (fucose), 2 (deOH-glucose); 3 (galactosamine); 4 (arabinose); 5 (galactose); 6 (glucose); 7 (lyxose); 8 (xylose); 9 (mannose), 10 (fructose); 11 (ribose), 12 (lactose); 13 (cellobiose);14 (maltose).

Figure 4 shows representative high performance liquid chromatography (HPLC) traces of hemicellulose samples subjected to acidic hydrolysis. Peak numbering: 1 (deOH-glucose); 2 (arabinose); 3 (galactose); 4 (glucose); 5 (xylose); 6 (mannose), 7 (fructose); 8 (ribose).

Figure 5 shows representative high performance liquid chromatography (HPLC) traces of hemicellulose samples subjected to acidic hydrolysis. 1 (deOH-glucose); 2 (arabinose); 3 (galactose); 4 (glucose); 5 (xylose); 6 (mannose).

Definitions

As used in this application, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a plant cell" also includes a plurality of plant cells.

As used herein, the term "comprising" means "including." Variations of the word "comprising", such as "comprise" and "comprises", have correspondingly varied meanings. Thus, for example, a polynucleotide "comprising" a sequence encoding a protein may consist exclusively of that sequence or may include one or more additional sequences.

As used herein, the term "saccharide" encompasses any molecule comprising one or more monosaccharide units. Examples of saccharides include, but are not limited to, cellulose, hemicellulose, polysaccharides, oligosaccharides, disaccharides and monosaccharides. Saccharides also include glycoconjugates, such as glycoproteins and

glycolipids. All stereoisomeric and enantiomeric forms of "saccharides" are encompassed by the term.

As used herein, the terms "saccharification" and "saccharifying" refer to the process of breaking down carbohydrate molecules into shorter sugar chains (e.g. shorter polysaccharides, oligosaccharides, disaccharides and/or monosaccharides).

A supercritical solvent is a solvent heated above its critical temperature and pressurized above its critical pressure such that it exhibits properties of both a gas and a liquid.

As used herein, a "supercritical" substance (e.g. a supercritical solvent) refers to a substance that is heated above its critical temperature and pressurised above its critical pressure (i.e. a substance at a temperature and pressure above its critical point). The term "supercritical" also encompasses conditions of temperature and/or pressure that are a small, although not substantial, amount (e.g. approximately 5%) below the supercritical point of the substance in question (i.e. "sub-critical"). Accordingly, the term "supercritical" also encompasses oscillatory behaviour around the supercritical point of a substance (i.e. movement from supercritical conditions to sub-critical conditions and *vice versa*). For example, a solvent having a supercritical point of 305 degrees Kelvin and 4.87 atmospheres may, for the purposes of the present invention, still be considered to be "supercritical" at a slightly lower temperature (e.g. between about 290 degrees and 305 degrees Kelvin) and/or a slightly lower pressure (e.g. between about 4.6 and about 4.87 atmospheres).

It will be understood that use the term "about" herein in reference to a recited numerical value (e.g. a reaction temperature, pressure or pH) includes the recited numerical value and numerical values within plus or minus ten percent of the recited value.

Any description of prior art documents herein, or statements herein derived from or based on those documents, is not an admission that the documents or derived statements are part of the common general knowledge of the relevant art in Australia or elsewhere.

For the purposes of description all documents referred to herein are incorporated by reference unless otherwise stated.

Detailed Description of the Invention

Described herein are methods for the fractionation of lignocellulosic matter into one or more of its core components (i.e. hemicellulose, cellulose and lignin).

Existing technologies have demonstrated that lignocellulosic biomass may be dissolved using supercritical solvents such as ethanol or water. However, solutions generated by treatment with supercritical solvents such as ethanol are difficult to process, and the efficiency of saccharification and/or fermentation is significantly reduced due to the presence of lignin. Treatment of lignocellulosic biomass with supercritical water requires the use of high reaction temperatures which induce dehydration of the sugars, creating double bonds and highly reactive cyclic molecules (e.g. furfural) that easily polymerise and yield tar-like compounds if not stabilised.

Existing methods have also attempted solubilisation of lignocellulosic biomass into separate fractions by sequential treatment with supercritical water and supercritical ethanol (or *vice versa*). Such methods generally involve dissolving the cellulose component of lignocellulosic biomass with supercritical water and solubilisation of lignin component with supercritical ethanol. However, this approach still experiences the drawback of undesirable tar formation in the resulting the cellulose solution as the hemicellulose present in the biomass reacts alot faster than the cellulose.

Advantageously, the present invention circumvents the above-mentioned problems by commencing the fractionation of lignocellulosic biomass with solubilisation of the hemicellulose component under mild conditions, thereby preventing dehydration and the undesirable formation of tar-like molecules. The prevention of tar formation during hemicellulose fractionation increases the yield of sugars obtained during the process. Furthermore, the initial separation of hemicellulose under mild conditions increases the efficiency of lignin solubilisation from the remaining solid matter. In general, separation of lignin from the sugar-containing component(s) of lignocellulosic biomass (i.e. hemicellulose and/or cellulose) requires treatment under high temperature and pressure. The initial removal of hemicellulose (the most reactive of the three components) under mild conditions provides a means of reducing tar-formation during solubilisation of the lignin. Furthermore, in accordance with the methods provided herein solubilisation of lignin may be performed with supercritical ethanol (which is substantially milder than supercritical water and preferentially dissolves lignin) in order to avoid the degradation of cellulose. Following solubilisation of lignin, the remaining solid matter (comprising predominantly cellulose) may be further processed and utilised.

Fractionated hemicellulose and/or cellulose components fractionated in accordance with the methods described herein may be used to generate fermentable sugars which in turn may be converted into a range of compounds. Non-limiting examples of such compounds include fuel products, food additives, and organic acids.

Lignin fractionated in accordance with the methods described herein may be used, for example, as a fuel additive, or further upgraded for use in the production of compounds including but not limited to resins, polymers and fragrances.

It will be understood that the invention also relates to fractionated hemicellulose, lignin and cellulose components produced in accordance with the methods described herein, along with products that may be produced from processing those components.

Fractionation of lignocellulosic matter

Lignocellulosic matter

In accordance with the methods described herein, lignocellulosic matter may be fractionated into one or more of its core components (i.e. hemicellulose, cellulose and/or lignin).

"Fractionation" of lignocellulosic matter as contemplated herein refers to a process whereby one or more components of lignocellulosic matter (e.g. cellulose, hemicellulose, lignin) is/are partially or wholly separated from other component(s) of that same matter. Fractionation of component(s) from modified forms of lignocellulosic matter is also contemplated (e.g. fractionation of lignin or cellulose from a modified sample of lignocellulosic matter from which hemicellulose has already been substantially or wholly removed).

In certain embodiments of the invention, fractionation is achieved by solvation of one component (e.g. hemicellulose) from other component(s) (e.g. lignin and cellulose) of lignocellulosic matter (or modified forms of lignocellulosic matter). "Solvation" as contemplated herein refers to an interaction between a solute (e.g. hemicellulose) and a solvent (e.g. water) leading to the stabilisation or partial stabilisation of the solute in solution with the solvent.

In general, lignocellulosic matter refers to a substance comprising the components of lignin, cellulose and hemicellulose.

The relative percentages of lignin, hemicellulose and cellulose in a given sample will depend on the nature of the lignocellulosic matter.

For example, in some embodiments, lignocellulosic matter may comprise 20-35% lignin, 20-45% cellulose and 20-35% hemicellulose.

In other embodiments, the content of lignin may be more than 35%, or less that 20%, the content of cellulose may be more the 45% or less than 20%, and the content of hemicellulose may be more than 35% or less than 20%.

In some embodiments, the lignocellulosic matter comprises at least about 10% lignin, at least about 15% cellulose, and at least about 10% hemicellulose.

In other embodiments, the lignocellulosic matter comprises at least about 15% lignin, at least about 20% cellulose, and at least about 15% hemicellulose.

In additional embodiments, the lignocellulosic matter comprises at least about 20% lignin, at least about 25% cellulose, and at least about 20% hemicellulose.

In some embodiments, the lignocellulosic matter comprises at least about 25% lignin, at least about 30% cellulose, and at least about 25% hemicellulose.

Lignocellulosic matter for use in accordance with the methods described herein may be derived from any source. For example, lignocellulosic matter may be derived from woody plant matter. Examples of suitable woody plants include, but are not limited to, pine (e.g. *Pinus radiata*), birch, eucalyptus, beech, spruce, fir, cedar, poplar and aspen.

Additionally or alternatively, lignocellulosic matter may be derived from fibrous plant matter, non-limiting examples of which include grass (e.g. switchgrass), grass clippings, flax, corn cobs, corn stover, reed, bamboo, bagasse, hemp, sisal, jute, cannibas, hemp, straw, wheat straw, abaca, cotton plant, kenaf, rice hulls, and coconut hair.

Additionally or alternatively, products and byproducts comprising lignocellulosic matter may be used for the methods described herein, non-limiting examples of which include wood-related materials (for example, sawmill and paper mill discards and offcuts, saw dust, particle board and leaves) and industrial products such as pulp, paper, papermaking sludge, textiles and cloths, dextran, and rayon.

Pre-treatment of lignocellulosic matter

Lignocellulosic matter may optionally be pre-treated prior to commencing fractionation of hemicellulose. For example, mechanical and/or chemical methods may be used to disrupt the structure of lignocellulosic matter prior to separating the hemicellulose component. Non-limiting examples of mechanical pre-treatment methods include pressure, grinding, agitation, shredding, milling, compression/expansion, or other types of

mechanical action. Pre-treatment of the lignocellulosic matter may be performed using a mechanical apparatus, for example, an extruder, a pressurized vessel, or a batch reactor.

Pre-treatment methods may include treatment with heat. For example, steam explosion pre-treatment methods may be used to disrupt the structure of lignocellulosic biomass. In general, steam explosion pre-treatment methods involve exposing the biomass to high pressure steam in a contained environment before the resulting product is explosively discharged to an atmospheric pressure. Pre-treatment with steam explosion may additionally involve agitation of the lignocellulosic matter.

In preferred embodiments, lignocellulosic matter for use in the methods of the invention is provided in the form of a slurry. The slurry may be generated, for example, by converting the lignocellulosic matter into a powder of appropriate particle size (e.g. by using grinding, agitation, shredding, milling, compression/expansion and/or other types of mechanical action) and mixing with an appropriate liquid (e.g. water).

The particle size of solid matter included in the slurry may be between about 10 microns and about 10,000 microns. For example, the particle size of solid matter included in the slurry may be at least about 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000 or 9000 microns. Alternatively, the particle size may be between about 10 microns and 50 microns, between about 10 microns and about 100 microns, between about 10 microns and about 400 microns, between about 10 microns and about 500 microns, between about 100 microns and about 200 microns, between about 100 microns and about 300 microns, between about 100 microns and about 500 microns, or between about 100 microns and about 1000 microns.

In one embodiment, the particle size is between about 100 microns and about 400 microns.

In another embodiment, the solid matter is wood flour and the particle size is between about 150 microns and about 300 microns.

The concentration of solid matter in the slurry may be high (e.g. above about 50% w/v). Alternatively, the concentration of solid matter in the slurry may be between about 1% and about 50%, between about 1% and about 40%, between about 1% and about 30%, between about 1% and about 20%, or between about 1% and about 10% w/v.

In certain embodiments, the concentration of solid matter in the slurry is between about 5% and about 20% w/v.

In one embodiment, the solid matter is wood flour and the concentration of solid matter in the slurry is about 10% w/v.

Fractionation of hemicellulose

In accordance with the methods described herein, fractionation of lignocellulosic matter commences with separation of the hemicellulose component.

Preferably, separation of the hemicellulose component involves the use of one or more solvents. Any solvent capable of solvating hemicellulose may be used, non-limiting examples of which include water, aqueous acidic solutions, aqueous alkaline solutions, and organic solvents.

For example, heated steam may be used to facilitate the separation of hemicellulose. Such methods are described, for example, in US Patent No. 1578609 and US Patent No. 2303345.

Additional examples of methods by which hemicellulose may be separated from lignocellulosic matter using solvents are described in U.S. Patent No. 2868778, U.S. Patent No. 2709699 and U.S. Patent No 7198695.

Suitable reaction conditions for the solvation of hemicellulose from lignocellulosic biomass will depend on the specific solvent or solvents used, and the nature of the lignocellulosic starting material.

In preferred embodiments, the hemicellulose component is solvated in aqueous solution. In general, solvation of hemicellulose in aqueous solution will typically also involve partial hydrolysis of the hemicellulose. Examples of suitable aqueous solutions for the solvation and partial hydrolysis of hemicellulose include aqueous acidic solutions, aqueous alkaline solutions, and aqueous solutions of neutral pH (*i.e.* pH of about 7.0).

A suitable alkaline aqueous solution will have a pH of above about 7.0, or above about 7.5. For example, a suitable alkaline aqueous solution may have a pH of between about 7.0 and about 11.0. In certain embodiments of the invention, a suitable alkaline aqueous solution may have a pH of between about 7.0 and about 10.5, between about 7.0 and about 10.0, between about 7.0 and about 9.5, between about 7.0 and about 9.0, between about 7.0 and about 8.5, between about 7.0 and about 8.0 and between about 7.0 and 7.5.

A suitable acidic aqueous solution will have a pH of below about 7.0, or below about 6.5. For example, a suitable acidic aqueous solution may have a pH of between about 2.0 and about 7.0, or between about 3.0 and about 7.0. In certain embodiments of the invention, a suitable acidic aqueous solution may have a pH of between about 3.5 and about 7.0, between about 4.0 and about 7.0, between about 4.5 and about 7.0, between

about 5.0 and about 7.0, between about 5.5 and about 7.0, between about 6.0 and about 7.0, and between about 6.5 and about 7.0.

In one preferred embodiment, hemicellulose is fractionated from lignocellulosic biomass in aqueous solution at neutral pH (i.e. pH 7.0) or substantially neutral pH.

In another preferred embodiment, hemicellulose is fractionated from lignocellulosic biomass in aqueous solution at a pH of between about 6.5 and 7.5.

In another preferred embodiment, hemicellulose is fractionated from lignocellulosic biomass in acidic aqueous solution at a pH of about 2.0.

In most cases, the pH of the reaction mixture can be adjusted by adding a suitable acid or base.

Non-limiting examples of suitable acids that may be used to adjust the pH of a reaction mixture include hydrochloric acid, trifluoroacetic acid, sulfuric acid, sulfurous acid and/or organic acids such as propionic acid, lactic acid, citric acid, or glycolic acid. Additionally or alternatively, carbon dioxide may be added to the aqueous mixture to obtain an acidic pH (i.e. a pH of below about 7.0)

Non-limiting examples of suitable bases that may be used to adjust the pH of a reaction mixture include sodium hydroxide, potassium hydroxide, ammonium hydroxide, carbonates or bicarbonates.

Methods by which the pH of a reaction mix may be determined are known in the art, and described, for example in Gallagher and Wiley (Eds) *Current Protocols Essential Laboratory Techniques* John Wiley & Sons, Inc (2008).

The solvation of hemicellulose in aqueous solution may be performed at any reaction temperature (at any of the pH ranges or values referred to above). For example, the solvation of hemicellulose in aqueous solution may be performed at a reaction temperature of between about 120°C and about 250°C. In certain embodiments of the invention, the reaction temperature is between about 130°C and about 250°C, between about 140°C and about 250°C, between about 150°C and about 250°C, between about 160°C and about 250°C, between about 170°C and about 250°C, between about 180°C and about 250°C, between about 190°C and about 250°C, between about 200°C and about 250°C, between about 210°C and about 250°C, between about 220°C and about 250°C, between about 230°C and about 250°C, between about 240°C and about 250°C, between about 120°C and about 240°C, between about 120°C and about 230°C, between about 120°C and about 220°C, between about 120°C and about 210°C, between about 120°C and about 200°C, between about 120°C and about 190°C, between about 120°C and about

180°C, between about 120°C and about 170°C, between about 120°C and about 160°C, between about 120°C and about 150°C, between about 120°C and about 140°C and between about 120°C and about 130°C.

In one preferred embodiment, hemicellulose is fractionated from the lignocellulosic matter at reaction temperatures ranging from about 120°C to about 190°C.

Suitable reaction temperatures may be obtained, for example, by performing the solvation of hemicellulose in a mechanical apparatus such as a batch reactor or pressurized vessel. Performing the solvation of hemicellulose in a mechanical apparatus, may also allow alteration of the pressure applied at the operating temperatures contemplated.

The solvation of hemicellulose in aqueous solution may be performed at any reaction pressure (in combination with any of the reaction temperatures and pH ranges/values referred to above).

For example, the solvation of hemicellulose in aqueous solution may be performed at a reaction pressure of between about 1 atmospheres (atm) and about 250 atm, between about 1atm and about 100 atm, between about 1 atm and about 50 atm, preferably between about 2 atm and about 50 atm, and more preferably between about 10 atm and about 40 atm.

In a preferred embodiment, hemicellulose is fractionated from lignocellulosic matter at a reaction pressure of between about 2 and about 50 atmospheres.

In another preferred embodiment, hemicellulose is fractionated from lignocellulosic matter at a reaction pressure of between about 10 and about 40 atmospheres.

In general, reactions are performed for a period of time sufficient to solvate substantially all of the hemicellulose, or, the majority of hemicellulose from the lignocellulosic matter.

For example, a reaction under conditions defined by a combination of any of the reaction temperatures, reaction pressures and reaction pH ranges or values referred to above may be performed for less than 20 minutes. In some embodiments, the reaction is performed for between about 2 minutes and about 20 minutes. In other embodiments, the reaction is performed from between about 5 minutes and about 15 minutes. In other embodiments, the reaction is performed for a period of more than 20 minutes.

Optimal reaction conditions for the solvation of hemicellulose from lignocellulosic matter will ultimately depend on factors including the type of lignocellulosic matter under treatment and the specific solvent used. For example, factors such as temperature and pH

of the reaction mixture, isotonicity, amount of lignocellulosic matter and solvent, and length of reaction time may be routinely varied in order to optimise the reaction.

Optimal reaction conditions will be readily apparent to the skilled addressee upon analysis of the solvated hemicellulose, which may be performed using standard methods generally known in the art. For example, solvated hemicellulose may be analysed using spectroscopy techniques. Suitable spectroscopy techniques include, but are not limited to, near infra red spectroscopy, fourier transform infrared spectroscopy, nuclear magnetic resonance spectroscopy, raman microscopy, UV microspectrophotometry and X-ray diffraction. Additionally or alternatively, solubilized hemicellulose may quantified by high performance liquid chromatography, for example, using methods described in Bjerre et al., *"Quantification of solubilized hemicellulose from pretreated lignocellulose by acid hydrolysis and high performance liquid chromatography"*, (1996) in publication Riso-R-855 (EN), Rise National Laboratory.

In one preferred embodiment, hemicellulose is fractionated from lignocellulosic matter at a reaction temperature of between about 100 and 250 degrees Celsius, and a reaction pressure of between about 2 and about 50 atmospheres. The pH of the reaction mix may be about 7.0, above about 7.0, or below about 7.0. The pH of the reaction mix may be about 2.0.

In another preferred embodiment, hemicellulose is fractionated from lignocellulosic matter at a reaction temperature of 100 and 250 degrees Celsius, and a reaction pressure of between about 10 and about 40 atmospheres. The pH of the reaction mix may be about 7.0, above about 7.0, or below about 7.0. The pH of the reaction mix may be about 2.0.

In anoother preferred embodiment, the hemicellulose component is solvated from the lignocellulosic matter using water at a reaction pH of about 7.0 and a reaction temperature of about 210°C. The pH of the reaction mix may be about 7.0, above about 7.0, or below about 7.0. The pH of the reaction mix may be about 2.0.

The solvated hemicellulose component may be removed from the remaining solid matter (which substantially comprises lignin and cellulose). Separation of the solvated hemicellulose fraction from the remaining solid matter may be accomplished, for example, by transferring the mixture to an apparatus capable of separating and/or washing the remaining solid matter. For example, solid matter may be physically retained by passing the mixture through one or more appropriately sized filter(s) through which the solvated hemicellulose fraction may pass. The solid matter may be retained on the filter(s) and washed if so desired.

Additionally or alternatively, centrifugation may be used to separate solvated hemicellulose from the remaining solid matter. In a continuous system, countercurrent flow of solids and liquid may be used to facilitate the separation.

In certain embodiments, a hydrocyclone apparatus is used to separate the solvated hemicellulose fraction from the remaining solid matter. A hydrocyclone is a static apparatus that applies centrifugal force to a liquid mixture so as to promote the separation of heavy components, in this case the remaining solid matter, from light components, in this case the solvated hemicellulose fraction. In general, a hydrocyclone may operate to separate hemicellulose from remaining solid matter as follows. The hydrocyclone directs inflow tangentially near the top of a vertical cylinder, converting the velocity of incoming material into a rotary motion thus creating centrifugal force. The remaining solid matter moves outward toward the wall of the cylinder where it agglomerates and spirals down the wall to an outlet. The solvated hemicellulose fraction moves toward the axis of the hydrocyclone and upwards to a different outlet.

Fractionation of lignin

In accordance with the methods described herein, solid matter remaining after the fractionation of hemicellulose from lignocellulosic matter may be further separated into lignin and cellulose components.

Separation of lignin from cellulose may be achieved using a supercritical solvent.

In general, a supercritical solvent is a solvent heated above its critical temperature and pressurized above its critical pressure such that it exhibits properties of both a gas and a liquid (i.e. a substance at a temperature and pressure above its critical point). The term "supercritical" also encompasses conditions of temperature and/or pressure that are a small, although not substantial, amount (e.g. approximately 5%) below the supercritical point of the substance in question (i.e. "sub-critical"). Accordingly, the term also encompasses oscillatory behaviour around the supercritical point of a substance (i.e. movement from supercritical conditions to sub-critical conditions and *vice versa*). For example, a solvent having a supercritical point of 305 degrees Kelvin and 4.87 atmospheres may, for the purposes of the present invention, still be considered to be "supercritical" at a slightly lower temperature (e.g. between about 290 degrees and 305 degrees Kelvin) and/or a slightly lower pressure (e.g. between about 4.6 and about 4.87 atmospheres).

The supercritical solvent may be any solvent capable of solvating lignin from the remaining solid material comprising lignin and cellulose. Non-limiting examples of supercritical solvents that may be used to separate lignin from solid matter (comprising lignin and cellulose) remaining after hemicellulose fractionation include nitrous oxide, sulfur dioxide, ammonia based solvents, amines, carbon dioxide, alcohols and mixtures thereof.

Solvation of lignin may be performed at a temperature and pressure approximating the supercritical point of the solvent selected, and preferably, at a temperature and pressure above the supercritical point of the solvent. Temperature, solvent composition, and pressure range during the solvation of lignin can be selected so as to maximise lignin fractionation and minimise processing time. Examples of supercritical temperatures and pressures for various solvents suitable for the solvation of lignin are provided in **Table 1**.

Table 1: non-limiting examples of various solvents that may be utilised to solvate lignin from cellulose following hemicellulose fractionation

Solvent	Molecular weight	Critical temperature	Critical pressure	Critical density
	g/mol	K	MPa (atm)	g/cm^3
Carbon dioxide (CO_2)	44.01	304.1	7.38 (72.8)	0.469
Water (H_2O)	18.02	647.3	22.12 (218.3)	0.348
Methane (CH_4)	16.04	190.4	4.60 (45.4)	0.162
Ethane (C_2H_6)	30.07	305.3	4.87 (48.1)	0.203
Propane (C_3H_8)	44.09	369.8	4.25 (41.9)	0.217
Ethylene (C_2H_4)	28.05	282.4	5.04 (49.7)	0.215
Propylene (C_3H_6)	42.08	364.9	4.60 (45.4)	0.232
Methanol (CH_3OH)	32.04	512.6	8.09 (79.8)	0.272
Ethanol (C_2H_5OH)	46.07	513.9	6.14 (60.6)	0.276

Supercritical conditions may be achieved, for example, by conducting the reaction in a suitable mechanical apparatus capable of maintaining increased temperature and/or increased pressure. A suitable mechanical apparatus will, in general, include any

apparatus provided with suitable heating means that is designed to withstand the pressures utilised. Non-limiting examples of a suitable mechanical apparatus include an autoclave and a supercritical reactor apparatus. In general, the mechanical apparatus will provide a means of mixing the supercritical solvent with the solid matter comprising lignin and cellulose and maintaining the solvent in a supercritical state.

In one embodiment of the invention, a supercritical alkylating agent is used to solvate the lignin component. The alkylating agent will, in general, comprise an alkyl chain bearing an appropriate leaving group. Without limitation to a particular mechanism or mode of action, it is postulated that the transfer of an alkyl chain from an alkylating agent to lignin facilitates solvation of lignin from the remaining solid matter.

Non-limiting examples of suitable alkylating agents include alkylhalides, alkylsulfates, olefins, alkylphosphates, and alcohols.

Non-limiting examples of alkylhalides include methyl chloride, isopropyl chloride, ethyl bromide, and methyl iodide.

Non-limiting examples of alkylaromatics include xylenes, and trimethylbenzenes.

Non-limiting examples of suitable olefins include monoolefins such as ethylene, propylene, n-butene, isobutylene, 1-pentene, 1-hexene, cyclohexene, and 1-octene.

A non-limiting example of a suitable diolefin is 1,3-Butadiene.

Preferably, alcohols suitable to solvate the lignin component have between about one and about ten carbon atoms. Non-limiting examples of suitable alcohols include methanol, ethanol, isopropyl alcohol, isobutyl alcohol, pentyl alcohol, hexanol and iso-hexanol. Supercritical treatment of solid matter comprising lignin and cellulose (i.e. matter remaining after hemicellulose fractionation) with alkylating agents such as ethanol and methanol may result in an alkylated lignin product. The alkylated lignin product may exhibit anisole-like properties.

In a preferred embodiment of the invention, lignin is separated from solid matter remaining after hemicellulose fractionation using supercritical ethanol. In general, ethanol may be brought into a supercritical state by heating the reaction above a temperature of about 514 Kelvin under pressure of about 6.14 Mpa (mega Pascal) (equivalent to about 60 atmospheres).

In one embodiment, lignin is separated from solid matter remaining after hemicellulose fractionation using supercritical ethanol as a solvent at a reaction temperature of above about 230°C and a pressure of above about 55 atmospheres (5500kPa). Preferably, the reaction is performed at a reaction temperature of above about

250°C, a pressure of above about 65 atmospheres (6500kPa). The reaction may be performed at a pH of between about 6.5 and 7.5. Preferably, the reaction is performed at a pH of about 7.0.In alternative embodiments the reaction may be performed at a pH of below about 6.5 or above about 7.5.

In certain embodiments, the reaction is performed for between about 2 and about 15 minutes. Preferably the reaction is performed for between about 3 and about 10 minutes.

Without limitation to a particular mechanism or mode of action, it is postulated that treatment with supercritical ethanol facilitates the transfer of the alkyl chain of ethanol to lignin (ethylation), thereby solvating lignin and fractionating it from the remaining solid matter. The resulting ethylated lignin may exhibit anisole-like properties. Ethylated lignin produced in accordance with the methods described herein may be suitable for use in transport fuels and/or further upgraded for use as specialty chemicals suitable for applications in the manufacture of fragrances.

The solvated lignin fraction may be removed from the remaining solid matter. Separation of the solvated lignin fraction from the remaining solid matter may be accomplished, for example, by way of a cyclone apparatus. In general, a cyclone apparatus will operate to separate lignin from remaining solid matter as follows. A high speed rotating air-flow comprising solvated lignin is established within a conical or cylindrical cyclone, the air flowing in a spiral pattern from an upper (wider) end to a lower (narrower) end. The air flow exits the cyclone in a straight stream through the center of the cyclone and out the upper portion. Particles of remaining solid matter in the rotating air stream have too much inertia to remain in the air stream, and fall to the bottom of lower end of the cyclone where they are removed.

Solvation of cellulose

In accordance with the methods described herein, fractionation of lignocellulosic matter commences with separation of the hemicellulose component. Solid matter remaining after the fractionation of hemicellulose may be further treated to solvate lignin.

Following solvation of the lignin fraction, the remaining solid matter comprising predominantly cellulose may be treated to solvate the cellulose. Any solvent capable of solvating cellulose may be used, non-limiting examples of which include water, aqueous acidic solutions, aqueous alkaline solutions, and organic solvents.

One example of a method by which cellulose may be solvated is hydrolytic disintegration by use of superheated steam at elevated pressure. Additionally or alternatively, cellulose may be solvated using ionic liquids, or tertiary amines.

Other non-limiting examples of methods by which cellulose may be solvated are described in U.S. Patent No. 2179181, U.S. Patent No. 3447939, U.S. Patent No. 4097666, U.S. Patent No. 4302252, U.S. Patent No. 5410034, and U.S. Patent No. 6824599.

In preferred embodiments, the cellulose component is solvated in aqueous solution. In general, solvation of cellulose in aqueous solution will typically also involve partial hydrolysis of the cellulose. Examples of suitable aqueous solutions for the solvation icellulose include aqueous acidic solutions, aqueous alkaline solutions, and aqueous solutions of neutral pH (*i.e.* pH of about 7.0).

A suitable alkaline aqueous solution may have a pH of greater than about 7.0. For example, a suitable alkaline aqueous solution may have a pH of between about 7.0 and about 11.0. In certain embodiments of the invention, a suitable alkaline aqueous solution may have a pH of between about 7.0 and about 10.5, between about 7.0 and about 10.0, between about 7.0 and about 9.5, between about 7.0 and about 9.0, between about 7.0 and about 8.5, between about 7.0 and about 8.0 and between about 7.0 and 7.5.

A suitable acidic aqueous solution may have a pH of less than about 7.0. For example, a suitable acidic aqueous solution may have a pH of between about 3.0 and about 7.0. In certain embodiments of the invention, a suitable acidic aqueous solution may have a pH of between about 3.5 and about 7.0, between about 4.0 and about 7.0, between about 4.5 and about 7.0, between about 5.0 and about 7.0, between about 5.5 and about 7.0, between about 6.0 and about 7.0, and between about 6.5 and about 7.0.

The solvation and partial hydrolysis of cellulose in aqueous solution may be performed at any reaction temperature (in combination with any of the pH ranges/values referred to above).

For example, the solvation and partial hydrolysis of cellulose in aqueous solution may be performed at a reaction temperature of between about 80°C and about 400°C. In certain embodiments of the invention, the reaction temperature is between about 100°C and about 400°C, between about 120°C and about 400°C, between about 140°C and about 400°C, between about 160°C and about 400°C, between about 180°C and about 400°C, between about 200°C and about 400°C, between about 220°C and about 400°C, between about 240°C and about 400°C, between about 260°C and about 400°C, between about

280°C and about 400°C, between about 300°C and about 400°C, between about 320°C and about 400°C, between about 340°C and about 400°C, between about 360°C and about 400°C, between about 380°C and about 400°C, between about 80°C and about 380°C, between about 80°C and about 360°C, between about 80°C and about 340°C, between about 80°C and about 320°C, between about 80°C and about 300°C, between about 80°C and about 280°C, between about 80°C and about 260°C, between about 80°C and about 240°C, between about 80°C and about 220°C, between about 80°C and about 200°C, between about 80°C and about 180°C, between about 80°C and about 160°C, between about 80°C and about 140°C, between about 80°C and about 120°C, between about 80°C and about 100°C, and between about 80°C and about 90°C.

The solvation of cellulose in aqueous solution may be performed at any reaction pressure (in combination with any of the reaction temperatures and pH ranges/values referred to above).

For example, the solvation of cellulose in aqueous solution may be performed at a reaction pressure of between about 1 atmospheres (atm) and about 250 atm, between about 1atm and about 100 atm, between about 1 atm and about 50 atm, preferably between about 2 atm and about 50 atm, and more preferably between about 10 atm and about 40 atm.

In general, reactions are performed for a period of time sufficient to solvate substantially all of the cellulose, or, the majority of cellulose.

For example, a reaction under conditions defined by a combination of any of the reaction temperatures, reaction pressures and reaction pH ranges or values referred to above may be performed for less than 20 minutes. In some embodiments, the reaction is performed for between about 2 minutes and about 20 minutes. In other embodiments, the reaction is performed from between about 5 minutes and about 15 minutes. In other embodiments, the reaction is performed for a period of more than 20 minutes.

In a preferred embodiment, the cellulose component is solvated and partially hydrolysed using water with a pH of about 7.0 at a reaction temperature of about 340°C.

Reaction conditions for the solvation of cellulose may be optimised by a person of ordinary skill in the field without the need for undue experimentation. Optimal reaction conditions will ultimately depend on factors including the specific solvent and the nature of the lignocellulosic matter subjected to fractionation in accordance with the methods described herein. For example, factors such as temperature and pH of the reaction

mixture, isotonicity, amount of lignocellulosic matter and solvent, and length of reaction time may be routinely varied in order to determine optimise the reaction.

In most cases, the pH of the reaction mixture can be adjusted by adding a suitable acid or base.

Non-limiting examples of suitable acids that may be used to adjust the pH of a reaction mixture include hydrochloric acid, trifluoroacetic acid, sulfuric acid, sulfurous acid and/or organic acids such as propionic acid, lactic acid, citric acid, or glycolic acid.

Additionally or alternatively, carbon dioxide may be added to the aqueous mixture to obtain an acidic pH (i.e. a pH of below about 7.0)

Non-limiting examples of suitable bases that may be used to adjust the pH of a reaction mixture include sodium hydroxide, potassium hydroxide, ammonium hydroxide, carbonates or bicarbonates.

Methods by which the pH of a reaction mix may be determined are known in the art, and described, for example in Gallagher and Wiley (Eds) *Current Protocols Essential Laboratory Techniques* John Wiley & Sons, Inc (2008).

Optimal reaction conditions will be readily apparent to the skilled addressee upon analysis of the solvated cellulose, which may be performed using standard methods generally known in the art. For example, solvated cellulose may be analysed using spectroscopy techniques. Suitable spectroscopy techniques include, but are not limited near infra red spectroscopy, fourier transform infrared spectroscopy, nuclear magnetic resonance spectroscopy, raman microscopy, UV microspectrophotometry and X-ray diffraction. A specific example of methodology suitable for the quantification of cellulose is described in Wickham et al. *"Quantification of cellulose forms in complex cellulose materials: a chemometric model"* (2001), 8:139-148.

In alternative embodiments of the invention, the solvation of cellulose may be performed after the fractionation of hemicellulose but prior to the solvation of lignin. For example, after the initial step of fractionating hemicellulose, the remaining solid matter may be treated with supercritical water in order to solvate the cellulose. In general, water may be brought into a supercritical state by heating the reaction above a temperature of about 643 Kelvin under pressure of about 22.1 Mpa (mega Pascal) (equivalent to about 218 atmospheres).

Supercritical conditions may be achieved, for example, by conducting the reaction in a suitable mechanical apparatus capable of maintaining increased temperature and/or increased pressure. A suitable mechanical apparatus will, in general, include any

apparatus provided with suitable heating means that is designed to withstand the pressures utilised. Non-limiting examples of a suitable mechanical apparatus include an autoclave and a supercritical reactor apparatus. In general, the mechanical apparatus will provide a means of mixing the supercritical solvent with the solid matter comprising lignin and cellulose and maintaining the solvent in a supercritical state.

Saccharification

Fractionated hemicellulose and/or cellulose components obtained from lignocellulosic matter in accordance with the methods described herein may be subjected to saccharification to produce fermentable sugars. For example, saccharification of fractionated hemicellulose and/or cellulose components may produce polysaccharides, oligosaccharides, disaccharides, monosaccharides or mixtures thereof. Preferably, saccharification of the hemicellulose and cellulose components producespolysaccharide chains comprising between about two and about 50 monosaccharide units. More preferably, saccharification of the hemicellulose and cellulose components produces polysaccharide chains comprising between about two and about 10 monosaccharide units, and/or between about five monosaccharide units and about two monosaccharide units. Most preferably, saccharification of the hemicellulose and cellulose components produces monosaccharides.

Production of shorter polysaccharide chains, oligosaccharides, disaccharides and/or monosaccharides may be achieved by the cleavage of one or more chemical bonds present in fractionated hemicellulose and/or cellulose components using any suitable means. Examples of preferred bonds within the structure of hemicellulose and/or cellulose that may be cleaved include S-glycosidic bonds, N-glycosidic bonds, C-glycosidic bonds, O-glycosidic bonds, α-glycosidic bonds, β-glycosidic bonds, 1,2-glycosidic bonds, 1,3-glycosidic bonds, 1,4-glycosidic bonds and 1,6-glycosidic bonds, ether bonds, hydrogen bonds and/or ester bonds.

Saccharification of fractionated cellulose and/or hemicellulose may be performed using any suitable method known in the art. For example, pyrolysis may be used to cleave chemical bonds in fractionated hemicellulose and/or cellulose to produce shorter polysaccharides, oligosaccharides, disaccharides, monosaccharides and/or mixtures thereof. In general, pyrolysis involves cleavage of chemical bonds by the application of heat. Non-limiting examples of pyrolysis techniques that may be utilised for the methods described herein include anhydrous pyrolysis (performed in the absence of oxygen),

hydrous pyrolysis (performed in the presence of water) and vacuum pyrolysis (performed in a vacuum). Methods by which heat may be provided for pyrolysis are generally known in the art and include, for example, direct heat transfer using a hot gas or circulating solids and indirect heat transfer using exchange surfaces such as walls or tubes. Suitable reactors for pyrolysis are described, for example in, U.S. Patent No. 3853498, U.S. Patent No. 4510021, Scott et al., *Canadian Journal of Chemical Engineering* (1984) 62: 404-412 and Scott et al., *Industrial and Engineering Chemistry Process and Development* (1985) 24: 581-588.

Additionally or alternatively, saccharification of fractionated cellulose and/or hemicellulose may be achieved by hydrolysis. For example, fractionated hemicellulose may be hydrolysed by the addition of a dilute acid (e.g. sulfuric acid), a dilute base, or pH neutral water, generally with the application of heat.

In accordance with the methods described herein, hemicellulose and/or cellulose fractionated from lignocellulosic matter may be hydrolysed by the use of one or more hydrolytic enzymes. Any enzyme capable of catalysing the hydrolysis of cellulose and/or hemicellulose to produce shorter polysaccharides, oligosaccharides, disaccharides, monosaccharides and/or mixtures thereof may be used. In general, hydrolytic enzymes suitable for use in the methods described herein are those classified under EC 3 (hydrolases) of the enzyme nomenclature of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) (http://www.chem.qmul.ac.uk/iubmb/) nomenclature as of the filing date of this application. Preferably, the hydrolytic enzymes utilised in the methods described herein are those classified under class EC 3.2 (glycosylases) of the NC-IUBMB enzyme nomenclature.

In certain embodiments, hydrolytic enzymes suitable for use in the methods described herein are those classified under subclass 3.2.1 (Glycosidases, *i.e.* enzymes hydrolysing O- and S-glycosyl compounds) of the NC-IUBMB nomenclature. In other embodiments, the enzymes that may be utilised are those classified under subclass EC 3.2.2 (Hydrolysing *N*-Glycosyl Compounds) of the NC-IUBMB nomenclature. In other embodiments, hydrolytic enzymes that may be utilised are those classified under subclass EC 3.2.3 (Hydrolysing *S*-Glycosyl Compounds) of the NC-IUBMB nomenclature.

Non-limiting examples of glycoside hydrolases and carbohydrases suitable for use in the methods described herein and commercial sources of those enzymes are described in US Patent Publication No. 20060073193. Preferred examples include cellulases,

xylanases, arabinosidases, β-glucosidases, β-xylosidases, mannanases, galactanases, dextranases, endoglucanases, and alpha-galactosidase.

Hydrolytic enzymes may be applied in a purified or substantially purified form, or in combination with other substances or compounds (e.g. as part of a culture supernatant). Additionally or alternatively, a hydrolytic enzyme-producing microorganism or mixtures of microorganisms capable of producing hydrolytic enzymes may be cultured in the presence of hemicellulose and/or cellulose fractionated in accordance with the methods described herein, thereby providing a source of hydrolytic enzymes.

Hydrolytic enzymes suitable for use in accordance with the methods described herein may be derived from any suitable microorganism, including but not limited to, bacteria and fungi. The microorganism may be a psychrophilic, mesophilic, thermophilic or extremely thermophilic organism, in accordance with the classification described in Brock, 1986, *"Thermophiles: General Molecular and Applied Microbiology"*, (T.D Brock, Ed) John Wiley and Sons, Inc. New York, and Bergquist et al., 1987, Biotechnol Genet. Eng. Rev. 5:199-244.

In one embodiment, enzymatic hydrolysis of fractionated cellulose and hemicellulose is performed using thermophilic hydrolytic enzymes. The use of thermostable hydrolytic enzymes for the hydrolysis of fractionated cellulose and/or hemicellulose offers several advantages over the use of hydrolytic enzymes that operate optimally at lower temperatures, including higher specific activity and higher stability. Typically, thermophilic hydrolytic enzymes display hydrolytic activity at elevated reaction temperatures. For example, a thermophilic hydrolytic enzyme will remain active at a reaction temperature of more than about 60°C.

Non-limiting examples of suitable bacteria from which hydrolytic enzymes may be derived include *Acidothermus* sp. (e.g. *A. cellulolyticus*), *Anaerocellum* sp. (e.g. *A. thermophilum*), *Bacillus* sp., *Butyrivibrio* sp. (e.g. *B. fibrisolvens*), *Cellulomonas* sp. (e.g. *C. fimi*), *Clostridium* sp. (e.g. *C. thermocellum, C. stercorarium*), *Erwinia* sp. (e.g. *E. chrysanthemi*), *Fibrobacter* sp. (e.g. *F. succinogenes*), *Micromonospora* sp., *Rhodothermus* sp. (e.g. *R. marinus*), *Ruminococcus* sp. (e.g. *R. albus*, *R. flavefaciens*), *Streptomyces* sp., *Thermotoga* sp. (e.g. *T. maritima*, *T. neapolitana*), *Xanthomonas* sp. (e.g. *X. campestris*) and *Zymomonas* sp. (e.g. *Z. mobilis*).

Non-limiting examples of suitable fungi from which hydrolytic enzymes may be derived include *Aureobasidium* sp., *Aspergillus* sp. (e.g. *A. awamori, A. niger* and *A. oryzae*), *Candida* sp., *Chaetomium* sp. (e.g. *C. thermophilum, C. thermophila*),

Chrysosporium sp. (e.g. *C. lucknowense*), *Corynascus* sp. (e.g. *C. thermophilus*), *Dictyoglomus* sp. (e.g. *D. thermophilum*), *Emericella* sp., *Fusarium* sp., *Gliocladium* sp., *Hansenula* sp., *Humicola* sp. (e.g. *H. insolens* and *H. grisea*), *Hypocrea* sp., *Kluyveromyces* sp., *Myceliophthera* sp., (e.g. *M. thermophila*), *Neurospora* sp., *Penicillium* sp., *Pichia* sp., *Rhizomucor* sp. (e.g. *R. pusillus*), *Saccharomyces* sp., *Schizosaccharomyces* sp., *Sporotrichum* sp., *Thermoanaerobacterium* sp. (e.g. *T. saccharolyticum*), *Thermoascus* sp. (e.g. *T. aurantiacus*, *T. lanuginosa*), *Thermomyces* sp., (e.g. *T. lanuginosa*), *Thermonospora* sp. (e.g. *T. curvata*, *T. fusca*), *Thielavia* sp. (e.g. *T. terrestris*) and *Trichoderma* sp. (e.g. *T. reesei, T. viride, T. koningii, T. harzianum*), and *Yarrowia* sp.

Suitable microorganisms that naturally produce hydrolytic enzymes, for example any of the fungi or bacteria referred to above, may be cultured under suitable conditions for propagation and/or expression of the hydrolytic enzyme or enzymes of interest. Methods and conditions suitable for the culture of microorganisms are generally known in the art and are described in, for example, *Current Protocols in Microbiology* (Coico et al (Eds), John Wiley and Sons, Inc, 2007).

Recombinant organisms may be used as a source of hydrolytic enzymes for use in accordance with the methods described herein. Additionally or alternatively, recombinant organisms capable of producing hydrolytic enzymes may be cultured with fractionated hemicellulose and/or cellulose. Recombinant microorganisms including bacterial and/or fungal strains may be generated expressing one or more hydrolytic enzymes derived from an exogenous source. Methods for the production of recombinant microorganisms are generally known in the art and are described, for example, in Ausubel et al., (Eds) *Current Protocols in Molecular Biology* (2007) John Wiley & Sons and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, (2000) 3rd Ed., Cold Spring Harbor Laboratory Press, *Molecular Cloning* (Maniatis et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982) and *Current Protocols in Microbiology* (Coico et al (Eds), John Wiley and Sons, Inc, 2007). In particular embodiments, one or more genes encoding hydrolytic enzymes may be cloned into a vector. The vector may be a plasmid vector, a viral vector, a phosmid, a cosmid or any other vector construct suitable for the insertion of foreign sequences, introduction into cells and subsequent expression of the introduced sequences. In a preferred embodiment, the vector is an expression vector comprising expression control and processing sequences such as a promoter, an enhancer, polyadenylation signals and/or transcription termination sequences.

The construct may also include a selectable marker, for example, an antibiotic-resistance gene such as chloramphenicol or tetracycline. Genetic material for insertion into the construct may be generated, for example, by chemical synthesis techniques such as the phosphodiester and phosphotriester methods (see for example Narang et al. *"Improved phosphotriester method for the synthesis of gene fragments"* (1979) Meth. Enzymol. 68:90; Brown, E. L. et al. *"Chemical synthesis and cloning of a tyrosine tRNA gene"* (1979) Meth. Enzymol. 68:109; and Unites States Patent No. 4356270), or the diethylphosphoramidite method (see Beaucage et al. *"Deoxynucleoside Phosphoramidites - A New Class of Key Intermediates for Deoxypolynucleotide Synthesis"* (1981) Tetrahedron Letters, 22:1859-1862). Genetic material for insertion into the construct may be amplified in number by performing polymerase chain reaction (PCR) assays on DNA or cDNA sequences encoding hydrolytic enzymes, or RT-PCR on RNA sequences encoding hydrolytic enzymes. The resulting nucleic acids may then be inserted into the construct, for example, by restriction-ligation reactions or by the TA cloning method.

Suitable methods for the introduction of vector constructs and other foreign nucleic acid material into host cells are generally known in the art, and are described, for example, in *Current Protocols in Molecular Biology*, Ausubel et al. (Eds), New York: John Wiley & Sons, 2007) and *Molecular Cloning: A Laboratory Manual*, (Sambrook et al. 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001).

By way of example, host cells may be transformed with vector constructs by the "heat shock" method. Under this method the cells are chilled in the presence of divalent cations such as Ca^{2+}, which causes cell wall permeability. Cells are incubated on ice with the construct and briefly heat shocked (e.g. at 42 °C for 0.5-2 minutes) causing the vector construct to enter the cell.

Alternatively, host cells may be transformed with vector constructs by electroporation, a method involving briefly shocking the cells with an electric field causing the cells to briefly develop holes through which the construct may enter the cell. Natural membrane-repair mechanisms rapidly close these holes after the shock.

Vector constructs may also be introduced into fungal cells (e.g. protoplast cells) using methods described in *"A versatile transformation system for the cellulolytic filamentous fungus Trichoderma reesei"* (1987), Pentillä et al., Gene, 61:155-164.

Following entry of the vector construct, the host cell may be cultured under conditions suitable to facilitate reproduction. Methods for the culture of microorganisms such as are well known in the art and described in, for example, *Current Protocols in*

Microbiology, (Coico, et al. (Eds), John Wiley & Sons, Inc., 2007). The culture may be performed in medium containing a substrate that facilitates the identification of transformed strains, for example, an antibiotic such as chloramphenicol, kanamycin or tetracycline.

Transformed host cells may be selected and propagated. For example, if the target vector contains one or more selectable markers, transformed host cells may be identified by expression of the marker or markers. Using the example of a drug resistance gene such as a chloramphenicol resistance gene, host cell transformants that grow in the selection medium containing chloramphenicol can be identified as transformants. In the case of host cell transformants expressing more than one selectable marker, double transformants may be identified by the ability to grow in the selection media containing multiple selection determinants.

Hydrolytic enzymes may be purified from the cultured microorganism. Any suitable methods of screening or purification may be used, taking into account various factors such as size and the structural, enzymatic and functional features of the desired hydrolytic enzyme. Methods and assays suitable for purification of the hydrolytic enzymes from microorganism cultures are known in the art, and are described, for example, in *Current Protocols in Protein Science*, Coligan *et al.*, (Eds) John Wiley and Sons, Inc. 2007. The screening and purification step may comprise, for example, chromatography methods, methods that can accelerate solvent extraction, or combinations thereof. Chromatography methods may include, for example, reverse phase chromatography, normal phase chromatography, affinity chromatography, thin layer chromatography, counter current chromatography, ion exchange chromatography and reverse phase chromatography. Examples of other methods include precipitation with ammonium sulphate, PEG, antibodies and the like, or heat denaturation, followed by centrifugation, isoelectric focusing, gel electrophoresis, selective precipitation techniques, and combinations of those and other techniques.

Hydrolytic enzymes produced in accordance with the methods described herein may also be genetically engineered to contain various affinity tags or carrier proteins to aid purification. For example, histidine and protein tags may be engineered into an expression vector comprising, for example, a streptavidin subunit to facilitate purification by its high non-covalent affinity to biotin, and histidine-tagged proteins can be purified using metal-chelate chromatography (MCAC) under either native and denaturing conditions. The

purification of secondary metabolites may also be "scaled-up" for large-scale production purposes.

The skilled addressee will recognise that general methods for the production, isolation and purification of enzymes will, in general, be suitable for the production, isolation and purification of hydrolytic enzymes.

The reaction conditions for enzymatic hydrolysis are typically based on consideration of the conditions suitable for the specific enzyme or mixture of enzymes. In general, typical conditions for enzymatic hydrolysis include a reaction temperature of between about 30°C and about 90°C, and a pH of between about 4.0 and about 8.0. Suitable reaction temperatures and pH for enzymatic hydrolysis are described, for example, in Viikari et al., "*Thermostable Enzymes in Lignocellulosic Hydrolysis*", 2007, 108:121-145.

Non-limiting examples of oligosaccharide fragments that may be produced by saccharification of cellulose and/or hemicellulose fractions derived from lignocellulosic matter using the methods described herein include oligosaccharides such as mannan-oligosaccharides, fructo-oligosaccharides and galacto-oligosaccharides.

Non-limiting examples of disaccharide fragments that may be produced by saccharification of cellulose and/or hemicellulose fractions derived from the methods described herein include sucrose, lactose, maltose, trehalose, cellobiose, laminaribiose, xylobiose, gentiobiose, isomaltose, mannobiose, kojibiose, rutinose, nigerose, and melibiose.

Non-limiting examples of monosaccharide fragments that may be produced by saccharification of cellulose and/or hemicellulose fractions derived from the methods described herein include trioses including aldotrioses (e.g. glyceraldehyde) and ketotrioses (e.g. dihydroxyacetone), tetroses including aldotetroses (e.g. threose and erythrose) and ketotetroses (e.g. erythrulose), pentoses including aldopentoses (e.g. lyxose, ribose, arabinose, deoxyribose) and ketopentoses (e.g. xylulose and ribulose), hexoses including aldohexoses (e.g. glucose, mannose, altrose, idose, galactose, allose, talsoe and gulose) and ketohexoses (e.g. fructose, psicose, tagatose and sorbose), heptoses including keto-heptoses (e.g. sedoheptulose and mannoheptulose), octoses including octolose and 2-keto-3-deoxy-manno-octonate and nonoses including sialose.

In a preferred embodiment, saccharification of the hemicellulose and/or cellulose fractions yields an aqueous solution comprising shorter length polysaccharide chains, oligosaccharides, disaccharides, monosaccharides, and/or mixtures thereof.

In an alternative embodiment of the invention, fractionated hemicellulose and/or cellulose components obtained in accordance with the methods described herein may be subjected to hydrothermal upgrading in sub-supercritical water to produce fermentable sugars. Methods for hydrothermal upgrading are known in the art and are described for example in Srokol et al., "*Hydrothermal upgrading of biomass to biofuel; studies on some monosaccharide model compounds*" Carbohydr Res. 2004 Jul 12;339(10):1717-26.

The invention also relates to saccharides produced from fractionated hemicellulose and cellulose components in accordance with the methods described herein.

Fermentation

In accordance with the methods described herein, sugars derived from fractionated hemicellulose and/or cellulose may be fermented to produce one or more fermented sugar products. For example, the microorganism may be capable of converting saccharide fragments into alcohols (e.g. ethanol), or organic acids (e.g. succinic acid and glutamic acid). The organic acids may be used in the production of other products such as, for example, biopolymers, amino acids and antibiotics. Suitable microorganisms for fermentation include, but are not limited to, yeasts, bacteria, fungi, and/or recombinant varieties of these organisms.

Fermentation may be performed directly on fractionated hemicellulose and/or cellulose components. Additionally or alternatively, fermentation may be performed on fragmented saccharides derived from saccharification of the fractionated hemicellulose and/or cellulose components. Additionally or alternatively, fermentation may be performed simultaneously with saccharification of fractionated hemicellulose and/or cellulose components. For example, a reaction mixture comprising hydrolytic enzymes and/or microorganisms capable of producing hydrolytic enzymes may be combined with microorganisms to ferment sugars and applied under suitable culture conditions to hemicellulose and/or cellulose fractionated in accordance with the methods described herein.

In certain embodiments, residual lignin may be removed from the fractionated hemicellulose and/or cellulose components prior to fermentation. Residual lignin may be removed, for example, using methods described in Mosier et al., "*Features of promising technologies for pretreatment of lignocellulosic biomass*", 2005, Bioresource Technology, 96:673-86.

In general, fermentation may be performed using any microorganism capable of converting saccharides into one or more desired fermented sugar products. For example, the microorganism may be capable of converting saccharides into alcohols (including ethanol), or organic acids (for example succinic acid and glutamic acid). The organic acids may be used in the production of other fermented sugar products, for example biopolymers, amino acids and antibiotics. Suitable microorganisms for fermentation include, but are not limited to, yeasts, bacteria, fungi, and/or recombinant varieties of these organisms.

In certain embodiments, the microorganism is capable of fermenting saccharides derived from fractionated hemicellulose and/or cellulose into one or more alcohols. Non-limiting examples of alcohols that may be produced in accordance with the methods described herein include xylitol, mannitol, arabinol, butanol and ethanol.

In a preferred embodiment, 5-carbon saccharides (pentoses) derived from saccharification of the hemicellulose fraction are fermented to produce alcohols, non-limiting examples of which include, but are not limited to, xylitol, mannitol, arbinol and ethanol. In another preferred embodiment, saccharides derived from saccharification of the cellulose fraction are fermented to produce alcohols, examples of which include, but are not limited to, ethanol and butanol.

Non-limiting examples of microorganisms capable of producing ethanol from saccharides include *Zymomonas* sp. (e.g. *Z. mobilis*), *Saccharomyces* sp. (e.g. *S. cerevisiae*), *Candida* sp. (e.g. *C. shehatae*), *Schizosaccharomyces* sp. (e.g. *S. pombe*), *Pachysolen* sp. (e.g. *P. tannophilus*), and *Pichia* sp. (e.g. *P. stipitis*).

Microorganisms suitable for the fermentation of saccharides to produce mannitol include, for example, yeast, fungi and lactic acid bacteria. Suitable microorganisms will in general express enzymes necessary for mannitol production, for example, mannitol dehydrogenase. Examples of bacterial species that may be used for the fermentation of saccharides to mannitol include *Leuconostoc* sp. (e.g. *Leuconostoc mesenteroides*) *Lactobacillus* sp. (e.g. *L. bevis, L. buchnei, L. fermeyitum, L. sanfranciscensis*), *Oenococcus* sp. (*e.g. O. oeni*), *Leuconostoc* sp. (e.g. *L. mesenteriode*) and *Mycobacterium* sp. (e.g. *M. smegmatis*).

Examples of yeasts suitable for the fermentation of saccharides to produce mannitol include, but are not limited to *Candida* sp. (e.g. *C. zeylannoide, C. lipolitica*), *Cryptococcus* sp. (e.g. *C. neoformans*) and *Torulopsis* sp. (e.g. *T. mannitofaciens*).

Suitable fungi for the fermentation of saccharides to produce mannitol include, but are not limited to *Basidiomycetes sp., Trichocladium sp., Geotrichum sp., Fusarium sp., Mucor* sp. (e.g. *M. rouxii*), *Aspergillus* sp. (e.g. *A. nidulans*), and *Penicillum* sp. (e.g. *P. scabrosum*). Methods for the fermentation of saccharides to produce mannitol are described, for example, in United States Patent No. 6528290 and PCT publication No. WO/2006/044608.

Microorganisms suitable for the fermentation of saccharides to produce xylitol include yeasts such as *Saccharomyces* sp., *Candida* sp. (e.g. *C. magnoliae, C. tropicalis C. guilliermondif*), *Pichia* sp., and *Debaryomyces* sp. (e.g. *D. hansenii*). Methods for the fermentation of xylitol from saccharides are described, for example, in U.S. Patent No. 5081026, U.S. Patent No. 5686277, U.S. Patent No. 5998181, U.S. Patent No. 6893849 and PCT publication No. WO 805467.

In preferred embodiments of the invention, fermentation of saccharides is performed using one or more recombinant microorganisms. Methods for the production of recombinant microorganisms are generally known in the art and are described, for example, in Ausubel et al., (Eds) *Current Protocols in Molecular Biology* (2007) John Wiley & Sons and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, (2000) 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. In general, recombinant microorganisms suitable for use in the methods described herein will express one or more genes encoding enzymes necessary for the conversion of saccharides to the desired target product.

Examples of preferred recombinant ethanologenic microorganisms are those which express alcohol dehydrogenase and pyruvate decarboxylase. Genes encoding alcohol dehydrogenase and pyruvate decarboxylase may be obtained, for example, from *Zymomonas mobilis*. Examples of recombinant microorganisms expressing one or both of these enzymes and methods for their generation are described, for example, in U.S. Patent No. 5000000, U.S. Patent No. 5028539, U.S. Patent No. 424202, and U.S. Patent No.5482846.

Suitable recombinant microorganisms may be capable of converting both pentoses and hexoses to ethanol. Recombinant microorganisms capable of converting pentoses and hexoses to ethanol are described, for example, in PCT Publication No. WO 95/13362 and U.S. Patent No. 5000000, U.S. Patent No. 5028539, U.S. Patent No. 5424202, U.S. Patent No. 5482846, and U.S. Patent No. 5514583.

Culture conditions suitable for the fermentation of saccharides to alcohols, organic acids and other fermented sugar products are generally known in the art, and are described in, for example, Bonifacino et al., (Eds) *Current Protocols in Cell Biology* (2007) John Wiley and Sons, Inc. and Coico et al., (Eds) *Current Protocols in Microbiology* (2007) John Wiley and Sons, Inc.. Generally, microorganisms may be cultured at a temperature of between about 30°C and about 40°C, and a pH of between about 5.0 and about 7.0. In may be advantageous to add cofactors for the fermenting enzymes and/or nutrients for the microorganisms to optimize the enzymatic fermentation. For example, cofactors such as NADPH and/or NAD may be added to the culture to assist the activity of fermenting enzymes (e.g. xylose reductase and xylitol dehydrogenase). Carbon, nitrogen and sulfur sources may also be included in the culture.

Fermented sugar products derived from fractionated hemicellulose and/or cellulose components may be further refined or processed. For example, organic acids derived from fermentation of cellulose saccharides may be refined to produce higher value chemicals such as biopolymers, and antibiotics.

Accordingly, the invention also relates to fermented sugar products derived from fractionated hemicellulose and cellulose components produced in accordance with the methods described herein.

Ethylated lignin products

In accordance with the methods described herein, hemicellulose may be fractionated from lignocellulosic matter. Following this, lignin may be separated from the remaining solid matter using a supercritical solvent. In certain embodiments of the invention, the supercritical solvent is an alkylating agent. The alkylating agent will, in general, comprise an alkyl chain bearing an appropriate leaving group, facilitating the alkylation of lignin. In a preferred embodiment, lignin is fractionated using supercritical ethanol, resulting in the ethylation and solvation of lignin.

The alkylated lignin may exhibit anisole-like properties. Alkylated lignin, and in particular, ethylated lignin exhibiting anisole-like properties may be used for transport fuel or further upgraded for use as specialty chemicals suitable for applications in the manufacture of fragrances, resins and/or polymers.

Accordingly, the invention also relates to fractionated lignin, and in particular alkylated lignin, produced in accordance with the methods described herein, and products derived therefrom.

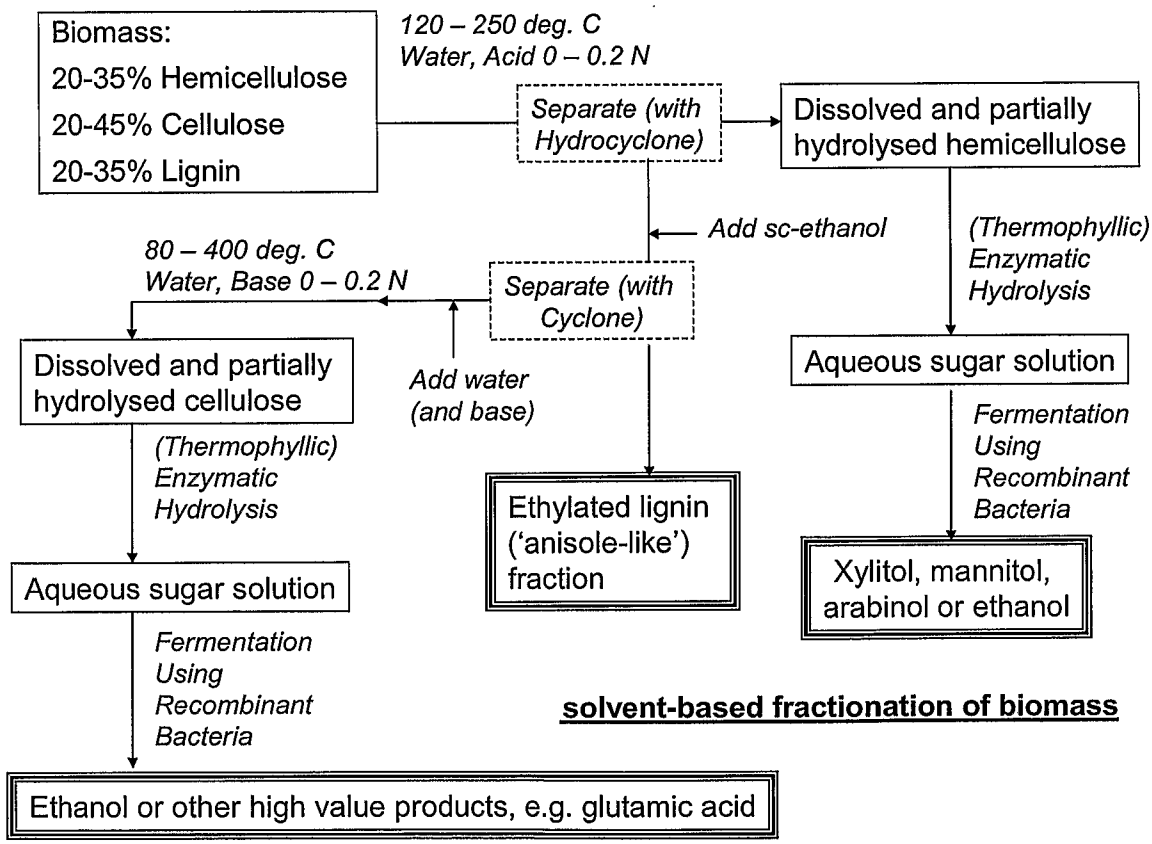
It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

The invention will now be described with reference to specific examples, which should not be construed as in any way limiting

Examples

Example 1: Overview

A flow diagram illustrating certain embodiments of the invention is provided below.



Example 2: Fractionation of hemicellulose liquor from Radiata Pine (*Pinus radiata*)

A series of different runs were performed in which hemicellulose liquor was extracted from Radiata pine (*Pinus radiata*). Different reaction conditions used for each of thirteen representative runs are described in **Table 2** below.

Wood flour was prepared (150-300 microns) and combined with water in a batch tank to produce a slurry (5%-10% v/v solids concentration) which was then pumped into a reactor. The slurry was steam-heated to a temperature of 120°C-210°C and hemicellulose extracted at neutral PH, or under acidic conditions afforded by the addition of sulphuric acid (0.1% - 0.4% wt) or carbon dioxide. Hemicellulose extraction reactions were performed for up to 10 minutes.

Upon completion of the reaction the mixture was passed through a filter to provide separate solid (lignin and cellulose) and liquid (hemicellulose and water) fractions. In some cases the solid fraction (filter cake) was washed to obtain residual hemicellulose liquor. The separated hemicellulose fraction was then analysed for sugar content as described in Example 3 below.

Table 2: Reaction conditions for hemicellulose extraction from *P. radiata*

Variable	Available range	Run Conditions					
		1	2	3	4	5	6
Pressure (bar)	22 – 60	40	40	40	30	30	30
Temperature (°C)	120 – 210	120 – 210	120 – 210	120 – 210	120 – 190	120 – 190	120 – 190
Solids Concentration (%)	5 – 15%	10	10	10	10	20	10
Retention Time (min)	0 – 10	10	5	0	10	20	10
pH	2 – 7	7	7	7	~2	7	~2
Additives	Ethanol, phosphoric acid, sulphuric acid, carbon dioxide	None	None	None	0.4% sulphuric acid	None	Carbon dioxide
Wood Flour Grade	180 and 300 micron	300	300	300	300	300	300
Wood Species	Radiata pine, Oak	Radiata pine	Radiata pine	Radiata pine	Radiata pine	Radiata pine	Radiata pine

Table 2 (cont): Reaction conditions for hemicellulose extraction from *P. radiata*

Variable	Available range	Run Conditions					
		7	**8**	**9**	**10**	**11**	**12**
Pressure (bar)	22 – 60	30	30	30	30	30	30
Temperature (°C)	120 – 210	120 – 190	160 – 190	160 – 190	190	140 – 160	190
Solids concentration (%)	5 – 15%	15% or greater	10	10	10	10	10
Retention Time (min)	0 – 10	20	2.5	5	5	5	5
pH	2 – 7	7	7	7	7	7	7
Additives	Ethanol, phosphoric acid, sulphuric acid, carbon dioxide	None	None	None	None	0.1% wt sulphuric acid	None
Wood Flour Grade	180 and 300 micron	300	300	300	150	300	150
Wood Species	Radiata pine, Oak	Radiata pine	Radiata pine	Radiata pine	Radiata pine	Radiata pine	Radiata pine

Example 3: production of reducing sugars from hemicellulose fraction using enzyme hydrolysis

Enzyme hydrolysis was conducted on hemicellulose liquor fractions obtained from Radiata pine samples by the process described in Example 2 above.

3.1 Materials and methods

Conditions for enzyme hydrolysis were as shown in **Table 3** below.

Table 3: Enzymatic hydrolysis of samples

Sample Number	pH (liquor samples)	pH (with enzyme + buffer)	Temp. during sampling (° C)	Extractives weight * (mg/mL)	Description / Comments
1.1	4.90	5.16	RT	5.2	10 % FS
1.2	3.82	4.8	190	12.0	
1.3	3.84	4.8	190	11.12	
1.4	4.12	4.96	150	11.68	
1.5	4.24	5.02	150	11.08	
1.6	4.37	5.11	130	7.76	
1.7	4.49	5.10	130	7.40	
2.1	4.75	5.15	RT	5.16	10 % FS
2.2	4.19	5.0	190	13.12	
2.3	4.21	5.0	190	13.08	
2.4	4.40	5.08	163	9.56	
2.5	4.46	5.12	163	8.44	
2.6	4.72	5.17	105	6.28	
2.7	4.62	5.20	105	5.68	

FS: feedstock slurry;
RT: room temperature
*: based on dry weight from 25 mL of clear liquor samples dried in petri dish at 70 °C, 14.5 hours

Buffers and pH

120mM of universal buffer (pH 6.5) was included in reaction mixes to provide optimal conditions for hydrolytic enzymes to act on hemicellulose present in the different fractions. The target pH during these assays was ~5-6. As shown in **Table 3** above, the pH of each sample was measured before and after the addition of buffer and enzyme samples.

Hydrolytic enzymes

A recombinant *Trichoderma reesei* strain was used to produce a mixture of hydrolytic enzymes comprising both hydrolytic fungal enzymes and a thermophilic xylanase (XynB).

Reaction mixes

Reaction mixes for enzyme hydrolysis were prepared as follows:

(i) Hemicellulose liquor samples

Substrate	500 µL
Enzyme	300 µL
Univ. buffer (pH 6.5)	200 µL

(ii) Substrate only control

Substrate	500 µL
Univ. buffer (pH 6.5)	200 µL
H_2O	300 µL

(iii) Enzyme only control:

Enzyme	300 µL
Univ. buffer (pH 6.5)	200 µL
H_2O	500 µL

All tubes were incubated at 50°C (with rotation) for 1.5 hours then removed and kept at 4 °C.

Colorimetric reducing sugar assay

A colourimetric dinitrosalicyclic acid (DNS)-reducing sugar assay was used as an indicator of enzyme hydrolysis (see Bailey and Poutanen (1989), "*Production of xylanases by strains of Aspergillus*", Appl. Microbiol. Biotechnol. 30: 5-10). The DNS reducing sugar method tests for the presence of free carbonyl groups (C=O), present on reducing sugars (e.g. glucose, xylose, mannose, etc). As a result, 3,5-dinitrosalicyclic acid (DNS) is reduced to 3-amino,5-nitrosalicyclic acid under alkaline conditions and an intense orange-brown colour is formed, indicative of reducing sugars, etc.

50 µL of sample was collected from each tube after enzyme hydrolysis, mixed with 75 µL DNS and boiled for 5 minutes. Absorbance I_{540} was read from 100 µL samples.

3.2 Results

Colorimetric reducing sugar assay

Absorbance readings obtained from the 100 µL samples labelled 1.1-1.7 and 2.1-2.7 were plotted and are shown in **Figure 1**. These results are indicative of the presence and subsequent increase in reducing ends following hydrolysis with the mixture of hydrolytic enzymes utilised.

Example 4: Total sugar-acid hydrolysis and analysis by high performance liquid chromatography (HPLC)

4.1 Materials and methods

Total sugar analysis was performed according to the Standard Test Method for Carbohydrate Distribution of Cellulosic Materials, TAPPI Standard, Designation: D 5896 - 96 (2007), with some minor modifications.

In brief, samples were prepared as follows:

1. Liquor samples (containing 100 mg total extractives, see **Table 3**) were transferred into a 20 x 150 mm glass culture tubes and dried using an oven set at 75 °C.
2. 1mL of cold 72 % sulfuric acid was added to each tube containing 100 mg of extractives/carbohydrate (bone dry basis), carefully mixed, then incubated in refrigerator overnight (4 °C).
3. Samples were heated at 30 °C for 1 hour followed by addition of 28 mL of MilliQ-H_2O
4. Samples were autoclaved at 121 °C for 1hour (wet run) then cooled to room temperature.
5. 20 - 25 mL supernatant was removed and centrifuged at 13,500 rpm for 30 - 60 minutes at room temperature.
6. Clear supernatant was removed for analysis, or stored at -20 °C.

High performance liquid chromatography was then performed at the Australian Proteome Analysis Facility (APF, www.proteome.org.au)

4.2 Results

Total sugar-acid hydrolysis and HPLC analysis

Tables 4 - 9 summarise total sugar concentration calculations and molecular ratios of the different types of mono sugars in hemicellulose liquor samples subjected to acid hydrolysis. Results are summarised in **Table 10**.

HPLC traces of standards and samples are shown in **Figures 2 to 6**.

Table 4. Detected amount by HPAEC-PAD (pmol)

	Sample (i)	Sample (i) (control)	Sample (ii)	Sample (ii) (control)
Ara	192	220	112	223
Gal	329	35	332	52
Glc	319	91	298	36
Xly	132	49	346	90
Man	465	21	756	29
Fru	0	87	0	0

Table 5. Concentration of sample diluted with water to 1/50 (uM)

	Sample (i)	Sample (i) (control)	Sample (ii)	Sample (ii) (control)
Ara	19	22	11	22
Gal	33	4	33	5
Glc	32	9	30	4
Xly	13	5	35	9
Man	46	2	76	3
Fru	0	9	0	0

Table 6. Concentration of sample (uM, x 50 dilution factor)

MW		Sample (i)	Sample (i) (control)	Sample (ii)	Sample (ii) (control)
150.13	Ara	959	1100	561	1117
180.16	Gal	1647	176	1658	261
180.2	Glc	1593	456	1489	182
150.1	Xly	661	247	1731	448
180.16	Man	2325	105	3780	147
	SUM	7184	2083	9218	2154

Table 7. Molecular ratio (%)

	Sample (i)	Sample (i) (control)	Sample (ii)	Sample (ii) (control)
Ara	13	53	6	52
Gal	23	8	18	12
Glc	22	22	16	8
Xly	9	12	19	21
Man	32	5	41	7
SUM	100	100	100	100

Table 8. Weight in 29 mL (mg)

	Sample (i)	Sample (i) (control)	Sample (ii)	Sample (ii) (control)
Ara	4.2	4.8	2.4	4.9
Gal	8.6	0.9	8.7	1.4
Glc	8.3	2.4	7.8	0.9
Xly	2.9	1.1	7.5	1.9
Man	12.1	0.5	19.7	0.8
SUM	36.1	9.7	46.2	9.9

Table 9. Weight ratio (%)

	Sample (i)	Sample (i) (control)	Sample (ii)	Sample (ii) (control)
Ara	12	49	5	49
Gal	24	9	19	14
Glc	23	25	17	10
Xly	8	11	16	20
Man	34	6	43	8
SUM	100	100	100	100

Table 10. Overview of total sugar-acid hydrolysis

Sugar	Concentration (μM)				Weight ratio (%)			
	Sample (i)	Sample (i) control	Sample (ii)	Sample (ii) control	Sample (i)	Sample (i) control	Sample (ii)	Sample (ii) control
Ara	959	1100	561	1117	12	49	5	49
Gal	1647	176	1658	261	24	9	19	14
Glc	1593	456	1489	182	23	25	17	10
Xyl	661	247	2325	105	8	11	16	20
Man	2325	105	3780	147	34	6	43	8
SUM	7184	2083	9218	2154	100	100	100	100

HPLC results demonstrated that each sample analysed was a hemicellulose fraction based on the type and ratio of mono sugars released following acid hydrolysis. Assuming a 100 mg of total mono sugars, the % ratios of the main sugars following acid hydrolysis for sample (ii) were as follows: Man:Gal:Glc:Xyl:Ara = 43:19:17:16:5.

Example 5: Fractionation of lignin from cellulose using supercritical ethanol

The initial separation of hemicellulose from lignocellulosic biomass under mild conditions as described in Example 2 above is envisaged to increase the efficiency of lignin solubilisation from the remaining solid matter (i.e. the solubilisation of lignin from cellulose). In general, separation of lignin from the sugar-containing component(s) of lignocellulosic biomass (i.e. hemicellulose and/or cellulose) requires treatment under high temperature and pressure. The initial removal of hemicellulose (the most reactive of the three components) under mild conditions is envisaged to provide a means of reducing tar-

formation during solubilisation of the lignin from the filter cake produced in Example 2 above.

Solubilisation of lignin from the filter cake product described in Example 2 will be performed in a continuous process using supercritical ethanol (which is substantially milder than supercritical water and preferentially dissolves lignin) in order to reduce degradation of cellulose.

Filter cake produced using the process described in Example 2 will be fed into a batch tank and mixed with aqueous ethanol (50-100% v/v ethanol, and preferably 90% - 95% v/v ethanol) and mixed by agitation to form a slurry. The percentage of solid material in the slurry will be in the range of 15%-30%. Slurry will be pumped from the batch tank into a supercritical reactor apparatus, and heated/pressurised above the supercritical point of the ethanol solvent (e.g. above approximately 250°C and 65 atm a (6500kP)). The pH of the reaction mix is envisaged to be neutral (i.e. pH 7.0) or close to neutral (i.e. in the range of pH 6.5 – 7.5). The reaction time will be 5-7 minutes at which point it is anticipated that greater than 90% of lignin present in the slurry will be solvated. However, the reaction time may be extended for up to 15 minutes if so desired.

Upon completion of the reaction, the material will be passed through a filter with appropriately sized pores to remove the solid fraction (containing predominantly cellulose) from the liquid fraction (containing solvated lignin). The filter cake produced will be washed to remove residual lignin from the cellulose cake.

It is anticipated that the yield and purity of the solid cellulose fraction produced in accordance with the methods of the invention will be substantially increased by the initial step of solvating hemicellulose (as described in Example 2) before treatment of the lignin-containing fraction with supercritical ethanol. Furthermore, it is envisaged that solvation of lignin from the lignin-containing fraction in accordance with the methods of the invention will yield ethylated lignin products exhibiting anisole-like properties. These will be used for transport fuel or further upgraded for use as specialty chemicals suitable for applications in the manufacture of fragrances, resins and/or polymers.

Incorporation by reference

This application claims priority from United States provisional application number 61/099,360 filed on 23 September 2008, the entire contents of which are incorporated herein by reference.

CLAIMS

1. A method of fractionating hemicellulose from lignocellulosic matter, said method comprising treating the lignocellulosic matter with a solvent at a reaction temperature of between about 100 and about 250 degrees Celsius, and a reaction pressure of between about 2 and about 50 atmospheres.

2. The method according to claim 1, wherein said lignocellulosic matter comprises at least about 10% lignin, at least about 15% cellulose, and at least about 10% hemicellulose.

3. The method according to claim 1, wherein said lignocellulosic matter comprises at least about 20% lignin, at least about 25% cellulose, and at least about 20% hemicellulose.

4. The method according to any one of claims 1 to 3, wherein the solvent is an aqueous acid and the treatment is performed at a pH of below about 6.5.

5. The method according to any one of claims 1 to 3, wherein the solvent is an aqueous base and the treatment is performed at a pH of above about 7.5.

6. The method according to any one of claims 1 to 3, wherein the solvent is water and the treatment is performed at a pH of between about 6.5 and about 7.5.

7. The method according to any one of claims 1 to 6, further comprising pre-treating the lignocellulosic matter prior to fractionating said hemicellulose.

8. The method according to claim 7, wherein said pre-treating comprises producing a slurry comprising a mixture of the solvent and particles derived from said lignocellulosic matter.

9. The method according to claim 8, wherein said particles are between about 100 microns and about 400 microns in size.

10. The method according to claim 8 or claim 9, wherein the slurry comprises between about 5% and about 20% lignocellulosic matter.

11. A method of fractionating lignocellulosic matter, the method comprising the steps of:

(a) solvating hemicellulose from said lignocellulosic matter using the method according to any one of claims 1 to 10,

(b) removing solvated hemicellulose from solid matter remaining after step (a),

(c) solvating lignin from the solid matter remaining after step (a) using a supercritical solvent.

12. The method of claim 11, wherein the supercritical solvent is ethanol and said solvating in step (c) is performed at a temperature of above about 230 degrees Celsius and a pressure of above about 55 atmospheres.

13. The method according to claim 11 or claim 12, wherein said supercritical solvent is ethanol and said solvating in step (c) is performed at a pH of between about 6.5 and 7.5.

14. The method according to any one of claims 11 to 13, comprising the further step of removing solvated lignin from solid matter remaining after step (c).

15. The method according to claim 14, comprising the further step of solvating cellulose from said solid matter remaining after step (c) to produce solvated cellulose.

16. The method according to any one of claims 11 to 14, comprising the step of saccharifying the solvated hemicellulose to produce a fermentable saccharide.

17. The method according to claim 16, comprising the step of saccharifying either or both of:

(a) the solvated hemicellulose,

(b) the solvated cellulose,

to produce a fermentable saccharide.

18. The method according to claim 17, comprising the step of fermenting said saccharide to produce a fermented sugar product.

19. The method according to claim 18, wherein said saccharifying and fermenting are performed simultaneously.

20. The method according to claim 18 or claim 19, wherein the fermented sugar product is an alcohol or an organic acid.

21. The method according to claim 20, wherein the alcohol is selected from the group consisting of ethanol, butanol, xylitol, mannitol, and arabinol.

22. The method according to claim 21, wherein the organic acid is glutamic acid or succinic acid.

23. A method of producing an alkylated lignin product from lignocellulosic matter, the method comprising fractionating lignocellulosic matter in accordance with the method of claim 11, wherein an alkylating agent is used as the supercritical solvent, and wherein said agent alkylates the lignin of step (c).

24. The method according to claim 23, wherein the supercritical solvent is ethanol and the alkylated lignin product is ethylated lignin.

25. A product obtained by the process of any one of claims 1 to 24.

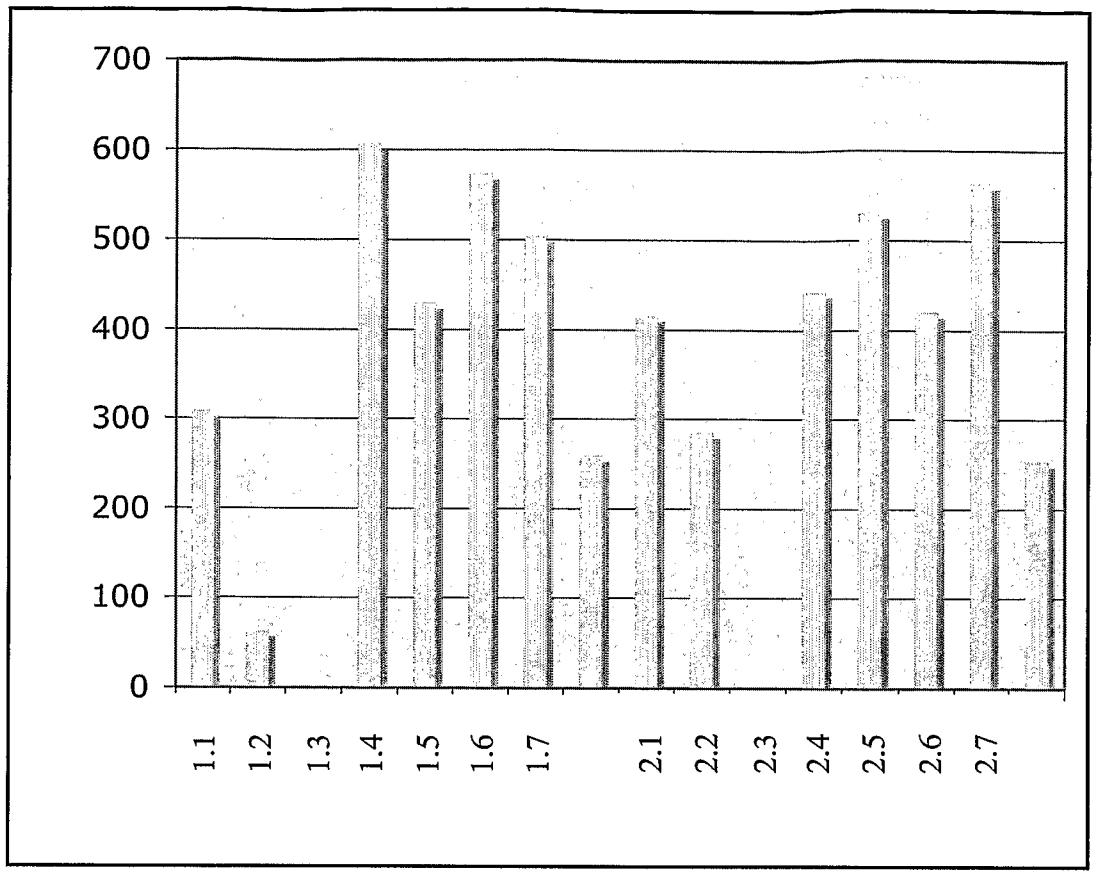

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FIGURE 1

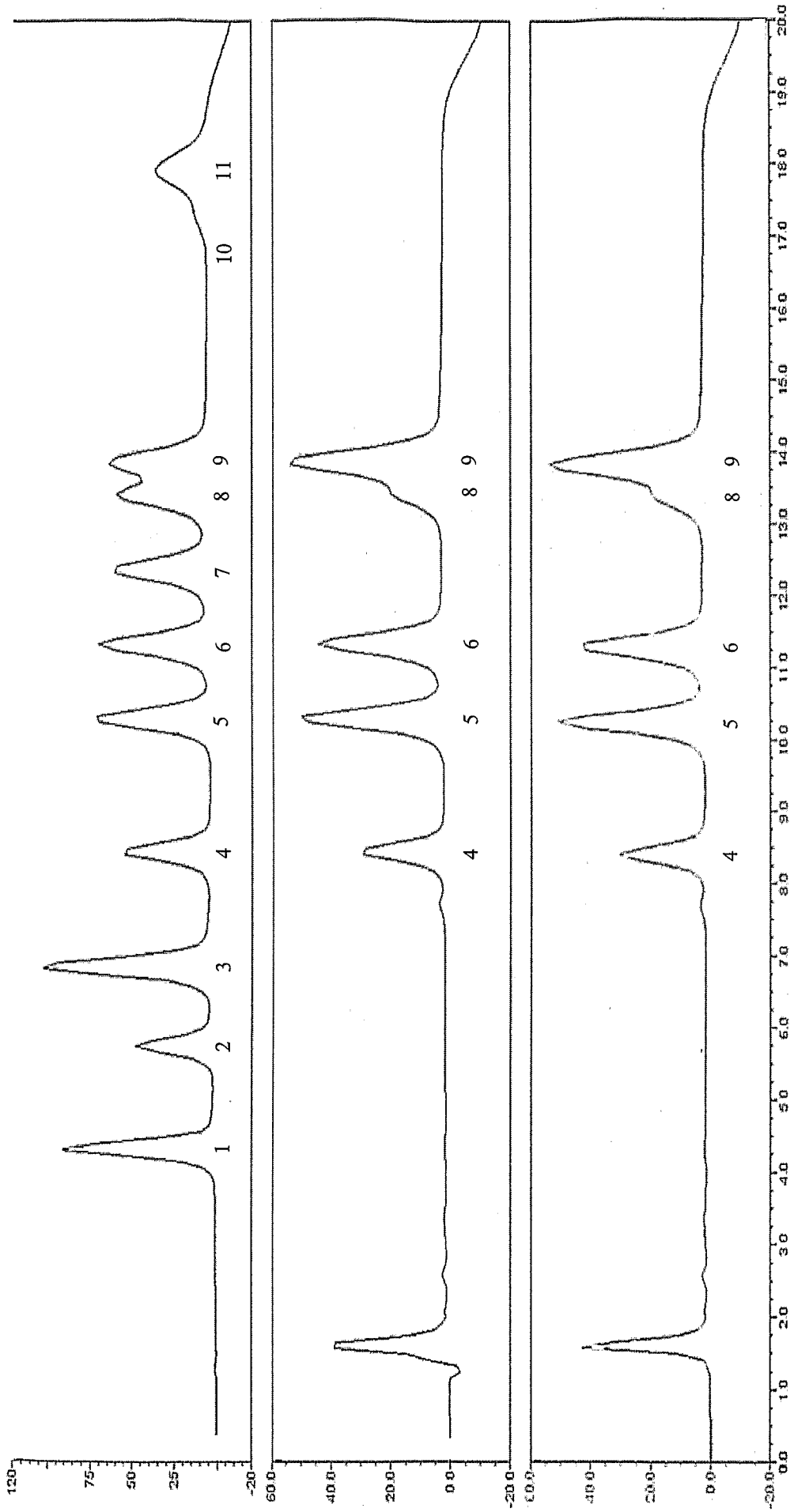
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FIGURE 2

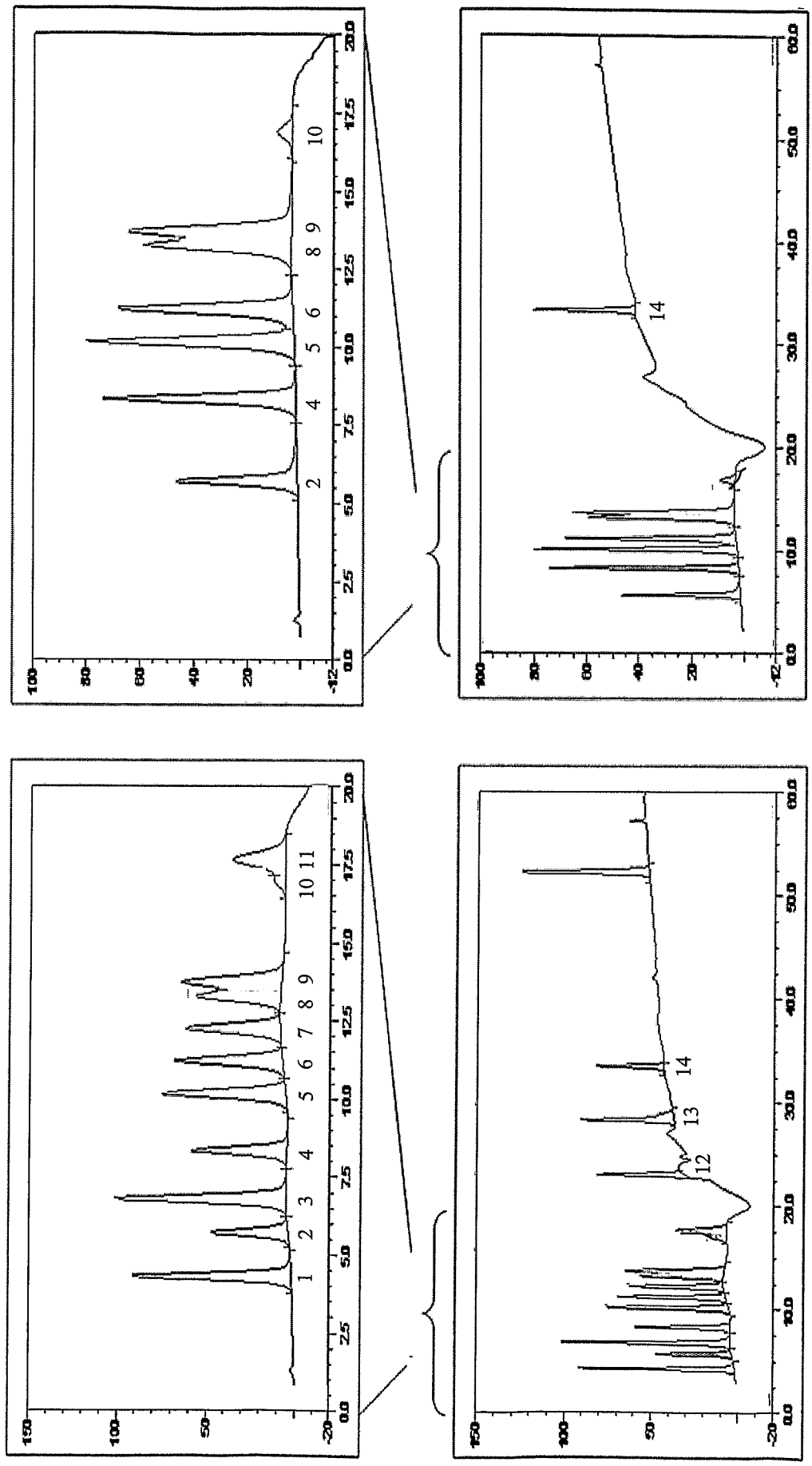

FIGURE 3

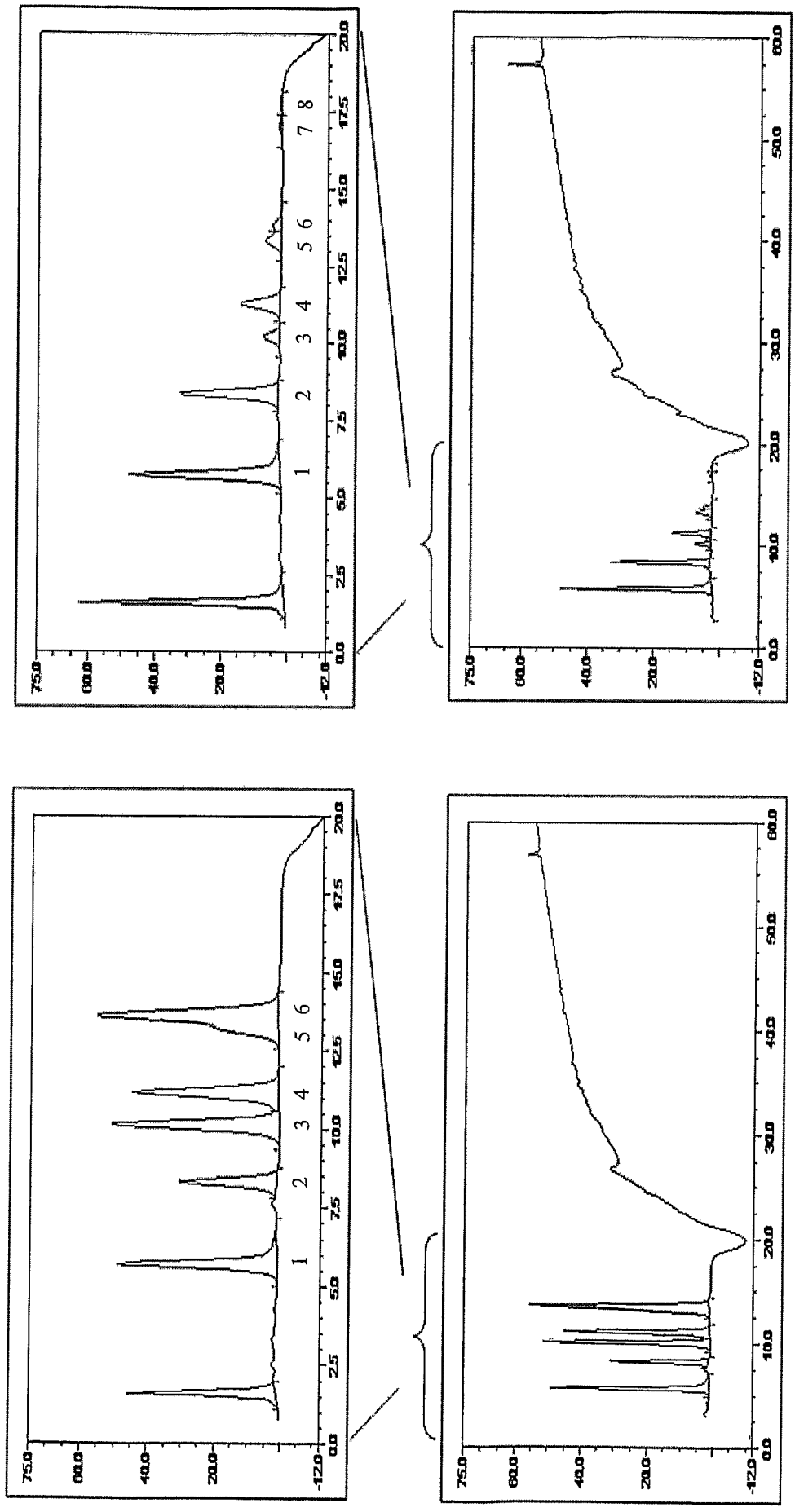

FIGURE 4

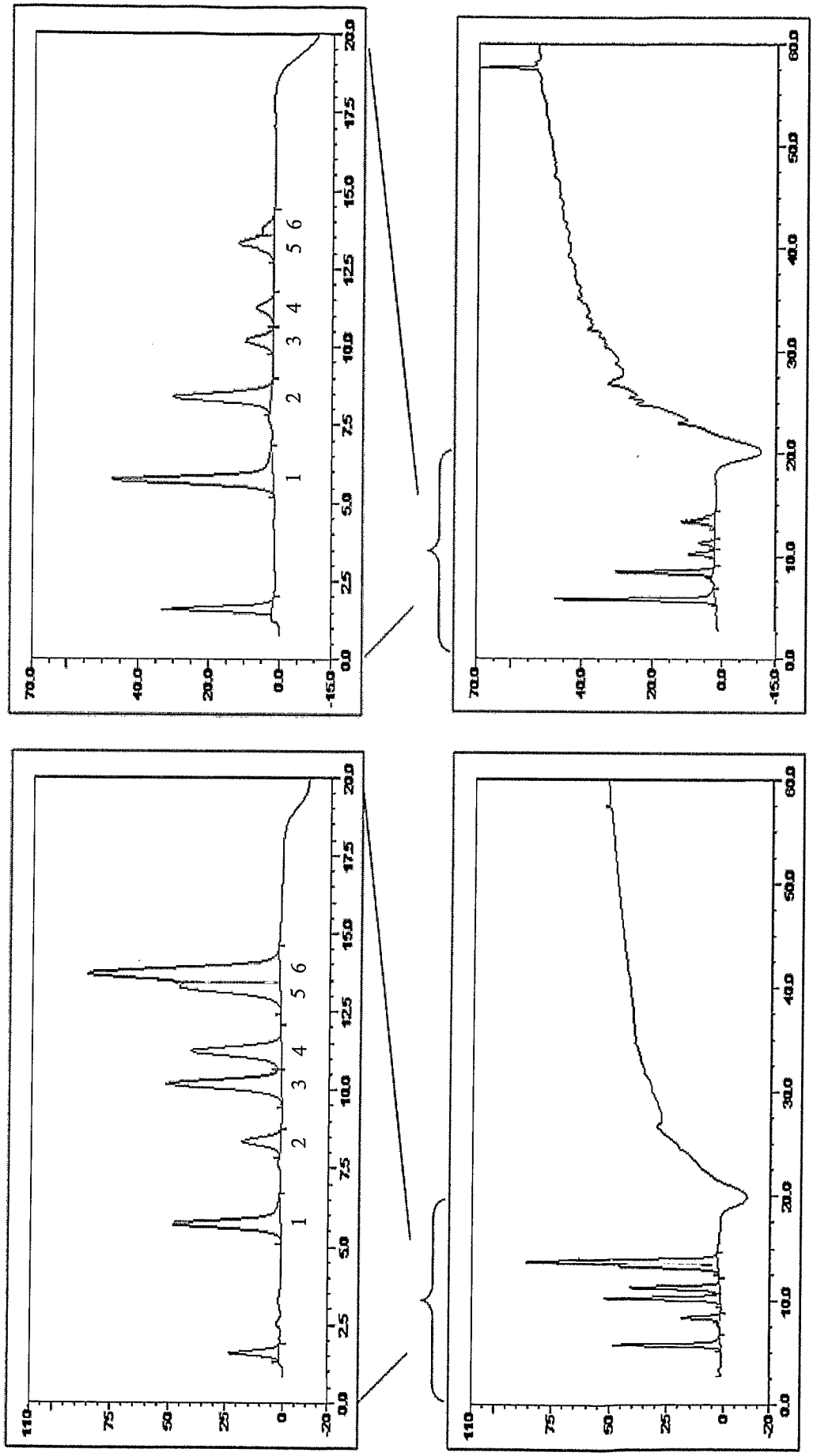

FIGURE 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2009/001260

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C07C 31/08 (2006.01) ***C12P 7/10*** (2006.01) ***D21C 3/26*** (2006.01)
C07C 29/84 (2006.01) ***D21C 3/02*** (2006.01)
C08B 37/14 (2006.01) ***D21C 3/04*** (2006.01)

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)

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)
EPODOC and WPIDs
Keywords: lignin; lignocellu+; hemicellulose+; Fraction+, purif+ separat+; distil+; extract+. remov+

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,411,594 A (BRELSFORD) 2 May 1995 See column 2, lines 56 to column 5, line 29 and abstract.	1-4,7-11,14-21,25
X	WO 2008/155639 A2 (NAGARJUNA ENERGY PRIVATE LIMITED) 24 December 2008 See summary of invention beginning on column 2 and abstract.	1-3,5,7-11,14-21,25
X	WO 2007/120210 A2 (NATUREWORKS LLC) 25 October 2007 See detailed description of the invention on page four and example 1	1-4,7-11,14-21,23-25
X	WO 2001/060752 A1 (FORSKNINGSCENTER RISØ) 23 August 2001 See page 3, lines 17-30; page 5, lines 24-29; page 7, lines 11-14.	1-3,6,7-11,13-21,25

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 November 2009	02 DEC 2009
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **KEITH WAGG** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 3 9935 9607

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2009/001260

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2,159,643 A (WEINGAND, R. ET AL.) 23 May 1939 See page 1, second column, last paragraph to page 2 first paragraph.	1-4,7-11,14-17
X	WO 2007/129921 A1 (BIOJOULE LTD) 15 November 2007 See whole document.	1-25

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2009/001260

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	5411594	NONE					
WO	2008/155639	NONE					
WO	2007/120210	CA	2631021	US	2009176286		
WO	2001/060752	AU	33621/01	CA	2400336	CN	1443141
		EP	1259466	US	6555350	US	2002192774
US	2159643	NONE					
WO	2007/129921	AU	2007248991	CA	2651628	EP	2027330
		US	2007259412	US	2009062516	US	2009069550
		WO	2009/028969				

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX