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(54) Title: PROCESSES FOR ENZYMIC REFINING OF PRETREATED CELLULOSIC MATERIAL FOR SACCHARIFICATION

(57) Abstract: The present invention relates to processes for enzymatic refining of a pretreated cellulosic material for saccharification.



PROCESSES FOR ENZYMATIC REFINING OF PRETREATED CELLULOSIC MATERIAL FOR SACCHARIFICATION

Reference to a Sequence Listing

5 This application contains a Sequence Listing in computer readable form. The computer readable form is incorporated herein by reference.

Background of the Invention

Field of the Invention

10 The present invention relates to processes for enzymatic refining of a pretreated cellulosic material using one or more esterase enzymes. The refined pretreated cellulosic material is suitable for saccharification.

Description of the Related Art

15 Cellulose is a polymer of the simple sugar glucose covalently linked by beta-1,4-bonds. Many microorganisms produce enzymes that hydrolyze beta-linked glucans. These enzymes include endoglucanases, cellobiohydrolases, and beta-glucosidases. Endoglucanases digest the cellulose polymer at random locations, opening it to attack by cellobiohydrolases. Cellobiohydrolases sequentially release molecules of cellobiose from the ends of the cellulose polymer. Cellobiose is a water-soluble beta-1,4-linked dimer of glucose. Beta-glucosidases hydrolyze cellobiose to glucose.

20 The conversion of lignocellulosic feedstocks into ethanol has the advantages of the ready availability of large amounts of feedstock, the desirability of avoiding burning or land filling the materials, and the cleanliness of the ethanol fuel. Wood, agricultural residues, herbaceous crops, and municipal solid wastes have been considered as feedstocks for ethanol production. These materials primarily consist of cellulose, hemicellulose, and lignin. 25 Once the lignocellulose is converted to fermentable sugars, *e.g.*, glucose, the fermentable sugars are easily fermented by yeast into ethanol. The sugars can also be catalytically converted or fermented to other chemicals besides ethanol.

30 The conversion of lignocellulosic feedstocks into sugars, typically, involves pretreatment of the cellulosic materials, followed by their enzymatic hydrolysis, prior to the conversion of the sugars into fermentation products or catalytically converted products. The pretreatments disrupt the lignocellulosic material, so enzymatic hydrolysis can take place efficiently.

35 However, pretreatment of cellulosic materials can produce impurities in the pretreated cellulosic materials having a deleterious effect on cellulase enzymes and/or decreases or inhibits enzymatic hydrolysis and/or saccharification.

 It would be advantageous to the art to be able to improve the pretreated cellulosic material for saccharification. For example, it would be advantageous in the art to improve

the enzymatic hydrolysis performance of pretreated cellulosic material such as biomass including corn stover, wood chips, switch grass, etc by reducing, eliminating or removing impurities such as esters that have a deleterious effect on the cellulase enzymes.

5 Prior art of interest includes WO 2009/042622 A2 which discloses a process for producing fermentation product from wood-containing material, wherein the process includes the steps of i) pre-treating wood-containing material; ii) hydrolyzing by subjecting the pre-treated wood-containing material to one or more cellulolytic enzymes; iii) fermenting using a fermenting organism, wherein the wood-containing material is subjected to one or more esterases before and/or during pre-treatment in step i) and/or hydrolysis in step ii) and/or 10 fermentation in step iii).

The present invention relates to processes for enzymatically assisted refining of a pretreated cellulosic material for saccharification.

Summary of the Invention

15 The present invention relates to a process for enzymatic refining of a pretreated cellulosic material, including: (a) contacting the pretreated cellulosic material with an esterase enzyme and/or esterase enzyme composition to form a refined pretreated cellulosic material.

20 The present invention also relates to a process for enzymatic refining of a pretreated cellulosic material, including:

(a) processing the pretreated cellulosic material to form a solid/liquid mixture of pretreated cellulosic material;

(b) separating a liquor from the solid/liquid mixture of pretreated cellulosic material; and

25 (c) treating the liquor with feruloyl esterase. In embodiments, the esterase or feruloyl esterase reduces and/or eliminates toxic esters that inhibit hydrolysis of pretreated cellulosic materials.

30 In one aspect, the processes of the present disclosure include an enzyme and/or enzyme composition including one or more (several) enzymes selected from the group consisting of an esterase and feruloyl esterase. Non-limiting examples of suitable feruloyl esterase for use in accordance with the present disclosure include enzymes having an amino acid with at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% sequence identity to the mature polypeptide of SEQ ID NOS: 133, 134, 135, 136 and 137 or fragments thereof with feruloyl esterase activity, alone or in combination.

35 In one aspect, the processes above further include recovering the refined pretreated cellulosic material.

In one aspect, the processes above further include separating liquor from the

pretreated cellulosic material.

In one aspect, the processes above further include contacting the liquor with feruloyl esterase and recycling the liquor so that it is contacted with cellulosic material, pretreated cellulosic material and/or refined pretreated cellulosic material.

5 In one aspect, the processes above further include recycling the liquor to a new pretreatment of a pretreated cellulosic material with the enzyme composition.

In one aspect, the processes above further include post-treating the refined pretreated cellulosic material with an enzymatic pre-treatment, chemical pre-treatment, mechanical pre-treatment and/or a physical pretreatment.

10 One aspect of the present disclosure relates to a process for hydrolyzing a pretreated cellulosic material, including saccharifying a pretreated cellulosic material treated and refined according to the present disclosure. In embodiments, the process includes recovering the saccharified pretreated cellulosic material from the saccharification. In embodiments, the saccharified cellulosic material is a sugar such as glucose, xylose, mannose, galactose, and
15 arabinose.

One aspect of the present disclosure relates to a process for producing a fermentation product, including:

(a) saccharifying a pretreated cellulosic material with an enzyme composition suitable for saccharification, wherein the pretreated cellulosic material is contacted or treated
20 according to the present disclosure with an enzyme including esterase and/or feruloyl esterase;

(b) fermenting the saccharified pretreated cellulosic material with one or more (several) fermenting microorganisms to produce the fermentation product; and

(c) recovering the fermentation product from the fermentation. In embodiments, 25 the esterase includes one or more (several) enzymes selected from the group consisting of an esterase and feruloyl esterase in accordance with the present disclosure. In embodiments, the esterase comprises or, consists of an amino acid with at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% sequence identity to the mature polypeptide of SEQ ID NOS: 133, 134, 135, 136 and 137, alone or in combination, or fragments thereof with esterase activity.
30 In embodiments, the esterase comprises or consists of the mature polypeptide from the amino acid of SEQ ID. NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136 or SEQ ID NO: 137 or fragments thereof having enzyme activity such as esterase activity. In embodiments the enzyme is selected from the group consisting of FAE(1), FAE-A, FAE-C, and FAE-D or fragments thereof having enzyme activity. In embodiments, the steps (a) (saccharifying a
35 pretreated cellulosic material with an enzyme composition, wherein the pretreated cellulosic material is contacted or treated according to the present disclosure with esterase and/or feruloyl esterase) and (b) (fermenting the saccharified pretreated cellulosic material with one

or more (several) fermenting microorganisms to produce the fermentation product) are performed simultaneously in a simultaneous saccharification and fermentation. In embodiments, the fermentation product is an alcohol, an organic acid, a ketone, an amino acid, an alkane, a cycloalkane, an alkene, or a gas. Suitable enzymes/enzyme compositions for saccharification include one or more (several) enzymes selected from the group consisting of a cellulase, a GH61 polypeptide having cellulolytic enhancing activity, a hemicellulase, an expansin, an esterase, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin. In embodiments, the cellulase is one or more (several) enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase. In embodiments, the hemicellulase is one or more (several) enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, a beta-xylosidase and a glucuronidase.

Another aspect of the present disclosure relates to a process for fermenting a pretreated cellulosic material, including: fermenting a pretreated cellulosic material with one or more (several) fermenting microorganisms, wherein the pretreated cellulosic material is treated, refined, and/or saccharified according to the present disclosure with esterase and/or feruloyl esterase or a composition thereof. In embodiments, the fermenting of the pretreated cellulosic material produces a fermentation product. In embodiments, the step of recovering the fermentation product from the fermentation is included. In embodiments, fermentation product is an alcohol, an organic acid, a ketone, an amino acid, an alkane, a cycloalkane, an alkene, or a gas.

In an embodiment, the pretreated cellulosic material is a non-woody substrate.

The present disclosure relates to a process for enzymatic refining of a pretreated cellulosic material, comprising or consisting of: (a) contacting the pretreated cellulosic material with one or more esterase enzymes to form a refined pretreated cellulosic material. In embodiments, the one or more esterase comprises or consists of one or more (several) enzymes selected from the group consisting of an esterase and feruloyl esterase. In embodiments, the esterase comprises or consists of an amino acid sequence having at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% sequence identity to the mature polypeptide of SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136 or SEQ ID NO: 137, or fragments thereof with feruloyl esterase activity. In embodiments, the esterase comprises or consists of SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136 or SEQ ID NO: 137 or the mature polypeptide thereof. In embodiments, the feruloyl esterase is one or more (several) enzymes selected from the group consisting of FAE(1), FAE-A, FAE-C, and FAE-D. In embodiments, the process comprises or consists of recovering the refined pretreated cellulosic material. In embodiments, the process comprises separating a liquor

from the pretreated cellulosic material. In embodiments, the process comprises contacting the liquor with feruloyl esterase and recycling the liquor so that it is contacted with pretreated cellulosic material or refined pretreated cellulosic material. In embodiments, the process comprises recycling the liquor to a new pretreatment of a pretreated cellulosic material with the esterase composition. In embodiments, the process comprises post-treating the refined pretreated cellulosic material with an enzymatic pre-treatment, chemical pre-treatment, mechanical pre-treatment and/or a physical pretreatment. In embodiments, the process comprises recovering the refined pretreated cellulosic material. In embodiments, the contacting with the esterase is performed with about 0.0005 to about 5 mg, e.g., about 0.001 to about 5 mg, about 0.0025 to about 5 mg, about 0.005 to about 5 mg, about 0.005 to about 4.5 mg, about 0.005 to about 4 mg, about 0.005 to about 3.5 mg, about 0.005 to about 3 mg, about 0.005 to about 2 mg, about 0.005 to about 1 mg, about 0.075 to about 1 mg, or about 0.1 to about 1 mg of esterase per g of pretreated cellulosic material. In embodiments, the contacting with esterase is performed with a total solids (TS) of about 1% to about 50%, e.g., about 2% to about 40%, about 2% to about 35%, about 3% to about 30%, about 3% to about 25%, about 4% to about 20%, or about 5% to about 10%. In embodiments, the contacting with esterase is performed at a pH of about 2 to about 9, e.g., about 3 to about 7.5, about 3.5 to about 7, about 4 to about 6.5, about 4.5 to about 6.5, about 4.5 to about 6.0, about 5 and about 6.0, or about 5 to about 5.5. In embodiments, the contacting is performed at a temperature in the range of about 20°C to about 70°C, e.g., about 25°C to about 65°C, about 30°C to about 65°C, about 35°C to about 65°C, about 40°C to about 60°C, about 45°C to about 55°C, or about 45°C to about 50°C. In embodiments, the contacting with esterase is performed for a period of time of 5 minutes to 35 hours, e.g., 10 minutes to 15 hours, 10 hours to 15 hours, 10 hours to 20 hours, 10 hours to 20 hours, 20 hours to 24 hours, 24 hours to 30 hours, 1 hour to 72 hours.

The present disclosure relates to a process for enzymatic refining of a pretreated cellulosic material, comprising: (a) processing the pretreated cellulosic material to form a solid/liquid mixture of pretreated cellulosic material; (b) separating a liquor from the solid/liquid mixture of pretreated cellulosic material; and (c) treating the liquor with a feruloyl esterase treatment. In embodiments, the process comprises returning the treated liquor to the pretreated cellulosic material. In embodiments, the process comprises treating with feruloyl esterase performed with about 0.0005 to about 5 mg, e.g., about 0.001 to about 5 mg, about 0.0025 to about 5 mg, about 0.005 to about 5 mg, about 0.005 to about 4.5 mg, about 0.005 to about 4 mg, about 0.005 to about 3.5 mg, about 0.005 to about 3 mg, about 0.005 to about 2 mg, about 0.005 to about 1 mg, about 0.075 to about 1 mg, or about 0.1 to about 1 mg of esterase per mL of liquor. In embodiments, the treating with feruloyl esterase is performed with a total solids (TS) of about 1% to about 50%, e.g., about 2% to about 40%,

about 2% to about 35%, about 3% to about 30%, about 3% to about 25%, about 4% to about 20%, or about 5% to about 10%. In embodiments, the treating with feruloyl esterase is performed at a pH of about 2 to about 9, e.g., about 3 to about 7.5, about 3.5 to about 7, about 4 to about 6.5, about 4.5 to about 6.5, about 4.5 to about 6.0, about 5 and about 6.0, or about 5 to about 5.5. In embodiments, the the treating with feruloyl esterase is performed at a temperature in the range of about 20°C to about 70°C, e.g., about 25°C to about 65°C, about 30°C to about 65°C, about 35°C to about 65°C, about 40°C to about 60°C, about 45°C to about 55°C, or about 45°C to about 50°C. In embodiments, the treating with feruloyl esterase is performed for a period of time of 5 minutes to 35 hours, e.g., 10 minutes to 15 hours, 10 hours to 15 hours, 10 hours to 20 hours, 10 hours to 20 hours, 20 hours to 24 hours, 24 hours to 30 hours, 1 hour to 72 hours.

The present disclosure further relates to a process for hydrolyzing a pretreated cellulosic material, comprising saccharifying a pretreated cellulosic material treated and refined with an esterase as described herein with an enzyme composition. In embodiments, the process includes esterase comprising or consisting of one or more (several) enzymes selected from the group consisting of an esterase and feruloyl esterase. In embodiments, the esterase comprises or consists of an amino acid sequence having at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% sequence identity to the mature polypeptide of SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136, SEQ ID NO: 137 or fragments thereof with feruloyl esterase activity. In embodiments, the esterase comprises or consists of SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136, SEQ ID NO: 137 or the mature polypeptide thereof. In embodiments, the feruloyl esterase is one or more (several) enzymes selected from the group consisting of FAE(1), FAE-A, FAE-C, and FAE-D. In embodiments, the enzyme composition comprises one or more (several) enzymes selected from the group consisting of a cellulase, a GH61 polypeptide having cellulolytic enhancing activity, a hemicellulase, an expansin, an esterase, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin. In embodiments, the cellulase is one or more (several) enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase. In embodiments, the hemicellulase is one or more (several) enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase. In embodiments, the process comprises recovering the saccharified pretreated cellulosic material from the saccharification. In embodiments, the saccharified cellulosic material is a sugar. In embodiments, the sugar is selected from the group consisting of glucose, xylose, mannose, galactose, and arabinose.

The present disclosure relates to a process for producing a fermentation product, comprising or consisting of: (a) saccharifying a pretreated cellulosic material with an

enzyme composition, wherein the pretreated cellulosic material is contacted or treated with an esterase according to the present disclosure; (b) fermenting the saccharified pretreated cellulosic material with one or more (several) fermenting microorganisms to produce the fermentation product; and (c) recovering the fermentation product from the fermentation. In
5 embodiments, the esterase comprises or consists of one or more (several) enzymes selected from the group consisting of an esterase and feruloyl esterase. In embodiments, the esterase composition comprises or consists of an amino acid sequence having at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% sequence identity to the mature polypeptide of SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136, SEQ ID NO: 137 or
10 fragments thereof with feruloyl esterase activity. In embodiments, the esterase composition consists of SEQ ID NO: 133, SEQ ID. NO: 134, SEQ ID NO: 135, SEQ ID NO: 136 or SEQ ID NO: 137 or the mature polypeptide thereof. In embodiments, the feruloyl esterase is one or more (several) enzymes selected from the group consisting of FAE(1), FAE-A, FAE-C, and FAE-D. In embodiments, the feruloyl esterase is FAE(1). In embodiments, the feruloyl esterase
15 is FAE-A. In embodiments, the feruloyl esterase is FAE-C. In embodiments, the feruloyl esterase FAE-D. In embodiments, the enzyme composition comprises one or more (several) enzymes selected from the group consisting of a cellulase, a GH61 polypeptide having cellulolytic enhancing activity, a hemicellulase, an expansin, an esterase, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin. In
20 embodiments, the cellulase is one or more (several) enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase. In embodiments, the hemicellulase is one or more (several) enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase. In embodiments, the steps (a) and
25 (b) are performed simultaneously in a simultaneous saccharification and fermentation. In embodiment, the fermentation product is an alcohol, an organic acid, a ketone, an amino acid, an alkane, a cycloalkane, an alkene, or a gas.

The present disclosure relates to a process for fermenting a pretreated cellulosic material, comprising: fermenting a pretreated cellulosic material with one or more (several)
30 fermenting microorganisms, wherein the pretreated cellulosic material is treated, refined, and/or saccharified in accordance with the present disclosure. In embodiments, the fermenting of the pretreated cellulosic material produces a fermentation product. In embodiments, the process comprises recovering the fermentation product from the fermentation. In embodiments, the fermentation product is an alcohol, an organic acid, a
35 ketone, an amino acid, an alkane, a cycloalkane, an alkene, or a gas.

Definitions

Cellulolytic enzyme or cellulase: The term “cellulolytic enzyme” or “cellulase” means one or more (several) enzymes that hydrolyze a cellulosic material. Such enzymes include endoglucanase(s), cellobiohydrolase(s), beta-glucosidase(s), or combinations thereof. The two basic approaches for measuring cellulolytic activity include: (1) measuring the total cellulolytic activity, and (2) measuring the individual cellulolytic activities (endoglucanases, cellobiohydrolases, and beta-glucosidases) as reviewed in Zhang *et al.*, *Outlook for cellulase improvement: Screening and selection strategies*, 2006, *Biotechnology Advances* 24: 452-481. Total cellulolytic activity is usually measured using insoluble substrates, including Whatman №1 filter paper, microcrystalline cellulose, bacterial cellulose, algal cellulose, cotton, pretreated lignocellulose, *etc.* The most common total cellulolytic activity assay is the filter paper assay using Whatman №1 filter paper as the substrate. The assay was established by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987, Measurement of cellulase activities, *Pure Appl. Chem.* 59: 257-68).

For purposes of the present disclosure, cellulolytic enzyme activity is determined by measuring the increase in hydrolysis of a cellulosic material by cellulolytic enzyme(s) under the following conditions: 1-20 mg of cellulolytic enzyme protein/g of cellulose in PCS for 3-7 days at 50°C compared to a control hydrolysis without addition of cellulolytic enzyme protein. Typical conditions are 1 ml reactions, washed or unwashed PCS, 5% insoluble solids, 50 mM sodium acetate pH 5, 1 mM MnSO₄, 50°C, 72 hours, sugar analysis by AMINEX® HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Endoglucanase: The term “endoglucanase” means an endo-1,4-(1,3;1,4)-beta-D-glucan 4-glucanohydrolase (E.C. 3.2.1.4), which catalyses endohydrolysis of 1,4-beta-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxymethyl cellulose and hydroxyethyl cellulose), lichenin, beta-1,4 bonds in mixed beta-1,3 glucans such as cereal beta-D-glucans or xyloglucans, and other plant material containing cellulosic components. Endoglucanase activity can be determined by measuring reduction in substrate viscosity or increase in reducing ends determined by a reducing sugar assay (Zhang *et al.*, 2006, *Biotechnology Advances* 24: 452-481). For purposes of the present invention, endoglucanase activity is determined using carboxymethyl cellulose (CMC) as substrate according to the procedure of Ghose, 1987, *Pure and Appl. Chem.* 59: 257-268, at pH 5, 40°C.

Cellobiohydrolase: The term “cellobiohydrolase” means a 1,4-beta-D-glucan cellobiohydrolase (E.C. 3.2.1.91), which catalyzes the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, celooligosaccharides, or any beta-1,4-linked glucose containing polymer, releasing cellobiose from the reducing or non-reducing ends of the chain (Teeri, 1997, Crystalline cellulose degradation: New insight into the function of cellobiohydrolases, *Trends in Biotechnology* 15: 160-167; Teeri *et al.*, 1998, *Trichoderma reesei*

cellobiohydrolases: why so efficient on crystalline cellulose?, *Biochem. Soc. Trans.* 26: 173-178). For purposes of the present invention, cellobiohydrolase activity is determined according to the procedures described by Lever *et al.*, 1972, *Anal. Biochem.* 47: 273-279; van Tilbeurgh *et al.*, 1982, *FEBS Letters*, 149: 152-156; van Tilbeurgh and Claeysens, 1985, *FEBS Letters*, 187: 283-288; and Tomme *et al.*, 1988, *Eur. J. Biochem.* 170: 575-581. In the present invention, the Lever *et al.* method can be employed to assess hydrolysis of cellulose in corn stover, while the methods of van Tilbeurgh *et al.* and Tomme *et al.* can be used to determine the cellobiohydrolase activity on a fluorescent disaccharide derivative, 4-methylumbelliferyl- β -D-lactoside.

Beta-glucosidase: The term "beta-glucosidase" means a beta-D-glucoside glucohydrolase (E.C. 3.2.1.21), which catalyzes the hydrolysis of terminal non-reducing beta-D-glucose residues with the release of beta-D-glucose. For purposes of the present invention, beta-glucosidase activity is determined according to the basic procedure described by Venturi *et al.*, 2002, Extracellular beta-D-glucosidase from *Chaetomium thermophilum* var. *coprophilum*: production, purification and some biochemical properties, *J. Basic Microbiol.* 42: 55-66. One unit of beta-glucosidase is defined as 1.0 μ mole of *p*-nitrophenolate anion produced per minute at 25°C, pH 4.8 from 1 mM *p*-nitrophenyl-beta-D-glucopyranoside as substrate in 50 mM sodium citrate containing 0.01% TWEEN® 20.

Polypeptide having cellulolytic enhancing activity: The term "polypeptide having cellulolytic enhancing activity" means a GH61 polypeptide that catalyzes the enhancement of the hydrolysis of a cellulosic material by enzyme having cellulolytic activity. For purposes of the present invention, cellulolytic enhancing activity is determined by measuring the increase in reducing sugars or the increase of the total of cellobiose and glucose from the hydrolysis of a cellulosic material by cellulolytic enzyme under the following conditions: 1-50 mg of total protein/g of cellulose in PCS, wherein total protein is comprised of 50-99.5% w/w cellulolytic enzyme protein and 0.5-50% w/w protein of a GH61 polypeptide having cellulolytic enhancing activity for 1-7 days at 50°C compared to a control hydrolysis with equal total protein loading without cellulolytic enhancing activity (1-50 mg of cellulolytic protein/g of cellulose in PCS). In a preferred aspect, a mixture of CELLUCLAST® 1.5L (Novozymes A/S, Bagsværd, Denmark) in the presence of 2-3% of total protein weight *Aspergillus oryzae* beta-glucosidase (recombinantly produced in *Aspergillus oryzae* according to WO 02/095014) or 2-3% of total protein weight *Aspergillus fumigatus* beta-glucosidase (recombinantly produced in *Aspergillus oryzae* as described in WO 2002/095014) of cellulase protein loading is used as the source of the cellulolytic activity.

The GH61 polypeptides having cellulolytic enhancing activity enhance the hydrolysis of a cellulosic material catalyzed by enzyme having cellulolytic activity by reducing the amount of cellulolytic enzyme required to reach the same degree of hydrolysis preferably at

least 1.01-fold, more preferably at least 1.05-fold, more preferably at least 1.10-fold, more preferably at least 1.25-fold, more preferably at least 1.5-fold, more preferably at least 2-fold, more preferably at least 3-fold, more preferably at least 4-fold, more preferably at least 5-fold, even more preferably at least 10-fold, and most preferably at least 20-fold.

5 **Family 61 glycoside hydrolase:** The term “Family 61 glycoside hydrolase” or “Family GH61” or “GH61” means a polypeptide falling into the glycoside hydrolase Family 61 according to Henrissat B., 1991, A classification of glycosyl hydrolases based on amino-acid sequence similarities, *Biochem. J.* 280: 309-316, and Henrissat B., and Bairoch A., 1996, Updating the sequence-based classification of glycosyl hydrolases, *Biochem. J.* 316: 695-10 696.

Hemicellulolytic enzyme or hemicellulase: The term “hemicellulolytic enzyme” or “hemicellulase” means one or more (several) enzymes that hydrolyze a hemicellulosic material. See, for example, Shallom, D. and Shoham, Y. Microbial hemicellulases. *Current Opinion In Microbiology*, 2003, 6(3): 219-228). Hemicellulases are key components in the degradation of plant biomass. Examples of hemicellulases include, but are not limited to, an acetylmannan esterase, an acetyxylan esterase, an arabinanase, an arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase. The substrates of these enzymes, the hemicelluloses, are a heterogeneous group of branched and linear polysaccharides that are bound via hydrogen bonds to the cellulose microfibrils in the plant cell wall, crosslinking them into a robust network. Hemicelluloses are also covalently attached to lignin, forming together with cellulose a highly complex structure. The variable structure and organization of hemicelluloses require the concerted action of many enzymes for its complete degradation. The catalytic modules of hemicellulases are either glycoside hydrolases (GHs) that hydrolyze glycosidic bonds, or carbohydrate esterases (CEs), which hydrolyze ester linkages of acetate or ferulic acid side groups. These catalytic modules, based on homology of their primary sequence, can be assigned into GH and CE families marked by numbers. Some families, with overall similar fold, can be further grouped into clans, marked alphabetically (e.g., GH-A). A most informative and updated classification of these and other carbohydrate active enzymes is available on the Carbohydrate-Active Enzymes (CAZy) database. Hemicellulolytic enzyme activities can be measured according to Ghose and Bisaria, 1987, *Pure & Appl. Chem.* 59: 1739-1752.

Xylan degrading activity or xylanolytic activity: The term “xylan degrading activity” or “xylanolytic activity” means a biological activity that hydrolyzes xylan-containing material. The two basic approaches for measuring xylanolytic activity include: (1) measuring the total xylanolytic activity, and (2) measuring the individual xylanolytic activities (e.g., endoxylanases, beta-xylosidases, arabinofuranosidases, alpha-glucuronidases, acetylxylan

esterases, feruloyl esterases, and alpha-glucuronyl esterases). Recent progress in assays of xylanolytic enzymes was summarized in several publications including Biely and Puchard, Recent progress in the assays of xylanolytic enzymes, 2006, *Journal of the Science of Food and Agriculture* 86(11): 1636-1647; Spanikova and Biely, 2006, Glucuronoyl esterase - Novel carbohydrate esterase produced by *Schizophyllum commune*, *FEBS Letters* 580(19): 4597-4601; Herrmann, Vrsanska, Jurickova, Hirsch, Biely, and Kubicek, 1997, The beta-D-xylosidase of *Trichoderma reesei* is a multifunctional beta-D-xylan xylohydrolase, *Biochemical Journal* 321: 375-381.

Total xylan degrading activity can be measured by determining the reducing sugars formed from various types of xylan, including, for example, oat spelt, beechwood, and larchwood xylans, or by photometric determination of dyed xylan fragments released from various covalently dyed xylans. The most common total xylanolytic activity assay is based on production of reducing sugars from polymeric 4-O-methyl glucuronoxylan as described in Bailey, Biely, Poutanen, 1992, Interlaboratory testing of methods for assay of xylanase activity, *Journal of Biotechnology* 23(3): 257-270. Xylanase activity can also be determined with 0.2% AZCL-arabinoxylan as substrate in 0.01% Triton X-100 and 200 mM sodium phosphate buffer pH 6 at 37°C. One unit of xylanase activity is defined as 1.0 µmole of azurine produced per minute at 37°C, pH 6 from 0.2% AZCL-arabinoxylan as substrate in 200 mM sodium phosphate pH 6 buffer.

For purposes of the present invention, xylan degrading activity is determined by measuring the increase in hydrolysis of birchwood xylan (Sigma Chemical Co., Inc., St. Louis, MO, USA) by xylan-degrading enzyme(s) under the following typical conditions: 1 ml reactions, 5 mg/ml substrate (total solids), 5 mg of xylanolytic protein/g of substrate, 50 mM sodium acetate pH 5, 50°C, 24 hours, sugar analysis using *p*-hydroxybenzoic acid hydrazide (PHBAH) assay as described by Lever, 1972, A new reaction for colorimetric determination of carbohydrates, *Anal. Biochem* 47: 273-279.

Xylanase: The term "xylanase" means a 1,4-beta-D-xylan-xylohydrolase (E.C. 3.2.1.8) that catalyzes the endohydrolysis of 1,4-beta-D-xylosidic linkages in xylans. For purposes of the present invention, xylanase activity is determined with 0.2% AZCL-arabinoxylan as substrate in 0.01% Triton X-100 and 200 mM sodium phosphate buffer pH 6 at 37°C. One unit of xylanase activity is defined as 1.0 µmole of azurine produced per minute at 37°C, pH 6 from 0.2% AZCL-arabinoxylan as substrate in 200 mM sodium phosphate pH 6 buffer.

Beta-xylosidase: The term "beta-xylosidase" means a beta-D-xyloside xylohydrolase (E.C. 3.2.1.37) that catalyzes the exo-hydrolysis of short beta-(4) - xylooligosaccharides, to remove successive D-xylose residues from the non-reducing

termini. For purposes of the present invention, one unit of beta-xylosidase is defined as 1.0 μ mole of *p*-nitrophenolate anion produced per minute at 40°C, pH 5 from 1 mM *p*-nitrophenyl-beta-D-xyloside as substrate in 100 mM sodium citrate containing 0.01% TWEEN® 20.

Esterase: The term “esterase” means a hydrolase enzyme that splits esters into an acid and an alcohol in a chemical reaction with water call hydrolysis. The term also refers to enzyme referred to as carboxylic ester hydrolyases, referring to enzymes acting on ester bonds, and includes enzymes classified in EC 3.1.1 carboxylic ester hydrolases according to Enzyme Nomenclature (available at <http://www.chem.qmw.ac.uk/iubmb/enzyme> or from Enzyme Nomenclature 1992, Academic Press, San Diego, California, with Supplement 1 (1993), Supplement 2 (1994), Supplement 3 (1995), Supplement 4 (1997) and Supplement 5, in Eur. J. Biochem. 1994, 223, 1-5; Eur. J. Biochem. 1995, 232, 1-6; Eur. J. Biochem. 1996, 237, 1-5; Eur. J. Biochem. 1997, 250; 1- 6, and Eur. J. Biochem. 1999, 264, 610-650; respectively). Non-limiting examples of esterases include carboxylesterase, arylesterase, triacylglycerol lipase, acylesterase, acetylcholinesterase, cholinesterase, tropinesterase, pectinesterase, sterol esterase, chlorophyllase, L- arabinonolactonase, gluconolactonase, uronolactonase, tannase, retinyl-palmitate esterase, hydroxybutyrate-dimer hydrolase, acylglycerol lipase, 3-oxoadipate enol-lactonase, 1,4- lactonase, galactolipase, 4-pyridoxolactonase, acylcarnitine hydrolase, aminoacyl-tRNA hydrolase, D-arabinonolactonase, 6-phosphogluconolactonase, phospholipase A1 , 6- acetylglucose deacetylase, lipoprotein lipase, dihydrocoumarin lipase, limonin-D-ring- lactonase, steroid-lactonase, triacetate-lactonase, actinomycin lactonase, orsellinate- depside hydrolase, cephalosporin-C deacetylase, chlorogenate hydrolase, alpha-amino-acid esterase, 4-methyloxaloacetate esterase, carboxymethylenebutenolidase, deoxylimonate A- ring-lactonase, 2-acetyl-1-alkylglycerophosphocholine esterase, fusarinine-C ornithinesterase, sinapine esterase, wax-ester hydrolase, phorbol-diester hydrolase, phosphatidylinositol deacylase, sialate O-acylesterase, acetoxylbutynylbithiophene deacetylase, acetylsalicylate deacetylase, methylumbelliferyl-acetate deacetylase, 2-pyrone- 4,6-dicarboxylate lactonase, N-acetylgalactosaminoglycan deacetylase, juvenile-hormone esterase, bis(2-ethylhexyl)phthalate esterase, protein-glutamate methylesterase, 11-cis- retinyl-palmitate hydrolase, all-trans-retinyl-palmitate hydrolase, L-rhamnono-1,4-lactonase, 5-(3,4-diacetoxybut-1-ynyl)-2,2'-bithiophene deacetylase, fatty-acyl-ethyl-ester synthase, xylonolactonase, N-acetylglucosaminylphosphatidylinositol deacetylase, cetaxate benzylesterase, acetylalkylglycerol acetylhydrolase, and acetylxylan esterase. Non-limiting examples of esterase include carboxylic ester hydrolases classified in EC 3.1.1.1 through and including EC3.1.1.85 according to the Enzyme Nomenclature (available at a website having the address www.chem.qmw.ac.uk/iubmb/enzyme). Esterases have wide specificity;

and also may hydrolyze vitamin A esters. Esterases may also come from microsomes that also catalyze the reactions of EC 3.1.1.2, EC 3.1.1.5, EC 3.1.1.6, EC 3.1.1.23, EC 3.1.1.28, EC 3.1.2.2, EC 3.5.1.4, and EC 3.5.1.13.

Acetylxyylan esterase: The term "acetylxyylan esterase" means a carboxylesterase (EC 3.1.1.72) that catalyses the hydrolysis of acetyl groups from polymeric xylan, acetylated xylose, acetylated glucose, alpha-naphthyl acetate, and *p*-nitrophenyl acetate. For purposes of the present invention, acetylxyylan esterase activity is determined using 0.5 mM *p*-nitrophenylacetate as substrate in 50 mM sodium acetate pH 5.0 containing 0.01% TWEEN™ 20. One unit of acetylxyylan esterase is defined as the amount of enzyme capable of releasing 1 μmole of *p*-nitrophenolate anion per minute at pH 5, 25°C.

Feruloyl esterase: The term "feruloyl esterase" means a 4-hydroxy-3-methoxycinnamoyl-sugar hydrolase (EC 3.1.1.73) that catalyzes the hydrolysis of the 4-hydroxy-3-methoxycinnamoyl (feruloyl) group from an esterified sugar, which is usually arabinose in "natural" substrates, to produce ferulate (4-hydroxy-3-methoxycinnamate). Feruloyl esterase is also known as ferulic acid esterase, hydroxycinnamoyl esterase, FAE-III, cinnamoyl ester hydrolase, FAEA, cinnAE, FAE-I, or FAE-II. Non-limiting examples of feruloyl esterase for use in accordance with the present disclosure are set forth below. For purposes of the present disclosure, feruloyl esterase activity is determined using 0.5 mM *p*-nitrophenylferulate as substrate in 50 mM sodium acetate pH 5.0. One unit of feruloyl esterase equals the amount of enzyme capable of releasing 1 μmole of *p*-nitrophenolate anion per minute at pH 5, 25°C.

Alpha-glucuronidase: The term "alpha-glucuronidase" means an alpha-D-glucosiduronate glucuronohydrolase (EC 3.2.1.139) that catalyzes the hydrolysis of an alpha-D-glucuronoside to D-glucuronate and an alcohol. For purposes of the present invention, alpha-glucuronidase activity is determined according to de Vries, 1998, *J. Bacteriol.* 180: 243-249. One unit of alpha-glucuronidase equals the amount of enzyme capable of releasing 1 μmole of glucuronic or 4-O-methylglucuronic acid per minute at pH 5, 40°C.

Alpha-L-arabinofuranosidase: The term "alpha-L-arabinofuranosidase" means an alpha-L-arabinofuranoside arabinofuranohydrolase (EC 3.2.1.55) that catalyzes the hydrolysis of terminal non-reducing alpha-L-arabinofuranoside residues in alpha-L-arabinosides. The enzyme acts on alpha-L-arabinofuranosides, alpha-L-arabinans containing (1,3)- and/or (1,5)-linkages, arabinoxylans, and arabinogalactans. Alpha-L-arabinofuranosidase is also known as arabinosidase, alpha-arabinosidase, alpha-L-arabinosidase, alpha-arabinofuranosidase, polysaccharide alpha-L-arabinofuranosidase, alpha-L-arabinofuranoside hydrolase, L-arabinosidase, or alpha-L-arabinanase. For purposes of the present invention, alpha-L-arabinofuranosidase activity is determined using

5 mg of medium viscosity wheat arabinoxylan (Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland) per ml of 100 mM sodium acetate pH 5 in a total volume of 200 µl for 30 minutes at 40°C followed by arabinose analysis by AMINEX® HPX-87H column chromatography (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cellulosic material: The cellulosic material can be any material containing cellulose. The predominant polysaccharide in the primary cell wall of biomass is cellulose, the second most abundant is hemicellulose, and the third is pectin. The secondary cell wall, produced after the cell has stopped growing, also contains polysaccharides and is strengthened by polymeric lignin covalently cross-linked to hemicellulose. Cellulose is a homopolymer of anhydrocellobiose and thus a linear beta-(1-4)-D-glucan, while hemicelluloses include a variety of compounds, such as xylans, xyloglucans, arabinoxylans, and mannans in complex branched structures with a spectrum of substituents. Although generally polymorphous, cellulose is found in plant tissue primarily as an insoluble crystalline matrix of parallel glucan chains. Hemicelluloses usually hydrogen bond to cellulose, as well as to other hemicelluloses, which help stabilize the cell wall matrix. Cells from dead plants can also be referred to as fibers or cellulosic fibers. The cell walls are composed by layers, which are assembled by fibrils, hemicellulose and lignin. The fibrils are mainly composed by cellulose with minor quantities of hemicellulose and lignin.

Cellulose is generally found, for example, in the stems, leaves, hulls, husks, and cobs of plants or leaves, branches, and wood of trees. The cellulosic material can be, but is not limited to, herbaceous material, agricultural residue, forestry residue, municipal solid waste, waste paper, and pulp and paper mill residue (see, for example, Wiseloge *et al.*, 1995, in Handbook on Bioethanol (Charles E. Wyman, editor), pp.105-118, Taylor & Francis, Washington D.C.; Wyman, 1994, *Bioresource Technology* 50: 3-16; Lynd, 1990, *Applied Biochemistry and Biotechnology* 24/25: 695-719; Mosier *et al.*, 1999, Recent Progress in Bioconversion of Lignocellulosics, in *Advances in Biochemical Engineering/Biotechnology*, T. Scheper, managing editor, Volume 65, pp.23-40, Springer-Verlag, New York). It is understood herein that the cellulose may be in the form of lignocellulose, a plant cell wall material containing lignin, cellulose, and hemicellulose in a mixed matrix. In a preferred aspect, the cellulosic material is lignocellulose.

In one aspect, the cellulosic material is herbaceous material. In another aspect, the cellulosic material is agricultural residue. In another aspect, the cellulosic material is forestry residue. In another aspect, the cellulosic material is municipal solid waste. In another aspect, the cellulosic material is waste paper. In another aspect, the cellulosic material is pulp and paper mill residue.

In another aspect, the cellulosic material is hardwood. In another aspect, the cellulosic material is hardwood chips. In another aspect, the cellulosic material is hardwood

pulp. In another aspect, the cellulosic material is softwood. In another aspect, the cellulosic material is softwood chips. In another aspect, the cellulosic material is softwood pulp.

In another aspect, the cellulosic material is a byproduct of the pulp and paper industry. In another aspect, the cellulosic material is a virgin fiber byproduct of the pulp and paper industry. In another aspect, the cellulosic material is a recycled byproduct of the pulp and paper industry.

In another aspect, the cellulosic material is a cellulose-containing fiber. In another aspect, the cellulosic material is a cellulose-containing fibrous material.

In another aspect, the cellulosic material is corn stover. In another aspect, the cellulosic material is corn fiber. In another aspect, the cellulosic material is corn cob. In another aspect, the cellulosic material is orange peel. In another aspect, the cellulosic material is rice straw. In another aspect, the cellulosic material is wheat straw. In another aspect, the cellulosic material is switch grass. In another aspect, the cellulosic material is miscanthus. In another aspect, the cellulosic material is bagasse.

In another aspect, the cellulosic material is microcrystalline cellulose. In another aspect, the cellulosic material is bacterial cellulose. In another aspect, the cellulosic material is algal cellulose. In another aspect, the cellulosic material is cotton linter. In another aspect, the cellulosic material is amorphous phosphoric-acid treated cellulose. In another aspect, the cellulosic material is filter paper.

The cellulosic material may be used as is or may be subjected to pretreatment, using conventional methods known in the art, as described herein. In a preferred aspect, the cellulosic material is pretreated.

The cellulosic material may have been dried, at least once, at any stage of its production, before being submitted to the enzymatically aided refining process. The cellulosic material may have been never dried, before being submitted to the enzymatically aided refining process.

Pretreated Cellulosic material: The term pretreated cellulosic material means any cellulosic material that has been treated in preparation for further processing. Non-limiting examples of pretreated cellulosic material includes cellulosic material treated by one or more chemical, enzymatic, mechanical, or physical pre-treatment steps in preparation for enzymatic hydrolysis.

Pretreated corn stover: The term "PCS" or "Pretreated Corn Stover" means a cellulosic material derived from corn stover by treatment with heat and dilute sulfuric acid.

Isolated or purified: The term "isolated" or "purified" means a polypeptide or polynucleotide that is removed from at least one component with which it is naturally associated. For example, a polypeptide may be at least 1% pure, e.g., at least 5% pure, at least 10% pure, at least 20% pure, at least 40% pure, at least 60% pure, at least 80% pure,

at least 90% pure, or at least 95% pure, as determined by SDS-PAGE, and a polynucleotide may be at least 1% pure, *e.g.*, at least 5% pure, at least 10% pure, at least 20% pure, at least 40% pure, at least 60% pure, at least 80% pure, at least 90% pure, or at least 95% pure, as determined by agarose electrophoresis.

Mature polypeptide: The term “mature polypeptide” means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. It is known in the art that a host cell may produce a mixture of two or more different mature polypeptides (*i.e.*, with a different C-terminal and/or N-terminal amino acid) expressed by the same polynucleotide. The mature polypeptide can be predicted using the SignalP program (Nielsen *et al.*, 1997, *Protein Engineering* 10: 1-6).

Mature polypeptide coding sequence: The term “mature polypeptide coding sequence” is defined herein as a nucleotide sequence that encodes a mature polypeptide having biological activity. The mature polypeptide coding sequence can be predicted using the SignalP program (Nielsen *et al.*, 1997, *supra*).

Sequence Identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter “sequence identity”.

For purposes of the present invention, the degree of sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled “longest identity” (obtained using the *-nobrief* option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Residues} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

For purposes of the present invention, the degree of sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled “longest identity” (obtained using the *-nobrief* option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Deoxyribonucleotides} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

Polypeptide fragment: The term “fragment” means a polypeptide having one or more (several) amino acids deleted from the amino and/or carboxyl terminus of a mature polypeptide; wherein the fragment has biological activity.

Subsequence: The term “subsequence” means a polynucleotide having one or more (several) nucleotides deleted from the 5' and/or 3' end of a mature polypeptide coding sequence, wherein the subsequence encodes a fragment having biological activity.

Allelic variant: The term “allelic variant” means any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

Coding sequence: The term “coding sequence” means a polynucleotide, which directly specifies the amino acid sequence of a polypeptide. The boundaries of the coding sequence are generally determined by an open reading frame, which usually begins with the ATG start codon or alternative start codons such as GTG and TTG and ends with a stop codon such as TAA, TAG, and TGA. The coding sequence may be a DNA, cDNA, synthetic, or recombinant polynucleotide.

cDNA: The term “cDNA” means a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic or prokaryotic cell. cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps, including splicing, before appearing as mature spliced mRNA.

Nucleic acid construct: The term “nucleic acid construct” means a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or is modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature or which is synthetic. The term nucleic acid construct is synonymous with the term “expression cassette” when the nucleic acid construct contains the control sequences required for expression of a coding sequence.

Control sequences: The term “control sequences” means all components necessary for the expression of a polynucleotide encoding a polypeptide. Each control sequence may be native or foreign to the polynucleotide encoding the polypeptide or native or foreign to each other. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control

sequences with the coding region of the polynucleotide encoding a polypeptide.

Operably linked: The term “operably linked” means a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of a polynucleotide such that the control sequence directs the expression of the coding sequence.

Expression: The term “expression” includes any step involved in the production of a polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

Expression vector: The term “expression vector” means a linear or circular DNA molecule that comprises a polynucleotide encoding a polypeptide and is operably linked to additional nucleotides that provide for its expression.

Host cell: The term “host cell” means any cell type that is susceptible to transformation, transfection, transduction, and the like with a nucleic acid construct or expression vector comprising a polynucleotide. The term “host cell” encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

Variant: The term “variant” means a polypeptide comprising an alteration, *i.e.*, a substitution, insertion, and/or deletion of one or more (several) amino acid residues at one or more (several) positions. A substitution means a replacement of an amino acid occupying a position with a different amino acid; a deletion means removal of an amino acid occupying a position; and an insertion means adding one or more (several) amino acids, *e.g.*, 1-5 amino acids, adjacent to an amino acid occupying a position.

Ester: The term “ester” refers to a radical having the structure $--C(O)O--$, $--C(O)O--R_j$, $--R_kC(O)O--R_j--$, or $--R_kC(O)O--$, where O is not bound to hydrogen, and R_j and R_k can independently be selected from alkoxy, aryloxy, alkyl, alkenyl, alkynyl, amide, amino, aryl, arylalkyl, cycloalkyl, ether, formyl, haloalkyl, halogen, heteroaryl, heterocyclyl, ketone, phosphate, sulfide, sulfinyl, sulfonyl, sulfonic acid and thioketone. R_k can be a hydrogen, but R_j cannot be hydrogen. The ester may be cyclic, for example the carbon atom and R_j , the oxygen atom and R_k , or R_j and R_k may be joined to form a 3- to 12-membered ring. Exemplary esters include, but are not limited to, alkyl esters wherein at least one of R_j or R_k is alkyl, such as $-alkyl-C(O)--O--$, $--C(O)--O-alkyl-$, $-alkyl-C(O)--O-alkyl-$, etc. Exemplary esters also include aryl or heteroaryl esters, *e.g.* wherein at least one of R_j or R_k is a heteroaryl group such as pyridine, pyridazine, pyrimidine and pyrazine, such as a nicotinate ester. Exemplary esters also include reverse esters having the structure $--R_kC(O)O--$, where the oxygen is bound to the parent molecular group. Exemplary reverse esters include succinate, D-argininate, L-argininate, L-lysinate and D-lysinate. Esters also include carboxylic acid anhydrides and acid halides.

Alkoxy: The term "alkoxy" as used herein refers to an alkyl group attached to an oxygen (--O-alkyl-). "Alkoxy" groups also include an alkenyl group attached to an oxygen ("alkenoxy") or an alkynyl group attached to an oxygen ("alkynoxy") groups. Exemplary alkoxy groups include, but are not limited to, groups with an alkyl, alkenyl or alkynyl group of 1-22, 1-8, or 1-6 carbon atoms, referred to herein as (C₁-C₂₂)alkoxy, (C₁-C₈)alkoxy, and (C₁-C₆)alkoxy, respectively. Exemplary alkoxy groups include, but are not limited to methoxy, ethoxy, etc

Aryloxy: as used herein refers to an aryl group attached to an oxygen atom. Exemplary aryloxy groups include, but are not limited to, aryloxys having a monocyclic aromatic ring system, wherein the ring comprises 6 carbon atoms, referred to herein as "(C₆)aryloxy."

Alkyl: The term "alkyl" as used herein refers to a saturated straight or branched hydrocarbon, such as a straight or branched group of 1-22, 1-8, or 1-6 carbon atoms, referred to herein as (C₁-C₂₂)alkyl, (C₁-C₈)alkyl, and (C₁-C₆)alkyl, respectively. Exemplary alkyl groups include, but are not limited to, methyl, ethyl, propyl, isopropyl, 2-methyl-1-propyl, 2-methyl-2-propyl, 2-methyl-1-butyl, 3-methyl-1-butyl, 2-methyl-3-butyl, 2,2-dimethyl-1-propyl, 2-methyl-1-pentyl, 3-methyl-1-pentyl, 4-methyl-1-pentyl, 2-methyl-2-pentyl, 3-methyl-2-pentyl, 4-methyl-2-pentyl, 2,2-dimethyl-1-butyl, 3,3-dimethyl-1-butyl, 2-ethyl-1-butyl, butyl, isobutyl, t-butyl, pentyl, isopentyl, neopentyl, hexyl, heptyl, octyl, etc.

Alkenyl: The term "alkenyl" as used herein refers to an unsaturated straight or branched hydrocarbon having at least one carbon-carbon double bond, such as a straight or branched group of 2-22, 2-8, or 2-6 carbon atoms, referred to herein as (C₁-C₂₂)alkenyl, (C₂-C₈)alkenyl, and (C₂-C₆)alkenyl, respectively. Exemplary alkenyl groups include, but are not limited to, vinyl, allyl, butenyl, pentenyl, hexenyl, butadienyl, pentadienyl, hexadienyl, 2-ethylhexenyl, 2-propyl-2-butenyl, 4-(2-methyl-3-butene)-pentenyl, etc.

Alkynyl: The term "alkynyl" as used herein refers to an unsaturated straight or branched hydrocarbon having at least one carbon-carbon triple bond, such as a straight or branched group of 2-22, 2-8, or 2-6 carbon atoms, referred to herein as (C₂-C₂₂)alkynyl, (C₂-C₈)alkynyl, and (C₂-C₆)alkynyl, respectively. Exemplary alkynyl groups include, but are not limited to, ethynyl, propynyl, butynyl, pentynyl, hexynyl, methylpropynyl, 4-methyl-1-butynyl, 4-propyl-2-pentynyl, and 4-butyl-2-hexynyl, etc.

Amide: The term "amide" as used herein refers to a radical of the form --R_aC(O)N(R_b)--, --R_aC(O)N(R_b)R_c--, or --C(O)NR_bR_c, wherein R_b and R_c are each independently selected from alkoxy, aryloxy, alkyl, alkenyl, alkynyl, amide, amino, aryl, arylalkyl, carbamate, carboxy, cyano, cycloalkyl, ester, ether, formyl, halogen, haloalkyl, heteroaryl, heterocyclyl, hydrogen, hydroxyl, ketone, nitro, phosphate, sulfide, sulfinyl, sulfonyl, sulfonic acid, sulfonamide and thioketone. The amide can be attached to another

group through the carbon, the nitrogen, R_b, R_c, or R_a. The amide also may be cyclic, for example R_b and R_c, R_a and R_b, or R_a and R_c may be joined to form a 3- to 12-membered ring, such as a 3- to 10-membered ring or a 5- to 6-membered ring. The term "amide" encompasses groups such as sulfonamide, urea, carbamate, carbamic acid, and cyclic versions thereof. The term "amide" also encompasses an amide group attached to a carboxy group, e.g., -amide-COOH or salts such as -amide-COONa, etc, an amino group attached to a carboxy group, e.g., -amino-COOH or salts such as -amino-COONa, etc.

Amino: The term "amine" or "amino" as used herein refers to a radical of the form -- N R_d R_e, --N(R_d) R_e--, or -- R_e N(R_d) R_f -- where R_d, R_e, and R_f are independently selected from alkoxy, aryloxy, alkyl, alkenyl, alkynyl, amide, amino, aryl, arylalkyl, carbamate, carboxy, cyano, cycloalkyl, ester, ether, formyl, halogen, haloalkyl, heteroaryl, heterocyclyl, hydrogen, hydroxyl, ketone, nitro, phosphate, sulfide, sulfinyl, sulfonyl, sulfonic acid, sulfonamide and thioketone. The amino can be attached to the parent molecular group through the nitrogen, R_d, R_e or R_f. The amino also may be cyclic, for example any two of R_a, R_b, and R_c may be joined together or with the N to form a 3- to 12-membered ring, e.g., morpholino or piperidinyl. The term amino also includes the corresponding quaternary ammonium salt of any amino group, e.g., --[N(R_d)(R_e)(R_f)]⁺. Exemplary amino groups include aminoalkyl groups, wherein at least one of R_d, R_e, or R_f is an alkyl group.

Aryl: The term "aryl" as used herein refers to a mono-, bi-, or other multi-carbocyclic, aromatic ring system. The aryl group can optionally be fused to one or more rings selected from aryls, cycloalkyls, and heterocyclyls. The aryl groups of this invention can be substituted with groups selected from alkoxy, aryloxy, alkyl, alkenyl, alkynyl, amide, amino, aryl, arylalkyl, carbamate, carboxy, cyano, cycloalkyl, ester, ether, formyl, halogen, haloalkyl, heteroaryl, heterocyclyl, hydroxyl, ketone, nitro, phosphate, sulfide, sulfinyl, sulfonyl, sulfonic acid, sulfonamide and thioketone. Exemplary aryl groups include, but are not limited to, phenyl, tolyl, anthracenyl, fluorenyl, indenyl, azulenyl, and naphthyl, as well as benzo-fused carbocyclic moieties such as 5,6,7,8-tetrahydronaphthyl. Exemplary aryl groups also include, but are not limited to a monocyclic aromatic ring system, wherein the ring comprises 6 carbon atoms, referred to herein as "(C₆)aryl."

Arylalkyl: The term "arylalkyl" as used herein refers to an aryl group having at least one alkyl substituent, e.g. -aryl-alkyl-. Exemplary arylalkyl groups include, but are not limited to, arylalkyls having a monocyclic aromatic ring system, wherein the ring comprises 6 carbon atoms, referred to herein as "(C₆)arylalkyl."

Cycloalkyl: The term "cycloalkyl" as used herein refers to a monovalent saturated or unsaturated cyclic, bicyclic, or bridged bicyclic hydrocarbon group of 3-12 carbons, or 3-8 carbons, referred to herein as "(C₃-C₈)cycloalkyl," derived from a cycloalkane. Exemplary cycloalkyl groups include, but are not limited to, cyclohexanes, cyclohexenes,

cyclopentanes, and cyclopentenenes. Cycloalkyl groups may be substituted with alkoxy, aryloxy, alkyl, alkenyl, alkynyl, amide, amino, aryl, arylalkyl, carbamate, carboxy, cyano, cycloalkyl, ester, ether, formyl, halogen, haloalkyl, heteroaryl, heterocyclyl, hydroxyl, ketone, nitro, phosphate, sulfide, sulfinyl, sulfonyl, sulfonic acid, sulfonamide and thioketone.

5 Cycloalkyl groups can be fused to other cycloalkyl, aryl, or heterocyclyl groups.

Ether: The term ("ether" refers to a radical having the structure $-R_1O-R_m-$, where R_1 and R_m can independently be alkyl, alkenyl, alkynyl, aryl, cycloalkyl, heterocyclyl, or ether. The ether can be attached to the parent molecular group through R_1 or R_m . Exemplary ethers include, but are not limited to, alkoxyalkyl and alkoxyaryl groups. Ethers also includes

10 polyethers, e.g., where one or both of R_1 and R_m are ethers.

Aldehyde or formyl: The term "aldehyde" or "formyl" as used herein refers to the radical $-CHO$.

Haloalkyl: The term "haloalkyl" as used herein refers to an alkyl group substituted with one or more halogen atoms. "Haloalkyls" also encompass alkenyl or alkynyl groups

15 substituted with one or more halogen atoms.

Halogen: The terms "halo" or "halogen" or "Hal" as used herein refer to F, Cl, Br, or I.

Heteroaryl: The term "heteroaryl" as used herein refers to a mono-, bi-, or multi-cyclic, aromatic ring system containing one or more heteroatoms, for example 1 to 3

20 heteroatoms, such as nitrogen, oxygen, and sulfur. Heteroaryls can be substituted with one or more substituents including alkoxy, aryloxy, alkyl, alkenyl, alkynyl, amide, amino, aryl, arylalkyl, carbamate, carboxy, cyano, cycloalkyl, ester, ether, formyl, halogen, haloalkyl, heteroaryl, heterocyclyl, hydroxyl, ketone, nitro, phosphate, sulfide, sulfinyl, sulfonyl, sulfonic acid, sulfonamide and thioketone. Heteroaryls can also be fused to non-aromatic rings.

25 Illustrative examples of heteroaryl groups include, but are not limited to, pyridinyl, pyridazinyl, pyrimidyl, pyrazyl, triazinyl, pyrrolyl, pyrazolyl, imidazolyl, (1,2,3,-) and (1,2,4)-triazolyl, pyrazinyl, pyrimidyl, tetrazolyl, furyl, thienyl, isoxazolyl, thiazolyl, furyl, phenyl, isoxazolyl, and oxazolyl. Exemplary heteroaryl groups include, but are not limited to, a monocyclic aromatic ring, wherein the ring comprises 2 to 5 carbon atoms and 1 to 3

30 heteroatoms, referred to herein as "(C₂-C₅)heteroaryl."

Heterocyclyl: The terms "heterocycle," "heterocyclyl," or "heterocyclic" as used herein refer to a saturated or unsaturated 3-, 4-, 5-, 6- or 7-membered ring containing one, two, or three heteroatoms independently selected from nitrogen, oxygen, and sulfur. Heterocycles can be aromatic (heteroaryls) or non-aromatic. Heterocycles can be

35 substituted with one or more substituents including alkoxy, aryloxy, alkyl, alkenyl, alkynyl, amide, amino, aryl, arylalkyl, carbamate, carboxy, cyano, cycloalkyl, ester, ether, formyl, halogen, haloalkyl, heteroaryl, heterocyclyl, hydroxyl, ketone, nitro, phosphate, sulfide,

sulfinyl, sulfonyl, sulfonic acid, sulfonamide and thioketone.

Ketone: The term "ketone" as used herein refers to a radical having the structure --C(O)--R_n (such as acetyl, --C(O)CH₃) or --R_n--C(O)--R_o--. The ketone can be attached to another group through R_n or R_o. R_n or R_o can be alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl or aryl, or R_n or R_o can be joined to form a 3- to 12-membered ring.

Phosphate: The term "phosphate" as used herein refers to a radical having the structure --OP(O)O₂--, --R_xOP(O)O₂--, --OP(O)O₂R_y--, or --R_xOP(O)O₂R_y--, wherein R_x and R_y can be alkyl, alkenyl, alkynyl, alkoxy, amide, amino, aryl, aryloxy, carboxy, cyano, cycloalkyl, ester, ether, halogen, heterocyclyl, hydrogen, hydroxy, ketone, nitro, sulfonate, sulfonyl, and thio.

Sulfide: The term "sulfide" as used herein refers to the radical having the structure R_zS--, where R_z can be alkoxy, aryloxy, alkyl, alkenyl, alkynyl, amide, amino, aryl, arylalkyl, carbamate, carboxy, cycloalkyl, ester, ether, formyl, haloalkyl, heteroaryl, heterocyclyl, and ketone. The term "alkylsulfide" as used herein refers to an alkyl group attached to a sulfur atom.

Sulfinyl: The term "sulfinyl" as used herein refers to a radical having the structure --S(O)O--, --R_p(O)O--, --R_pS(O)OR_q--, or --S(O)OR_q--, wherein R_p and R_s can be alkoxy, aryloxy, alkyl, alkenyl, alkynyl, amide, amino, aryl, arylalkyl, cycloalkyl, ester, ether, formyl, halogen, haloalkyl, heteroaryl, heterocyclyl, hydroxyl, ketone, nitro, phosphate, sulfide, sulfonyl, sulfonic acid, sulfonamide and thioketone. Exemplary sulfinyl groups include, but are not limited to, alkylsulfinyls wherein at least one of R_p or R_q is alkyl, alkenyl or alkynyl.

Sulfonyl: The term "sulfonyl" as used herein refers to a radical having the structure R_uSO₂--, where R_u can be alkyl, alkenyl, alkynyl, amino, amide, aryl, cycloalkyl, and heterocyclyl, e.g., alkylsulfonyl. The term "alkylsulfonyl" as used herein refers to an alkyl group attached to a sulfonyl group. "Alkylsulfonyl" groups can optionally contain alkenyl or alkynyl groups.

Sulfonic acid: The term "sulfonic acid" refers to the radical --SO₃H-- and its corresponding salts, e.g. --SO₃K--, --SO₃Na--.

Thioketone: The term "thioketone" refers to a radical having the structure --R_v--C(S)--R_w--. The ketone can be attached to another group through R_v or R_w. R_v or R_w can be alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl or aryl, or R_v or R_w can be joined to form a 3- to 12-membered ring.

Hydroxy and hydroxyl: The terms "hydroxy" and "hydroxyl" as used herein refers to the radical --OH.

Saturated and unsaturated hydrocarbons: "Alkyl," "alkenyl," and "alkynyl" groups, collectively referred to as "saturated and unsaturated hydrocarbons," can be substituted with or interrupted by at least one group selected from alkoxy, aryloxy, alkyl, alkenyl, alkynyl,

amide, amino, aryl, arylalkyl, carbamate, carboxy, cyano, cycloalkyl, ester, ether, formyl, halogen, haloalkyl, heteroaryl, heterocyclyl, hydroxyl, ketone, nitro, phosphate, sulfide, sulfinyl, sulfonyl, sulfonic acid, sulfonamide, thioketone, and N.

Detailed Description of the Invention

The present invention relates to a process for enzymatic refining of a pretreated cellulosic material, including: (a) contacting the pretreated cellulosic material with an esterase and/or esterase enzyme composition to form a refined pretreated cellulosic material. In embodiments, a preferred esterase or esterase enzyme composition is a feruloyl esterase and/or feruloyl esterase composition.

The present invention also relates to a process for enzymatic refining of a pretreated cellulosic material, including:

- a) processing the pretreated cellulosic material to form a solid/liquid mixture of pretreated cellulosic material;
- b) separating a liquor from the solid/liquid mixture of pretreated cellulosic material; and
- c) treating the liquor with a feruloyl esterase treatment.

In one aspect, the processes of the present disclosure include an enzyme composition including one or more (several) enzymes selected from the group consisting of an esterase and/or feruloyl esterase. Non-limiting examples of suitable feruloyl esterase include enzymes having an amino acid with at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% sequence identity to the mature polypeptide of SEQ ID NOS: 133, 134, 135, 136 or 137 or fragments thereof with feruloyl esterase activity. Non-limiting examples of suitable feruloyl esterase include enzymes having an amino acid comprising or consisting of the mature polypeptide of SEQ ID NOS: 133, 134, 135, 136 or 137 or fragments thereof with feruloyl esterase activity.

One aspect of the present disclosure relates to a process for hydrolyzing a pretreated cellulosic material, including saccharifying a pretreated cellulosic material treated and refined according to the present disclosure with an enzyme or enzyme composition including esterase and/or feruloyl esterase. In embodiments, the process includes recovering the saccharified pretreated cellulosic material from the saccharification. In embodiments, the saccharified cellulosic material is a sugar such as glucose, xylose, mannose, galactose, and arabinose.

One aspect of the present disclosure relates to a process for producing a fermentation product, including:

- (a) saccharifying a pretreated cellulosic material with an enzyme composition, wherein the pretreated cellulosic material is treated and refined according to the present

disclosure with an esterase and/or feruloyl esterase;

(b) fermenting the saccharified pretreated cellulosic material with one or more (several) fermenting microorganisms to produce the fermentation product; and

(c) recovering the fermentation product from the fermentation. In embodiments, the enzyme composition includes one or more (several) enzymes selected from the group consisting of a cellulase, a GH61 polypeptide having cellulolytic enhancing activity, a hemicellulase, an expansin, an esterase, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin. In embodiments, the cellulase is one or more (several) enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase. In embodiments, the hemicellulase is one or more (several) enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase.

Another aspect of the present disclosure relates to a process for fermenting a pretreated cellulosic material, including: fermenting a pretreated cellulosic material with one or more (several) fermenting microorganisms, wherein the pretreated cellulosic material is treated, refined, and/or saccharified according to the present disclosure with esterase and/or feruloyl esterase. In embodiments, the fermenting of the pretreated cellulosic material produces a fermentation product. In embodiments, the step of recovering the fermentation product from the fermentation is included. In embodiments, fermentation product is an alcohol, an organic acid, a ketone, an amino acid, an alkane, a cycloalkane, an alkene, or a gas.

Pretreatment of cellulosic material

Cellulosic material suitable for use in accordance with the present disclosure is pretreated with any suitable method known in the art. In embodiments, the cellulosic material and/or the lignocellulose-containing material may according to the present disclosure be pretreated before being hydrolyzed and fermented. The goal of pretreatment is to separate and/or release cellulose, hemicellulose and/or lignin and this way improve the rate of enzymatic hydrolysis.

Without wishing to be bound by the present disclosure it is believed that conventional pretreatment produces impurities in the pretreated cellulosic materials and have a deleterious effect on cellulase enzymes and/or enzyme hydrolysis. Non-limiting examples of impurities or toxins to the cellulase enzymes include one or more esters in the pretreated cellulosic material. The present disclosure provides processes for processing pretreated cellulosic material to improve hydrolysis of the pretreated cellulosic material.

Pretreatment of cellulosic material refers to any conventional pre-treatment step

known in the art. Pre-treatment may take place in aqueous slurry or may be directly applied to the cellulosic material in raw form. In embodiments, the cellulose containing material may during pretreatment be present in an amount between 10-80 wt. %, for example between 20-50 wt.-% of the total weight of the pretreatment reaction.

5

Chemical, Mechanical and/or Biological Pre-treatment

Non-limiting examples of pretreatment of cellulosic material according to the present disclosure includes chemically, mechanically and/or biologically pre-treating cellulosic material before hydrolysis and/or fermentation. Mechanical treatment (often referred to as physical pre-
10 treatment) may be used alone or in combination with subsequent or simultaneous hydrolysis, especially enzymatic hydrolysis, to promote the separation and/or release of cellulose, hemicellulose and/or lignin.

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

In an embodiment of the present disclosure the pre-treated cellulosic material is washed and/or detoxified in accordance with the present disclosure before, during or after the hydrolysis
20 step. This may improve the fermentability of, e.g., dilute-acid hydrolyzed lignocellulose-containing material, such as corn stover. In accordance with the present disclosure detoxification is carried out by contacting the pretreated cellulosic material with an enzyme or enzyme composition including an esterase and/or feruloyl esterase to form a refined (enzyme) pretreated cellulosic material.

25 In accordance with the present disclosure detoxification is carried out by contacting the pretreated cellulosic material with an enzyme or enzyme composition including an esterase and/or feruloyl esterase to form a refined (enzyme) pretreated cellulosic material.

Chemical Pre-treatment

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

In embodiments, the chemical pre-treatment is acid treatment, for example, a continuous dilute and/or mild acid treatment, such as, treatment with sulfuric acid, or another

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

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

Alkaline chemical pre-treatment with base, e.g., NaOH, Na₂CO₃ and/or ammonia or the like, is also within the scope of the present disclosure. Pre-treatment methods using ammonia are described in, e.g., WO 2006/110891, WO 2006/11899, WO 2006/11900, WO 2006/110901, which are hereby incorporated by reference in their entirety.

Wet oxidation techniques involve use of oxidizing agents, such as: sulphite based oxidizing agents or the like. Non-limiting examples of solvent pre-treatments include treatment with DMSO (Dimethyl Sulfoxide) or the like. Chemical pre-treatment is generally carried out for 1 to 60 minutes, such as from 5 to 30 minutes, but may be carried out for shorter or longer periods of time dependent on the material to be pre-treated.

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

In accordance with the present disclosure detoxification is carried out by contacting the pretreated cellulosic material with an enzyme or enzyme composition including an esterase and/or feruloyl esterase to form a refined (enzyme) pretreated cellulosic material.

Mechanical Pre-treatment

As used in context of the present disclosure the term "mechanical pre-treatment" refers to any mechanical or physical pre-treatment which promotes the separation and/or release of cellulose, hemicellulose and/or lignin from lignocellulose-containing material. Non-limiting examples of mechanical pre-treatment includes various types of milling, irradiation,

steaming/steam explosion, and hydrothermolysis.

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

In accordance with the present disclosure detoxification is carried out by contacting the pretreated cellulosic material with an enzyme or enzyme composition including an esterase and/or feruloyl esterase to form a refined (enzyme) pretreated cellulosic material.

Combined Chemical and Mechanical Pre-treatment

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

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

In embodiments the pre-treatment is carried out as a dilute and/or mild acid steam explosion step. In embodiments, pre-treatment is carried out as an ammonia fiber explosion step (or AFEX pretreatment step).

In accordance with the present disclosure detoxification is carried out by contacting the pretreated cellulosic material with an enzyme or enzyme composition including an esterase and/or feruloyl esterase to form a refined (enzyme) pretreated cellulosic material.

Biological Pre-treatment

As used in the present disclosure the term "biological pre-treatment" refers to any biological pre-treatment which promotes the separation and/or release of cellulose, hemicellulose, and/or lignin from the lignocellulose-containing material. Biological pre-treatment techniques can involve applying lignin-solubilizing microorganisms (see, for example, Hsu, T.-A., 1996, Pretreatment of biomass, in *Handbook on Bioethanol: Production and Utilization*, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212; Ghosh, P.,

and Singh, A., 1993, Physicochemical and biological treatments for enzymatic/microbial conversion of lignocellulosic biomass, *Adv. Appl. Microbiol.* 39: 295-333; McMillan, J. D., 1994, Pretreating lignocellulosic biomass: a review, in *Enzymatic Conversion of Biomass for Fuels Production*, Himmel, M. E., Baker, J. O., and Overend, R. P., eds., ACS Symposium Series 566, American Chemical Society, Washington, DC, chapter 15; Gong, C. S., Cao, N. J., Du, J., and Tsao, G. T., 1999, Ethanol production from renewable resources, in *Advances in Biochemical Engineering/Biotechnology*, Scheper, T., ed., Springer-Verlag Berlin Heidelberg, Germany, 65: 207-241; Olsson, L., and Hahn-Hagerdal, B., 1996, Fermentation of lignocellulosic hydrolysates for ethanol production, *Enz. Microb. Tech.* 18: 312-331; and Vallander, L., and Eriksson, K.-E. L., 1990, Production of ethanol from lignocellulosic materials: State of the art, *Adv. Biochem. Eng./Biotechnol.* 42: 63-95).

In embodiments, biological pre-treatment involves applying lignin degrading enzymes to lignin or pretreated material. Non-limiting examples of suitable lignin degrading enzymes include one or more lignolytic enzymes, one or more oxidoreductases, and combinations thereof. Non-limiting examples of lignolytic enzymes include manganese peroxidase, lignin peroxidase and cellobiose dehydrogenase, and combinations thereof. Non-limiting examples of suitable pretreatment enzymes also include one or more laccases, cellobiose dehydrogenases and combinations thereof.

In embodiments, lignin peroxidase such as "ligninase", EC number 1.14.99, is suitable for use in accordance with the present disclosure.

In one embodiment, Ethazyme™ Pre available from Zymetis is suitable for use in pretreatment in accordance with the present disclosure.

In accordance with the present disclosure detoxification is carried out by contacting the pretreated cellulosic material with an enzyme and/or enzyme composition including an esterase and/or feruloyl esterase to form a refined (enzyme) pretreated cellulosic material.

Treatment of pretreated cellulosic material

The present disclosure relates to a process for enzymatic refining of a pretreated cellulosic material, comprising or consisting of (a) contacting the pretreated cellulosic material with an esterase enzyme or esterase enzyme composition to form a refined pretreated cellulosic material. Without wishing to be bound by the present disclosure it is believed that the pretreatment of cellulosic material increases impurities within the cellulosic material that can diminish enzyme hydrolysis of the material. For examples, esters can be present in the pretreated cellulosic material in an amount sufficient to have a negative affect on the cellulase enzymes used in hydrolysis. The present disclosure provides enzymes and enzyme compositions and methods for treating, removing or eliminating toxins in the pretreated cellulosic material. The process includes applying a predetermined amount of

esterase enzyme to pretreated cellulosic material in need of treatment, including pretreated cellulosic material with cellulase inhibiting amounts of esters therein.

Accordingly, esterase(s) and/or esterase compositions in accordance with the present disclosure provide a treatment of esters and/or toxins in which the major active ingredient is esterase enzyme such as feruloyl esterase. In embodiments, compositions in accordance with the present disclosure include feruloyl esterase in a commercially available form.

In embodiments, esterase or esterase compositions in accordance with the present disclosure can be applied to pretreated cellulosic material in need of improvement e.g., such as the reduction or elimination of an undesirable toxin such one or more esters. As used herein the word "treat," "treating" or "treatment" refers to using the one or more esterase or esterase compositions of the present disclosure prophylactically to prevent toxins such as esters from accumulating in pretreated cellulosic material, or to ameliorate an existing toxic condition, and/or promote or extend the cellulase activity of cellulase enzyme used to hydrolyze the pretreated cellulosic material. A number of different treatments are now possible, which reduce and/or eliminate toxins from the pretreated cellulose material and/or liquor separated from the pretreated cellulosic material.

Treatments in accordance with the present disclosure contact pretreated cellulose material, or a portion isolated there from such as a liquid stream, with one or more active esterase enzymes such as feruloyl esterase in accordance with the present disclosure in an effective amount to improve the toxic conditions. In embodiments, pretreated cellulosic material or a portion isolated therefrom is/are treated by contacting the toxic material with one or more feruloyl esterase in accordance with the present disclosure. The esterase ingredient or composition is applied until the treatment goals are obtained. However, the duration of the treatment can vary depending on the severity of the toxic condition or amount of ester(s) present in the sample. For example, treatments can last several minutes to days depending on whether the goal of treatment is to reduce or eliminate the toxic condition.

In embodiments, the esterase enzyme or esterase composition comprises, or consists of one or more (several) enzymes selected from the group consisting of an esterase and feruloyl esterase. The esterase compositions can comprise any esterase protein that is useful in detoxifying a pretreated cellulosic material.

In embodiments, feruloyl esterase is suitable for use in accordance with the present disclosure which refers generally to a 4-hydroxy-3-methoxycinnamoyl-sugar hydrolase (EC 3.1.1.73) that catalyzes the hydrolysis of the 4-hydroxy-3-methoxycinnamoyl (feruloyl) group from an esterified sugar, which is usually arabinose in "natural" substrates, to produce ferulate (4-hydroxy-3-methoxycinnamate). Feruloyl esterase is also known as ferulic acid esterase, hydroxycinnamoyl esterase, FAE-III, cinnamoyl ester hydrolase, FAEA, cinnAE,

FAE-I, or FAE-II. Non-limiting examples of feruloyl esterase for use in accordance with the present disclosure are set forth below. For purposes of the present invention, feruloyl esterase activity is determined using 0.5 mM *p*-nitrophenylferulate as substrate in 50 mM sodium acetate pH 5.0. One unit of feruloyl esterase equals the amount of enzyme capable of releasing 1 μ mole of *p*-nitrophenolate anion per minute at pH 5, 25°C.

Non-limiting examples of esterase useful in the present invention include, but are not limited to, FAE-I from *Humicola*, FAE from *Asp. Niger*, FAE from *Asp. Oryzae*, FAE from *Chaetomium globusam*, and esterase from *Asp. Oryzae* or fragments of these with esterase or feruloyl esterase activity.

Additional non-limiting examples of esterase for use in accordance with the present disclosure include feruloyl esterase as described in US Patent Publication No. 2010/0122380 A1, EP1752533-A1 and WO9814594-A2 all of which are herein incorporated by reference in their entirety. Suitable esterase comprises amino acid sequences that have a degree of sequence identity to the feruloyl esterase of each of US Patent Publication No. 2010/0122380 A1, EP1752533-A1 and WO9814594-A2 of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, or at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100% or an active fragment thereof having feruloyl esterase activity.

In an aspect of the present disclosure, the esterase comprises an amino acid sequence that has a degree of sequence identity to the mature polypeptide of SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136 or SEQ ID NO: 137 of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, or at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100% or an active fragment thereof having esterase activity or feruloyl esterase activity. In embodiments, suitable esterase for use in accordance with the present disclosure include the mature polypeptide of SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136 or SEQ ID NO: 137 or an active fragment thereof having esterase activity. In embodiments, suitable esterase for use in accordance with the present disclosure include 2 or more of the mature polypeptide of SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136 or SEQ ID NO: 137 in combination.

In an aspect, the esterase of the present disclosure is an artificial variant comprising a substitution, deletion, and/or insertion of one or more (or several) amino acids of the mature polypeptide of SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136 or SEQ ID NO: 137, or a homologous sequence thereof.

Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the

protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, *In, The Proteins*, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

Alternatively, the amino acid changes are of such a nature that the physico-chemical properties of the polypeptides are altered. For example, amino acid changes may improve the thermal stability of the polypeptide, alter the substrate specificity, change the pH optimum, and the like.

Essential amino acids in a parent polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for cellulolytic enhancing activity to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Lett.* 309: 59-64. The identities of essential amino acids can also be inferred from analysis of identities with polypeptides that are related to the parent polypeptide.

Single or multiple amino acid substitutions, deletions, and/or insertions can be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone

PCR, phage display (e.g., Lowman *et al.*, 1991, *Biochemistry* 30: 10832-10837; U.S. Patent No. 5,223,409; WO 92/06204), and region-directed mutagenesis (Derbyshire *et al.*, 1986, *Gene* 46: 145; Ner *et al.*, 1988, *DNA* 7: 127).

Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells (Ness *et al.*, 1999, *Nature Biotechnology* 17: 893-896). Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide.

In embodiments, the total number of amino acid substitutions, deletions and/or insertions of the mature polypeptide of SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136 or SEQ ID NO: 137 is not more than 10, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

In embodiments, suitable esterase enzyme composition for use in accordance with the present disclosure comprises, or consists of SEQ ID. NO: 133, SEQ ID. NO: 134, SEQ ID. NO: 135, SEQ ID. NO: 136 or SEQ ID NO: 137 or active fragments thereof having esterase activity.

In embodiments, suitable esterase enzyme for use in accordance with the present disclosure comprises, or consists of FAE(1), FAE-A, FAE-C, and FAE-D or an active fragment thereof having esterase activity.

One or more (several) components of the esterase enzyme or esterase composition for use in accordance with the present disclosure may be wild-type proteins, recombinant proteins, or a combination of wild-type proteins and recombinant proteins. For example, one or more (several) components may be native proteins of a cell, which is used as a host cell to express recombinantly one or more (several) other components of the esterase composition. One or more (several) components of the esterase composition may be produced as monocomponents, which are then combined to form the enzyme composition. The enzyme composition may be a combination of multicomponent and monocomponent protein preparations.

The esterases used in the processes of the present disclosure may be in any form suitable for use, such as, for example, a crude fermentation broth with or without cells removed, a cell lysate with or without cellular debris, a semi-purified or purified enzyme preparation, or a host cell as a source of the esterases. The esterase composition may be a dry powder or granulate, a non-dusting granulate, a liquid, a stabilized liquid, or a stabilized protected enzyme. Liquid esterase preparations may, for instance, be stabilized by adding stabilizers such as a sugar, a sugar alcohol or another polyol, and/or lactic acid or another organic acid according to established processes.

The esterase can be derived or obtained from any suitable origin, including, bacterial,

5 fungal, yeast, plant, or mammalian origin. The term "obtained" means herein that the esterase may have been isolated from an organism that naturally produces the esterase as a native enzyme. The term "obtained" also means herein that the enzyme may have been produced recombinantly in a host organism employing methods described herein, wherein
10 the recombinantly produced esterase is either native or foreign to the host organism or has a modified amino acid sequence, *e.g.*, having one or more (several) amino acids that are deleted, inserted and/or substituted, *i.e.*, a recombinantly produced enzyme that is a mutant and/or a fragment of a native amino acid sequence or an enzyme produced by nucleic acid shuffling processes known in the art. Encompassed within the meaning of a native enzyme
15 are natural variants and within the meaning of a foreign enzyme are variants obtained recombinantly, such as by site-directed mutagenesis or shuffling.

In embodiments, the treatment in accordance with the present disclosure includes a step of separating a liquor or liquid portion from the pretreated cellulosic material. Without wishing to be bound by the present disclosure, it is believed that a liquid fraction or liquor
20 from the pretreated cellulosic material may contain the toxins such as esters toxic to the hydrolysis and/or cellulase enzymes. In accordance with the present disclosure, esterase enzyme composition comprises, or consists of one or more (several) enzymes selected from the group consisting of an esterase and feruloyl esterase, or active fragments thereof having esterase activity can be applied to liquid isolate or liquor. This liquor can then be added back to
25 the pre-treated cellulosic material for an effective treatment thereof. For example, it is possible to contact the liquor with feruloyl esterase and recycle the liquor. The recycled and treated liquor can be recycled to a new pretreatment of pretreated cellulosic material with the esterase composition or used alone for the purpose of hydrolysis and/or fermentation.

Treatments in accordance with the present disclosure are not limited to direct
30 treatment of the pretreated cellulosic material. It is envisioned that processes in accordance with the present disclosure include, comprise, or consist of post-treating the refined pretreated cellulosic material with an enzymatic pre-treatment, chemical pre-treatment, mechanical pre-treatment and/or a physical pretreatment.

In embodiments, the treatments in accordance with the present disclosure include,
35 comprise or consist of recovering the refined enzyme pretreated cellulosic material. The refined pretreated cellulosic material has improved qualities as a substrate for hydrolysis.

In embodiments, the contacting or treating with enzyme compositions such as esterase and feruloyl esterase is performed with a sufficient amount of enzyme per gram (g) of pretreated cellulosic material. In embodiments, compositions for use in accordance with
40 the present invention contain esterase enzyme such as feruloyl esterase in an effective amount to improve hydrolysis of the pretreated cellulosic material. As used herein "effective amount" refers to an amount of esterase or esterase composition having esterase

constituents in accordance with the present disclosure sufficient to induce a particular positive benefit to the pretreated cellulosic material composition or portion thereof. The positive benefit can be toxin related, or it can relate more to the nature of enzyme hydrolysis, or it may be a combination of the two. In embodiments, the positive benefit is achieved by contacting the pretreated cellulosic material or a portion thereof with an esterase or esterase composition to improve the pretreated cellulosic materials condition in order to improve its hydrolysis performance. For example, the amount of enzyme added in accordance with the present disclosure includes an amount sufficient to detoxify the pretreated cellulosic material such that hydrolysis thereof can be improved. Non-limiting examples of improvements include a reduction of ester toxins in the pretreated cellulosic material and/or an increase in the amount of sugar formed during the hydrolysis of the material. In embodiments, the amount of esters and/or toxins in the pretreated cellulosic material is reduced by an amount of 1-10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%. The reduction of esters and toxins can be measured by any suitable analytical method known in the art such as HPLC. In embodiments, the amount of esters and/or toxins in the pretreated cellulosic material is reduced by an amount of: 10-30% of the total amount of esters or toxins present, 20-40% of the total amount of esters or toxins present, 40-50% of the total amount of esters or toxins present, 50-60% of the total amount of esters or toxins present, 60-70% of the total amount of esters or toxins present, 70-80% of the total amount of esters or toxins present, 80-90% of the total amount of esters or toxins present, or 90-100% of the total amount of esters or toxins present. In embodiments, the improvement could refer to an increased amount of hydrolysis product, such as sugar, greater than the amount of hydrolysis product produced compared to the use of pretreated cellulosic material hydrolyzed but not treated in accordance with the present disclosure. In embodiments, the amount of hydrolysis product or sugar is increased 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9 or 2 times more than the amount of hydrolysis product produced compared to use of pretreated cellulosic material hydrolyzed, but not treated in accordance with the present disclosure. In embodiments, the amount of hydrolysis product or sugar is increased 3, 4, 5, 6, 7, 8, 8, 9 or 10 times more than the amount of hydrolysis product produced compared to use of pretreated cellulosic material hydrolyzed but not treated in accordance with the present disclosure. Non-limiting suitable amounts of enzyme such as esterase and feruloyl esterase for use in accordance with the present disclosure include about 0.0005 to about 5 mg, e.g., about 0.001 to about 5 mg, about 0.0025 to about 5 mg, about 0.005 to about 5 mg, about 0.005 to about 4.5 mg, about 0.005 to about 4 mg, about 0.005 to about 3.5 mg, about 0.005 to about 3 mg, about 0.005 to about 2 mg, about 0.005 to about 1 mg, about 0.075 to about 1 mg, or about 0.1 to about 1 mg of enzyme per g of pretreated cellulosic material, or per mL of liquor separated from the pretreated cellulosic material. In embodiments, suitable amounts of enzyme such as

esterase and feruloyl esterase for use in accordance with the present disclosure include about 0.0005 to about 5 mg, *e.g.*, about 0.001 to about 5 mg, about 0.0025 to about 5 mg, about 0.005 to about 5 mg, about 0.005 to about 4.5 mg, about 0.005 to about 4 mg, about 0.005 to about 3.5 mg, about 0.005 to about 3 mg, about 0.005 to about 2 mg, about 0.005 to about 1 mg, about 0.075 to about 1 mg, or about 0.1 to about 1 mg of enzyme per mL of liquor separated from the pretreated cellulosic material.

In embodiments, treatments in accordance with the present invention comprise, consist of, or include an amount of pretreated cellulosic material sufficient to be useful in additional reactions. For example, treatments and contacting of pretreated cellulosic material is performed with an amount of pretreated cellulosic material such that treatments are performed with a total solids (TS) of about 1% to about 50% *e.g.*, about 2% to about 40%, about 2% to about 35%, about 3% to about 30%, about 3% to about 25%, about 4% to about 20%, or about 5% to about 10%. In embodiments, treatments are performed with a total solids (TS) of about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49% or 50%.

In embodiments, the contacting or treating the pretreated cellulosic material with esterase enzyme or esterase composition is performed at a pH suitable for the esterase enzyme. Non-limiting examples of suitable pH's include contacting or the treating with the esterase enzyme composition at a pH of about 2 to about 9, *e.g.*, about 3 to about 8, about 3 to about 7.5, about 3.5 to about 7, about 4 to about 6.5, about 4.5 to about 6.5, about 4.5 to about 6.0, about 5 to about 6.0, or about 5 to about 5.5. In embodiments the pH is 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9 or 9.0. In embodiments, examples of suitable pH's include contacting or the treating with the esterase enzyme composition at a pH of 2 to 9, *e.g.*, 3 to 8, 3 to 7.5, 3.5 to 7, 4 to 6.5, 4.5 to 6.5, 4.5 to 6.0, 5 to 6.0, or 5 to 5.5.

In embodiments, the contacting or treating the pretreated cellulosic material with esterase enzyme composition is performed at a temperature suitable for the esterase enzyme. Non-limiting examples of suitable temperatures include a temperature in the range of about 20°C to about 70°C, *e.g.*, about 25°C to about 65°C, about 30°C to about 65°C, about 35°C to about 65°C, about 40°C to about 60°C, about 45°C to about 55°C, or about 45°C to about 50°C. In embodiments, a suitable temperature is 46°C, 47°C, 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C, 70°C.

In embodiments, the contacting or treating the pretreated cellulosic material with esterase enzyme composition is performed for a duration suitable for the esterase enzyme to react on esters or toxins in the pretreated cellulosic material composition. Non-limiting examples of suitable durations include a period of time of 5 minutes to 35 hours, *e.g.*, 10 minutes to 15 hours, 10 hours to 15 hours, 10 hours to 20 hours, 10 hours to 24 hours, 20 hours to 24 hours, 24 hours to 30 hours. In embodiments, the duration is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 48, 72 hours.

The esterase treatment is generally performed in tanks under controlled pH, temperature, and conditions as described herein. In embodiments, the contacting or treating the pretreated cellulosic material with esterase enzyme or enzyme composition is performed with an amount of pretreated cellulosic material described herein, with an amount of enzyme such as esterase and feruloyl esterase described herein, at a pH, temperature and duration in accordance with the present disclosure. Various modification of the amounts used herein can be used to optimize the performance of the esterase in removing the toxins and/or increasing enzyme hydrolysis yields. In embodiments, esterase treatment is preferably carried out in a suitable aqueous environment under conditions that can be readily determined by one skilled in the art. In a preferred aspect, esterase treatment is performed under conditions suitable for the activity of the esterase(s), *i.e.*, optimal for the esterase(s). The esterase treatment can be carried out as a fed batch or continuous process where the pretreated cellulosic material (substrate) is fed gradually to, for example, an esterase containing solution.

In embodiments, the process of the present disclosure includes a process for enzymatic refining of a pretreated cellulosic material, comprising, consisting of or including:

- (a) processing the pretreated cellulosic material to form a solid/liquid mixture of pretreated cellulosic material;
- (b) separating a liquor from the solid/liquid mixture of pretreated cellulosic material; and
- (c) treating the liquor with a feruloyl esterase treatment.

In embodiments, the process further includes, comprises, or consists of returning the treated liquor to the pretreated cellulosic material. Here, the process of the present disclosure can be repeated, such that various liquor isolates can be consolidated to form a batch.

In embodiments the present disclosure relates to a process for hydrolyzing a pretreated cellulosic material, comprising saccharifying a pretreated cellulosic material treated and refined according to the processes of the present disclosure including an esterase enzyme or esterase composition of the present disclosure. In embodiments the hydrolysis is carried out using enzyme composition including one or more (several) enzymes

selected from the group consisting of a cellulase, a GH61 polypeptide having cellulolytic enhancing activity, a hemicellulase, an expansin, an esterase, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin. In embodiments, the cellulase is one or more (several) enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase. In embodiments, the hemicellulase is one or more (several) enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, a beta-xylosidase and a glucuronidase.

In embodiments, the process further includes the steps comprising or consisting of recovering the saccharified pretreated cellulosic material from the saccharification. In embodiments, the saccharified cellulosic material is a sugar. Non-limiting examples of sugars include glucose, xylose, mannose, galactose, and arabinose.

In embodiments, the present disclosure relates to a process for producing a fermentation product, comprising or consisting of: (a) saccharifying a pretreated cellulosic material, treated with an esterase or esterase enzyme composition in accordance with the present disclosure. Here saccharification is performed using an enzyme composition suitable for saccharification. In embodiments, enzymes suitable for saccharification include one or more (several) enzymes selected from the group consisting of a cellulase, a GH61 polypeptide having cellulolytic enhancing activity, a hemicellulase, an expansin, an esterase, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin. In embodiments, cellulase is one or more (several) enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase. In embodiments, hemicellulase is one or more (several) enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase. The next step includes (b) fermenting the saccharified pretreated cellulosic material with one or more (several) fermenting microorganisms to produce the fermentation product; and (c) recovering the fermentation product from the fermentation. In embodiments, the steps (a) (saccharifying a pretreated cellulosic material (wherein the pretreated cellulosic material is contacted, treated or refined in accordance with the present disclosure using esterase) and (b) (fermenting the saccharified pretreated cellulosic material with one or more (several) fermenting microorganisms to produce the fermentation product) are performed simultaneously in a simultaneous saccharification and fermentation. In embodiments, the fermentation product is an alcohol, an organic acid, a ketone, an amino acid, an alkane, a cycloalkane, an alkene, or a gas.

The present disclosure further relates to a process for fermenting a pretreated cellulosic material, comprising or consisting of: fermenting a pretreated cellulosic material

with one or more (several) fermenting microorganisms, wherein the pretreated cellulosic material is treated, refined, and/or saccharified according to the present disclosure. In embodiments, the fermenting of the pretreated cellulosic material produces a fermentation product. In embodiments, the process comprises or consists of a step of recovering the fermentation product from the fermentation. In embodiments, the fermentation product is an alcohol, an organic acid, a ketone, an amino acid, an alkane, a cycloalkane, an alkene, or a gas.

In embodiments, processes of the present disclosure are preferably used on non-woody pretreated cellulosic materials or non-woody feedstock. Non-limiting examples of non-woody pretreated cellulosic materials include pretreated stems, leaves, hulls, husks, and cobs of plants or leaves. The non-woody cellulosic material can be, but is not limited to, herbaceous material, agricultural residue, dedicated energy crop, municipal solid waste, waste paper, and pulp and paper mill residue. In one aspect, the cellulosic material is a non-woody herbaceous material. In another aspect, the cellulosic material is a non-woody agricultural residue. In one aspect, the cellulosic material is a non-woody energy crop. In one aspect of the invention, woody feedstock is excluded from use as a suitable feedstock or cellulosic material in accordance with the present disclosure.

Saccharification

In the hydrolysis step, also known as saccharification, the cellulosic material, *i.e.*, pretreated, is hydrolyzed to break down cellulose and alternatively also hemicellulose to fermentable sugars, such as glucose, cellobiose, xylose, xylulose, arabinose, mannose, galactose, and/or soluble oligosaccharides. The hydrolysis is performed enzymatically by an enzyme composition. The enzyme and protein components of the compositions can be added sequentially.

Enzymatic hydrolysis is preferably carried out in a suitable aqueous environment under conditions that can be readily determined by one skilled in the art. In a preferred aspect, hydrolysis is performed under conditions suitable for the activity of the enzyme(s), *i.e.*, optimal for the enzyme(s). The hydrolysis can be carried out as a fed batch or continuous process where the pretreated cellulosic material (substrate) is fed gradually to, for example, an enzyme containing hydrolysis solution.

The saccharification is generally performed in stirred-tank reactors or fermentors under controlled pH, temperature, and mixing conditions. Suitable process time, temperature and pH conditions can readily be determined by one skilled in the art. For example, the saccharification can last up to 200 hours, but is typically performed for preferably about 12 to about 96 hours, more preferably about 16 to about 72 hours, and most preferably about 24 to about 48 hours. The temperature is in the range of preferably about 25°C to about 70°C,

more preferably about 30°C to about 65°C, and more preferably about 40°C to 60°C, in particular about 50°C. The pH is in the range of preferably about 3 to about 8, more preferably about 3.5 to about 7, and most preferably about 4 to about 6, in particular about pH 5. The dry solids content is in the range of preferably about 5 to about 50 wt %, more preferably about 10 to about 40 wt %, and most preferably about 20 to about 30 wt %.

The optimum amounts of the enzymes depend on several factors including, but not limited to, the mixture of component cellulolytic enzymes, the cellulosic material, the concentration of the cellulosic material, the pretreatment(s) of the cellulosic material, temperature, time, pH, and inclusion of fermenting organism (*e.g.*, yeast for Simultaneous Saccharification and Fermentation).

In one aspect, an effective amount of cellulolytic or hemicellulolytic enzyme protein to cellulosic material is about 0.5 to about 50 mg, preferably at about 0.5 to about 40 mg, more preferably at about 0.5 to about 25 mg, more preferably at about 0.75 to about 20 mg, more preferably at about 0.75 to about 15 mg, even more preferably at about 0.5 to about 10 mg, and most preferably at about 2.5 to about 10 mg per g of cellulosic material.

In another aspect, an effective amount of a GH61 polypeptide having cellulolytic enhancing activity to cellulosic material is about 0.01 to about 50.0 mg, preferably about 0.01 to about 40 mg, more preferably about 0.01 to about 30 mg, more preferably about 0.01 to about 20 mg, more preferably about 0.01 to about 10 mg, more preferably about 0.01 to about 5 mg, more preferably at about 0.025 to about 1.5 mg, more preferably at about 0.05 to about 1.25 mg, more preferably at about 0.075 to about 1.25 mg, more preferably at about 0.1 to about 1.25 mg, even more preferably at about 0.15 to about 1.25 mg, and most preferably at about 0.25 to about 1.0 mg per g of cellulosic material.

In another aspect, an effective amount of a GH61 polypeptide having cellulolytic enhancing activity to cellulolytic enzyme protein is about 0.005 to about 1.0 g, preferably at about 0.01 to about 1.0 g, more preferably at about 0.15 to about 0.75 g, more preferably at about 0.15 to about 0.5 g, more preferably at about 0.1 to about 0.5 g, even more preferably at about 0.1 to about 0.5 g, and most preferably at about 0.05 to about 0.2 g per g of cellulolytic enzyme protein.

Fermentation

The fermentable sugars obtained from the hydrolyzed cellulosic material can be fermented by one or more (several) fermenting microorganisms capable of fermenting the sugars directly or indirectly into a desired fermentation product. "Fermentation" or "fermentation process" refers to any fermentation process or any process comprising a fermentation step. Fermentation processes also include fermentation processes used in the

consumable alcohol industry (e.g., beer and wine), dairy industry (e.g., fermented dairy products), leather industry, and tobacco industry. The fermentation conditions depend on the desired fermentation product and fermenting organism and can easily be determined by one skilled in the art.

5 In the fermentation step, sugars, released from cellulosic material as a result of the pretreatment and enzymatic hydrolysis steps, are fermented to a product, e.g., ethanol, by a fermenting organism, such as yeast. Hydrolysis (saccharification) and fermentation can be separate or simultaneous, as described herein.

10 The term "fermentation medium" is understood herein to refer to a medium before the fermenting microorganism(s) is(are) added, such as, a medium resulting from a saccharification process, as well as a medium used in a simultaneous saccharification and fermentation process (SSF).

15 "Fermenting microorganism" refers to any microorganism, including bacterial and fungal organisms, suitable for use in a desired fermentation process to produce a fermentation product. The fermenting organism can be C₆ and/or C₅ fermenting organisms, or a combination thereof. Both C₆ and C₅ fermenting organisms are well known in the art. Suitable fermenting microorganisms are able to ferment, i.e., convert, sugars, such as glucose, xylose, xylulose, arabinose, maltose, mannose, galactose, or oligosaccharides, directly or indirectly into the desired fermentation product.

20 Examples of bacterial and fungal fermenting organisms producing ethanol are described by Lin *et al.*, 2006, *Appl. Microbiol. Biotechnol.* 69: 627-642.

Examples of fermenting microorganisms that can ferment C₆ sugars include bacterial and fungal organisms, such as yeast. Preferred yeast includes strains of the *Saccharomyces* spp., preferably *Saccharomyces cerevisiae*.

25 Examples of fermenting organisms that can ferment C₅ sugars include bacterial and fungal organisms, such as some yeast. Preferred C₅ fermenting yeast include strains of *Pichia*, preferably *Pichia stipitis*, such as *Pichia stipitis* CBS 5773; strains of *Candida*, preferably *Candida boidinii*, *Candida brassicae*, *Candida sheatae*, *Candida diddensii*, *Candida pseudotropicalis*, or *Candida utilis*.

30 Other fermenting organisms include strains of *Zymomonas*, such as *Zymomonas mobilis*; *Hansenula*, such as *Hansenula anomala*; *Kluyveromyces*, such as *K. fragilis*; *Schizosaccharomyces*, such as *S. pombe*; *E. coli*, especially *E. coli* strains that have been genetically modified to improve the yield of ethanol; *Clostridium*, such as *Clostridium acetobutylicum*, *Chlostridium thermocellum*, and *Chlostridium phytofermentans*; *Geobacillus* sp.; *Thermoanaerobacter*, such as *Thermoanaerobacter saccharolyticum*; and *Bacillus*, such as *Bacillus coagulans*.

35 In a preferred aspect, the yeast is a *Saccharomyces* spp. In a more preferred aspect,

the yeast is *Saccharomyces cerevisiae*. In another more preferred aspect, the yeast is *Saccharomyces distaticus*. In another more preferred aspect, the yeast is *Saccharomyces uvarum*. In another preferred aspect, the yeast is a *Kluyveromyces*. In another more preferred aspect, the yeast is *Kluyveromyces marxianus*. In another more preferred aspect, the yeast is *Kluyveromyces fragilis*. In another preferred aspect, the yeast is a *Candida*. In another more preferred aspect, the yeast is *Candida boidinii*. In another more preferred aspect, the yeast is *Candida brassicae*. In another more preferred aspect, the yeast is *Candida diddensii*. In another more preferred aspect, the yeast is *Candida pseudotropicalis*. In another more preferred aspect, the yeast is *Candida utilis*. In another preferred aspect, the yeast is a *Clavispora*. In another more preferred aspect, the yeast is *Clavispora lusitaniae*. In another more preferred aspect, the yeast is *Clavispora opuntiae*. In another preferred aspect, the yeast is a *Pachysolen*. In another more preferred aspect, the yeast is *Pachysolen tannophilus*. In another preferred aspect, the yeast is a *Pichia*. In another more preferred aspect, the yeast is a *Pichia stipitis*. In another preferred aspect, the yeast is a *Bretannomyces*. In another more preferred aspect, the yeast is *Bretannomyces clausenii* (Philippidis, G. P., 1996, Cellulose bioconversion technology, in *Handbook on Bioethanol: Production and Utilization*, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212).

Bacteria that can efficiently ferment hexose and pentose to ethanol include, for example, *Zymomonas mobilis*, *Clostridium acetobutylicum*, *Clostridium thermocellum*, *Chlostridium phytofermentans*, *Geobacillus* sp., *Thermoanaerobacter saccharolyticum*, and *Bacillus coagulans* (Philippidis, 1996, *supra*).

In a preferred aspect, the bacterium is a *Zymomonas*. In a more preferred aspect, the bacterium is *Zymomonas mobilis*. In another preferred aspect, the bacterium is a *Clostridium*. In another more preferred aspect, the bacterium is *Clostridium thermocellum*.

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

In a preferred aspect, the fermenting microorganism has been genetically modified to provide the ability to ferment pentose sugars, such as xylose utilizing, arabinose utilizing, and xylose and arabinose co-utilizing microorganisms.

The cloning of heterologous genes into various fermenting microorganisms has led to the construction of organisms capable of converting hexoses and pentoses to ethanol (cofermentation) (Chen and Ho, 1993, Cloning and improving the expression of *Pichia stipitis* xylose reductase gene in *Saccharomyces cerevisiae*, *Appl. Biochem. Biotechnol.* 39-40:

135-147; Ho *et al.*, 1998, Genetically engineered *Saccharomyces* yeast capable of effectively cofermenting glucose and xylose, *Appl. Environ. Microbiol.* 64: 1852-1859; Kotter and Ciriacy, 1993, Xylose fermentation by *Saccharomyces cerevisiae*, *Appl. Microbiol. Biotechnol.* 38: 776-783; Walfridsson *et al.*, 1995, Xylose-metabolizing *Saccharomyces cerevisiae* strains overexpressing the TKL1 and TAL1 genes encoding the pentose phosphate pathway enzymes transketolase and transaldolase, *Appl. Environ. Microbiol.* 61: 4184-4190; Kuyper *et al.*, 2004, Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle, *FEMS Yeast Research* 4: 655-664; Beall *et al.*, 1991, Parametric studies of ethanol production from xylose and other sugars by recombinant *Escherichia coli*, *Biotech. Bioeng.* 38: 296-303; Ingram *et al.*, 1998, Metabolic engineering of bacteria for ethanol production, *Biotechnol. Bioeng.* 58: 204-214; Zhang *et al.*, 1995, Metabolic engineering of a pentose metabolism pathway in ethanologenic *Zymomonas mobilis*, *Science* 267: 240-243; Deanda *et al.*, 1996, Development of an arabinose-fermenting *Zymomonas mobilis* strain by metabolic pathway engineering, *Appl. Environ. Microbiol.* 62: 4465-4470; WO 2003/062430, xylose isomerase).

In a preferred aspect, the genetically modified fermenting microorganism is *Saccharomyces cerevisiae*. In another preferred aspect, the genetically modified fermenting microorganism is *Zymomonas mobilis*. In another preferred aspect, the genetically modified fermenting microorganism is *Escherichia coli*. In another preferred aspect, the genetically modified fermenting microorganism is *Klebsiella oxytoca*. In another preferred aspect, the genetically modified fermenting microorganism is *Kluyveromyces* sp.

It is well known in the art that the organisms described above can also be used to produce other substances, as described herein.

The fermenting microorganism is typically added to the degraded lignocellulose or hydrolysate and the fermentation is performed for about 8 to about 96 hours, such as about 24 to about 60 hours. The temperature is typically between about 26°C to about 60°C, in particular about 32°C or 50°C, and at about pH 3 to about pH 8, such as around pH 4-5, 6, or 7.

In a preferred aspect, the yeast and/or another microorganism is applied to the degraded cellulosic material and the fermentation is performed for about 12 to about 96 hours, such as typically 24-60 hours. In a preferred aspect, the temperature is preferably between about 20°C to about 60°C, more preferably about 25°C to about 50°C, and most preferably about 32°C to about 50°C, in particular about 32°C or 50°C, and the pH is generally from about pH 3 to about pH 7, preferably around pH 4-7. However, some fermenting organisms, e.g., bacteria, have higher fermentation temperature optima. Yeast or another microorganism is preferably applied in amounts of approximately 10^5 to 10^{12} , preferably from approximately 10^7 to 10^{10} , especially approximately 2×10^8 viable cell count

per ml of fermentation broth. Further guidance in respect of using yeast for fermentation can be found in, e.g., "The Alcohol Textbook" (Editors K. Jacques, T.P. Lyons and D.R. Kelsall, Nottingham University Press, United Kingdom 1999), which is hereby incorporated by reference.

For ethanol production, following the fermentation the fermented slurry is distilled to extract the ethanol. The ethanol obtained according to the processes of the invention can be used as, e.g., fuel ethanol, drinking ethanol, *i.e.*, potable neutral spirits, or industrial ethanol.

A fermentation stimulator can be used in combination with any of the processes described herein to further improve the fermentation process, and in particular, the performance of the fermenting microorganism, such as, rate enhancement and ethanol yield. A "fermentation stimulator" refers to stimulators for growth of the fermenting microorganisms, in particular, yeast. Preferred fermentation stimulators for growth include vitamins and minerals. Examples of vitamins include multivitamins, biotin, pantothenate, nicotinic acid, meso-inositol, thiamine, pyridoxine, para-aminobenzoic acid, folic acid, riboflavin, and Vitamins A, B, C, D, and E. See, for example, Alfenore *et al.*, Improving ethanol production and viability of *Saccharomyces cerevisiae* by a vitamin feeding strategy during fed-batch process, Springer-Verlag (2002), which is hereby incorporated by reference. Examples of minerals include minerals and mineral salts that can supply nutrients comprising P, K, Mg, S, Ca, Fe, Zn, Mn, and Cu.

Fermentation Products

The fermentation product can be any substance derived from the fermentation. The fermentation product can be, without limitation, an alcohol (e.g., arabinitol, butanol, ethanol, glycerol, methanol, 1,3-propanediol, sorbitol, and xylitol); an organic acid (e.g., acetic acid, acetic acid, adipic acid, ascorbic acid, citric acid, 2,5-diketo-D-gluconic acid, formic acid, fumaric acid, glucaric acid, gluconic acid, glucuronic acid, glutaric acid, 3-hydroxypropionic acid, itaconic acid, lactic acid, malic acid, malonic acid, oxalic acid, oxaloacetic acid, propionic acid, succinic acid, and xylonic acid); a ketone (e.g., acetone); an amino acid (e.g., aspartic acid, glutamic acid, glycine, lysine, serine, and threonine); an alkane (e.g., pentane, hexane, heptane, octane, nonane, decane, undecane, and dodecane), a cycloalkane (e.g., cyclopentane, cyclohexane, cycloheptane, and cyclooctane), an alkene (e.g. pentene, hexene, heptene, and octene); and a gas (e.g., methane, hydrogen (H₂), carbon dioxide (CO₂), and carbon monoxide (CO)). The fermentation product can also be protein as a high value product.

In a preferred aspect, the fermentation product is an alcohol. It will be understood that the term "alcohol" encompasses a substance that contains one or more hydroxyl

moieties. In a more preferred aspect, the alcohol is arabinitol. In another more preferred aspect, the alcohol is butanol. In another more preferred aspect, the alcohol is ethanol. In another more preferred aspect, the alcohol is glycerol. In another more preferred aspect, the alcohol is methanol. In another more preferred aspect, the alcohol is 1,3-propanediol. In another more preferred aspect, the alcohol is sorbitol. In another more preferred aspect, the alcohol is xylitol. See, for example, Gong, C. S., Cao, N. J., Du, J., and Tsao, G. T., 1999, Ethanol production from renewable resources, in *Advances in Biochemical Engineering/Biotechnology*, Scheper, T., ed., Springer-Verlag Berlin Heidelberg, Germany, 65: 207-241; Silveira, M. M., and Jonas, R., 2002, The biotechnological production of sorbitol, *Appl. Microbiol. Biotechnol.* 59: 400-408; Nigam, P., and Singh, D., 1995, Processes for fermentative production of xylitol – a sugar substitute, *Process Biochemistry* 30 (2): 117-124; Ezeji, T. C., Qureshi, N. and Blaschek, H. P., 2003, Production of acetone, butanol and ethanol by *Clostridium beijerinckii* BA101 and *in situ* recovery by gas stripping, *World Journal of Microbiology and Biotechnology* 19 (6): 595-603.

In another preferred aspect, the fermentation product is an organic acid. In another more preferred aspect, the organic acid is acetic acid. In another more preferred aspect, the organic acid is acetonic acid. In another more preferred aspect, the organic acid is adipic acid. In another more preferred aspect, the organic acid is ascorbic acid. In another more preferred aspect, the organic acid is citric acid. In another more preferred aspect, the organic acid is 2,5-diketo-D-gluconic acid. In another more preferred aspect, the organic acid is formic acid. In another more preferred aspect, the organic acid is fumaric acid. In another more preferred aspect, the organic acid is glucaric acid. In another more preferred aspect, the organic acid is gluconic acid. In another more preferred aspect, the organic acid is glucuronic acid. In another more preferred aspect, the organic acid is glutaric acid. In another preferred aspect, the organic acid is 3-hydroxypropionic acid. In another more preferred aspect, the organic acid is itaconic acid. In another more preferred aspect, the organic acid is lactic acid. In another more preferred aspect, the organic acid is malic acid. In another more preferred aspect, the organic acid is malonic acid. In another more preferred aspect, the organic acid is oxalic acid. In another more preferred aspect, the organic acid is propionic acid. In another more preferred aspect, the organic acid is succinic acid. In another more preferred aspect, the organic acid is xylonic acid. See, for example, Chen, R., and Lee, Y. Y., 1997, Membrane-mediated extractive fermentation for lactic acid production from cellulosic biomass, *Appl. Biochem. Biotechnol.* 63-65: 435-448.

In another preferred aspect, the fermentation product is a ketone. It will be understood that the term “ketone” encompasses a substance that contains one or more ketone moieties. In another more preferred aspect, the ketone is acetone. See, for example, Qureshi and Blaschek, 2003, *supra*.

In another preferred aspect, the fermentation product is an amino acid. In another more preferred aspect, the organic acid is aspartic acid. In another more preferred aspect, the amino acid is glutamic acid. In another more preferred aspect, the amino acid is glycine. In another more preferred aspect, the amino acid is lysine. In another more preferred aspect, the amino acid is serine. In another more preferred aspect, the amino acid is threonine. See, for example, Richard, A., and Margaritis, A., 2004, Empirical modeling of batch fermentation kinetics for poly(glutamic acid) production and other microbial biopolymers, *Biotechnology and Bioengineering* 87 (4): 501-515.

In another preferred aspect, the fermentation product is an alkane. The alkane can be an unbranched or a branched alkane. In another more preferred aspect, the alkane is pentane. In another more preferred aspect, the alkane is hexane. In another more preferred aspect, the alkane is heptane. In another more preferred aspect, the alkane is octane. In another more preferred aspect, the alkane is nonane. In another more preferred aspect, the alkane is decane. In another more preferred aspect, the alkane is undecane. In another more preferred aspect, the alkane is dodecane.

In another preferred aspect, the fermentation product is a cycloalkane. In another more preferred aspect, the cycloalkane is cyclopentane. In another more preferred aspect, the cycloalkane is cyclohexane. In another more preferred aspect, the cycloalkane is cycloheptane. In another more preferred aspect, the cycloalkane is cyclooctane.

In another preferred aspect, the fermentation product is an alkene. The alkene can be an unbranched or a branched alkene. In another more preferred aspect, the alkene is pentene. In another more preferred aspect, the alkene is hexene. In another more preferred aspect, the alkene is heptene. In another more preferred aspect, the alkene is octene.

In another preferred aspect, the fermentation product is a gas. In another more preferred aspect, the gas is methane. In another more preferred aspect, the gas is H₂. In another more preferred aspect, the gas is CO₂. In another more preferred aspect, the gas is CO. See, for example, Kataoka, N., A. Miya, and K. Kiriya, 1997, Studies on hydrogen production by continuous culture system of hydrogen-producing anaerobic bacteria, *Water Science and Technology* 36 (6-7): 41-47; and Gunaseelan V.N. in *Biomass and Bioenergy*, Vol. 13 (1-2), pp. 83-114, 1997, Anaerobic digestion of biomass for methane production: A review.

The fermentation product(s) can be optionally recovered from the fermentation medium using any method known in the art including, but not limited to, chromatography, electrophoretic procedures, differential solubility, distillation, or extraction. For example, alcohol is separated from the fermented cellulosic material and purified by conventional methods of distillation. Ethanol with a purity of up to about 96 vol.% can be obtained, which can be used as, for example, fuel ethanol, drinking ethanol, *i.e.*, potable neutral spirits, or

industrial ethanol.

Hydrolysis Enzyme Compositions

The enzyme compositions can comprise any protein that is useful in saccharifying a
5 cellulosic material.

In one aspect, the enzyme composition comprises or further comprises one or more
(several) proteins selected from the group consisting of a cellulase, a GH61 polypeptide
having cellulolytic enhancing activity, a hemicellulase, an expansin, an esterase, a laccase,
10 a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin. In another
aspect, the cellulase is preferably one or more (several) enzymes selected from the group
consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase. In another
aspect, the hemicellulase is preferably one or more (several) enzymes selected from the
group consisting of an acetylmannan esterase, an acetyxylan esterase, an arabinanase, an
15 arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a
glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a
xylosidase.

In another aspect, the enzyme composition comprises one or more (several)
cellulolytic enzymes. In another aspect, the enzyme composition comprises or further
comprises one or more (several) hemicellulolytic enzymes. In another aspect, the enzyme
20 composition comprises one or more (several) cellulolytic enzymes and one or more (several)
hemicellulolytic enzymes. In another aspect, the enzyme composition comprises one or
more (several) enzymes selected from the group of cellulolytic enzymes and hemicellulolytic
enzymes. In another aspect, the enzyme composition comprises an endoglucanase. In
another aspect, the enzyme composition comprises a cellobiohydrolase. In another aspect,
25 the enzyme composition comprises a beta-glucosidase. In another aspect, the enzyme
composition comprises a polypeptide having cellulolytic enhancing activity. In another
aspect, the enzyme composition comprises an endoglucanase and a polypeptide having
cellulolytic enhancing activity. In another aspect, the enzyme composition comprises a
cellobiohydrolase and a polypeptide having cellulolytic enhancing activity. In another aspect,
30 the enzyme composition comprises a beta-glucosidase and a polypeptide having cellulolytic
enhancing activity. In another aspect, the enzyme composition comprises an endoglucanase
and a cellobiohydrolase. In another aspect, the enzyme composition comprises an
endoglucanase and a beta-glucosidase. In another aspect, the enzyme composition
comprises a cellobiohydrolase and a beta-glucosidase. In another aspect, the enzyme
35 composition comprises an endoglucanase, a cellobiohydrolase, and a polypeptide having
cellulolytic enhancing activity. In another aspect, the enzyme composition comprises an

endoglucanase, a beta-glucosidase, and a polypeptide having cellulolytic enhancing activity. In another aspect, the enzyme composition comprises a cellobiohydrolase, a beta-glucosidase, and a polypeptide having cellulolytic enhancing activity. In another aspect, the enzyme composition comprises an endoglucanase, a cellobiohydrolase, and a beta-glucosidase, and a polypeptide having cellulolytic enhancing activity.

In another aspect, the enzyme composition comprises an acetylmannan esterase. In another aspect, the enzyme composition comprises an acetyxylan esterase. In another aspect, the enzyme composition comprises an arabinanase (*e.g.*, alpha-L-arabinanase). In another aspect, the enzyme composition comprises an arabinofuranosidase (*e.g.*, alpha-L-arabinofuranosidase). In another aspect, the enzyme composition comprises a coumaric acid esterase. In another aspect, the enzyme composition comprises a feruloyl esterase. In another aspect, the enzyme composition comprises a galactosidase (*e.g.*, alpha-galactosidase and/or beta-galactosidase). In another aspect, the enzyme composition comprises a glucuronidase (*e.g.*, alpha-D-glucuronidase). In another aspect, the enzyme composition comprises a glucuronoyl esterase. In another aspect, the enzyme composition comprises a mannanase. In another aspect, the enzyme composition comprises a mannosidase (*e.g.*, beta-mannosidase). In another aspect, the enzyme composition comprises a xylanase. In a preferred aspect, the xylanase is a Family 10 xylanase. In another aspect, the enzyme composition comprises a xylosidase (*e.g.*, beta-xylosidase). In another aspect, the enzyme composition comprises an expansin. In another aspect, the enzyme composition comprises an esterase. In another aspect, the enzyme composition comprises a laccase. In another aspect, the enzyme composition comprises a ligninolytic enzyme. In a preferred aspect, the ligninolytic enzyme is a manganese peroxidase. In another preferred aspect, the ligninolytic enzyme is a lignin peroxidase. In another preferred aspect, the ligninolytic enzyme is a H₂O₂-producing enzyme. In another aspect, the enzyme composition comprises a pectinase. In another aspect, the enzyme composition comprises a peroxidase. In another aspect, the enzyme composition comprises a protease. In another aspect, the enzyme composition comprises a swollenin.

In the processes of the present invention, the enzyme(s) can be added prior to or during fermentation, *e.g.*, during saccharification or during or after propagation of the fermenting microorganism(s).

One or more (several) components of the enzyme composition may be wild-type proteins, recombinant proteins, or a combination of wild-type proteins and recombinant proteins. For example, one or more (several) components may be native proteins of a cell, which is used as a host cell to express recombinantly one or more (several) other components of the enzyme composition. One or more (several) components of the enzyme composition may be produced as monocomponents, which are then combined to form the

enzyme composition. The enzyme composition may be a combination of multicomponent and monocomponent protein preparations.

The enzymes used in the processes of the present invention may be in any form suitable for use, such as, for example, a crude fermentation broth with or without cells removed, a cell lysate with or without cellular debris, a semi-purified or purified enzyme preparation, or a host cell as a source of the enzymes. The enzyme composition may be a dry powder or granulate, a non-dusting granulate, a liquid, a stabilized liquid, or a stabilized protected enzyme. Liquid enzyme preparations may, for instance, be stabilized by adding stabilizers such as a sugar, a sugar alcohol or another polyol, and/or lactic acid or another organic acid according to established processes.

The enzymes can be derived or obtained from any suitable origin, including, bacterial, fungal, yeast, plant, or mammalian origin. The term "obtained" means herein that the enzyme may have been isolated from an organism that naturally produces the enzyme as a native enzyme. The term "obtained" also means herein that the enzyme may have been produced recombinantly in a host organism employing methods described herein, wherein the recombinantly produced enzyme is either native or foreign to the host organism or has a modified amino acid sequence, e.g., having one or more (several) amino acids that are deleted, inserted and/or substituted, i.e., a recombinantly produced enzyme that is a mutant and/or a fragment of a native amino acid sequence or an enzyme produced by nucleic acid shuffling processes known in the art. Encompassed within the meaning of a native enzyme are natural variants and within the meaning of a foreign enzyme are variants obtained recombinantly, such as by site-directed mutagenesis or shuffling.

The polypeptide having enzyme activity may be a bacterial polypeptide. For example, the polypeptide may be a gram positive bacterial polypeptide such as a *Bacillus*, *Streptococcus*, *Streptomyces*, *Staphylococcus*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Clostridium*, *Geobacillus*, or *Oceanobacillus* polypeptide having enzyme activity, or a Gram negative bacterial polypeptide such as an *E. coli*, *Pseudomonas*, *Salmonella*, *Campylobacter*, *Helicobacter*, *Flavobacterium*, *Fusobacterium*, *Ilyobacter*, *Neisseria*, or *Ureaplasma* polypeptide having enzyme activity.

In a preferred aspect, the polypeptide is a *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, or *Bacillus thuringiensis* polypeptide having enzyme activity.

In another preferred aspect, the polypeptide is a *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, or *Streptococcus equi* subsp. *Zooepidemicus* polypeptide having enzyme activity.

In another preferred aspect, the polypeptide is a *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, or *Streptomyces lividans* polypeptide having enzyme activity.

The polypeptide having enzyme activity may also be a fungal polypeptide, and more preferably a yeast polypeptide such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* polypeptide having enzyme activity; or more preferably a filamentous fungal polypeptide such as an *Acremonium*, *Agaricus*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Botryosphaeria*, *Ceriporiopsis*, *Chaetomidium*, *Chrysosporium*, *Claviceps*, *Cochliobolus*, *Coprinopsis*, *Coptotermes*, *Corynascus*, *Cryphonectria*, *Cryptococcus*, *Diplodia*, *Exidia*, *Filibasidium*, *Fusarium*, *Gibberella*, *Holomastigotoides*, *Humicola*, *Irpex*, *Lentinula*, *Leptosphaeria*, *Magnaporthe*, *Melanocarpus*, *Meripilus*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Piromyces*, *Poitrasia*, *Pseudoplectania*, *Pseudotrichonympha*, *Rhizomucor*, *Schizophyllum*, *Scytalidium*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trichoderma*, *Trichophaea*, *Verticillium*, *Volvariella*, or *Xylaria* polypeptide having enzyme activity.

In a preferred aspect, the polypeptide is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, or *Saccharomyces oviformis* polypeptide having enzyme activity.

In another preferred aspect, the polypeptide is an *Acremonium cellulolyticus*, *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus fumigatus*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium tropicum*, *Chrysosporium merdarium*, *Chrysosporium inops*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium zonatum*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulfureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola grisea*, *Humicola insolens*, *Humicola lanuginosa*, *Irpex lacteus*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium funiculosum*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Thielavia achromatica*, *Thielavia albomyces*, *Thielavia albopilosa*, *Thielavia australeinsis*, *Thielavia fimeti*, *Thielavia microspora*, *Thielavia ovispora*, *Thielavia peruviana*, *Thielavia spededonium*, *Thielavia setosa*, *Thielavia subthermophila*, *Thielavia terrestris*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, *Trichoderma viride*, or *Trichophaea saccata* polypeptide having enzyme activity.

Chemically modified or protein engineered mutants of the polypeptides having enzyme activity may also be used.

One or more (several) components of the enzyme composition may be a recombinant component, *i.e.*, produced by cloning of a DNA sequence encoding the single component and subsequent cell transformed with the DNA sequence and expressed in a host (see, for example, WO 91/17243 and WO 91/17244). The host is preferably a heterologous host (enzyme is foreign to host), but the host may under certain conditions also be a homologous host (enzyme is native to host). Monocomponent cellulolytic enzymes may also be prepared by purifying such a protein from a fermentation broth.

In one aspect, the one or more (several) cellulolytic enzymes comprise a commercial cellulolytic enzyme preparation. Examples of commercial cellulolytic enzyme preparations suitable for use in the present invention include, for example, CELLIC™ CTec (Novozymes A/S), CELLIC™ CTec2 (Novozymes A/S), CELLUCLAST™ (Novozymes A/S), NOVOZYM™ 188 (Novozymes A/S), CELLUZYME™ (Novozymes A/S), CEREFLO™ (Novozymes A/S), and ULTRAFLO™ (Novozymes A/S), ACCELERASE™ (Genencor Int.), LAMINEX™ (Genencor Int.), SPEZYME™ CP (Genencor Int.), ROHAMENT™ 7069 W (Röhm GmbH), FIBREZYME® LDI (Dyadic International, Inc.), FIBREZYME® LBR (Dyadic International, Inc.), or VISCOSTAR® 150L (Dyadic International, Inc.). The cellulase enzymes are added in amounts effective from about 0.001 to about 5.0 wt % of solids, more preferably from about 0.025 to about 4.0 wt % of solids, and most preferably from about 0.005 to about 2.0 wt % of solids. The cellulase enzymes are added in amounts effective from about 0.001 to about 5.0 wt % of solids, more preferably from about 0.025 to about 4.0 wt % of solids, and most preferably from about 0.005 to about 2.0 wt % of solids.

In the processes of the present invention, any GH61 polypeptide having cellulolytic enhancing activity can be used, such as those polypeptides described *supra*.

Examples of bacterial endoglucanases that can be used in the processes of the present invention, include, but are not limited to, an *Acidothermus cellulolyticus* endoglucanase (WO 91/05039; WO 93/15186; U.S. Patent No. 5,275,944; WO 96/02551; U.S. Patent No. 5,536,655, WO 00/70031, WO 05/093050); *Thermobifida fusca* endoglucanase III (WO 05/093050); and *Thermobifida fusca* endoglucanase V (WO 05/093050).

Examples of fungal endoglucanases that can be used in the present invention include, but are not limited to, a *Trichoderma reesei* endoglucanase I (Penttilä *et al.*, 1986, *Gene* 45: 253-263; *Trichoderma reesei* Cel7B endoglucanase I; GENBANK™ accession no. M15665; SEQ ID NO: 2); *Trichoderma reesei* endoglucanase II (Saloheimo, *et al.*, 1988, *Gene* 63:11-22; *Trichoderma reesei* Cel5A endoglucanase II; GENBANK™ accession no.

M19373; SEQ ID NO: 4); *Trichoderma reesei* endoglucanase III (Okada *et al.*, 1988, *Appl. Environ. Microbiol.* 64: 555-563; GENBANK™ accession no. AB003694; SEQ ID NO: 6); *Trichoderma reesei* endoglucanase V (Saloheimo *et al.*, 1994, *Molecular Microbiology* 13: 219-228; GENBANK™ accession no. Z33381; SEQ ID NO: 8); *Aspergillus aculeatus* endoglucanase (Ooi *et al.*, 1990, *Nucleic Acids Research* 18: 5884); *Aspergillus kawachii* endoglucanase (Sakamoto *et al.*, 1995, *Current Genetics* 27: 435-439); *Erwinia carotovora* endoglucanase (Saarilahti *et al.*, 1990, *Gene* 90: 9-14); *Fusarium oxysporum* endoglucanase (GENBANK™ accession no. L29381); *Humicola grisea* var. *thermoidea* endoglucanase (GENBANK™ accession no. AB003107); *Melanocarpus albomyces* endoglucanase (GENBANK™ accession no. MAL515703); *Neurospora crassa* endoglucanase (GENBANK™ accession no. XM_324477); *Humicola insolens* endoglucanase V (SEQ ID NO: 10); *Myceliophthora thermophila* CBS 117.65 endoglucanase (SEQ ID NO: 12); basidiomycete CBS 495.95 endoglucanase (SEQ ID NO: 14); basidiomycete CBS 494.95 endoglucanase (SEQ ID NO: 16); *Thielavia terrestris* NRRL 8126 CEL6B endoglucanase (SEQ ID NO: 18); *Thielavia terrestris* NRRL 8126 CEL6C endoglucanase (SEQ ID NO: 20); *Thielavia terrestris* NRRL 8126 CEL7C endoglucanase (SEQ ID NO: 22); *Thielavia terrestris* NRRL 8126 CEL7E endoglucanase (SEQ ID NO: 24); *Thielavia terrestris* NRRL 8126 CEL7F endoglucanase (SEQ ID NO: 26); *Cladorrhinum foecundissimum* ATCC 62373 CEL7A endoglucanase (SEQ ID NO: 28); and *Trichoderma reesei* strain No. VTT-D-80133 endoglucanase (SEQ ID NO: 30; GENBANK™ accession no. M15665). The endoglucanases of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, and SEQ ID NO: 30, described above are encoded by the mature polypeptide coding sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, and SEQ ID NO: 29, respectively.

Examples of cellobiohydrolases useful in the present invention include, but are not limited to, *Trichoderma reesei* cellobiohydrolase I (SEQ ID NO: 32); *Trichoderma reesei* cellobiohydrolase II (SEQ ID NO: 34); *Humicola insolens* cellobiohydrolase I (SEQ ID NO: 36); *Myceliophthora thermophila* cellobiohydrolase II (SEQ ID NO: 38 and SEQ ID NO: 40); *Thielavia terrestris* cellobiohydrolase II (CEL6A) (SEQ ID NO: 42); *Chaetomium thermophilum* cellobiohydrolase I (SEQ ID NO: 44); and *Chaetomium thermophilum* cellobiohydrolase II (SEQ ID NO: 46), *Aspergillus fumigatus* cellobiohydrolase I (SEQ ID NO: 48), and *Aspergillus fumigatus* cellobiohydrolase II (SEQ ID NO: 50). The cellobiohydrolases of SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ

ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, and SEQ ID NO: 50, described above are encoded by the mature polypeptide coding sequence of SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, and SEQ ID NO: 49, respectively.

5 Examples of beta-glucosidases useful in the present invention include, but are not limited to, *Aspergillus oryzae* beta-glucosidase (SEQ ID NO: 52); *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 54); *Penicillium brasilianum* IBT 20888 beta-glucosidase (SEQ ID NO: 56); *Aspergillus niger* beta-glucosidase (SEQ ID NO: 58); and *Aspergillus aculeatus* beta-glucosidase (SEQ ID NO: 60). The beta-glucosidases of SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, and SEQ ID NO: 60, described above are encoded by the mature polypeptide coding sequence of SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, and SEQ ID NO: 59, respectively.

15 Examples of other beta-glucosidases useful in the present invention include a *Aspergillus oryzae* beta-glucosidase variant fusion protein of SEQ ID NO: 62 or the *Aspergillus oryzae* beta-glucosidase fusion protein of SEQ ID NO: 64. The beta-glucosidase fusion proteins of SEQ ID NO: 62 and SEQ ID NO: 64 are encoded by SEQ ID NO: 61 and SEQ ID NO: 63, respectively.

20 The *Aspergillus oryzae* beta-glucosidase can be obtained according to WO 2002/095014. The *Aspergillus fumigatus* beta-glucosidase can be obtained according to WO 2005/047499. The *Penicillium brasilianum* beta-glucosidase can be obtained according to WO 2007/019442. The *Aspergillus niger* beta-glucosidase can be obtained according to Dan *et al.*, 2000, *J. Biol. Chem.* 275: 4973-4980. The *Aspergillus aculeatus* beta-glucosidase can be obtained according to Kawaguchi *et al.*, 1996, *Gene* 173: 287-288.

25 Other useful endoglucanases, cellobiohydrolases, and beta-glucosidases are disclosed in numerous Glycosyl Hydrolase families using the classification according to Henrissat B., 1991, A classification of glycosyl hydrolases based on amino-acid sequence similarities, *Biochem. J.* 280: 309-316, and Henrissat B., and Bairoch A., 1996, Updating the sequence-based classification of glycosyl hydrolases, *Biochem. J.* 316: 695-696.

30 Other cellulolytic enzymes that may be useful in the present invention are described in EP 495,257, EP 531,315, EP 531,372, WO 89/09259, WO 94/07998, WO 95/24471, WO 96/11262, WO 96/29397, WO 96/034108, WO 97/14804, WO 98/08940, WO 98/012307, WO 98/13465, WO 98/015619, WO 98/015633, WO 98/028411, WO 99/06574, WO 99/10481, WO 99/025846, WO 99/025847, WO 99/031255, WO 2000/009707, WO 2002/050245, WO 2002/0076792, WO 2002/101078, WO 2003/027306, WO 2003/052054, WO 2003/052055, WO 2003/052056, WO 2003/052057, WO 2003/052118, WO 2004/016760, WO 2004/043980, WO 2004/048592, WO 2005/001065, WO 2005/028636, WO 2005/093050, WO 2005/093073, WO 2006/074005, WO 2006/117432, WO

2007/071818, WO 2007/071820, WO 2008/008070, WO 2008/008793, U.S. Patent No. 4,435,307, U.S. Patent No. 5,457,046, U.S. Patent No. 5,648,263, U.S. Patent No. 5,686,593, U.S. Patent No. 5,691,178, U.S. Patent No. 5,763,254, and U.S. Patent No. 5,776,757.

In the processes of the present invention, any GH61 polypeptide having cellulolytic enhancing activity can be used.

In a first aspect, the polypeptide having cellulolytic enhancing activity comprises the following motifs:

[ILMV]-P-X(4,5)-G-X-Y-[ILMV]-X-R-X-[EQ]-X(4)-[HNQ] and [FW]-[TF]-K-[AIV],

wherein X is any amino acid, X(4,5) is any amino acid at 4 or 5 contiguous positions, and X(4) is any amino acid at 4 contiguous positions.

The polypeptide comprising the above-noted motifs may further comprise:

H-X(1,2)-G-P-X(3)-[YW]-[AILMV],

[EQ]-X-Y-X(2)-C-X-[EHQN]-[FILV]-X-[ILV], or

H-X(1,2)-G-P-X(3)-[YW]-[AILMV] and [EQ]-X-Y-X(2)-C-X-[EHQN]-[FILV]-X-[ILV],

wherein X is any amino acid, X(1,2) is any amino acid at 1 position or 2 contiguous positions, X(3) is any amino acid at 3 contiguous positions, and X(2) is any amino acid at 2 contiguous positions. In the above motifs, the accepted IUPAC single letter amino acid abbreviation is employed.

In a preferred aspect, the polypeptide having cellulolytic enhancing activity further comprises H-X(1,2)-G-P-X(3)-[YW]-[AILMV]. In another preferred aspect, the isolated polypeptide having cellulolytic enhancing activity further comprises [EQ]-X-Y-X(2)-C-X-[EHQN]-[FILV]-X-[ILV]. In another preferred aspect, the polypeptide having cellulolytic enhancing activity further comprises H-X(1,2)-G-P-X(3)-[YW]-[AILMV] and [EQ]-X-Y-X(2)-C-X-[EHQN]-[FILV]-X-[ILV].

In a second aspect, the polypeptide having cellulolytic enhancing activity comprises the following motif:

[ILMV]-P-x(4,5)-G-x-Y-[ILMV]-x-R-x-[EQ]-x(3)-A-[HNQ],

wherein x is any amino acid, x(4,5) is any amino acid at 4 or 5 contiguous positions, and x(3) is any amino acid at 3 contiguous positions. In the above motif, the accepted IUPAC single letter amino acid abbreviation is employed.

In a third aspect, the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence that has a degree of identity to the mature polypeptide of SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 88, SEQ ID NO: 90, SEQ ID NO: 92, SEQ ID NO: 94, SEQ ID NO: 96, SEQ ID NO: 98, SEQ ID NO: 100, SEQ ID NO: 102, SEQ ID NO: 104, SEQ ID NO: 106, SEQ ID NO: 108, SEQ ID

NO: 110, SEQ ID NO: 112, SEQ ID NO: 114, SEQ ID NO: 116, SEQ ID NO: 118, SEQ ID NO: 120, SEQ ID NO: 122, SEQ ID NO: 124, SEQ ID NO: 126, or SEQ ID NO: 128 of at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, or at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100%.

In a fourth aspect, the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide that hybridizes under at least very low stringency conditions, preferably at least low stringency conditions, more preferably at least medium stringency conditions, more preferably at least medium-high stringency conditions, even more preferably at least high stringency conditions, and most preferably at least very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO: 79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, SEQ ID NO: 109, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, SEQ ID NO: 119, SEQ ID NO: 121, SEQ ID NO: 123, SEQ ID NO: 125, or SEQ ID NO: 127, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, or SEQ ID NO: 79, or the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 77, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, SEQ ID NO: 109, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, SEQ ID NO: 119, SEQ ID NO: 121, SEQ ID NO: 123, SEQ ID NO: 125, or SEQ ID NO: 127, (iii) a subsequence of (i) or (ii), or (iv) a full-length complementary strand of (i), (ii), or (iii) (J. Sambrook, E.F. Fritsch, and T. Maniatus, 1989, *supra*). A subsequence of the mature polypeptide coding sequence of SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO: 79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, SEQ ID NO: 109, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, SEQ ID NO: 119, SEQ ID NO: 121, SEQ ID NO: 123, SEQ ID NO: 125, or SEQ ID NO: 127 contains at least 100 contiguous nucleotides or preferably at least 200 contiguous nucleotides. Moreover, the subsequence may encode a polypeptide fragment that has cellulolytic enhancing activity.

In a fifth aspect, the polypeptide having cellulolytic enhancing activity is encoded by a

polynucleotide comprising or consisting of a nucleotide sequence that has a degree of identity to the mature polypeptide coding sequence of SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO: 79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, SEQ ID NO: 109, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, SEQ ID NO: 119, SEQ ID NO: 121, SEQ ID NO: 123, SEQ ID NO: 125, or SEQ ID NO: 127 of preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 91%, at least 92%, at least 93%, at least 94%, or at least 95%, and even most preferably at least 96%, at least 97%, at least 98%, at least 99%, or at least 100%.

In a sixth aspect, the polypeptide having cellulolytic enhancing activity is an artificial variant comprising a substitution, deletion, and/or insertion of one or more (or several) amino acids of the mature polypeptide of SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 88, SEQ ID NO: 90, SEQ ID NO: 92, SEQ ID NO: 94, SEQ ID NO: 96, SEQ ID NO: 98, SEQ ID NO: 100, SEQ ID NO: 102, SEQ ID NO: 104, SEQ ID NO: 106, SEQ ID NO: 108, SEQ ID NO: 110, SEQ ID NO: 112, SEQ ID NO: 114, SEQ ID NO: 116, SEQ ID NO: 118, SEQ ID NO: 120, SEQ ID NO: 122, SEQ ID NO: 124, SEQ ID NO: 126, or SEQ ID NO: 128; or a homologous sequence thereof.

Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, *In, The Proteins*, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr,

Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

Alternatively, the amino acid changes are of such a nature that the physico-chemical properties of the polypeptides are altered. For example, amino acid changes may improve the thermal stability of the polypeptide, alter the substrate specificity, change the pH optimum, and the like.

Essential amino acids in a parent polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for cellulolytic enhancing activity to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Lett.* 309: 59-64. The identities of essential amino acids can also be inferred from analysis of identities with polypeptides that are related to the parent polypeptide.

Single or multiple amino acid substitutions, deletions, and/or insertions can be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR, phage display (e.g., Lowman *et al.*, 1991, *Biochemistry* 30: 10832-10837; U.S. Patent No. 5,223,409; WO 92/06204), and region-directed mutagenesis (Derbyshire *et al.*, 1986, *Gene* 46: 145; Ner *et al.*, 1988, *DNA* 7: 127).

Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells (Ness *et al.*, 1999, *Nature Biotechnology* 17: 893-896). Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide.

The total number of amino acid substitutions, deletions and/or insertions of the mature polypeptide of SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 88, SEQ ID NO: 90, SEQ ID NO: 92, SEQ ID NO: 94,

SEQ ID NO: 96, SEQ ID NO: 98, SEQ ID NO: 100, SEQ ID NO: 102, SEQ ID NO: 104, SEQ ID NO: 106, SEQ ID NO: 108, SEQ ID NO: 110, SEQ ID NO: 112, SEQ ID NO: 114, SEQ ID NO: 116, SEQ ID NO: 118, SEQ ID NO: 120, SEQ ID NO: 122, SEQ ID NO: 124, SEQ ID NO: 126, or SEQ ID NO: 128 is not more than 4, e.g., 1, 2, 3, or 4.

5 In one aspect, the GH61 polypeptide having cellulolytic enhancing activity is used in the presence of a soluble activating divalent metal cation according to WO 2008/151043, e.g., manganese sulfate.

10 In one aspect, the GH61 polypeptide having cellulolytic enhancing activity is used in the presence of a dioxy compound, a bicyclic compound, a heterocyclic compound, a nitrogen-containing compound, a sulfur-containing compound, or a liquor obtained from a pretreated cellulosic material such as pretreated corn stover (PCS).

15 The dioxy compound may include any suitable compound containing two or more oxygen atoms. In some aspects, the dioxy compounds contain a substituted aryl moiety as described herein. The dioxy compounds may comprise one or more (several) hydroxyl and/or hydroxyl derivatives, but also include substituted aryl moieties lacking hydroxyl and hydroxyl derivatives. Non-limiting examples of dioxy compounds include pyrocatechol or catechol; caffeic acid; 3,4-dihydroxybenzoic acid; 4-tert-butyl-5-methoxy-1,2-benzenediol; pyrogallol; gallic acid; methyl-3,4,5-trihydroxybenzoate; 2,3,4-trihydroxybenzophenone; 2,6-dimethoxyphenol; sinapinic acid; 3,5-dihydroxybenzoic acid; 4-chloro-1,2-benzenediol; 4-nitro-1,2-benzenediol; tannic acid; ethyl gallate; methyl glycolate; dihydroxyfumaric acid; 2-butene-1,4-diol; (croconic acid; 1,3-propanediol; tartaric acid; 2,4-pentanediol; 3-ethoxy-1,2-propanediol; 2,4,4'-trihydroxybenzophenone; cis-2-butene-1,4-diol; 3,4-dihydroxy-3-cyclobutene-1,2-dione; dihydroxyacetone; acrolein acetal; methyl-4-hydroxybenzoate; 4-hydroxybenzoic acid; and methyl-3,5-dimethoxy-4-hydroxybenzoate; or a salt or solvate thereof.

25 The bicyclic compound may include any suitable substituted fused ring system as described herein. The compounds may comprise one or more (several) additional rings, and are not limited to a specific number of rings unless otherwise stated. In one aspect, the bicyclic compound is a flavonoid. In another aspect, the bicyclic compound is an optionally substituted isoflavonoid. In another aspect, the bicyclic compound is an optionally substituted flavylum ion, such as an optionally substituted anthocyanidin or optionally substituted anthocyanin, or derivative thereof. Non-limiting examples of bicyclic compounds include epicatechin; quercetin; myricetin; taxifolin; kaempferol; morin; acacetin; naringenin; isorhamnetin; apigenin; cyanidin; cyanin; kuromanin; (keracyanin; or a salt or solvate thereof.

35 The heterocyclic compound may be any suitable compound, such as an optionally substituted aromatic or non-aromatic ring comprising a heteroatom, as described herein. In

one aspect, the heterocyclic is a compound comprising an optionally substituted heterocycloalkyl moiety or an optionally substituted heteroaryl moiety. In another aspect, the optionally substituted heterocycloalkyl moiety or optionally substituted heteroaryl moiety is an optionally substituted 5-membered heterocycloalkyl or an optionally substituted 5-membered heteroaryl moiety. In another aspect, the optionally substituted heterocycloalkyl or optionally substituted heteroaryl moiety is an optionally substituted moiety selected from pyrazolyl, furanyl, imidazolyl, isoxazolyl, oxadiazolyl, oxazolyl, pyrrolyl, pyridyl, pyrimidyl, pyridazinyl, thiazolyl, triazolyl, thienyl, dihydrothieno-pyrazolyl, thianaphthenyl, carbazolyl, benzimidazolyl, benzothienyl, benzofuranyl, indolyl, quinoliny, benzotriazolyl, benzothiazolyl, benzooxazolyl, benzimidazolyl, isoquinoliny, isoindolyl, acridinyl, benzoisazolyl, dimethylhydantoin, pyrazinyl, tetrahydrofuranyl, pyrrolinyl, pyrrolidinyl, morpholinyl, indolyl, diazepinyl, azepinyl, thiepinyl, piperidinyl, and oxepinyl. In another aspect, the optionally substituted heterocycloalkyl moiety or optionally substituted heteroaryl moiety is an optionally substituted furanyl. Non-limiting examples of heterocyclic compounds include (1,2-dihydroxyethyl)-3,4-dihydroxyfuran-2(5H)-one; 4-hydroxy-5-methyl-3-furanone; 5-hydroxy-2(5H)-furanone; [1,2-dihydroxyethyl]furan-2,3,4(5H)-trione; α -hydroxy- γ -butyrolactone; ribonic γ -lactone; aldohexuronic acid γ -lactone; gluconic acid δ -lactone; 4-hydroxycoumarin; dihydrobenzofuran; 5-(hydroxymethyl)furfural; furoin; 2(5H)-furanone; 5,6-dihydro-2H-pyran-2-one; and 5,6-dihydro-4-hydroxy-6-methyl-2H-pyran-2-one; or a salt or solvate thereof.

The nitrogen-containing compound may be any suitable compound with one or more nitrogen atoms. In one aspect, the nitrogen-containing compound comprises an amine, imine, hydroxylamine, or nitroxide moiety. Non-limiting examples of nitrogen-containing compounds include acetone oxime; violuric acid; pyridine-2-aldoxime; 2-aminophenol; 1,2-benzenediamine; 2,2,6,6-tetramethyl-1-piperidinyloxy; 5,6,7,8-tetrahydrobiopterin; 6,7-dimethyl-5,6,7,8-tetrahydropterine; and maleamic acid; or a salt or solvate thereof.

The quinone compound may be any suitable compound comprising a quinone moiety as described herein. Non-limiting examples of quinone compounds include 1,4-benzoquinone; 1,4-naphthoquinone; 2-hydroxy-1,4-naphthoquinone; 2,3-dimethoxy-5-methyl-1,4-benzoquinone or coenzyme Q₀; 2,3,5,6-tetramethyl-1,4-benzoquinone or duroquinone; 1,4-dihydroxyanthraquinone; 3-hydroxy-1-methyl-5,6-indolinedione or adrenochrome; 4-tert-butyl-5-methoxy-1,2-benzoquinone; pyrroloquinoline quinone; or a salt or solvate thereof.

The sulfur-containing compound may be any suitable compound comprising one or more sulfur atoms. In one aspect, the sulfur-containing comprises a moiety selected from thionyl, thioether, sulfinyl, sulfonyl, sulfamide, sulfonamide, sulfonic acid, and sulfonic ester. Non-limiting examples of sulfur-containing compounds include ethanethiol; 2-propanethiol; 2-

propene-1-thiol; 2-mercaptoethanesulfonic acid; benzenethiol; benzene-1,2-dithiol; cysteine; methionine; glutathione; cystine; or a salt or solvate thereof.

In one aspect, an effective amount of such a compound described above to cellulosic material as a molar ratio to glucosyl units of cellulose is about 10^{-6} to about 10, *e.g.*, about 10^{-6} to about 7.5, about 10^{-6} to about 5, about 10^{-6} to about 2.5, about 10^{-6} to about 1, about 10^{-5} to about 1, about 10^{-5} to about 10^{-1} , about 10^{-4} to about 10^{-1} , about 10^{-3} to about 10^{-1} , and about 10^{-3} to about 10^{-2} . In another aspect, an effective amount of such a compound described above is about 0.1 μ M to about 1 M, *e.g.*, about 0.5 μ M to about 0.75 M, about 0.75 μ M to about 0.5 M, about 1 μ M to about 0.25 M, about 1 μ M to about 0.1 M, about 5 μ M to about 50 mM, about 10 μ M to about 25 mM, about 50 μ M to about 25 mM, about 10 μ M to about 10 mM, about 5 μ M to about 5 mM, and about 0.1 mM to about 1 mM.

In embodiments, the term liquor refers to the solution phase, either aqueous, organic, or a combination thereof, arising from treatment of a lignocellulose and/or hemicellulose material in a slurry, or monosaccharides thereof, *e.g.*, xylose, arabinose, mannose, *etc.*, under conditions as described herein, and the soluble contents thereof. A liquor for cellulolytic enhancement of a GH61 polypeptide can be produced by treating a lignocellulose or hemicellulose material (or feedstock) by applying heat and/or pressure, optionally in the presence of a catalyst, *e.g.*, acid, optionally in the presence of an organic solvent, and optionally in combination with physical disruption of the material, and then separating the solution from the residual solids. Such conditions determine the degree of cellulolytic enhancement obtainable through the combination of liquor and a GH61 polypeptide during hydrolysis of a cellulosic substrate by a cellulase preparation. The liquor can be separated from the treated material using methods standard in the art, such as filtration, sedimentation, or centrifugation.

In one aspect, an effective amount of the liquor to cellulose is about 10^{-6} to about 10 g per g of cellulose, *e.g.*, about 10^{-6} to about 7.5 g, about 10^{-6} to about 5, about 10^{-6} to about 2.5 g, about 10^{-6} to about 1 g, about 10^{-5} to about 1 g, about 10^{-5} to about 10^{-1} g, about 10^{-4} to about 10^{-1} g, about 10^{-3} to about 10^{-1} g, and about 10^{-3} to about 10^{-2} g per g of cellulose.

In one aspect, the one or more (several) hemicellulolytic enzymes comprise a commercial hemicellulolytic enzyme preparation. Examples of commercial hemicellulolytic enzyme preparations suitable for use in the present invention include, for example, SHEARZYME™ (Novozymes A/S), CELLIC™ HTec (Novozymes A/S), CELLIC™ HTec2 (Novozymes A/S), VISCOZYME® (Novozymes A/S), ULTRAFLO® (Novozymes A/S), PULPZYME® HC (Novozymes A/S), MULTIFECT® Xylanase (Genencor), ECOPULP® TX-200A (AB Enzymes), HSP 6000 Xylanase (DSM), DEPOL™ 333P (Biocatalysts Limit, Wales, UK), DEPOL™ 740L (Biocatalysts Limit, Wales, UK), and DEPOL™ 762P (Biocatalysts Limit, Wales, UK).

Examples of xylanases useful in the processes of the present invention include, but are not limited to, *Aspergillus aculeatus* xylanase (GeneSeqP:AAR63790; WO 94/21785), *Aspergillus fumigatus* xylanases (WO 2006/078256; xyl 3 SEQ ID NO: 129 [DNA sequence] and SEQ ID NO: 130 [deduced amino acid sequence]), and *Thielavia terrestris* NRRL 8126 xylanases (WO 2009/079210).

Examples of beta-xylosidases useful in the processes of the present invention include, but are not limited to, *Trichoderma reesei* beta-xylosidase (UniProtKB/TrEMBL accession number Q92458; SEQ ID NO: 131 [DNA sequence] and SEQ ID NO: 132 [deduced amino acid sequence]), *Talaromyces emersonii* (SwissProt accession number Q8X212), and *Neurospora crassa* (SwissProt accession number Q7SOW4).

Examples of acetylxyylan esterases useful in the processes of the present invention include, but are not limited to, *Hypocrea jecorina* acetylxyylan esterase (WO 2005/001036), *Neurospora crassa* acetylxyylan esterase (UniProt accession number q7s259), *Thielavia terrestris* NRRL 8126 acetylxyylan esterase (WO 2009/042846), *Chaetomium globosum* acetylxyylan esterase (Uniprot accession number Q2GWX4), *Chaetomium gracile* acetylxyylan esterase (GeneSeqP accession number AAB82124), *Phaeosphaeria nodorum* acetylxyylan esterase (Uniprot accession number Q0UHH1), and *Humicola insolens* DSM 1800 acetylxyylan esterase (WO 2009/073709).

Examples of ferulic acid esterases useful in the processes of the present invention include, but are not limited to, *Humicola insolens* DSM 1800 feruloyl esterase (WO 2009/076122), *Neurospora crassa* feruloyl esterase (UniProt accession number Q9HGR3), and *Neosartorya fischeri* feruloyl esterase (UniProt Accession number A1D9T4).

Examples of arabinofuranosidases useful in the processes of the present invention include, but are not limited to, *Humicola insolens* DSM 1800 arabinofuranosidase (WO 2009/073383) and *Aspergillus niger* arabinofuranosidase (GeneSeqP accession number AAR94170).

Examples of alpha-glucuronidases useful in the processes of the present invention include, but are not limited to, *Aspergillus clavatus* alpha-glucuronidase (UniProt accession number alcc12), *Trichoderma reesei* alpha-glucuronidase (Uniprot accession number Q99024), *Talaromyces emersonii* alpha-glucuronidase (UniProt accession number Q8X211), *Aspergillus niger* alpha-glucuronidase (Uniprot accession number Q96WX9), *Aspergillus terreus* alpha-glucuronidase (SwissProt accession number Q0CJP9), and *Aspergillus fumigatus* alpha-glucuronidase (SwissProt accession number Q4WW45).

The enzymes and proteins used in the processes of the present invention may be produced by fermentation of the above-noted microbial strains on a nutrient medium containing suitable carbon and nitrogen sources and inorganic salts, using procedures known in the art (see, e.g., Bennett, J.W. and LaSure, L. (eds.), *More Gene Manipulations in*

Fungi, Academic Press, CA, 1991). Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). Temperature ranges and other conditions suitable for growth and enzyme production are known in the art (see, e.g., Bailey, J.E., and Ollis, D.F.,
5 *Biochemical Engineering Fundamentals*, McGraw-Hill Book Company, NY, 1986).

The fermentation can be any method of cultivation of a cell resulting in the expression or isolation of an enzyme. Fermentation may, therefore, be understood as comprising shake flask cultivation, or small- or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial
10 fermentors performed in a suitable medium and under conditions allowing the enzyme to be expressed or isolated. The resulting enzymes produced by the methods described above may be recovered from the fermentation medium and purified by conventional procedures.

Nucleic Acid Constructs

An isolated polynucleotide encoding a polypeptide, e.g., a GH61 polypeptide having cellulolytic enhancing activity, a cellulolytic enzyme, a hemicellulolytic enzyme, etc., may be manipulated in a variety of ways to provide for expression of the polypeptide by constructing a nucleic acid construct comprising an isolated polynucleotide encoding the polypeptide operably linked to one or more (several) control sequences that direct the expression of the
20 coding sequence in a suitable host cell under conditions compatible with the control sequences. Manipulation of the polynucleotide's sequence prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotide sequences utilizing recombinant DNA methods are well known in the art.

The control sequence may be a promoter sequence, a polynucleotide that is recognized by a host cell for expression of a polynucleotide encoding a polypeptide. The promoter sequence contains transcriptional control sequences that mediate the expression of the polypeptide. The promoter may be any polynucleotide that shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may
30 be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs in the present invention in a bacterial host cell are the promoters obtained from the *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus*
35 *stearothermophilus* maltogenic amylase gene (*amyM*), *Bacillus subtilis* levansucrase gene

(*sacB*), *Bacillus subtilis xylA* and *xylB* genes, *E. coli lac* operon, *Streptomyces coelicolor* agarase gene (*dagA*), and prokaryotic beta-lactamase gene (Villa-Kamaroff *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75: 3727-3731), as well as the *tac* promoter (DeBoer *et al.*, 1983, *Proc. Natl. Acad. Sci. USA* 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in Gilbert *et al.*, 1980, *Scientific American*, 242: 74-94; and in Sambrook *et al.*, 1989, *supra*.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs in the present invention in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Aspergillus oryzae* TAKA amylase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Fusarium oxysporum* trypsin-like protease (WO 96/00787), *Fusarium venenatum* amyloglucosidase (WO 00/56900), *Fusarium venenatum* Daria (WO 00/56900), *Fusarium venenatum* Quinn (WO 00/56900), *Rhizomucor miehei* lipase, *Rhizomucor miehei* aspartic proteinase, *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase IV, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* beta-xylosidase, as well as the NA2-tpi promoter (a modified promoter from a gene encoding a neutral alpha-amylase in *Aspergilli* in which the untranslated leader has been replaced by an untranslated leader from a gene encoding triose phosphate isomerase in *Aspergilli*; non-limiting examples include modified promoters from the gene encoding neutral alpha-amylase in *Aspergillus niger* in which the untranslated leader has been replaced by an untranslated leader from the gene encoding triose phosphate isomerase in *Aspergillus nidulans* or *Aspergillus oryzae*); and mutant, truncated, and hybrid promoters thereof.

In a yeast host, useful promoters are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* galactokinase (GAL1), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH1, ADH2/GAP), *Saccharomyces cerevisiae* triose phosphate isomerase (TPI), *Saccharomyces cerevisiae* metallothionein (CUP1), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase. Other useful promoters for yeast host cells are described by Romanos *et al.*, 1992, *Yeast* 8: 423-488.

The control sequence may also be a suitable transcription terminator sequence, which is recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3'-terminus of the polynucleotide encoding the polypeptide. Any

terminator that is functional in the host cell of choice may be used in the present invention.

Preferred terminators for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase, *Aspergillus oryzae* TAKA amylase, and *Fusarium oxysporum* trypsin-like protease.

Preferred terminators for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be a suitable leader sequence, when transcribed is a nontranslated region of an mRNA that is important for translation by the host cell. The leader sequence is operably linked to the 5'-terminus of the polynucleotide encoding the polypeptide. Any leader sequence that is functional in the host cell of choice may be used.

Preferred leaders for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase and *Aspergillus nidulans* triose phosphate isomerase.

Suitable leaders for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, *Saccharomyces cerevisiae* alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3'-terminus of the polynucleotide and, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence that is functional in the host cell of choice may be used.

Preferred polyadenylation sequences for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Fusarium oxysporum* trypsin-like protease, and *Aspergillus niger* alpha-glucosidase.

Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, *Mol. Cellular Biol.* 15: 5983-5990.

The control sequence may also be a signal peptide coding region that encodes a signal peptide linked to the N-terminus of a polypeptide and directs the polypeptide into the cell's secretory pathway. The 5'-end of the coding sequence of the polynucleotide may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the polypeptide. Alternatively, the 5'-end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. The foreign signal peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding

sequence. Alternatively, the foreign signal peptide coding sequence may simply replace the natural signal peptide coding sequence in order to enhance secretion of the polypeptide. However, any signal peptide coding sequence that directs the expressed polypeptide into the secretory pathway of a host cell of choice may be used.

Effective signal peptide coding sequences for bacterial host cells are the signal peptide coding sequences obtained from the genes for *Bacillus* NCIB 11837 maltogenic amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus stearothermophilus* neutral proteases (*nprT*, *nprS*, *nprM*), and *Bacillus subtilis* *prsA*. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57: 109-137.

Effective signal peptide coding sequences for filamentous fungal host cells are the signal peptide coding sequences obtained from the genes for *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Aspergillus oryzae* TAKA amylase, *Humicola insolens* cellulase, *Humicola insolens* endoglucanase V, *Humicola lanuginosa* lipase, and *Rhizomucor miehei* aspartic proteinase.

Useful signal peptides for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase. Other useful signal peptide coding sequences are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be a propeptide coding sequence that encodes a propeptide positioned at the N-terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to an active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding sequence may be obtained from the genes for *Bacillus subtilis* alkaline protease (*aprE*), *Bacillus subtilis* neutral protease (*nprT*), *Myceliophthora thermophila* laccase (WO 95/33836), *Rhizomucor miehei* aspartic proteinase, and *Saccharomyces cerevisiae* alpha-factor.

Where both signal peptide and propeptide sequences are present at the N-terminus of a polypeptide, the propeptide sequence is positioned next to the N-terminus of a polypeptide and the signal peptide sequence is positioned next to the N-terminus of the propeptide sequence.

It may also be desirable to add regulatory sequences that allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those that cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems include the *lac*, *tac*, and *trp* operator systems. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the *Aspergillus niger* glucoamylase promoter, *Aspergillus oryzae* TAKA alpha-amylase promoter, and

Aspergillus oryzae glucoamylase promoter may be used. Other examples of regulatory sequences are those that allow for gene amplification. In eukaryotic systems, these regulatory sequences include the dihydrofolate reductase gene that is amplified in the presence of methotrexate, and the metallothionein genes that are amplified with heavy metals. In these cases, the polynucleotide encoding the polypeptide would be operably linked with the regulatory sequence.

Expression Vectors

The various nucleotide and control sequences described above may be joined together to produce a recombinant expression vector that may include one or more (several) convenient restriction sites to allow for insertion or substitution of a polynucleotide encoding a polypeptide, e.g., a GH61 polypeptide having cellulolytic enhancing activity, a cellulolytic enzyme, a hemicellulolytic enzyme, etc., at such sites. Alternatively, the polynucleotide may be expressed by inserting the polynucleotide or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

The recombinant expression vector may be any vector (e.g., a plasmid or virus) that can be conveniently subjected to recombinant DNA procedures and can bring about expression of the polynucleotide. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vector may be a linear or closed circular plasmid.

The vector may be an autonomously replicating vector, i.e., a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one that, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

The vector preferably contains one or more (several) selectable markers that permit easy selection of transformed, transfected, transduced, or the like cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

Examples of bacterial selectable markers are the *dal* genes from *Bacillus subtilis* or

Bacillus licheniformis, or markers that confer antibiotic resistance such as ampicillin, chloramphenicol, kanamycin, or tetracycline resistance. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for use in a filamentous fungal host cell include, but are not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in an *Aspergillus* cell are the *amdS* and *pyrG* genes of *Aspergillus nidulans* or *Aspergillus oryzae* and the *bar* gene of *Streptomyces hygroscopicus*.

The vector preferably contains an element(s) that permits integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome.

For integration into the host cell genome, the vector may rely on the polynucleotide's sequence encoding the polypeptide or any other element of the vector for integration into the genome by homologous or non-homologous recombination. Alternatively, the vector may contain additional polynucleotides for directing integration by homologous recombination into the genome of the host cell at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, 400 to 10,000 base pairs, and 800 to 10,000 base pairs, which have a high degree of sequence identity to the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding polynucleotides. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in a cell. The term "origin of replication" or "plasmid replicator" means a polynucleotide that enables a plasmid or vector to replicate *in vivo*.

Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*, and pUB110, pE194, pTA1060, and pAM β 1 permitting replication in *Bacillus*.

Examples of origins of replication for use in a yeast host cell are the 2 micron origin of replication, ARS1, ARS4, the combination of ARS1 and CEN3, and the combination of ARS4 and CEN6.

Examples of origins of replication useful in a filamentous fungal cell are AMA1 and

ANS1 (Gems *et al.*, 1991, *Gene* 98: 61-67; Cullen *et al.*, 1987, *Nucleic Acids Res.* 15: 9163-9175; WO 00/24883). Isolation of the AMA1 gene and construction of plasmids or vectors comprising the gene can be accomplished according to the methods disclosed in WO 00/24883.

5 More than one copy of a polynucleotide may be inserted into a host cell to increase production of a polypeptide. An increase in the copy number of the polynucleotide can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the polynucleotide where cells containing amplified copies of the selectable marker gene, and thereby additional
10 copies of the polynucleotide, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

The procedures used to ligate the elements described above to construct the recombinant expression vectors are well known to one skilled in the art (see, *e.g.*, Sambrook *et al.*, 1989, *supra*).

Host Cells

15 Recombinant host cells comprising a polynucleotide encoding a polypeptide, *e.g.*, a GH61 polypeptide having cellulolytic enhancing activity, a cellulolytic enzyme, a hemicellulolytic enzyme, etc., can be advantageously used in the recombinant production of the polypeptide. A construct or vector comprising such a polynucleotide is introduced into a host cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The choice of a host cell will to a large extent depend upon the gene
25 encoding the polypeptide and its source.

The host cell may be any cell useful in the recombinant production of a polypeptide, *e.g.*, a prokaryote or a eukaryote.

The prokaryotic host cell may be any gram-positive or gram-negative bacterium. Gram-positive bacteria include, but not limited to, *Bacillus*, *Clostridium*, *Enterococcus*,
30 *Geobacillus*, *Lactobacillus*, *Lactococcus*, *Oceanobacillus*, *Staphylococcus*, *Streptococcus*, and *Streptomyces*. Gram-negative bacteria include, but not limited to, *Campylobacter*, *E. coli*, *Flavobacterium*, *Fusobacterium*, *Helicobacter*, *Ilyobacter*, *Neisseria*, *Pseudomonas*, *Salmonella*, and *Ureaplasma*.

The bacterial host cell may be any *Bacillus* cell including, but not limited to, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*,
35 *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*,

Bacillus megaterium, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis* cells.

The bacterial host cell may also be any *Streptococcus* cell including, but not limited to, *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, and *Streptococcus equi* subsp. *Zooepidemicus* cells.

The bacterial host cell may also be any *Streptomyces* cell including, but not limited to, *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, and *Streptomyces lividans* cells.

The introduction of DNA into a *Bacillus* cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Mol. Gen. Genet.* 168: 111-115), by using competent cells (see, e.g., Young and Spizizen, 1961, *J. Bacteriol.* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *J. Mol. Biol.* 56: 209-221), by electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or by conjugation (see, e.g., Koehler and Thorne, 1987, *J. Bacteriol.* 169: 5271-5278). The introduction of DNA into an *E. coli* cell may, for instance, be effected by protoplast transformation (see, e.g., Hanahan, 1983, *J. Mol. Biol.* 166: 557-580) or electroporation (see, e.g., Dower *et al.*, 1988, *Nucleic Acids Res.* 16: 6127-6145). The introduction of DNA into a *Streptomyces* cell may, for instance, be effected by protoplast transformation and electroporation (see, e.g., Gong *et al.*, 2004, *Folia Microbiol. (Praha)* 49: 399-405), by conjugation (see, e.g., Mazodier *et al.*, 1989, *J. Bacteriol.* 171: 3583-3585), or by transduction (see, e.g., Burke *et al.*, 2001, *Proc. Natl. Acad. Sci. USA* 98: 6289-6294). The introduction of DNA into a *Pseudomonas* cell may, for instance, be effected by electroporation (see, e.g., Choi *et al.*, 2006, *J. Microbiol. Methods* 64: 391-397) or by conjugation (see, e.g., Pinedo and Smets, 2005, *Appl. Environ. Microbiol.* 71: 51-57). The introduction of DNA into a *Streptococcus* cell may, for instance, be effected by natural competence (see, e.g., Perry and Kuramitsu, 1981, *Infect. Immun.* 32: 1295-1297), by protoplast transformation (see, e.g., Catt and Jollick, 1991, *Microbios* 68: 189-207), by electroporation (see, e.g., Buckley *et al.*, 1999, *Appl. Environ. Microbiol.* 65: 3800-3804) or by conjugation (see, e.g., Clewell, 1981, *Microbiol. Rev.* 45: 409-436). However, any method known in the art for introducing DNA into a host cell can be used.

The host cell may also be a eukaryote, such as a mammalian, insect, plant, or fungal cell.

The host cell may be a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth *et al.*, In, *Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth *et al.*, 1995, *supra*, page 171) and all mitosporic fungi (Hawksworth *et al.*, 1995, *supra*).

The fungal host cell may be a yeast cell. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner, F.A., Passmore, S.M., and Davenport, R.R., eds, Soc. App. Bacteriol. Symposium Series No. 9, 1980).

The yeast host cell may be a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell such as a *Kluyveromyces lactis*, *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, *Saccharomyces oviformis*, or *Yarrowia lipolytica* cell.

The fungal host cell may be a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are generally characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

The filamentous fungal host cell may be an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, or *Trichoderma* cell.

For example, the filamentous fungal host cell may be an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor*

miehei, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

5 Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described in EP 238023, Yelton *et al.*, 1984, *Proc. Natl. Acad. Sci. USA* 81: 1470-1474, and Christensen *et al.*, 1988, *Bio/Technology* 6: 1419-1422. Suitable methods for transforming
10 *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156, and WO 96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *J. Bacteriol.* 153: 163; and Hinnen *et al.*, 1978, *Proc. Natl. Acad. Sci.*
15 *USA* 75: 1920.

Methods of Production

Methods for producing a polypeptide, e.g., a GH61 polypeptide having cellulolytic enhancing activity, a cellulolytic enzyme, a hemicellulolytic enzyme, etc., comprise (a)
20 cultivating a cell, which in its wild-type form is capable of producing the polypeptide, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide. In a preferred aspect, the cell is of the genus *Aspergillus*. In a more preferred aspect, the cell is *Aspergillus fumigatus*.

Alternatively, methods for producing a polypeptide, e.g., a GH61 polypeptide having
25 cellulolytic enhancing activity, a cellulolytic enzyme, a hemicellulolytic enzyme, etc., comprise (a) cultivating a recombinant host cell under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

In the production methods, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods well known in the art. For example, the cell may
30 be cultivated by shake flask cultivation, and small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures
35 known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type

Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

The polypeptide may be detected using methods known in the art that are specific for the polypeptides. These detection methods may include use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the polypeptide. The polypeptides having cellulolytic enhancing activity are detected using the methods described herein.

The resulting broth may be used as is or the polypeptide may be recovered using methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation.

The polypeptides may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989) to obtain substantially pure polypeptides.

In an alternative aspect, the polypeptide is not recovered, but rather a host cell expressing a polypeptide is used as a source of the polypeptide.

The following non-limiting examples further illustrate compositions, methods, and treatments in accordance with the present disclosure. It should be noted that the disclosure is not limited to the specific details embodied in the examples.

Examples

Example 1: Esterase(s) as detoxification for hydrolysis of pretreated cellulosic material.

Pretreated cellulosic material

Corn stover was pretreated at the U.S. Department of Energy National Renewable Energy Laboratory (NREL) using dilute sulfuric acid. According to NREL, the water-insoluble solids in the pretreated corn stover (PCS) contained 57.5% cellulose, 7.0% hemicellulose and 27.2% lignin, which were determined by the NREL Laboratory Analytical Procedure (LAP) "Determination of Structural Carbohydrates and Lignin in Biomass". The total solids (TS) of the PCS was 28.9% and the fraction of insoluble solids (FIS), as the fraction (% w/w) of insoluble solids in the total solids, was 61.5%. The TS and FIS were determined based on

the NREL LAP "Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples".

Collection of Pretreatment Liquor

5 Liquor was collected from acid, steam exploded corn stover. PCS was slurried in water to a final total solids (TS) level of 15 wt. % with mixing at ambient temperature for 5 hour, and liquor was collected by vacuum filtration through a glass fiber filter (Whatman GF/D). Prior to the experiments, the pH was adjusted to 5.0.

10 Washed PCS

 Washed PCS (wPCS) was obtained from a thoroughly wash of PCS, until the filtrated presented neutral pH.

Detoxification of the PCS liquor with Esterases

15 A 20 mL volume of PCS liquor was treated with : (1) ferulic acid esterase (FAE) from *Humicola*, (2) FAE from *Aspergillus niger* (SEQ ID NO: 133), (3) FAE from *Aspergillus oryzae* (SEQ ID NO: 134), (4) FAE from *Chaetomium globusam* (SEQ ID NO: 135) or (5) esterase from *Aspergillus oryzae* (SEQ ID NO: 136) at 100 ppm (protein in the enzyme solution vs. liquor), pH 5.0 for 16 hours in 50 mL glass flasks.

20

Hydrolysis of wPCS in the presence of detoxified pretreated liquor

 Enzymatic hydrolysis of wPCS in 75 mmol/L acetate buffer and in the presence of the enzymatically detoxified treated liquors and corresponding control (untreated liquor) was performed in a 24 well (5 mL) polypropylene cell growth plate (Whatman Uniplate). A 2.5 g
25 quantity of 8% wPCS slurry, with pH pre-adjusted to 5 and pre-mixed in 150 mmol/L acetate buffer, was mixed with 2.5 mL of the enzymatically detoxified treated liquors and 62 µL of cellulolytic preparation comprising *Trichoderma reesei* cellulases, *Thermoascus aurantiacus* GH61 polypeptide having cellulolytic enhancing activity (WO 2005/074656 A2), and *Aspergillus oryzae* beta-glucosidase fusion protein (WO 2008/057637), for a final
30 concentration of 5 mg-protein/g-cellulose. Hydrolysis reactions at 4% TS were incubated at 50°C with shaking (150 rpm) for 120 hours. All experiments reported were performed in triplicate.

Glucose yield determination

35 Following hydrolysis, samples were filtered using a 0.20 µm syringe filters (Millipore, Bedford, MA, USA) and filtrates analyzed for sugar content as described below. When not used immediately, filtered aliquots were frozen at -20°C. The sugar concentrations of

samples diluted in 0.005 M H₂SO₄ were measured using a 4.6 x 250 mm AMINEX® HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, USA) by elution with 0.005 M H₂SO₄ at 65°C at a flow rate of 0.6 ml per minute, and quantitation by integration of the glucose, cellobiose, and xylose signals from refractive index detection (CHEMSTATION®,
5 AGILENT® 1100 HPLC, Agilent Technologies, Santa Clara, CA, USA) calibrated by pure sugar samples. The resultant glucose and cellobiose equivalents were used to calculate the percentage of cellulose conversion for each reaction.

Glucose, cellobiose, and xylose were measured individually. Measured sugar concentrations were adjusted for the appropriate dilution factor. In the cases, the net
10 concentrations of enzymatically-produced sugars were determined by adjusting the measured sugar concentrations for corresponding background sugar concentrations at zero time point. All HPLC data processing was performed using MICROSOFT EXCEL™ software (Microsoft, Richland, WA, USA).

The degree of cellulose conversion to glucose was calculated using the following
15 equation: % glucose yield = glucose concentration / glucose concentration in a limit digest. The glucose concentration in a limit digest was determined taking into account the pretreated material composition analysis, the insoluble solids and the liquid volume in hydrolysis. It was used the following equation:

Glucose concentration in a limit digest = [Weight of pretreated biomass x % Insoluble
20 Solids in pretreated biomass x % cellulose x 1.111 x 0.1] / [Weight of the slurry in hydrolysis – total solids in hydrolysis].

The 1.111 factor takes into account the increase in mass when cellulose is converted to glucose. The 0.1 factor is to transform % into g/L. In the denominator, we have the weight of the liquor in hydrolysis and assuming density as one, it is the volume of the hydrolysis
25 liquor that contains the sugars. Duplicate data points were averaged and standard deviation was calculated.

Results

The improvements in the enzymatic hydrolysis of wPCS in the presence of the PCS
30 liquors treated with esterases of the present disclosure are presented in Table 1 (Hydrolysis of wPCS in the presence of PCS liquors treated with esterases, with 5 mg-protein/g-cellulose at 4% TS wPCS, pH 5.5 and 50°C for 120 hours.).

Table 1

	Glucose released after 5 days hydrolysis (g/L)	Error
Control	12.83	0.16
FAE from <i>Humicola</i>	13.16	0.16
FAE from <i>Aspergillus niger</i>	13.18	0.19
FAE from <i>Aspergillus Oryzae</i>	13.80	0.15
FAE from <i>Chaetomium globusam</i>	13.85	0.25
Esterase from <i>Aspergillus Oryzae</i>	14.02	0.11

5 It will be understood that various modifications may be made to the embodiments disclosed herein. Therefore, the above description should not be construed as limiting, but merely as exemplifications of embodiments. Those skilled in art will envision other modifications within the scope and spirit of the claims appended hereto.

What is claimed is:

1. A process for enzymatic refining of a pretreated cellulosic material, comprising:
(a) contacting the pretreated cellulosic material with one or more esterase
5 enzymes to form a refined pretreated cellulosic material.
2. The process of claim 1, wherein the esterase comprises one or more (several) enzymes selected from the group consisting of an esterase and feruloyl esterase.
- 10 3. The process of claim 1, where the esterase comprises an amino acid sequence having at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% sequence identity to the mature polypeptide of SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136 or SEQ ID NO: 137, or fragments thereof with feruloyl esterase activity.
- 15 4. The process of claim 1, wherein the enzyme comprises or consists of SEQ ID. NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136 or SEQ ID NO: 137 or the mature polypeptide thereof.
5. The process of claim 2, wherein the feruloyl esterase is one or more (several)
20 enzymes selected from the group consisting of FAE(1), FAE-A, FAE-C, and FAE-D.
6. The process of claim 1 comprising: (b) recovering the refined pretreated cellulosic material.
- 25 7. The process of any of claims 1-4, further comprising separating a liquor from the pretreated cellulosic material.
8. The process of claim 7, further comprising contacting the liquor with feruloyl esterase and recycling the liquor so that it is contacted with pretreated cellulosic material or refined
30 pretreated cellulosic material.
9. The process of claim 7, further comprising recycling the liquor to a new pretreatment of a pretreated cellulosic material with the esterase composition.
- 35 10. The process of any of claims 1-9 further comprising post-treating the refined pretreated cellulosic material with an enzymatic pre-treatment, chemical pre-treatment,

mechanical pre-treatment and/or a physical pretreatment.

11. The process of any of claims 1-5 and 7-9, further comprising recovering the refined pretreated cellulosic material.

5

12. The process of any of claims 1-11, wherein the contacting with the esterase is performed with about 0.0005 to about 5 mg, *e.g.*, about 0.001 to about 5 mg, about 0.0025 to about 5 mg, about 0.005 to about 5 mg, about 0.005 to about 4.5 mg, about 0.005 to about 4 mg, about 0.005 to about 3.5 mg, about 0.005 to about 3 mg, about 0.005 to about 2 mg, about 0.005 to about 1 mg, about 0.075 to about 1 mg, or about 0.1 to about 1 mg of esterase per g of pretreated cellulosic material.

10

13. The process of any of claims 1-12, wherein the contacting with esterase is performed with a total solids (TS) of about 1% to about 50%, *e.g.*, about 2% to about 40%, about 2% to about 35%, about 3% to about 30%, about 3% to about 25%, about 4% to about 20%, or about 5% to about 10%.

15

14. The process of any of claims 1-13, wherein the contacting with esterase is performed at a pH of about 2 to about 9, *e.g.*, about 3 to about 7.5, about 3.5 to about 7, about 4 to about 6.5, about 4.5 to about 6.5, about 4.5 to about 6.0, about 5 and about 6.0, or about 5 to about 5.5.

20

15. The process of any of claims 1-14, wherein the contacting is performed at a temperature in the range of about 20°C to about 70°C, *e.g.*, about 25°C to about 65°C, about 30°C to about 65°C, about 35°C to about 65°C, about 40°C to about 60°C, about 45°C to about 55°C, or about 45°C to about 50°C.

25

16. The process of any of claims 1-15, wherein the contacting with esterase is performed for a period of time of 5 minutes to 35 hours, *e.g.*, 10 minutes to 15 hours, 10 hours to 15 hours, 10 hours to 20 hours, 10 hours to 20 hours, 20 hours to 24 hours, 24 hours to 30 hours, 1 hour to 72 hours.

30

17. A process for enzymatic refining of a pretreated cellulosic material, comprising:
(a) processing the pretreated cellulosic material to form a solid/liquid mixture of pretreated cellulosic material;

35

(b) separating a liquor from the solid/liquid mixture of pretreated cellulosic material; and

(c) treating the liquor with a feruloyl esterase treatment.

5 18. A process for hydrolyzing a pretreated cellulosic material, comprising saccharifying a pretreated cellulosic material treated and refined with an esterase according to any of claims 1-5 with an enzyme composition.

10 19. The process of claim 18, further comprising recovering a saccharified pretreated cellulosic material from the saccharification.

20. A process for producing a fermentation product, comprising:

15 (a) saccharifying a pretreated cellulosic material with an enzyme composition, wherein the pretreated cellulosic material is contacted or treated with an esterase according to claims 1-5;

(b) fermenting the saccharified pretreated cellulosic material with one or more (several) fermenting microorganisms to produce the fermentation product; and

(c) recovering the fermentation product from the fermentation.

20 21. A process for fermenting a pretreated cellulosic material, comprising: fermenting a pretreated cellulosic material with one or more (several) fermenting microorganisms, wherein the pretreated cellulosic material is treated, refined, and/or saccharified according to any of claims 1-20.

25 22. The process of claim 21, wherein the fermenting of the pretreated cellulosic material produces a fermentation product.

23. The process of claim 21, further comprising recovering the fermentation product from the fermentation.

30