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(54) **Title:** METHODS FOR INCREASING ENZYMATIC HYDROLYSIS OF CELLULOSIC MATERIAL IN THE PRESENCE OF A PEROXIDASE

(57) **Abstract:** The present invention relates to methods for increasing hydrolysis of a cellulosic material, comprising: hydrolyzing the cellulosic material with an enzyme composition in the presence of a polypeptide having peroxidase activity.

METHODS FOR INCREASING ENZYMATIC HYDROLYSIS OF CELLULOSIC MATERIAL IN THE PRESENCE OF A PEROXIDASE

5 Reference to a Sequence Listing

This application contains a Sequence Listing filed electronically by EFS, which is incorporated herein by reference.

Background of the Invention

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Field of the Invention

The present invention relates to methods for increasing hydrolysis of cellulosic material with an enzyme composition.

15 Description of the Related Art

Cellulose is a polymer of the simple sugar glucose 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.

25 It is well known in the art that oxidation of biomolecules such as DNA, lipids, or protein is a significant issue in biological systems. Consequently, treatment with a peroxidase may improve the performance of cellulose-hydrolyzing enzyme systems.

Different peroxide-decomposing enzymes often have different specificities and potencies. For example, catalase is very efficient only at high levels of hydrogen peroxide (0.1 M or above) because of its high Michaelis constant, K_m , on this substance (K_m ranges from 0.1 to 1 M: see Nicholls *et al.*, 2001, *Advances in Inorg. Chem.* 51: 52-106; and Masaki *et al.*, 1998, *Archives of Dermatological Research* 290: 113-118). At low peroxide levels, a peroxidase can be significantly more efficient (than catalase) to decompose the peroxide, because of the enzyme's high affinity (sub-mM ranges) for the peroxide. For example, horseradish peroxidase, an archetypical peroxidase, has a K_m of 0.02 mM on hydrogen peroxide or ethyl hydroperoxide (Kedderis and Hollenberg, 1983, *J. Biol. Chem.* 258: 8129-8138), and glutathione peroxidase has a K_m of 0.025-0.06 mM (Masaki *et al.*, 1998, *supra*). Since many biomass conversion techniques are prone to generate low level peroxide, peroxidase may be more effective than catalase to remove the peroxide to improve cellulose

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hydrolysis.

The present invention provides methods for increasing hydrolysis of cellulosic materials with enzyme compositions.

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Summary of the Invention

The present invention relates to methods for degrading or converting a cellulosic material, comprising: treating the cellulosic material with an enzyme composition in the presence of a polypeptide having peroxidase activity.

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The present invention also relates to methods for producing a fermentation product, comprising:

(a) saccharifying a cellulosic material with an enzyme composition in the presence of a polypeptide having peroxidase activity;

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

(c) recovering the fermentation product from the fermentation.

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The present invention further relates to methods of fermenting a cellulosic material, comprising: fermenting the cellulosic material with one or more (several) fermenting microorganisms, wherein the cellulosic material is hydrolyzed with an enzyme composition in the presence of a polypeptide having peroxidase activity.

Brief Description of the Figures

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Figure 1 shows horseradish peroxidase mitigation of cellulase-inhibition by cellobiose dehydrogenase. Fractional cellulose conversion is plotted for various reaction conditions. Solid bars: 1 day of hydrolysis; hatched bars: 3 days of hydrolysis.

Figure 2 shows horseradish peroxidase enhancement of PCS hydrolysis. Circles: 1 day of hydrolysis; squares: 3 days of hydrolysis.

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Figure 3 shows the effect of various peroxidases on PCS hydrolysis. Solid bars: 1 day of hydrolysis; hatched bars: 3 days of hydrolysis. Numbers indicate the volumes of the stock peroxidases added, as indicated in the text. Mn-perox: manganese peroxidase; lignin-perox: lignin-peroxidase; and cellulase: *Trichoderma reesei* cellulase composition.

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Definitions

Peroxide-generating system: The term "peroxide-generating system" is defined herein as either a peroxide generating enzyme as defined below, or as a chemical reaction

leading to production of peroxide. Common examples of chemical methods of peroxide generation include, but are not limited to, UV-irradiation of Rose Bengal; the Reidl-Pfleiderer process of autooxidation of 2-ethyl-9,10-dihydroxyanthracene + O₂ to 2-ethylanthraquinone + H₂O₂; reaction of singlet state molecular oxygen ¹O₂ with ascorbate; the oxidation of organic alcohols by molecular oxygen in the presence of various metal and metal complex catalysts; and the oxidation of unsaturated lipid by oxygen (after a radical initiation) to form lipid peroxide.

Peroxide-generating enzyme: The term “peroxide-generating enzyme” is defined herein as an donor:oxygen oxidoreductase (E.C. number 1.1.3.x) that catalyzes the reaction reduced substrate(2e⁻) + O₂ → oxidized substrate + H₂O₂, such as glucose oxidase that catalyzes the reaction glucose + O₂ → gluconolactone + H₂O₂; and a donor: superoxide oxidoreductase (E.C. 1.15.1.x), such as superoxide dismutase that catalyzes the reaction 2O₂⁻ + 2H⁺ → O₂ + H₂O₂. Other examples of peroxide-generating enzymes are provided herein. Alternatively oxidoreductases with side activities, wherein molecular oxygen can be used as electron acceptor by the enzyme, are also included within the term hydrogen-peroxide-generating enzyme. In addition to hydrogen peroxide, other peroxides may also be generated by these enzymes.

Peroxidase activity: The term “peroxidase activity” is defined herein as an enzyme activity that converts a peroxide, e.g., hydrogen peroxide, to a less oxidative species, e.g., water. It is understood herein that a polypeptide having peroxidase activity encompasses a peroxide-decomposing enzyme (defined below).

Peroxide-decomposing enzyme: The term “peroxide-decomposing enzyme” is defined herein as an donor:peroxide oxidoreductase (E.C. number 1.11.1.x) that catalyzes the reaction reduced substrate(2e⁻) + ROOR' → oxidized substrate + ROH + R'OH; such as horseradish peroxidase that catalyzes the reaction phenol + H₂O₂ → quinone + H₂O, and catalase that catalyzes the reaction H₂O₂ + H₂O₂ → O₂ + 2H₂O. In addition to hydrogen peroxide, other peroxides may also be decomposed by these enzymes.

Cellobiose dehydrogenase: The term “cellobiose dehydrogenase” is defined herein as a cellobiose:acceptor 1-oxidoreductase (E.C. 1.1.99.18) that catalyzes the conversion of cellobiose in the presence of an acceptor to cellobiono-1,5-lactone and a reduced acceptor. 2,6-Dichloroindophenol can act as acceptor, as can iron, especially Fe(SCN)₃, molecular oxygen, ubiquinone, or cytochrome C, and likely many other polyphenolics. Substrates of the enzyme include cellobiose, cello-oligosaccharides, lactose, and D-glucosyl-1,4-β-D-mannose, glucose, maltose, mannobiose, thiocellobiose, galactosyl-mannose, xylobiose, and xylose. Electron donors are preferably beta-1-4 dihexoses with glucose or mannose at the reducing end, though alpha-1-4 hexosides, hexoses, pentoses, and beta-1-4 pentomers have also been shown to act as substrates for these enzymes

(Henriksson *et al.*, 1998, *Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology*; 1383: 48-54; and Schou *et al.*, 1998, *Biochem. J.* 330: 565-571).

Cellulose dehydrogenases comprise two families, 1 and 2, differentiated by the presence of a cellulose binding motif (CBM). The 3-dimensional structure of cellulose dehydrogenase features two globular domains, each containing one of two cofactors: a heme or a flavin. The active site lies at a cleft between the two domains. The catalytic cycle of cellulose dehydrogenase follows an ordered sequential mechanism. Oxidation of cellulose occurs via 2-electron transfer from cellulose to the flavin, generating cellulobionolactone and reduced flavin. The active FAD is regenerated by electron transfer to the heme group, leaving a reduced heme. The native state heme is regenerated by reaction with the oxidizing substrate at the second active site.

The oxidizing substrate is preferentially iron ferricyanide, cytochrome C, or an oxidized phenolic compound such as dichloroindophenol (DCIP), a substrate commonly used for colorimetric assays. Metal ions and O₂ are also substrates, but for most cellulose dehydrogenases the reaction rate for these substrates is several orders of magnitude lower than that observed for iron or organic oxidants. Following cellulobionolactone release, the product may undergo spontaneous ring-opening to generate cellulobionic acid (Hallberg *et al.*, 2003, *J. Biol. Chem.* 278: 7160-7166).

Cellulolytic activity: The term "cellulolytic activity" is defined herein as a biological activity that hydrolyzes a cellulosic material. 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 No. 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 No. 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 invention, cellulolytic 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 protein/g of cellulose in PCS for 3-7 days at 50-65°C compared to a control hydrolysis without addition of cellulolytic protein. Typical conditions are 1 ml reactions, washed or unwashed PCS, 5% insoluble solids, 50 mM sodium acetate pH 5, 1 mM MnSO₄, 50-65°C, 72 hours, sugar analysis by AMINEX® HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Endoglucanase: The term “endoglucanase” is defined herein as 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 based on a 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) hydrolysis according to the procedure of Ghose, 1987, *Pure and Appl. Chem.* 59: 257-268.

Cellobiohydrolase: The term “cellobiohydrolase” is defined herein as 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, cellooligosaccharides, 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 using a fluorescent disaccharide derivative 4-methylumbelliferyl- β -D-lactoside according to the procedures described by van Tilbeurgh *et al.*, 1982, *FEBS Letters* 149: 152-156 and van Tilbeurgh and Claeysens, 1985, *FEBS Letters* 187: 283-288.

Beta-glucosidase: The term “beta-glucosidase” is defined herein as 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 activity is defined as 1.0 μ mole of p-nitrophenol produced per minute at 40°C, pH 5 from 1 mM p-nitrophenyl-beta-D-glucopyranoside as substrate in 100 mM sodium citrate containing 0.01% TWEEN® 20.

Cellulolytic enhancing activity: The term “cellulolytic enhancing activity” is defined herein as a biological activity that enhances the hydrolysis of a cellulosic material by polypeptides 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 protein 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 protein and 0.5-50%

w/w protein of cellulolytic enhancing activity for 1-7 day at 50-65°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 3% of total protein weight *Aspergillus oryzae* beta-glucosidase (recombinantly produced in *Aspergillus oryzae* according to WO 02/095014) or 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 polypeptides having cellulolytic enhancing activity enhance the hydrolysis of a cellulosic material catalyzed by proteins 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.

Family 61 glycoside hydrolase: The term "Family 61 glycoside hydrolase" or "Family GH61" is defined herein as 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: 695-696. Presently, Henrissat lists the GH61 Family as unclassified indicating that properties such as mechanism, catalytic nucleophile/base, and catalytic proton donors are not known for polypeptides belonging to this family.

Xylan degrading activity: The terms "xylan degrading activity" or "xylanolytic activity" are defined herein as 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 (endoxylanases, beta-xylosidases, arabinofuranosidases, alpha-glucuronidases, acetylxylan esterases, feruloyl esterases, and alpha-glucuronoyl 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 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
5 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.

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.
10 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.

15 **Xylanase activity:** The term "xylanase activity" is defined herein as a 1,4-beta-D-xylan-xylohydrolase activity (E.C. 3.2.1.8) that catalyzes the endo-hydrolysis of 1,4-beta-D-xylosidic linkages in xylans. For purposes of the present invention, xylanase activity is determined using birchwood xylan as substrate. One unit of xylanase activity is defined as 1.0 μ mole of reducing sugar (measured in glucose equivalents as described by Lever, 1972,
20 A new reaction for colorimetric determination of carbohydrates, *Anal. Biochem* 47: 273-279) produced per minute during the initial period of hydrolysis at 50°C, pH 5 from 2 g of birchwood xylan per liter as substrate in 50 mM sodium acetate containing 0.01% TWEEN® 20.

25 **Beta-xylosidase activity:** The term "beta-xylosidase activity" is defined herein as a beta-D-xyloside xylohydrolase (E.C. 3.2.1.37) that catalyzes the exo-hydrolysis of short beta (1→4)-xylooligosaccharides, to remove successive D-xylose residues from the non-reducing termini. For purposes of the present invention, one unit of beta-xylosidase activity is defined as 1.0 μ mole of p-nitrophenol 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.

30 **Acetylxylan esterase activity:** The term "acetylxylan esterase activity" is defined herein as a carboxylesterase activity (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, acetylxylan esterase activity is determined using 0.5 mM p-nitrophenylacetate as substrate in 50 mM sodium
35 acetate pH 5.0 containing 0.01% TWEEN™ 20. One unit of acetylxylan esterase activity is defined as the amount of enzyme capable of releasing 1 μ mole of p-nitrophenolate anion per minute at pH 5, 25°C.

Feruloyl esterase activity: The term "feruloyl esterase activity" is defined herein as a 4-hydroxy-3-methoxycinnamoyl-sugar hydrolase activity (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. 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 activity equals the amount of enzyme capable of releasing 1 μ mole of *p*-nitrophenolate anion per minute at pH 5, 25°C.

Alpha-glucuronidase activity: The term "alpha-glucuronidase activity" is defined herein as an alpha-D-glucosiduronate glucuronohydrolase activity (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 activity equals the amount of enzyme capable of releasing 1 μ mole of glucuronic or 4-O-methylglucuronic acid per minute at pH 5, 40°C.

Alpha-L-arabinofuranosidase activity: The term "alpha-L-arabinofuranosidase activity" is defined herein as an alpha-L-arabinofuranoside arabinofuranohydrolase activity (EC 3.2.1.55) that catalyzes the hydrolysis of terminal non-reducing alpha-L-arabinofuranoside residues in alpha-L-arabinosides. The enzyme activity 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 μ l 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
5 hemicelluloses, which help stabilize the cell wall matrix.

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, Wiselogel *et al.*,
10 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
15 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
20 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 corn stover. In another aspect, the cellulosic material is corn fiber. In another aspect, the cellulosic material is corn cob. In
25 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
30 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
35 conventional methods known in the art, as described herein. In a preferred aspect, the cellulosic material is pretreated.

Pretreated corn stover: The term “PCS” or “Pretreated Corn Stover” is defined herein as a cellulosic material derived from corn stover by treatment with heat and dilute sulfuric acid.

Xylan-containing material: The term “xylan-containing material” is defined herein as any material comprising a plant cell wall polysaccharide containing a backbone of beta-(1-4)-linked xylose residues. Xylans of terrestrial plants are heteropolymers possessing a beta-(1-4)-D-xylopyranose backbone, which is branched by short carbohydrate chains. They comprise D-glucuronic acid or its 4-O-methyl ether, L-arabinose, and/or various oligosaccharides, composed of D-xylose, L-arabinose, D- or L-galactose, and D-glucose. Xylan-type polysaccharides can be divided into homoxylans and heteroxylans, which include glucuronoxylans, (arabino)glucuronoxylans, (glucurono)arabinoxylans, arabinoxylans, and complex heteroxylans. See, for example, Ebringerova *et al.*, 2005, *Adv. Polym. Sci.* 186: 1–67.

In the methods of the present invention, any material containing xylan may be used. In a preferred aspect, the xylan-containing material is lignocellulose.

Isolated polypeptide: The term “isolated polypeptide” as used herein refers to a polypeptide that is isolated from a source. In a preferred aspect, the polypeptide is at least 1% pure, preferably at least 5% pure, more preferably at least 10% pure, more preferably at least 20% pure, more preferably at least 40% pure, more preferably at least 60% pure, even more preferably at least 80% pure, and most preferably at least 90% pure, as determined by SDS-PAGE.

Substantially pure polypeptide: The term “substantially pure polypeptide” denotes herein a polypeptide preparation that contains at most 10%, preferably at most 8%, more preferably at most 6%, more preferably at most 5%, more preferably at most 4%, more preferably at most 3%, even more preferably at most 2%, most preferably at most 1%, and even most preferably at most 0.5% by weight of other polypeptide material with which it is natively or recombinantly associated. It is, therefore, preferred that the substantially pure polypeptide is at least 92% pure, preferably at least 94% pure, more preferably at least 95% pure, more preferably at least 96% pure, more preferably at least 97% pure, more preferably at least 98% pure, even more preferably at least 99% pure, most preferably at least 99.5% pure, and even most preferably 100% pure by weight of the total polypeptide material present in the preparation. The polypeptides are preferably in a substantially pure form, *i.e.*, that the polypeptide preparation is essentially free of other polypeptide material with which it is natively or recombinantly associated. This can be accomplished, for example, by preparing the polypeptide by well-known recombinant methods or by classical purification methods.

Mature polypeptide: The term “mature polypeptide” is defined herein as a

polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc.

Mature polypeptide coding sequence: The term “mature polypeptide coding sequence” is defined herein as a nucleotide sequence that encodes a mature polypeptide.

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

For purposes of the present invention, the degree of 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.*, 10 2000, *Trends in Genetics* 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:

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

For purposes of the present invention, the degree of 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.*, 20 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:

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

30 **Homologous sequence:** The term “homologous sequence” is defined herein as a predicted protein having an E value (or expectancy score) of less than 0.001 in a tfasty search (Pearson, W.R., 1999, in *Bioinformatics Methods and Protocols*, S. Misener and S. A. Krawetz, ed., pp. 185-219) with a polypeptide of interest.

Polypeptide fragment: The term “polypeptide fragment” is defined herein as a polypeptide having one or more (several) amino acids deleted from the amino and/or carboxyl terminus of a mature polypeptide or a homologous sequence thereof, wherein the 35 fragment has biological activity.

Subsequence: The term “subsequence” is defined herein as a nucleotide sequence having one or more (several) nucleotides deleted from the 5' and/or 3' end of a mature

polypeptide coding sequence or a homologous sequence thereof, wherein the subsequence encodes a polypeptide fragment having biological activity.

Allelic variant: The term “allelic variant” denotes herein 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.

Isolated polynucleotide: The term “isolated polynucleotide” as used herein refers to a polynucleotide that is isolated from a source. In a preferred aspect, the polynucleotide is at least 1% pure, preferably at least 5% pure, more preferably at least 10% pure, more preferably at least 20% pure, more preferably at least 40% pure, more preferably at least 60% pure, even more preferably at least 80% pure, and most preferably at least 90% pure, as determined by agarose electrophoresis.

Substantially pure polynucleotide: The term “substantially pure polynucleotide” as used herein refers to a polynucleotide preparation free of other extraneous or unwanted nucleotides and in a form suitable for use within genetically engineered protein production systems. Thus, a substantially pure polynucleotide contains at most 10%, preferably at most 8%, more preferably at most 6%, more preferably at most 5%, more preferably at most 4%, more preferably at most 3%, even more preferably at most 2%, most preferably at most 1%, and even most preferably at most 0.5% by weight of other polynucleotide material with which it is natively or recombinantly associated. A substantially pure polynucleotide may, however, include naturally occurring 5' and 3' untranslated regions, such as promoters and terminators. It is preferred that the substantially pure polynucleotide is at least 90% pure, preferably at least 92% pure, more preferably at least 94% pure, more preferably at least 95% pure, more preferably at least 96% pure, more preferably at least 97% pure, even more preferably at least 98% pure, most preferably at least 99% pure, and even most preferably at least 99.5% pure by weight. The polynucleotides are preferably in a substantially pure form, *i.e.*, that the polynucleotide preparation is essentially free of other polynucleotide material with which it is natively or recombinantly associated. The polynucleotides may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

Coding sequence: When used herein the term “coding sequence” means a nucleotide sequence, which directly specifies the amino acid sequence of its protein product. 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 nucleotide sequence.

cDNA: The term "cDNA" is defined herein as a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic 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 before appearing as mature spliced mRNA. These steps include the removal of intron sequences by a process called splicing. cDNA derived from mRNA lacks, therefore, any intron sequences.

Nucleic acid construct: The term "nucleic acid construct" as used herein refers to a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or which 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" is defined herein to include all components necessary for the expression of a polynucleotide encoding a polypeptide. Each control sequence may be native or foreign to the nucleotide sequence 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 nucleotide sequence encoding a polypeptide.

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

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" is defined herein as 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", as used herein, includes any cell type that is susceptible to transformation, transfection, transduction, and the like with a nucleic acid construct or expression vector comprising a polynucleotide of the present invention.

Modification: The term "modification" means herein any chemical modification of a

polypeptide, as well as genetic manipulation of the DNA encoding the polypeptide. The modification can be a substitution, a deletion and/or an insertion of one or more (several) amino acids as well as replacements of one or more (several) amino acid side chains.

Artificial variant: When used herein, the term "artificial variant" means a polypeptide produced by an organism expressing a modified polynucleotide sequence encoding a polypeptide variant. The modified nucleotide sequence is obtained through human intervention by modification of the polynucleotide sequence.

Detailed Description of the Invention

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The present invention relates to methods for degrading or converting a cellulosic material, comprising: treating the cellulosic material with an enzyme composition in the presence of a polypeptide having peroxidase activity. In one aspect, the method further comprises recovering the degraded or converted cellulosic material.

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The present invention also relates to methods for producing a fermentation product, comprising: (a) saccharifying a cellulosic material with an enzyme composition in the presence of a polypeptide having peroxidase activity; (b) fermenting the saccharified cellulosic material with one or more (several) fermenting microorganisms to produce the fermentation product; and (c) recovering the fermentation product from the fermentation.

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The present invention further relates to methods of fermenting a cellulosic material, comprising: fermenting the cellulosic material with one or more (several) fermenting microorganisms, wherein the cellulosic material is hydrolyzed with an enzyme composition in the presence of a polypeptide having peroxidase activity. In one aspect, the fermenting of the cellulosic material produces a fermentation product. In another aspect, the method further comprises recovering the fermentation product from the fermentation.

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In each of the methods described above, the presence of the polypeptide having peroxidase activity increases the hydrolysis of the cellulosic material compared to the absence of the polypeptide having peroxidase activity.

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Preferably, the K_m of the peroxide-decomposing enzyme or peroxidase is in the range of preferably 0.0001 to 50 mM, more preferably 0.001 to 10 mM, even more preferably 0.005 to 1 mM, and most preferably 0.01 to 0.1 mM. In one aspect, the K_m of the peroxide-decomposing enzyme or peroxidase is in the range of 0.0001 to 50 mM. In another aspect, the K_m of the peroxide-decomposing enzyme or peroxidase is in the range of 0.001 to 10 mM. In another aspect, the K_m of the peroxide-decomposing enzyme or peroxidase is in the range of 0.005 to 1 mM. In another aspect, the K_m of the peroxide-decomposing enzyme or peroxidase is in the range of 0.01 to 0.1 mM.

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In one aspect, in each of the methods described above, the enzyme composition

further comprises a peroxide-generating system. In another aspect, the cellulosic material comprises a peroxide-generating system. The presence of the peroxide-generating system and the polypeptide having peroxidase activity increases the hydrolysis of the cellulosic material compared to the presence of the peroxide-generating system and the absence of the polypeptide having peroxidase activity.

In another aspect, the peroxide-generating system is a hydrogen peroxide-generating enzyme.

The methods of the present invention can be used to saccharify a cellulosic material to fermentable sugars and convert the fermentable sugars to many useful substances, e.g., fuel, potable ethanol, and/or fermentation products (e.g., acids, alcohols, ketones, gases, and the like). The production of a desired fermentation product from cellulosic material typically involves pretreatment, enzymatic hydrolysis (saccharification), and fermentation.

The processing of cellulosic material according to the present invention can be accomplished using processes conventional in the art. Moreover, the methods of the present invention can be implemented using any conventional biomass processing apparatus configured to operate in accordance with the invention.

Hydrolysis (saccharification) and fermentation, separate or simultaneous, include, but are not limited to, separate hydrolysis and fermentation (SHF); simultaneous saccharification and fermentation (SSF); simultaneous saccharification and cofermentation (SSCF); hybrid hydrolysis and fermentation (HHF); separate hydrolysis and co-fermentation (SHCF); hybrid hydrolysis and fermentation (HHCF); and direct microbial conversion (DMC). SHF uses separate process steps to first enzymatically hydrolyze cellulosic material to fermentable sugars, e.g., glucose, cellobiose, cellotriose, and pentose sugars, and then ferment the fermentable sugars to ethanol. In SSF, the enzymatic hydrolysis of cellulosic material and the fermentation of sugars to ethanol are combined in one step (Philippidis, G. P., 1996, Cellulose bioconversion technology, in *Handbook on Bioethanol: Production and Utilization*, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212). SSCF involves the cofermentation of multiple sugars (Sheehan, J., and Himmel, M., 1999, Enzymes, energy and the environment: A strategic perspective on the U.S. Department of Energy's research and development activities for bioethanol, *Biotechnol. Prog.* 15: 817-827). HHF involves a separate hydrolysis step, and in addition a simultaneous saccharification and hydrolysis step, which can be carried out in the same reactor. The steps in an HHF process can be carried out at different temperatures, i.e., high temperature enzymatic saccharification followed by SSF at a lower temperature that the fermentation strain can tolerate. DMC combines all three processes (enzyme production, hydrolysis, and fermentation) in one or more (several) steps where the same organism is used to produce the enzymes for conversion of the cellulosic material to fermentable sugars and to convert the fermentable

sugars into a final product (Lynd, L. R., Weimer, P. J., van Zyl, W. H., and Pretorius, I. S., 2002, Microbial cellulose utilization: Fundamentals and biotechnology, *Microbiol. Mol. Biol. Reviews* 66: 506-577). It is understood herein that any method known in the art comprising pretreatment, enzymatic hydrolysis (saccharification), fermentation, or a combination thereof, can be used in the practicing the methods of the present invention.

A conventional apparatus can include a fed-batch stirred reactor, a batch stirred reactor, a continuous flow stirred reactor with ultrafiltration, and/or a continuous plug-flow column reactor (Fernanda de Castilhos Corazza, Flávio Faria de Moraes, Gisella Maria Zanin and Ivo Neitzel, 2003, Optimal control in fed-batch reactor for the cellobiose hydrolysis, *Acta Scientiarum. Technology* 25: 33-38; Gusakov, A. V., and Sinitsyn, A. P., 1985, Kinetics of the enzymatic hydrolysis of cellulose: 1. A mathematical model for a batch reactor process, *Enz. Microb. Technol.* 7: 346-352), an attrition reactor (Ryu, S. K., and Lee, J. M., 1983, Bioconversion of waste cellulose by using an attrition bioreactor, *Biotechnol. Bioeng.* 25: 53-65), or a reactor with intensive stirring induced by an electromagnetic field (Gusakov, A. V., Sinitsyn, A. P., Davydkin, I. Y., Davydkin, V. Y., Protas, O. V., 1996, Enhancement of enzymatic cellulose hydrolysis using a novel type of bioreactor with intensive stirring induced by electromagnetic field, *Appl. Biochem. Biotechnol.* 56: 141-153). Additional reactor types include: fluidized bed, upflow blanket, immobilized, and extruder type reactors for hydrolysis and/or fermentation.

Pretreatment. In practicing the methods of the present invention, any pretreatment process known in the art can be used to disrupt plant cell wall components of cellulosic material (Chandra *et al.*, 2007, Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics? *Adv. Biochem. Engin./Biotechnol.* 108: 67-93; Galbe and Zacchi, 2007, Pretreatment of lignocellulosic materials for efficient bioethanol production, *Adv. Biochem. Engin. / Biotechnol.* 108: 41-65; Hendriks and Zeeman, 2009, Pretreatments to enhance the digestibility of lignocellulosic biomass, *Bioresource Technol.* 100: 10-18; Mosier *et al.*, 2005, Features of promising technologies for pretreatment of lignocellulosic biomass, *Bioresource Technol.* 96: 673-686; Taherzadeh and Karimi, 2008, Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review, *Int. J. of Mol. Sci.* 9: 1621-1651; Yang and Wyman, 2008, Pretreatment: the key to unlocking low-cost cellulosic ethanol, *Biofuels Bioproducts and Biorefining-Biofpr.* 2: 26-40).

The cellulosic material can also be subjected to particle size reduction, pre-soaking, wetting, washing, or conditioning prior to pretreatment using methods known in the art.

Conventional pretreatments include, but are not limited to, steam pretreatment (with or without explosion), dilute acid pretreatment, hot water pretreatment, alkaline pretreatment, lime pretreatment, wet oxidation, wet explosion, ammonia fiber explosion, organosolv pretreatment, and biological pretreatment. Additional pretreatments include ammonia

percolation, ultrasound, electroporation, microwave, supercritical CO₂, supercritical H₂O, ozone, and gamma irradiation pretreatments.

The cellulosic material can be pretreated before hydrolysis and/or fermentation. Pretreatment is preferably performed prior to the hydrolysis. Alternatively, the pretreatment can be carried out simultaneously with enzyme hydrolysis to release fermentable sugars, such as glucose, xylose, and/or cellobiose. In most cases the pretreatment step itself results in some conversion of biomass to fermentable sugars (even in absence of enzymes).

Steam Pretreatment. In steam pretreatment, cellulosic material is heated to disrupt the plant cell wall components, including lignin, hemicellulose, and cellulose to make the cellulose and other fractions, e.g., hemicellulose, accessible to enzymes. Cellulosic material is passed to or through a reaction vessel where steam is injected to increase the temperature to the required temperature and pressure and is retained therein for the desired reaction time. Steam pretreatment is preferably done at 140-230°C, more preferably 160-200°C, and most preferably 170-190°C, where the optimal temperature range depends on any addition of a chemical catalyst. Residence time for the steam pretreatment is preferably 1-15 minutes, more preferably 3-12 minutes, and most preferably 4-10 minutes, where the optimal residence time depends on temperature range and any addition of a chemical catalyst. Steam pretreatment allows for relatively high solids loadings, so that cellulosic material is generally only moist during the pretreatment. The steam pretreatment is often combined with an explosive discharge of the material after the pretreatment, which is known as steam explosion, that is, rapid flashing to atmospheric pressure and turbulent flow of the material to increase the accessible surface area by fragmentation (Duff and Murray, 1996, *Bioresource Technology* 855: 1-33; Galbe and Zacchi, 2002, *Appl. Microbiol. Biotechnol.* 59: 618-628; U.S. Patent Application No. 20020164730). During steam pretreatment, hemicellulose acetyl groups are cleaved and the resulting acid autocatalyzes partial hydrolysis of the hemicellulose to monosaccharides and oligosaccharides. Lignin is removed to only a limited extent.

A catalyst such as H₂SO₄ or SO₂ (typically 0.3 to 3% w/w) is often added prior to steam pretreatment, which decreases the time and temperature, increases the recovery, and improves enzymatic hydrolysis (Ballesteros *et al.*, 2006, *Appl. Biochem. Biotechnol.* 129-132: 496-508; Varga *et al.*, 2004, *Appl. Biochem. Biotechnol.* 113-116: 509-523; Sassner *et al.*, 2006, *Enzyme Microb. Technol.* 39: 756-762).

Chemical Pretreatment: The term "chemical treatment" refers to any chemical pretreatment that promotes the separation and/or release of cellulose, hemicellulose, and/or lignin. Examples of suitable chemical pretreatment processes include, for example, dilute acid pretreatment, lime pretreatment, wet oxidation, ammonia fiber/freeze explosion (AFEX), ammonia percolation (APR), and organosolv pretreatments.

In dilute acid pretreatment, cellulosic material is mixed with dilute acid, typically H₂SO₄, and water to form a slurry, heated by steam to the desired temperature, and after a residence time flashed to atmospheric pressure. The dilute acid pretreatment can be performed with a number of reactor designs, e.g., plug-flow reactors, counter-current reactors, or continuous counter-current shrinking bed reactors (Duff and Murray, 1996, *supra*; Schell *et al.*, 2004, *Bioresource Technol.* 91: 179-188; Lee *et al.*, 1999, *Adv. Biochem. Eng. Biotechnol.* 65: 93-115).

Several methods of pretreatment under alkaline conditions can also be used. These alkaline pretreatments include, but are not limited to, lime pretreatment, wet oxidation, ammonia percolation (APR), and ammonia fiber/freeze explosion (AFEX).

Lime pretreatment is performed with calcium carbonate, sodium hydroxide, or ammonia at low temperatures of 85-150°C and residence times from 1 hour to several days (Wyman *et al.*, 2005, *Bioresource Technol.* 96: 1959-1966; Mosier *et al.*, 2005, *Bioresource Technol.* 96: 673-686). WO 2006/110891, WO 2006/11899, WO 2006/11900, and WO 2006/110901 disclose pretreatment methods using ammonia.

Wet oxidation is a thermal pretreatment performed typically at 180-200°C for 5-15 minutes with addition of an oxidative agent such as hydrogen peroxide or over-pressure of oxygen (Schmidt and Thomsen, 1998, *Bioresource Technol.* 64: 139-151; Palonen *et al.*, 2004, *Appl. Biochem. Biotechnol.* 117: 1-17; Varga *et al.*, 2004, *Biotechnol. Bioeng.* 88: 567-574; Martin *et al.*, 2006, *J. Chem. Technol. Biotechnol.* 81: 1669-1677). The pretreatment is performed at preferably 1-40% dry matter, more preferably 2-30% dry matter, and most preferably 5-20% dry matter, and often the initial pH is increased by the addition of alkali such as sodium carbonate.

A modification of the wet oxidation pretreatment method, known as wet explosion (combination of wet oxidation and steam explosion), can handle dry matter up to 30%. In wet explosion, the oxidizing agent is introduced during pretreatment after a certain residence time. The pretreatment is then ended by flashing to atmospheric pressure (WO 2006/032282).

Ammonia fiber explosion (AFEX) involves treating cellulosic material with liquid or gaseous ammonia at moderate temperatures such as 90-100°C and high pressure such as 17-20 bar for 5-10 minutes, where the dry matter content can be as high as 60% (Gollapalli *et al.*, 2002, *Appl. Biochem. Biotechnol.* 98: 23-35; Chundawat *et al.*, 2007, *Biotechnol. Bioeng.* 96: 219-231; Alizadeh *et al.*, 2005, *Appl. Biochem. Biotechnol.* 121: 1133-1141; Teymouri *et al.*, 2005, *Bioresource Technol.* 96: 2014-2018). AFEX pretreatment results in the depolymerization of cellulose and partial hydrolysis of hemicellulose. Lignin-carbohydrate complexes are cleaved.

Organosolv pretreatment delignifies cellulosic material by extraction using aqueous ethanol (40-60% ethanol) at 160-200°C for 30-60 minutes (Pan *et al.*, 2005, *Biotechnol. Bioeng.*

90: 473-481; Pan *et al.*, 2006, *Biotechnol. Bioeng.* 94: 851-861; Kurabi *et al.*, 2005, *Appl. Biochem. Biotechnol.* 121: 219-230). Sulphuric acid is usually added as a catalyst. In organosolv pretreatment, the majority of hemicellulose is removed.

Other examples of suitable pretreatment methods are described by Schell *et al.*, 2003, *Appl. Biochem. and Biotechnol.* Vol. 105-108, p. 69-85, and Mosier *et al.*, 2005, *Bioresource Technology* 96: 673-686, and U.S. Published Application 2002/0164730.

In one aspect, the chemical pretreatment is preferably carried out as an acid treatment, and more preferably as a continuous dilute and/or mild acid treatment. The acid is typically sulfuric acid, but other acids can also be used, such as acetic acid, citric acid, nitric acid, phosphoric acid, tartaric acid, succinic acid, hydrogen chloride, or mixtures thereof. Mild acid treatment is conducted in the pH range of preferably 1-5, more preferably 1-4, and most preferably 1-3. In one aspect, the acid concentration is in the range from preferably 0.01 to 20 wt % acid, more preferably 0.05 to 10 wt % acid, even more preferably 0.1 to 5 wt % acid, and most preferably 0.2 to 2.0 wt % acid. The acid is contacted with cellulosic material and held at a temperature in the range of preferably 160-220°C, and more preferably 165-195°C, for periods ranging from seconds to minutes to, *e.g.*, 1 second to 60 minutes.

In another aspect, pretreatment is carried out as an ammonia fiber explosion step (AFEX pretreatment step).

In another aspect, pretreatment takes place in an aqueous slurry. In preferred aspects, cellulosic material is present during pretreatment in amounts preferably between 10-80 wt%, more preferably between 20-70 wt%, and most preferably between 30-60 wt%, such as around 50 wt%. The pretreated cellulosic material can be unwashed or washed using any method known in the art, *e.g.*, washed with water.

Mechanical Pretreatment: The term "mechanical pretreatment" refers to various types of grinding or milling (*e.g.*, dry milling, wet milling, or vibratory ball milling).

Physical Pretreatment: The term "physical pretreatment" refers to any pretreatment that promotes the separation and/or release of cellulose, hemicellulose, and/or lignin from cellulosic material. For example, physical pretreatment can involve irradiation (*e.g.*, microwave irradiation), steaming/steam explosion, hydrothermolysis, and combinations thereof.

Physical pretreatment can involve high pressure and/or high temperature (steam explosion). In one aspect, high pressure means pressure in the range of preferably about 300 to about 600 psi, more preferably about 350 to about 550 psi, and most preferably about 400 to about 500 psi, such as around 450 psi. In another aspect, high temperature means temperatures in the range of about 100 to about 300°C, preferably about 140 to about 235°C. In a preferred aspect, mechanical pretreatment is performed in a batch-process, steam gun hydrolyzer system that uses high pressure and high temperature as defined above, *e.g.*, a Sunds Hydrolyzer available from Sunds Defibrator AB, Sweden.

Combined Physical and Chemical Pretreatment: Cellulosic material can be pretreated both physically and chemically. For instance, the pretreatment step can involve dilute or mild acid treatment and high temperature and/or pressure treatment. The physical and chemical pretreatments can be carried out sequentially or simultaneously, as desired. A mechanical pretreatment can also be included.

Accordingly, in a preferred aspect, cellulosic material is subjected to mechanical, chemical, or physical pretreatment, or any combination thereof, to promote the separation and/or release of cellulose, hemicellulose, and/or lignin.

Biological Pretreatment: The term "biological pretreatment" refers to any biological pretreatment that promotes the separation and/or release of cellulose, hemicellulose, and/or lignin from cellulosic material. Biological pretreatment 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 and Singh, 1993, Physicochemical and biological treatments for enzymatic/microbial conversion of cellulosic 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 and Hahn-Hagerdal, 1996, Fermentation of lignocellulosic hydrolysates for ethanol production, *Enz. Microb. Tech.* 18: 312-331; and Vallander and Eriksson, 1990, Production of ethanol from lignocellulosic materials: State of the art, *Adv. Biochem. Eng./Biotechnol.* 42: 63-95).

Saccharification. In the hydrolysis step, also known as saccharification, the pretreated cellulosic material 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 in the presence of a polypeptide having peroxidase activity of the present invention. The composition can further comprise one or more (several) hemicellulolytic enzymes. The enzymes of the compositions can also 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 and polypeptides having cellulolytic enhancing activity depend on several factors including, but not limited to, the mixture of component cellulolytic enzymes, the cellulosic substrate, the concentration of cellulosic substrate, the pretreatment(s) of the cellulosic substrate, temperature, time, pH, and inclusion of fermenting organism (e.g., yeast for Simultaneous Saccharification and Fermentation).

In a preferred aspect, an effective amount of cellulolytic enzyme(s) 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 preferred aspect, an effective amount of a polypeptide having peroxidase activity to cellulosic material is about 0.001 to about 50 mg, preferably at about 0.01 to about 40 mg, more preferably at about 0.02 to about 25 mg, more preferably at about 0.03 to about 20 mg, more preferably at about 0.04 to about 15 mg, even more preferably at about 0.04 to about 10 mg, and most preferably at about 0.05 to about 5 mg per g of cellulosic material.

In another preferred aspect, an effective amount of polypeptide(s) 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 preferred aspect, an effective amount of a polypeptide having peroxidase activity to a hydrogen peroxide-generating enzyme 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 hydrogen peroxide-generating enzyme.

In another preferred aspect, an effective amount of polypeptide(s) having cellulolytic enhancing activity to cellulolytic protein(s) 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 protein(s).

Fermentation. The fermentable sugars obtained from the pretreated and 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.

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.

Any suitable hydrolyzed cellulosic material can be used in the fermentation step in practicing the present invention. The material is generally selected based on the desired fermentation product, i.e., the substance to be obtained from the fermentation, and the process employed, as is well known in the art.

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).

"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.

5 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*.

10 Examples of fermenting organisms that can ferment C₅ sugars include bacterial and fungal organisms, such as 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*.

15 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*; and *E. coli*, especially *E. coli* strains that have been genetically modified to improve the yield of ethanol.

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).

35 Bacteria that can efficiently ferment hexose and pentose to ethanol include, for example, *Zymomonas mobilis* and *Clostridium thermocellum* (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 methods 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: A 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, acetonic 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); 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 more 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,
5 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
10 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
15 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 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*,
20 Vol. 13 (1-2), pp. 83-114, 1997, Anaerobic digestion of biomass for methane production: A review.

Recovery. 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.
30 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.

35 **Polypeptides Having Cellulolytic Enhancing Activity and Polynucleotides Thereof**

In the methods of the present invention, any 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],

5 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],

10 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].

20 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],

25 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: 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, or SEQ ID NO: 16 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 95%, and even most preferably at least 96%, at least 97%, at least 98%, or at least 99% (hereinafter "homologous polypeptides"). In a preferred aspect, the mature polypeptide sequence is amino acids 20 to 326 of SEQ ID NO: 2, amino acids 18 to 239 of SEQ ID NO: 4, amino acids 20 to 258 of SEQ ID NO: 6, amino acids 19 to 226 of SEQ ID NO: 8, amino acids 20 to 304 of SEQ ID NO: 10, amino acids 16 to 317 of SEQ ID NO: 12, amino acids 23 to 250 of SEQ ID NO: 14, or amino acids 20 to 249 of SEQ ID NO: 16.

A polypeptide having cellulolytic enhancing activity preferably comprises the amino acid sequence of SEQ ID NO: 2 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises the amino acid sequence of SEQ ID NO: 2. In another preferred aspect, the polypeptide comprises the mature polypeptide of SEQ ID NO: 2. In another preferred aspect, the polypeptide comprises amino acids 20 to 326 of SEQ ID NO: 2, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises amino acids 20 to 326 of SEQ ID NO: 2. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 2 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 2. In another preferred aspect, the polypeptide consists of the mature polypeptide of SEQ ID NO: 2. In another preferred aspect, the polypeptide consists of amino acids 20 to 326 of SEQ ID NO: 2 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide consists of amino acids 20 to 326 of SEQ ID NO: 2.

A polypeptide having cellulolytic enhancing activity preferably comprises the amino acid sequence of SEQ ID NO: 4 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises the amino acid sequence of SEQ ID NO: 4. In another preferred aspect, the polypeptide comprises the mature polypeptide of SEQ ID NO: 4. In another preferred aspect, the polypeptide comprises amino acids 18 to 239 of SEQ ID NO: 4, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises amino acids 18 to 239 of SEQ ID NO: 4. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 4 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 4. In another preferred aspect, the polypeptide consists of the mature polypeptide of SEQ ID NO: 4. In another preferred aspect, the polypeptide consists of amino acids 18 to 239 of SEQ ID NO: 4 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide consists of amino acids 18 to 239 of SEQ ID NO: 4.

A polypeptide having cellulolytic enhancing activity preferably comprises the amino acid sequence of SEQ ID NO: 6 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises the amino acid sequence of SEQ ID NO: 6. In another preferred aspect, the polypeptide comprises the mature polypeptide of SEQ ID NO: 6. In another preferred aspect, the polypeptide

comprises amino acids 20 to 258 of SEQ ID NO: 6, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises amino acids 20 to 258 of SEQ ID NO: 6. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 6 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 6. In another preferred aspect, the polypeptide consists of the mature polypeptide of SEQ ID NO: 6. In another preferred aspect, the polypeptide consists of amino acids 20 to 258 of SEQ ID NO: 6 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide consists of amino acids 20 to 258 of SEQ ID NO: 6.

A polypeptide having cellulolytic enhancing activity preferably comprises the amino acid sequence of SEQ ID NO: 8 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises the amino acid sequence of SEQ ID NO: 8. In another preferred aspect, the polypeptide comprises the mature polypeptide of SEQ ID NO: 8. In another preferred aspect, the polypeptide comprises amino acids 19 to 226 of SEQ ID NO: 8, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises amino acids 19 to 226 of SEQ ID NO: 8. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 8 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 8. In another preferred aspect, the polypeptide consists of the mature polypeptide of SEQ ID NO: 8. In another preferred aspect, the polypeptide consists of amino acids 19 to 226 of SEQ ID NO: 8 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide consists of amino acids 19 to 226 of SEQ ID NO: 8.

A polypeptide having cellulolytic enhancing activity preferably comprises the amino acid sequence of SEQ ID NO: 10 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises the amino acid sequence of SEQ ID NO: 10. In another preferred aspect, the polypeptide comprises the mature polypeptide of SEQ ID NO: 10. In another preferred aspect, the polypeptide comprises amino acids 20 to 304 of SEQ ID NO: 10, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises amino acids 20 to 304 of SEQ ID NO: 10. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 10 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another

preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 10. In another preferred aspect, the polypeptide consists of the mature polypeptide of SEQ ID NO: 10. In another preferred aspect, the polypeptide consists of amino acids 20 to 304 of SEQ ID NO: 10 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide consists of amino acids 20 to 304 of SEQ ID NO: 10.

A polypeptide having cellulolytic enhancing activity preferably comprises the amino acid sequence of SEQ ID NO: 12 or an allelic variant thereof; or a fragment thereof having cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises the amino acid sequence of SEQ ID NO: 12. In another preferred aspect, the polypeptide comprises the mature polypeptide of SEQ ID NO: 12. In another preferred aspect, the polypeptide comprises amino acids 16 to 317 of SEQ ID NO: 12, or an allelic variant thereof; or a fragment thereof having cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises amino acids 16 to 317 of SEQ ID NO: 12. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 12 or an allelic variant thereof; or a fragment thereof having cellulolytic enhancing activity. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 12. In another preferred aspect, the polypeptide consists of the mature polypeptide of SEQ ID NO: 12. In another preferred aspect, the polypeptide consists of amino acids 16 to 317 of SEQ ID NO: 12 or an allelic variant thereof; or a fragment thereof having cellulolytic enhancing activity. In another preferred aspect, the polypeptide consists of amino acids 16 to 317 of SEQ ID NO: 12.

A polypeptide having cellulolytic enhancing activity preferably comprises the amino acid sequence of SEQ ID NO: 14 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises the amino acid sequence of SEQ ID NO: 14. In another preferred aspect, the polypeptide comprises the mature polypeptide of SEQ ID NO: 14. In another preferred aspect, the polypeptide comprises amino acids 23 to 250 of SEQ ID NO: 14, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises amino acids 23 to 250 of SEQ ID NO: 14. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 14 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 14. In another preferred aspect, the polypeptide consists of the mature polypeptide of SEQ ID NO: 14. In another preferred aspect, the polypeptide consists of amino acids 23 to 250 of SEQ ID NO: 14 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide consists of amino acids 23 to 250 of

SEQ ID NO: 14.

A polypeptide having cellulolytic enhancing activity preferably comprises the amino acid sequence of SEQ ID NO: 16 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises the amino acid sequence of SEQ ID NO: 16. In another preferred aspect, the polypeptide comprises the mature polypeptide of SEQ ID NO: 16. In another preferred aspect, the polypeptide comprises amino acids 20 to 249 of SEQ ID NO: 16, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises amino acids 20 to 249 of SEQ ID NO: 16. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 16 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 16. In another preferred aspect, the polypeptide consists of the mature polypeptide of SEQ ID NO: 16. In another preferred aspect, the polypeptide consists of amino acids 20 to 249 of SEQ ID NO: 16 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide consists of amino acids 20 to 249 of SEQ ID NO: 16.

Preferably, a fragment of the mature polypeptide of SEQ ID NO: 2 contains at least 277 amino acid residues, more preferably at least 287 amino acid residues, and most preferably at least 297 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 4 contains at least 185 amino acid residues, more preferably at least 195 amino acid residues, and most preferably at least 205 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 6 contains at least 200 amino acid residues, more preferably at least 212 amino acid residues, and most preferably at least 224 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 8 contains at least 175 amino acid residues, more preferably at least 185 amino acid residues, and most preferably at least 195 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 10 contains at least 240 amino acid residues, more preferably at least 255 amino acid residues, and most preferably at least 270 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 12 contains at least 255 amino acid residues, more preferably at least 270 amino acid residues, and most preferably at least 285 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 14 contains at least 175 amino acid residues, more preferably at least 190 amino acid residues, and most preferably at least 205 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 16 contains at least 200 amino acid residues, more preferably at least 210 amino acid residues, and most preferably at least 220 amino acid residues.

Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 1 contains at least 831 nucleotides, more preferably at least 861 nucleotides, and most preferably at least 891 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 3 contains at least 555 nucleotides, more preferably at least 585 nucleotides, and most preferably at least 615 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 5 contains at least 600 nucleotides, more preferably at least 636 nucleotides, and most preferably at least 672 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 7 contains at least 525 nucleotides, more preferably at least 555 nucleotides, and most preferably at least 585 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 9 contains at least 720 nucleotides, more preferably at least 765 nucleotides, and most preferably at least 810 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 11 contains at least 765 nucleotides, more preferably at least 810 nucleotides, and most preferably at least 855 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of nucleotides 67 to 796 of SEQ ID NO: 13 contains at least 525 nucleotides, more preferably at least 570 nucleotides, and most preferably at least 615 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 15 contains at least 600 nucleotides, more preferably at least 630 nucleotides, and most preferably at least 660 nucleotides.

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: 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, or SEQ ID NO: 15, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, or SEQ ID NO: 13, or the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 15, (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: 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, or SEQ ID NO: 15 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 preferred aspect, the mature polypeptide coding sequence is

nucleotides 388 to 1332 of SEQ ID NO: 1, nucleotides 98 to 821 of SEQ ID NO: 3, nucleotides 126 to 978 of SEQ ID NO: 5, nucleotides 55 to 678 of SEQ ID NO: 7, nucleotides 58 to 912 of SEQ ID NO: 9, nucleotides 46 to 951 of SEQ ID NO: 11, nucleotides 67 to 796 of SEQ ID NO: 13, or nucleotides 77 to 766 of SEQ ID NO: 15.

5 The nucleotide 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, or SEQ ID NO: 15, or a subsequence thereof; as well as the amino acid sequence 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, or SEQ ID NO: 16, or a fragment thereof, may be used to design a nucleic acid probe to identify and
10 clone DNA encoding polypeptides having cellulolytic enhancing activity from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire
15 sequence, but should be at least 14, preferably at least 25, more preferably at least 35, and most preferably at least 70 nucleotides in length. It is, however, preferred that the nucleic acid probe is at least 100 nucleotides in length. For example, the nucleic acid probe may be at least 200 nucleotides, preferably at least 300 nucleotides, more preferably at least 400 nucleotides, or most preferably at least 500 nucleotides in length. Even longer probes may
20 be used, *e.g.*, nucleic acid probes that are preferably at least 600 nucleotides, more preferably at least 700 nucleotides, even more preferably at least 800 nucleotides, or most preferably at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with ³²P, ³H, ³⁵S, biotin, or avidin). Such probes are encompassed by the present invention.

25 A genomic DNA or cDNA library prepared from such other strains may, therefore, be screened for DNA that hybridizes with the probes described above and encodes a polypeptide having cellulolytic enhancing activity. Genomic or other DNA from such other strains may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to
30 and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA that is homologous with SEQ ID NO: 1, or a subsequence thereof, the carrier material is preferably used in a Southern blot.

 For purposes of the present invention, hybridization indicates that the nucleotide sequence hybridizes to a labeled nucleic acid probe corresponding to the mature
35 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, or SEQ ID NO: 15 the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ

ID NO: 5, or SEQ ID NO: 13, or the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 15, its full-length complementary strand, or a subsequence thereof, under very low to very high stringency conditions, as described *supra*.

5 In a preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 1. In another preferred aspect, the nucleic acid probe is nucleotides 388 to 1332 of SEQ ID NO: 1. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 2, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 1.
10 In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pEJG120 which is contained in *E. coli* NRRL B-30699, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pEJG120 which is contained in *E. coli* NRRL B-30699.

15 In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 3. In another preferred aspect, the nucleic acid probe is nucleotides 98 to 821 of SEQ ID NO: 3. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 4, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 3.
20 In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pTter61C which is contained in *E. coli* NRRL B-30813, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pTter61C which is contained in *E. coli* NRRL B-30813.

25 In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 5. In another preferred aspect, the nucleic acid probe is nucleotides 126 to 978 of SEQ ID NO: 5. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 6, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 5.
30 In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pTter61D which is contained in *E. coli* NRRL B-30812, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pTter61D which is contained in *E. coli* NRRL B-30812.

35 In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 7. In another preferred aspect, the nucleic acid probe is nucleotides 55 to 678 of SEQ ID NO: 7. In another preferred aspect, the nucleic acid probe

is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 8, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 7. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pTter61E which is contained in *E. coli* NRRL B-30814, wherein the polynucleotide
5 sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pTter61E which is contained in *E. coli* NRRL B-30814.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 9. In another preferred aspect, the nucleic acid probe is
10 nucleotides 58 to 912 of SEQ ID NO: 9. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 10, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 9. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pTter61G which is contained in *E. coli* NRRL B-30811, wherein the polynucleotide
15 sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pTter61G which is contained in *E. coli* NRRL B-30811.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 11. In another preferred aspect, the nucleic acid probe is
20 nucleotides 46 to 951 of SEQ ID NO: 11. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 12, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 11. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pTter61F which is contained in *E. coli* NRRL B-50044, wherein the polynucleotide
25 sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding region contained in plasmid pTter61F which is contained in *E. coli* NRRL B-50044.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 13. In another preferred aspect, the nucleic acid probe is
30 nucleotides 67 to 796 of SEQ ID NO: 13. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 14, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 13. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pDZA2-7 which is contained in *E. coli* NRRL B-30704, wherein the polynucleotide
35 sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pDZA2-7 which is contained in *E. coli* NRRL B-30704.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 15. In another preferred aspect, the nucleic acid probe is nucleotides 77 to 766 of SEQ ID NO: 15. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 16, or a
5 subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 15. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pTr333 which is contained in *E. coli* NRRL B-30878, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence
10 contained in plasmid pTr333 which is contained in *E. coli* NRRL B-30878.

For long probes of at least 100 nucleotides in length, very low to very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 µg/ml sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50%
15 formamide for high and very high stringencies, following standard Southern blotting procedures for 12 to 24 hours optimally.

For long probes of at least 100 nucleotides in length, the carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS preferably at 45°C (very low stringency), more preferably at 50°C (low stringency), more preferably at 55°C (medium stringency), more preferably at 60°C (medium-high stringency), even more preferably at
20 65°C (high stringency), and most preferably at 70°C (very high stringency).

For short probes of about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as prehybridization, hybridization, and washing post-hybridization at about 5°C to about 10°C below the calculated T_m using the calculation according to Bolton and McCarthy (1962, *Proceedings of the National Academy of Sciences USA* 48:1390) in
25 0.9 M NaCl, 0.09 M Tris-HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1X Denhardt's solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per ml following standard Southern blotting procedures for 12 to 24 hours optimally.

30 For short probes of about 15 nucleotides to about 70 nucleotides in length, the carrier material is washed once in 6X SSC plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6X SSC at 5°C to 10°C below the calculated T_m .

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
35 identity to 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, or SEQ ID NO: 15 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 95%, and even most preferably at least 96%, at least 97%, at least 98%, or at least 99%.

5 In a preferred aspect, the mature polypeptide coding sequence is nucleotides 388 to 1332 of SEQ ID NO: 1, nucleotides 98 to 821 of SEQ ID NO: 3, nucleotides 126 to 978 of SEQ ID NO: 5, nucleotides 55 to 678 of SEQ ID NO: 7, nucleotides 58 to 912 of SEQ ID NO: 9, nucleotides 46 to 951 of SEQ ID NO: 11, nucleotides 67 to 796 of SEQ ID NO: 13, or nucleotides 77 to 766 of SEQ ID NO: 15.

10 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: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, or SEQ ID NO: 14, or SEQ ID NO: 16; or a homologous sequence thereof. Methods for preparing such an artificial variant is described *supra*.

15 The total number of amino acid substitutions, deletions and/or insertions of the mature polypeptide 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, or SEQ ID NO: 14, or SEQ ID NO: 16, is 10, preferably 9, more preferably 8, more preferably 7, more preferably at most 6, more preferably 5, more preferably 4, even more preferably 3, most preferably 2, and even most preferably 1.

20 A polypeptide having cellulolytic enhancing activity may be obtained from microorganisms of any genus. In a preferred aspect, the polypeptide obtained from a given source is secreted extracellularly.

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

30 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 cellulolytic enhancing activity.

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

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

The polypeptide having cellulolytic enhancing 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 cellulolytic enhancing 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*, *Pseudotriconympha*, *Rhizomucor*, *Schizophyllum*, *Scytalidium*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trichoderma*, *Trichophaea*, *Verticillium*, *Volvariella*, or *Xylaria* polypeptide having cellulolytic enhancing 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 cellulolytic enhancing 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 sarcochromum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *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 cellulolytic enhancing activity.

It will be understood that for the aforementioned species the invention encompasses both the perfect and imperfect states, and other taxonomic equivalents, e.g., anamorphs, regardless of the species name by which they are known. Those skilled in the art will readily recognize the identity of appropriate equivalents.

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

Furthermore, polypeptides having cellulolytic enhancing activity may be identified and obtained from other sources including microorganisms isolated from nature (e.g., soil, composts, water, etc.) using the above-mentioned probes. Techniques for isolating microorganisms from natural habitats are well known in the art. The polynucleotide may then be obtained by similarly screening a genomic or cDNA library of such a microorganism. Once a polynucleotide encoding a polypeptide has been detected with the probe(s), the polynucleotide can be isolated or cloned by utilizing techniques that are well known to those of ordinary skill in the art (see, e.g., Sambrook *et al.*, 1989, *supra*)

Polynucleotides comprising nucleotide sequences that encode polypeptide having cellulolytic enhancing activity can be isolated and utilized to express the polypeptide having cellulolytic enhancing activity for evaluation in the methods of the present invention, as described herein.

The polynucleotides comprise nucleotide sequences that have a degree of identity to 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, or SEQ ID NO: 15 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 95%, and even most preferably at least 96%, at least 97%, at least 98%, or at least 99%, which encode a polypeptide having cellulolytic enhancing activity.

The polynucleotide may also be a polynucleotide encoding a polypeptide having cellulolytic enhancing activity 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: 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, or SEQ ID NO: 15, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, or SEQ ID NO: 13, or the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 15, or (iii) a full-length complementary strand of (i) or (ii); or allelic variants and subsequences thereof (Sambrook *et al.*, 1989, *supra*), as defined herein. In a preferred aspect, the mature polypeptide coding sequence is nucleotides 388 to 1332 of SEQ ID NO: 1, nucleotides 98 to 821 of SEQ ID NO: 3, nucleotides 126 to 978 of SEQ ID NO: 5, nucleotides 55 to 678 of SEQ ID NO: 7, nucleotides 58 to 912 of SEQ ID NO: 9, nucleotides 46 to 951 of SEQ ID NO: 11, nucleotides 67 to 796 of SEQ ID NO: 13, or nucleotides 77 to 766 of SEQ ID NO: 15.

As described earlier, the techniques used to isolate or clone a polynucleotide encoding a polypeptide are known in the art and include isolation from genomic DNA, preparation from cDNA, or a combination thereof.

15

Peroxide-Generating Enzymes

In the methods of the present invention, the peroxide-generating enzyme can be any peroxide-generating enzyme. The peroxide-generating enzyme, *e.g.*, hydrogen peroxide-generating enzyme, may be present as an enzyme activity in the enzyme composition, a component in one or more (several) proteins added to the composition, and/or an enzyme component present in the cellulosic material. In one aspect, the peroxide is hydrogen peroxide.

Examples of peroxide-generating enzymes include the following:

E.C. 1.1.3.x - donor:oxygen oxidoreductase, glucose oxidase (E.C. 1.1.3.4), hexose oxidase (E.C. 1.1.3.5), aryl-alcohol oxidase (E.C. 1.1.3.7), D-arabinono-1,4-lactone oxidase (E.C. 1.1.3.37), vanillyl-alcohol oxidase (E.C. 1.1.3.38), xylitol oxidase (E.C. 1.1.3.41)

E.C. 1.1.99.8 - alcohol dehydrogenase

E.C. 1.1.99.18 - cellobiose dehydrogenase

E.C. 1.2.3.x - aldehyde oxidase (E.C. 1.2.3.1), aryl-aldehyde oxidase (E.C. 1.2.3.9)

E.C. 1.3.3.x - dihydroorotate oxidase (E.C. 1.3.3.1), pyrroloquinoline-quinone synthase (E.C. 1.3.3.11)

E.C. 1.4.3.x - L-amino acid oxidase (E.C. 1.4.3.2), L-glutamate oxidase (E.C. 1.4.3.11)

E.C. 1.5.3.x - polyamine oxidase (1.5.3.11)

E.C. 1.6.3.1 - NADPH oxidase

E.C. 1.7.3.x - urate oxidase (E.C. 1.7.3.3), hydroxylamine oxidase (E.C. 1.7.3.4)

E.C. 1.8.3.x - thiol oxidase (E.C. 1.8.3.2), glutathione oxidase (E.C. 1.8.3.3)

E.C. 1.9.3.1 - cytochrome c oxidase
E.C. 1.13.11.12 lipoxygenase
E.C. 1.13.11.31 arachidonate 12-lipoxygenase
E.C. 1.13.11.33 arachidonate 15-lipoxygenase
5 E.C. 1.13.11.34 arachidonate 5-lipoxygenase
E.C. 1.13.11.40 arachidonate 8-lipoxygenase
E.C. 1.13.11.45 linoleate 11-lipoxygenase
E.C. 1.15.1.1 - superoxide dismutase
E.C. 1.17.3.x - xanthine oxidase (E.C. 1.17.3.2)

10 The peroxide-generating enzyme may be obtained from microorganisms of any genus. In one aspect, the polypeptide obtained from a given source is secreted extracellularly.

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

In one aspect, the peroxide-generating enzyme 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*
25 peroxide-generating enzyme.

In another aspect, the peroxide-generating enzyme is a *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, or *Streptococcus equi* subsp. *Zooepidemicus* peroxide-generating enzyme.

In another aspect, the peroxide-generating enzyme is a *Streptomyces*
30 *achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, or *Streptomyces lividans* peroxide-generating enzyme.

The peroxide-generating enzyme may also be a fungal peroxide-generating enzyme, and more preferably a yeast peroxide-generating enzyme such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* peroxide-
35 generating enzyme; or more preferably a filamentous fungal peroxide-generating enzyme 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*,
5 *Pseudotriconympha*, *Rhizomucor*, *Schizophyllum*, *Scytalidium*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trichoderma*, *Trichophaea*, *Verticillium*, *Volvariella*,
or *Xylaria* peroxide-generating enzyme.

In another aspect, the peroxide-generating enzyme is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces*
10 *douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, or *Saccharomyces oviformis*
peroxide-generating enzyme.

In another aspect, the peroxide-generating enzyme is an *Acremonium cellulolyticus*,
Aspergillus aculeatus, *Aspergillus awamori*, *Aspergillus fumigatus*, *Aspergillus foetidus*,
Aspergillus japonicus, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*,
15 *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*,
20 *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium*
sporotrichioides, *Fusarium sulphureum*, *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*
25 *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*, or
Trichoderma viride peroxide-generating enzyme.

30 Examples of peroxide-generating enzymes and their sources include Cohn, 1958,
The enzymatic formation of oxalacetic acid by nonpyridine nucleotide malic dehydrogenase
of *Micrococcus lysodeikticus*, *J. Biol. Chem.* 233: 299-304; Yamashita *et al.*, 2000, Isolation,
characterization and molecular cloning of a thermostable xylitol oxidase from *Streptomyces*
sp. IKD472, *J. Biosci. Bioeng.* 89: 350-360 (Accession No. Q9KX73); Seo *et al.*, 2000, The
35 *Arabidopsis* aldehyde oxidase 3 (AAO3) gene product catalyzes the final step in abscisic
acid biosynthesis in leaves, *Proc. Natl. Acad. Sci. USA* 97: 12908-12913 (Accession No.
Q7G191); Aurich *et al.*, 1972, Purification and properties of L-amino acid oxidase from

Neurospora crassa, *Acta Biol. Med. Ger.* 28: 209-220 (Accession No. P23623); Hooper and Thorpe, 2002, Flavin-dependent sulfhydryl oxidases in protein disulfide bond formation, *Methods Enzymol.* 348: 30-34; Baum and Scandalios, 1981, Isolation and characterization of the cytosolic and mitochondrial superoxide dismutases of maize, *Arch. Biochem. Biophys.* 206: 249-64 (Accession No. P09233); Holdom *et al.*, 1996, The Cu,Zn superoxide dismutases of *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, and *Aspergillus terreus*: purification and biochemical comparison with the *Aspergillus fumigatus* Cu,Zn superoxide dismutase, *Infect. Immun.* 64: 3326-3332 (Accession No. Q3MSU9); Lamarre *et al.*, 2001, *Candida albicans* expresses an unusual cytoplasmic manganese-containing superoxide dismutase (SOD3 gene product) upon the entry and during the stationary phase, *J. Biol. Chem.* 276: 43784-43791 (Accession No. O13401); Dufernez *et al.*, 2006, The presence of four iron-containing superoxide dismutase isozymes in trypanosomatidae: characterization, subcellular localization, and phylogenetic origin in *Trypanosoma brucei*, *Free Radic. Biol. Med.* 40(2):193-5 (Accession Nos. AY894557, AY894558, AY894559 and AY894560); Hjalmarsson *et al.*, 1987, Isolation and sequence of complementary DNA encoding human extracellular superoxide dismutase *Proc. Natl. Acad. Sci. USA* 84:6340-6344 (Accession No. J02947); Yamada *et al.*, 1999 Sequence and analysis of chromosome 2 of the plant *Arabidopsis Thaliana*, *Nature* 402: 761-768 (Accession No. Q9ZPY2); Kriechbaum *et al.*, 1989, Cloning and DNA sequence analysis of the glucose oxidase gene from *Aspergillus niger* NRRL-3, *FEBS Lett.* 255 (1): 63-66 (Accession No. P13006); Kiess *et al.*, 1998) Glucose oxidase from *Penicillium amagasakiense*. Primary structure and comparison with other glucose-methanol-choline (GMC) oxidoreductases *Eur. J. Biochem.* 252 (1): 90-99 (Accession No. P81156); Nierman *et al.*, 2005, Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus* *Nature* 438: 1151-1156 (Accession No. Q4WZA6); Toyama *et al.*, 2005, Molecular cloning and structural analysis of quinohemoprotein alcohol dehydrogenase ADH-IIIG from *Pseudomonas putida* HK5 *J. Mol. Biol.* 352 (1): 91-104 (Accession No. Q4W6GO).

Examples of cellobiose dehydrogenase and their sources include Xu *et al.*, 2001, *Humicola insolens* cellobiose dehydrogenase: cloning, redox chemistry, and "logic gate"-like dual functionality, *Enz. Microb. Technol.* 28: 744-753 (Accession No. Q9P8H5); Nozaki *et al.*, 1999, Cloning and expression of cellobiose dehydrogenase from *Irpex lacteus*. Submitted (AUG-2004) to the EMBL/GenBank/DDBJ databases (Accession No. Q6AW20); Moukha *et al.*, 1999, Cloning and analysis of *Pycnoporus cinnabarinus* cellobiose dehydrogenase, *Gene* 234: 23-33 (Accession No. O74253); Li *et al.*, 1996, Cloning of a cDNA encoding cellobiose dehydrogenase, a hemoflavoenzyme from *Phanerochaete chrysosporium*, *Appl. Environ. Microbiol.* 62: 1329-1335 (Accession No. Q01738); Kajisa *et al.*, 2004, Characterization and molecular cloning of cellobiose dehydrogenase from the

brown-rot fungus *Coniophora puteana*, *Biosci. Bioeng.* 98: 57-63 (Accession No. Q6BDD5); Zamocky *et al.*, Phylogenetic analysis of cellobiose dehydrogenases. Submitted (NOV-2002) to the EMBL/GenBank/DDBJ databases (Accession No. Q7Z975); Yoshida *et al.*, 2002, Molecular cloning and characterization of a cDNA encoding cellobiose dehydrogenase from the wood-rotting fungus *Grifola frondosa*, *FEMS Microbiol. Lett.* 217: 225-230 (Accession No. Q8J2T4); Stapleton *et al.*, 2004, Molecular cloning of the cellobiose dehydrogenase gene from *Trametes versicolor* and expression in *Pichia pastoris*, *Enzyme Microb. Technol.* 34: 55-63 (Accession No. Q875J3); Dumonceaux *et al.*, 1998, Cloning and sequencing of a gene encoding cellobiose dehydrogenase from *Trametes versicolor*, *Gene* 210: 211-219 (Accession No. O42729); Nierman *et al.*, 2005, Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*, *Nature* 438: 1151-1156 (Accession No. Q4WIN9); Raices *et al.*, 1995, Cloning and characterization of a cDNA encoding a cellobiose dehydrogenase from the white rot fungus *Phanerochaete chrysosporium*, *FEBS Lett.* 369: 233-238 (Accession No. Q12661); Zamocky *et al.*, 2008, Cloning, sequence analysis and heterologous expression in *Pichia pastoris* of a gene encoding a thermostable cellobiose dehydrogenase from *Myriococcum thermophilum*, *Protein Expr. Purif.* 59: 258-265 (Accession No. A9XK88); Fedorova *et al.*, Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*, *PLoS* (Accession No. A1CFV0); Subramaniam *et al.*, Biochemical and molecular biological characterization of cellobiose dehydrogenase from *Sporotrichum thermophilum*, Submitted (JUN-1998) to the EMBL/GenBank/DDBJ databases (Accession No. O74240); Fedorova *et al.*, Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*, *PLoS* (Accession No. A1CYG2); Fedorova *et al.*, Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*, *PLoS* (Accession No. B0XVQ8); Fedorova *et al.*, Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*, *PLoS* (Accession No. A1C890); Fedorova *et al.*, Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*, *PLoS* (Accession No. A1DIY3); Zamocky *et al.*, 2008, Cloning, sequence analysis and heterologous expression in *Pichia pastoris* of a gene encoding a thermostable cellobiose dehydrogenase from *Myriococcum thermophilum*, *Protein Expr. Purif.* 59: 258-265 (Accession No. A9XK87); Birren *et al.*, The Broad Institute Genome Sequencing Platform "Genome Sequence of *Pyrenophora tritici-repentis*, Submitted (MAR-2007) to the EMBL/GenBank/DDBJ databases (Accession No. B2WHI7); Birren *et al.*, The Broad Institute Genome Sequencing Platform "Genome Sequence of *Pyrenophora tritici-repentis*, Submitted (MAR-2007) to the EMBL/GenBank/DDBJ databases (Accession No. B2WJX3); Fedorova *et al.*, Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*, *PLoS* (Accession No. Q4WC40); and Pel *et al.*, 2007, Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88, *Nat. Biotechnol.* 25: 221-

231 (Accession No. A2QD75).

In one aspect, the cellobiose dehydrogenase is a *Humicola insolens* cellobiose dehydrogenase. In another aspect, the cellobiose dehydrogenase is a *Humicola insolens* DSM 1800 cellobiose dehydrogenase, e.g., the polypeptide comprising SEQ ID NO: 18 encoded by SEQ ID NO: 17, or a fragment thereof having cellobiose dehydrogenase activity (see U.S. Patent No. 6,280,976).

In another aspect, the cellobiose dehydrogenase is a *Myceliophthora thermophila* cellobiose dehydrogenase. In another aspect, the cellobiose dehydrogenase is a *Myceliophthora thermophila* CBS 117.65 cellobiose dehydrogenase.

It will be understood that for the aforementioned species the invention encompasses both the perfect and imperfect states, and other taxonomic equivalents, e.g., anamorphs, regardless of the species name by which they are known. Those skilled in the art will readily recognize the identity of appropriate equivalents.

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

Non-Enzymatic Peroxide-Generating Systems

In the present invention, the peroxide generation system can be any peroxide-generating chemical reaction or system of reactions. The peroxide-generating system may be present as a reaction between components in the enzyme composition and/or one or more (several) of the components of the biomass and/or one or more (several) chemical components added to the composition. In one aspect, the peroxide is hydrogen peroxide.

Examples of peroxide generating systems include, but are not limited to, UV-irradiation of Rose Bengal (Wright *et al.*, 2000, Singlet Oxygen-Mediated Protein Oxidation: Evidence for the Formation of Reactive Peroxides, *Redox Report*, 5:159-161); the Reidl-Pfleiderer process of autooxidation of 2-ethyl-9,10-dihydroxyanthracene + O₂ to 2-ethylanthraquinone + H₂O₂; Eul *et al.*, 2001, Hydrogen peroxide, in *Kirk-Othmer Encyclopedia of Chemical Technology* Wiley, New York; reaction of singlet state molecular oxygen ¹O₂ with ascorbate (Kramarenko *et al.*, 2006, Ascorbate Reacts with Singlet Oxygen to Produce Hydrogen Peroxide, *Photochem. Photobiol.* 82(6):1634-1637); the oxidation of unsaturated lipid (after radical initiation) to lipid peroxide (Benzie, 1996, Lipid peroxidation: A review of causes, consequences, measurement and dietary influences, *International Journal of Food Sciences and Nutrition* 47(3): 233-261); and the oxidation of organic alcohols by molecular oxygen in the presence of various metal and metal complex catalysts (Bortolo *et*

al., 2000, Production of Hydrogen Peroxide from Oxygen and Alcohols, Catalyzed by Palladium Complexes, *J. Mol. Cat. A. Chem.*, 153:25-29).

Polypeptides Having Peroxidase Activity

5 In the methods of the present invention, the polypeptide having peroxidase activity can be any polypeptide having peroxidase activity. The polypeptide having peroxidase activity may be present as an enzyme activity in the enzyme composition and/or as one or more (several) protein components added to the composition. In a preferred aspect, the polypeptide having peroxidase activity is foreign to one or more (several) components of the
10 enzyme composition.

 Examples of peroxidase or peroxide-decomposing enzymes include, but are not limited to, the following:

- E.C. 1.11.1.1 NADH peroxidase
- E.C. 1.11.1.2 NADPH peroxidase
- 15 E.C. 1.11.1.3 fatty acid peroxidase
- E.C. 1.11.1.5 di-heme cytochrome c peroxidase
- E.C. 1.11.1.5 cytochrome c peroxidase
- E.C. 1.11.1.6 catalase
- E.C. 1.11.1.6 manganese catalase
- 20 E.C. 1.11.1.7 invertebrate peroxinectin
- E.C. 1.11.1.7 eosinophil peroxidase
- E.C. 1.11.1.7 lactoperoxidase
- E.C. 1.11.1.7 myeloperoxidase
- E.C. 1.11.1.8 thyroid peroxidase
- 25 E.C. 1.11.1.9 glutathione peroxidase
- E.C. 1.11.1.10 chloride peroxidase
- E.C. 1.11.1.11 ascorbate peroxidase
- E.C. 1.11.1.12 other glutathione peroxidase
- E.C. 1.11.1.13 manganese peroxidase
- 30 E.C. 1.11.1.14 lignin peroxidase
- E.C. 1.11.1.15 cysteine peroxiredoxin
- E.C. 1.11.1.16 versatile peroxidase
- E.C. 1.11.1.B2 chloride peroxidase
- E.C. 1.11.1.B4 haloperoxidase
- 35 E.C. 1.11.1.B4 no-heme vanadium haloperoxidase
- E.C. 1.11.1.B6 iodide peroxidase
- E.C. 1.11.1.B7 bromide peroxidase

E.C. 1.11.1.B8 iodide peroxidase

In one aspect, the peroxidase is a NADH peroxidase. In another aspect, the peroxidase is a NADPH peroxidase. In another aspect, the peroxidase is a fatty acid peroxidase. In another aspect, the peroxidase is a di-heme cytochrome c peroxidase. In another aspect, the peroxidase is a cytochrome c peroxidase. In another aspect, the peroxidase is a catalase. In another aspect, the peroxidase is a manganese catalase. In another aspect, the peroxidase is an invertebrate peroxinectin. In another aspect, the peroxidase is an eosinophil peroxidase. In another aspect, the peroxidase is a lactoperoxidase. In another aspect, the peroxidase is a myeloperoxidase. In another aspect, the peroxidase is a thyroid peroxidase. In another aspect, the peroxidase is a glutathione peroxidase. In another aspect, the peroxidase is a chloride peroxidase. In another aspect, the peroxidase is an ascorbate peroxidase. In another aspect, the peroxidase is a glutathione peroxidase. In another aspect, the peroxidase is a manganese peroxidase. In another aspect, the peroxidase is a lignin peroxidase. In another aspect, the peroxidase is a cysteine peroxiredoxin. In another aspect, the peroxidase is a versatile peroxidase. In another aspect, the peroxidase is a chloride peroxidase. In another aspect, the peroxidase is a haloperoxidase. In another aspect, the peroxidase is a no-heme vanadium haloperoxidase. In another aspect, the peroxidase is an iodide peroxidase. In another aspect, the peroxidase is a bromide peroxidase. In another aspect, the peroxidase is a iodide peroxidase.

Examples of polypeptides having peroxidase activity include, but are not limited to, *Coprinus cinereus* peroxidase (Baunsgaard *et al.*, 1993, Amino acid sequence of *Coprinus macrorhizus* peroxidase and cDNA sequence encoding *Coprinus cinereus* peroxidase. A new family of fungal peroxidases, *Eur. J. Biochem.* 213 (1): 605-611 (Accession number P28314); horseradish peroxidase (Fujiyama *et al.*, 1988, Structure of the horseradish peroxidase isozyme C genes, *Eur. J. Biochem.* 173 (3): 681-687 (Accession number P15232); peroxiredoxin (Singh and Shichi, 1998, A novel glutathione peroxidase in bovine eye. Sequence analysis, mRNA level, and translation, *J. Biol. Chem.* 273 (40): 26171-26178 (Accession number O77834); lactoperoxidase (Dull *et al.*, 1990, Molecular cloning of cDNAs encoding bovine and human lactoperoxidase, *DNA Cell Biol.* 9 (7): 499-509 (Accession number P80025); Eosinophil peroxidase (Fornhem *et al.*, 1996, Isolation and characterization of porcine cationic eosinophil granule proteins, *Int. Arch. Allergy Immunol.* 110 (2): 132-142 (Accession number P80550); versatile peroxidase (Ruiz-Duenas *et al.*, 1999, Molecular characterization of a novel peroxidase isolated from the ligninolytic fungus *Pleurotus eryngii*, *Mol. Microbiol.* 31 (1): 223-235 (Accession number O94753); turnip peroxidase (Mazza and Welinder, 1980, Covalent structure of turnip peroxidase 7. Cyanogen bromide fragments, complete structure and comparison to horseradish

peroxidase C, *Eur. J. Biochem.* 108 (2): 481-489 (Accession number P00434); myeloperoxidase (Morishita *et al.*, 1987, Chromosomal gene structure of human myeloperoxidase and regulation of its expression by granulocyte colony-stimulating factor, *J. Biol. Chem.* 262 (31): 15208-15213 (Accession number P05164); peroxidasin and peroxidasin homologs (Horikoshi *et al.*, 1999, Isolation of differentially expressed cDNAs from p53-dependent apoptotic cells: activation of the human homologue of the Drosophila peroxidasin gene, *Biochem. Biophys. Res. Commun.* 261 (3): 864-869 (Accession number Q92626); lignin peroxidase (Tien and Tu, 1987, Cloning and sequencing of a cDNA for a ligninase from *Phanerochaete chrysosporium*, *Nature* 326 (6112): 520-523 (Accession number P06181); Manganese peroxidase (Orth *et al.*, 1994, Characterization of a cDNA encoding a manganese peroxidase from *Phanerochaete chrysosporium*: genomic organization of lignin and manganese peroxidase-encoding genes, *Gene* 148 (1): 161-165 (Accession number P78733); alpha-dioxygenase, dual oxidase, peroxidasin, invertebrate peroxinectin, short peroxidockerin, lactoperoxidase, myeloperoxidase, non-mammalian vertebrate peroxidase, catalase, catalase-lipoxygenase fusion, di-heme cytochrome c peroxidase, methylamine utilization protein, DyP-type peroxidase, haloperoxidase, ascorbate peroxidase, catalase peroxidase, hybrid ascorbate-cytochrome c peroxidase, lignin peroxidase, manganese peroxidase, versatile peroxidase, other class II peroxidase, class III peroxidase, alkylhydroperoxidase D, other alkylhydroperoxidases, no-heme, no metal haloperoxidase, no-heme vanadium haloperoxidase, manganese catalase, NADH peroxidase, glutathione peroxidase, cysteine peroxiredoxin, thioredoxin-dependent thiol peroxidase, and AhpE-like peroxiredoxin (Passard *et al.*, 2007, *Phytochemistry* 68:1605-1611.

The polypeptide having peroxidase activity may be obtained from microorganisms of any genus. In one aspect, the polypeptide obtained from a given source is secreted extracellularly.

The polypeptide having peroxidase 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 peroxidase activity, or a Gram negative bacterial polypeptide such as an *E. coli*, *Pseudomonas*, *Salmonella*, *Campylobacter*, *Helicobacter*, *Flavobacterium*, *Fusobacterium*, *Ilyobacter*, *Neisseria*, or *Ureaplasma* polypeptide having peroxidase activity.

In one 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

peroxidase activity.

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

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

The polypeptide having peroxidase activity may also be a fungal polypeptide, and more preferably a yeast polypeptide such as a *Candida*, *Kluyveromyces*, *Pichia*,
 10 *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* polypeptide having peroxidase 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*,
 15 *Holomastigotoides*, *Humicola*, *Irpex*, *Lentinula*, *Leptosphaeria*, *Magnaporthe*, *Melanocarpus*, *Meripilus*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Piromyces*, *Poitrasia*, *Pseudoplectania*, *Pseudotriconympha*, *Rhizomucor*, *Schizophyllum*, *Scytalidium*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trichoderma*, *Trichophaea*, *Verticillium*, *Volvariella*, or *Xylaria* polypeptide having peroxidase
 20 activity.

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

25 In another 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*,
 30 *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochromum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*,
 35 *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*, or
5 *Trichoderma viride* polypeptide having peroxidase activity.

In another aspect, the peroxidase is horseradish peroxidase. In another aspect, the peroxidase is *Coprinus cinereus* peroxidase.

Techniques used to isolate or clone a polynucleotide encoding a polypeptide having peroxidase activity are known in the art and include isolation from genomic DNA, preparation
10 from cDNA, or a combination thereof. The cloning of the polynucleotides of the present invention from such genomic DNA can be effected, e.g., by using the well known polymerase chain reaction (PCR) or antibody screening of expression libraries to detect cloned DNA fragments with shared structural features. See, e.g., Innis *et al.*, 1990, *PCR: A Guide to Methods and Application*, Academic Press, New York. Other nucleic acid amplification
15 procedures such as ligase chain reaction (LCR), ligation activated transcription (LAT) and nucleotide sequence-based amplification (NASBA) may be used.

Enzyme Compositions

In the methods of the present invention, the enzyme composition may comprise any
20 protein involved in the processing of a cellulose-containing material to glucose and/or cellobiose, or hemicellulose to xylose, mannose, galactose, and/or arabinose.

The enzyme composition preferably comprises enzymes having cellulolytic activity and/or xylan degrading activity. In one aspect, the enzyme composition comprises one or more (several) cellulolytic enzymes. In another aspect, the enzyme composition comprises
25 one or more (several) xylan degrading enzymes. In another aspect, the enzyme composition comprises one or more (several) cellulolytic enzymes and one or more (several) xylan degrading enzymes.

The one or more (several) cellulolytic enzymes are preferably selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase. The
30 one or more (several) xylan degrading enzymes are preferably selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase.

In another aspect, the enzyme composition may further or even further comprise one or more (several) additional enzyme activities to improve the degradation of the cellulose-
35 containing material. Preferred additional enzymes are hemicellulases (e.g., alpha-D-glucuronidases, alpha-L-arabinofuranosidases, endo-mannanases, beta-mannosidases, alpha-galactosidases, endo-alpha-L-arabinanases, beta-galactosidases), carbohydrate-

esterases (e.g., acetyl-xylan esterases, acetyl-mannan esterases, ferulic acid esterases, coumaric acid esterases, glucuronoyl esterases), pectinases, proteases, ligninolytic enzymes (e.g., laccases, manganese peroxidases, lignin peroxidases, H₂O₂-producing enzymes, oxidoreductases), expansins, swollenins, or mixtures thereof. In the methods of the present invention, the additional 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 methods of the present invention may be in any form suitable for use in the processes described herein, 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.

A polypeptide having cellulolytic enzyme activity or xylan degrading 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 cellulolytic enzyme activity or xylan degrading activity, or a Gram negative bacterial polypeptide such as an *E. coli*, *Pseudomonas*, *Salmonella*, *Campylobacter*, *Helicobacter*, *Flavobacterium*, *Fusobacterium*, *Ilyobacter*, *Neisseria*, or *Ureaplasma* polypeptide having cellulolytic enzyme activity or xylan degrading 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 cellulolytic enzyme activity or xylan degrading activity.

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

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

The polypeptide having cellulolytic enzyme activity or xylan degrading activity may also be a fungal polypeptide, and more preferably a yeast polypeptide such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* polypeptide
10 having cellulolytic enzyme activity or xylan degrading 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*,
15 *Lentinula*, *Leptosphaeria*, *Magnaporthe*, *Melanocarpus*, *Meripilus*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Piromyces*, *Poitrasia*, *Pseudoplectania*, *Pseudotriconympha*, *Rhizomucor*, *Schizophyllum*, *Scytalidium*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trichoderma*, *Trichophaea*, *Verticillium*, *Volvariella*, or *Xylaria* polypeptide having cellulolytic enzyme activity or xylan
20 degrading 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 cellulolytic enzyme activity or xylan degrading activity.

25 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*,
30 *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochromum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*,
35 *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*,
5 *Trichoderma viride*, or *Trichophaea saccata* polypeptide having cellulolytic enzyme activity or xylan degrading activity.

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

One or more (several) components of the enzyme composition may be a
10 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 proteins may
15 also be prepared by purifying such a protein from a fermentation broth.

Examples of commercial cellulolytic protein preparations suitable for use in the present invention include, for example, CELLIC™ Ctec (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™
20 (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,
25 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.

Examples of bacterial endoglucanases that can be used in the methods of the
30 present invention, include, but are not limited to, an *Acidotherrmus 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 methods of the present
35 invention, include, but are not limited to, a *Trichoderma reesei* endoglucanase I (Penttila *et al.*, 1986, *Gene* 45: 253-263; GENBANK™ accession no. M15665); *Trichoderma reesei*

endoglucanase II (Saloheimo, *et al.*, 1988, *Gene* 63:11-22; GENBANK™ accession no. M19373); *Trichoderma reesei* endoglucanase III (Okada *et al.*, 1988, *Appl. Environ. Microbiol.* 64: 555-563; GENBANK™ accession no. AB003694); *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: 20); *Myceliophthora thermophila* CBS 117.65 endoglucanase (SEQ ID NO: 22); basidiomycete CBS 495.95 endoglucanase (SEQ ID NO: 24); basidiomycete CBS 494.95 endoglucanase (SEQ ID NO: 26); *Thielavia terrestris* NRRL 8126 CEL6B endoglucanase (SEQ ID NO: 28); *Thielavia terrestris* NRRL 8126 CEL6C endoglucanase (SEQ ID NO: 30); *Thielavia terrestris* NRRL 8126 CEL7C endoglucanase (SEQ ID NO: 32); *Thielavia terrestris* NRRL 8126 CEL7E endoglucanase (SEQ ID NO: 34); *Thielavia terrestris* NRRL 8126 CEL7F endoglucanase (SEQ ID NO: 36); *Cladorrhinum foecundissimum* ATCC 62373 CEL7A endoglucanase (SEQ ID NO: 38); and *Trichoderma reesei* strain No. VTT-D-80133 endoglucanase (SEQ ID NO: 40; GENBANK™ accession no. M15665). The endoglucanases of SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, and SEQ ID NO: 40 described above are encoded by the mature polypeptide coding sequence of SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, respectively.

Examples of cellobiohydrolases useful in the methods of the present invention include, but are not limited to, *Trichoderma reesei* cellobiohydrolase I (SEQ ID NO: 42); *Trichoderma reesei* cellobiohydrolase II (SEQ ID NO: 44); *Humicola insolens* cellobiohydrolase I (SEQ ID NO: 46), *Myceliophthora thermophila* cellobiohydrolase II (SEQ ID NO: 48 and SEQ ID NO: 50), *Thielavia terrestris* cellobiohydrolase II (CEL6A) (SEQ ID NO: 52), *Chaetomium thermophilum* cellobiohydrolase I (SEQ ID NO: 54), and *Chaetomium thermophilum* cellobiohydrolase II (SEQ ID NO: 56). The cellobiohydrolases of SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, and SEQ ID NO: 54 described above are encoded by the mature polypeptide coding sequence of SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, and SEQ ID NO: 55, respectively.

Examples of beta-glucosidases useful in the methods of the present invention include, but are not limited to, *Aspergillus oryzae* beta-glucosidase (SEQ ID NO: 58); *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 60); *Penicillium brasilianum* IBT 20888 beta-glucosidase (SEQ ID NO: 62); *Aspergillus niger* beta-glucosidase (SEQ ID NO: 64);
5 and *Aspergillus aculeatus* beta-glucosidase (SEQ ID NO: 66). The beta-glucosidases of SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, and SEQ ID NO: 66 described above are encoded by the mature polypeptide coding sequence of SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, and SEQ ID NO: 65, respectively.

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

The beta-glucosidase may be a fusion protein. In one aspect, the beta-glucosidase is the *Aspergillus oryzae* beta-glucosidase variant BG fusion protein of SEQ ID NO: 68 or the *Aspergillus oryzae* beta-glucosidase fusion protein of SEQ ID NO: 70. In another aspect, the
20 *Aspergillus oryzae* beta-glucosidase variant BG fusion protein is encoded by the polynucleotide of SEQ ID NO: 67 or the *Aspergillus oryzae* beta-glucosidase fusion protein is encoded by the polynucleotide of SEQ ID NO: 69.

Other endoglucanases, cellobiohydrolases, and beta-glucosidases are disclosed in numerous Glycosyl Hydrolase families using the classification according to Henrissat B.,
25 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.

Other cellulolytic enzymes that may be used 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
30 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
35 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.

5 Examples of commercial xylan degrading enzyme preparations suitable for use in the present invention include, for example, SHEARZYME™ (Novozymes A/S), CELLIC™ Htec (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
10 (Biocatalysts Limit, Wales, UK).

Examples of xylanases useful in the methods of the present invention include, but are not limited to, *Aspergillus aculeatus* xylanase (GeneSeqP:AAR63790; WO 94/21785), *Aspergillus fumigatus* xylanases (WO 2006/078256), and *Thielavia terrestris* NRRL 8126 xylanases (WO 2009/079210).

15 Examples of beta-xylosidases useful in the methods of the present invention include, but are not limited to, *Trichoderma reesei* beta-xylosidase (UniProtKB/TrEMBL accession number Q92458), *Talaromyces emersonii* (SwissProt accession number Q8X212), and *Neurospora crassa* (SwissProt accession number Q7SOW4).

20 Examples of acetylxylan esterases useful in the methods of the present invention include, but are not limited to, *Hypocrea jecorina* acetylxylan esterase (WO 2005/001036), *Neurospora crassa* acetylxylan esterase (UniProt accession number q7s259), *Thielavia terrestris* NRRL 8126 acetylxylan esterase (WO 2009/042846), *Chaetomium globosum* acetylxylan esterase (Uniprot accession number Q2GWX4), *Chaetomium gracile* acetylxylan esterase (GeneSeqP accession number AAB82124), *Phaeosphaeria nodorum* acetylxylan
25 esterase (Uniprot accession number Q0UHJ1), and *Humicola insolens* DSM 1800 acetylxylan esterase (WO 2009/073709).

30 Examples of ferulic acid esterases useful in the methods 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 methods 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).

35 Examples of alpha-glucuronidases useful in the methods 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).

5 The enzymes and proteins used in the methods 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
10 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., *Biochemical Engineering Fundamentals*, McGraw-Hill Book Company, NY, 1986).

 The fermentation can be any method of cultivation of a cell resulting in the
15 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 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
20 may be recovered from the fermentation medium and purified by conventional procedures.

Nucleic Acid Constructs

 Nucleic acid constructs comprising an isolated polynucleotide encoding a polypeptide of interest, e.g., one or more (several) cellulolytic enzymes, a polypeptide having peroxidase
25 activity, or a polypeptide having cellulolytic enhancing activity, operably linked to one or more (several) control sequences may be constructed that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

 The isolated polynucleotide may be manipulated in a variety of ways to provide for
30 expression of the polypeptide. 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 an appropriate promoter sequence, a nucleotide
35 sequence that is recognized by a host cell for expression of a polynucleotide encoding a polypeptide of the present invention. The promoter sequence contains transcriptional control sequences that mediate the expression of the polypeptide. The promoter may be any

nucleotide sequence that shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

5 Examples of suitable promoters for directing the transcription of the nucleic acid constructs of the present invention, especially in a bacterial host cell, are the promoters obtained from the *E. coli lac* operon, *Streptomyces coelicolor* agarase gene (*dagA*), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus subtilis xylA* and *xylB* genes, and prokaryotic beta-lactamase gene (Villa-Kamaroff *et al.*, 10 1978, *Proceedings of the National Academy of Sciences USA* 75: 3727-3731), as well as the *tac* promoter (DeBoer *et al.*, 1983, *Proceedings of the National Academy of Sciences USA* 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242: 74-94; and in Sambrook *et al.*, 1989, *supra*.

15 Examples of suitable promoters for directing the transcription of the nucleic acid constructs in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *Aspergillus oryzae* 20 alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase, *Fusarium venenatum* amyloglucosidase (WO 00/56900), *Fusarium venenatum* Daria (WO 00/56900), *Fusarium venenatum* Quinn (WO 00/56900), *Fusarium oxysporum* trypsin-like protease (WO 96/00787), *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* 25 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 including a gene encoding a neutral alpha-amylase in *Aspergilli* in which the untranslated leader has been replaced by 30 an untranslated leader from a gene encoding triose phosphate isomerase in *Aspergilli*; non-limiting examples include modified promoters including 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.

35 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.

5 The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleotide sequence encoding the polypeptide. Any terminator that is functional in the host cell of choice may be used in the present invention.

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

15 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*.

20 The control sequence may also be a suitable leader sequence, 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 nucleotide sequence encoding the polypeptide. Any leader sequence that is functional in the host cell of choice may be used in the present invention.

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

25 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).

30 The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3' terminus of the nucleotide sequence 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 in the present invention.

35 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, *Molecular Cellular Biology* 15: 5983-5990.

The control sequence may also be a signal peptide coding sequence that encodes a signal peptide linked to the amino terminus of a polypeptide and directs the encoded polypeptide into the cell's secretory pathway. The 5' end of the coding sequence of the nucleotide sequence may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the secreted 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 native 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, *i.e.*, secreted into a culture medium, may be used in the present invention.

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 stearothermophilus* alpha-amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *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 oryzae* TAKA amylase, *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Rhizomucor miehei* aspartic proteinase, *Humicola insolens* cellulase, *Humicola insolens* endoglucanase V, and *Humicola lanuginosa* lipase.

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 amino terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propeptide is generally inactive and can be converted to a mature 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*), *Saccharomyces cerevisiae* alpha-factor, *Rhizomucor miehei* aspartic proteinase, and *Myceliophthora thermophila* laccase (WO 95/33836).

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

5 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
10 yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the TAKA alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used as regulatory sequences. 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
15 presence of methotrexate, and the metallothionein genes that are amplified with heavy metals. In these cases, the nucleotide sequence encoding the polypeptide would be operably linked with the regulatory sequence.

Expression Vectors

20 Recombinant expression vectors comprising a polynucleotide encoding a polypeptide of interest, *e.g.*, one or more (several) cellulolytic enzymes, a polypeptide having peroxidase activity, or a polypeptide having cellulolytic enhancing activity, a promoter, and transcriptional and translational stop signals may be constructed for expression of the polypeptide in a suitable host cell. The various nucleic acids and control sequences
25 described herein 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 the nucleotide sequence encoding the polypeptide at such sites. Alternatively, a polynucleotide sequence may be expressed by inserting the nucleotide sequence or a nucleic acid construct comprising the sequence into an appropriate vector for
30 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
35 expression of the nucleotide sequence. 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 vectors may be linear or closed circular plasmids.

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 vectors preferably contain 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, kanamycin, chloramphenicol, 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 vectors preferably contain 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 nonhomologous recombination. Alternatively, the vector may contain additional nucleotide sequences 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 preferably contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, preferably 400 to 10,000 base pairs, and most preferably 800 to 10,000 base pairs, which have a high degree of 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 nucleotide sequences. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

5 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" is defined herein as a nucleotide sequence that enables a plasmid or vector to replicate *in vivo*.

10 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.

15 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 Research* 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.

20 More than one copy of a polynucleotide may be inserted into a host cell to increase production of the gene product. 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
25 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*).

30

Host Cells

The nucleic acid constructs or expression vectors comprising an isolated polynucleotide encoding a polypeptide of interest, *e.g.*, one or more (several) cellulolytic enzymes, a polypeptide having peroxidase activity, or a polypeptide having cellulolytic
35 enhancing activity, may be introduced into recombinant host cells for the recombinant production of the polypeptides. A vector comprising 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 encoding the polypeptide and its source.

5 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 bacterium or a Gram negative bacterium. Gram positive bacteria include, but not limited to, *Bacillus*, *Streptococcus*, *Streptomyces*, *Staphylococcus*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Clostridium*,
10 *Geobacillus*, and *Oceanobacillus*. Gram negative bacteria include, but not limited to, *E. coli*, *Pseudomonas*, *Salmonella*, *Campylobacter*, *Helicobacter*, *Flavobacterium*, *Fusobacterium*, *Ilyobacter*, *Neisseria*, and *Ureaplasma*.

The bacterial host cell may be any *Bacillus* cell. *Bacillus* cells include, but are not limited to, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus*
15 *circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis* cells.

In a preferred aspect, the bacterial host cell is a *Bacillus amyloliquefaciens*, *Bacillus*
lentus, *Bacillus licheniformis*, *Bacillus stearothermophilus* or *Bacillus subtilis* cell. In a more
20 preferred aspect, the bacterial host cell is a *Bacillus amyloliquefaciens* cell. In another more preferred aspect, the bacterial host cell is a *Bacillus clausii* cell. In another more preferred aspect, the bacterial host cell is a *Bacillus licheniformis* cell. In another more preferred aspect, the bacterial host cell is a *Bacillus subtilis* cell.

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

In a preferred aspect, the bacterial host cell is a *Streptococcus equisimilis* cell. In
another preferred aspect, the bacterial host cell is a *Streptococcus pyogenes* cell. In
another preferred aspect, the bacterial host cell is a *Streptococcus uberis* cell. In another
30 preferred aspect, the bacterial host cell is a *Streptococcus equi* subsp. *Zooepidemicus* cell.

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

In a preferred aspect, the bacterial host cell is a *Streptomyces achromogenes* cell. In
35 another preferred aspect, the bacterial host cell is a *Streptomyces avermitilis* cell. In another preferred aspect, the bacterial host cell is a *Streptomyces coelicolor* cell. In another preferred aspect, the bacterial host cell is a *Streptomyces griseus* cell. In another preferred

aspect, the bacterial host cell is a *Streptomyces lividans* cell.

The introduction of DNA into a *Bacillus* cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), by using competent cells (see, e.g., Young and Spizizen, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 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, *Journal of Bacteriology* 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.

In a preferred aspect, the host cell is 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*).

In a more preferred aspect, the fungal host cell is a yeast cell. "Yeast" as used herein includes ascosporegenous yeast (Endomycetales), basidiosporegenous 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).

In an even more preferred aspect, the yeast host cell is a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell.

In a most preferred aspect, the yeast host cell is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*,
5 *Saccharomyces kluyveri*, *Saccharomyces norbensis*, or *Saccharomyces oviformis* cell. In another most preferred aspect, the yeast host cell is a *Kluyveromyces lactis* cell. In another most preferred aspect, the yeast host cell is a *Yarrowia lipolytica* cell.

In another more preferred aspect, the fungal host cell is a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota
10 (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
15 be fermentative.

In an even more preferred aspect, the filamentous fungal host cell is an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*,
20 *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, or *Trichoderma* cell.

In a most preferred aspect, the filamentous fungal host cell is an *Aspergillus awamori*, *Aspergillus fumigatus*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or *Aspergillus oryzae* cell. In another most preferred aspect, the
25 filamentous fungal host cell is a *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*, or *Fusarium venenatum* cell. In another most preferred aspect, the filamentous fungal host cell is a
30 *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium tropicum*, *Chrysosporium merdarium*, *Chrysosporium inops*,
35 *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *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.

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 238 023 and Yelton *et al.*, 1984, *Proceedings of the National Academy of Sciences USA* 81: 1470-1474. Suitable methods for transforming *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, *Journal of Bacteriology* 153: 163; and Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920.

15

Methods of Production

A polypeptide of interest, *e.g.*, one or more (several) cellulolytic enzymes, a polypeptide having peroxidase activity, or a polypeptide having cellulolytic enhancing activity, can be produced by (a) cultivating a cell, which in its wild-type form produces the polypeptide, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

20

The polypeptide of interest can also be produced by (a) cultivating a recombinant host cell, as described herein, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

25

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 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 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 into the medium, it can be recovered from cell lysates.

30

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The polypeptides 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 as described herein.

5 The resulting 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.

10 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.

15

The present invention is further described by the following examples that should not be construed as limiting the scope of the invention.

20

Examples

Example 1: Growth of *Myceliophthora thermophila* CBS 117.65

25 Two plugs from a PDA plate of *Myceliophthora thermophila* CBS 117.65 were inoculated into a 500 ml shake flask containing 100 ml of shake flask medium to obtain culture broth for the purification of a cellobiose dehydrogenase. PDA plates were composed of 39 g of potato dextrose agar and deionized water to 1 liter. The shake flask medium was composed of 15 g of glucose, 4 g of K_2HPO_4 , 1 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2 g of MES free acid, 1 g of Bacto Peptone, 5 g of yeast extract, 2.5 g of citric acid, 0.2 g of $CaCl_2 \cdot 2H_2O$, 5 g of NH_4NO_3 , 1 ml of trace elements solution, and deionized water to 1 liter. The trace

30 elements solution was composed of 1.2 g of $FeSO_4 \cdot 7H_2O$, 10 g of $ZnSO_4 \cdot 7H_2O$, 0.7 g of $MnSO_4 \cdot H_2O$, 0.4 g of $CuSO_4 \cdot 5H_2O$, 0.4 g of $Na_2B_4O_7 \cdot 10H_2O$, 0.8 g of $Na_2MoO_4 \cdot 2H_2O$, and deionized water to 1 liter. The shake flask was incubated at 45°C on an orbital shaker at 200 rpm for 48 hours. Fifty ml of the shake flask broth was used to inoculate a 2 liter fermentation vessel.

35

A total of 1.8 liters of the fermentation batch medium was added to a two liter glass jacketed fermentor (Applikon Biotechnology, Schiedam, Netherlands). Fermentation feed medium was dosed at a rate of 4 g/l/hour for a period of 72 hours. Fermentation batch

medium was composed of 5 g of yeast extract, 176 g of powdered cellulose, 2 g of glucose, 1 g of NaCl, 1 g of Bacto Peptone, 4 g of K₂HPO₄, 0.2 g of CaCl₂·2H₂O, 0.2 g of MgSO₄·7H₂O, 2.5 g of citric acid, 5 g of NH₄NO₃, 1.8 ml of anti-foam, 1 ml of trace elements solution, and deionized water to 1 liter. The fermentation vessel was maintained at a temperature of 45°C and pH was controlled using an Applikon 1030 control system (Applikon Biotechnology, Schiedam, Netherlands) to a set-point of 5.6 +/- 0.1. Air was added to the vessel at a rate of 1 vvm and the broth was agitated by Rushton impeller rotating at 1100 to 1300 rpm. At the end of the fermentation, whole broth was harvested from the vessel and centrifuged at 3000 x g to remove the biomass.

10

Example 2: Purification of *Myceliophthora thermophila* CBS 117.65 cellobiose dehydrogenase

The *Myceliophthora thermophila* CBS 117.65 harvested broth described in Example 1 was centrifuged in 500 ml bottles at 13,000 x g for 20 minutes at 4°C and then sterile filtered using a 0.22 µm polyethersulfone membrane (Millipore, Bedford, MA, USA). The filtered broth was concentrated and buffer exchanged with 20 mM Tris-HCl pH 8.5 using a tangential flow concentrator (Pall Filtron, Northborough, MA, USA) equipped with a 10 kDa polyethersulfone membrane (Pall Filtron, Northborough, MA, USA). To decrease the amount of pigment, the concentrate was applied to a 60 ml Q-SEPHAROSE BIG BEAD™ column (GE Healthcare, Piscataway, NJ, USA) equilibrated with 20 mM Tris-HCl pH 8.5, and eluted stepwise with equilibration buffer containing 600 mM NaCl. Flow-through and eluate fractions were analyzed by SDS-PAGE using 8-16% CRITERION™ SDS-PAGE gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and stained with GELCODE™ Blue protein stain (Thermo Fisher Scientific, Waltham, MA, USA). The eluate fraction contained cellobiose dehydrogenase (CBDH) as judged by the presence of a band corresponding to the apparent molecular weight of approximately 100 kDa by SDS-PAGE (Schou *et al.*, 1998, *Biochem. J.* 330: 565–571).

25

The eluate fraction was concentrated using an AMICON™ ultrafiltration device (Millipore, Bedford, MA, USA) equipped with a 10 kDa polyethersulfone membrane, and buffer-exchanged into 20 mM Tris-HCl pH 8.5 using a HIPREP® 26/10 desalting column (GE Healthcare, Piscataway, NJ, USA). The desalted material was loaded onto a MONO Q® column (HR 16/10, GE Healthcare, Piscataway, NJ, USA) equilibrated with 20 mM Tris-HCl pH 8.5. Bound proteins were eluted with a linear NaCl gradient from 0 to 500 mM (18 column volumes) in 20 mM Tris-HCl pH 8.5. Fractions were analyzed by SDS-PAGE as described above and the cellobiose dehydrogenase eluted at approximately 350-400 mM NaCl.

30

35

Fractions containing cellobiose dehydrogenase were pooled (60 ml) and mixed with

an equal volume of 20 mM Tris-HCl pH 7.5 containing 3.4 M ammonium sulfate to yield a final concentration of 1.7 M ammonium sulfate. The sample was filtered (0.2 µM syringe filter, polyethersulfone membrane, Whatman, Maidstone, United Kingdom) to remove particulate matter prior to loading onto a Phenyl Superose column (HR 16/10, GE Healthcare, Piscataway, NJ, USA) equilibrated with 1.7 M ammonium sulfate in 20 mM Tris-HCl pH 7.5. Bound proteins were eluted with a decreasing 1.7→0 M ammonium sulfate gradient (12 column volumes) in 20 mM Tris-HCl pH 7.5. Fractions were analyzed by SDS-PAGE as described above and the cellobiose dehydrogenase eluted at approximately 800 mM ammonium sulfate. The cellobiose dehydrogenase fraction was >90% pure as judged by SDS-PAGE. CBDH activity was confirmed by a 2,6-dichlorophenolindophenol (DCIP) reduction assay in the presence of cellobiose, as described by Schou *et al.*, 1998, *supra*.

Fractions containing cellobiose dehydrogenase were pooled, concentrated, and buffer exchanged into 20 mM Tris-HCl pH 7.5 by centrifugal concentration in a SORVALL® RT7 centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) using VIVASPIN™ 20 centrifugal concentrators. (10 kDa polyethersulfone membrane; Sartorius, Göttingen, Germany) at 1877 x g. Protein concentration was determined using a Microplate BCA™ Protein Assay Kit (Thermo Fischer Scientific, Waltham, MA, USA) in which bovine serum albumin was used as a protein standard.

20 **Example 3: Pretreatment of corn stover**

Corn stover was pretreated at the U.S. Department of Energy National Renewable Energy Laboratory (NREL) using dilute sulfuric acid. The following conditions were used for the pretreatment: 1.4 wt % sulfuric acid at 165°C and 107 psi for 8 minutes. According to NREL, the water-insoluble solids in the pretreated corn stover (PCS) contained 56.5% cellulose, 4.6% hemicellulose and 28.4% lignin. Cellulose and hemicellulose were determined by a two-stage sulfuric acid hydrolysis with subsequent analysis of sugars by high performance liquid chromatography using NREL Standard Analytical Procedure #002. Lignin was determined gravimetrically after hydrolyzing the cellulose and hemicellulose fractions with sulfuric acid using NREL Standard Analytical Procedure #003. The PCS was washed with a large volume of DDI water on a glass filter.

Example 4: The effect of peroxidase on hydrolysis of pretreated corn stover (PCS) in the presence and absence of cellobiose dehydrogenase

The effect of horseradish peroxidase (HRP) on hydrolysis of PCS was evaluated in the presence and absence of *Myceliophthora thermophila* CBS 117.65 cellobiose dehydrogenase (CDH).

The hydrolysis of PCS was performed using 2.2 ml deep-well plates (Axygen, Union

City, CA, USA) in a total reaction volume of 1.0 ml. The hydrolysis was performed with 50 mg of unwashed PCS per ml of 50 mM sodium acetate pH 5.0 buffer containing 1 mM manganese sulfate and a *Trichoderma reesei* cellulase composition (CELLUCLAST® supplemented with *Aspergillus oryzae* beta-glucosidase available from Novozymes A/S, Bagsvaerd, Denmark; the cellulase composition is designated herein in the Examples as “*Trichoderma reesei* cellulase composition”) at 4 mg per g of PCS. Cellobiose dehydrogenase was added at concentrations between 0 and 10% (w/w) of total protein. The plate was then sealed using an ALPS-300™ plate heat sealer (Abgene, Epsom, United Kingdom), mixed thoroughly, and incubated at 50°C for 72 hours with shaking at 150 rpm. All experiments were performed in triplicate.

At various time points between 24 and 72 hours of incubation, 100 µl aliquots were removed and the extent of hydrolysis was assayed by HPLC using the protocol described below. Cellobiose dehydrogenase-dependent cellulase inhibition was established, and then horseradish peroxidase was added to eliminate any peroxide produced. Horseradish peroxidase (Invitrogen, Carlsbad, CA, USA) was added at final concentrations indicated in Figure 1 from a stock solution of 1 unit per µl. One unit of horseradish peroxidase is defined as that quantity of enzyme necessary to form 1.0 mg of purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C. High concentrations of horseradish peroxidase were added to ensure sufficient peroxidase activity was present. No Amplex Red or other electron acceptor was added.

For HPLC analysis, samples were filtered with a 0.45 µm MULTISCREEN® 96-well filter plate (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 0.5% w/w benzoic acid-5 mM H₂SO₄ at a flow rate of 0.6 ml per minute at 65°C for 11 minutes, and quantitation by integration of glucose and cellobiose signals from refractive index detection (CHEMSTATION®, AGILENT® 1100 HPLC, Agilent Technologies, Santa Clara, CA, USA) calibrated by pure sugar samples. The resultant equivalents were used to calculate the percentage of cellulose conversion for each reaction. The extent of each hydrolysis was determined as the fraction of total cellulose converted to cellobiose + glucose, and were not corrected for soluble sugars present in PCS liquor.

All HPLC data processing was performed using Kaleidagraph software (Synergy software, Reading, PA, USA). Measured sugar concentrations were adjusted for the appropriate dilution factor. Glucose and cellobiose were chromatographically separated and integrated and their respective concentrations determined independently. However, to calculate total conversion the glucose and cellobiose values were combined. Fractional

hydrolysis was determined as the overall mass conversion to [glucose+cellobiose]/[total cellulose]. Triplicate data points were averaged and standard deviation was calculated.

The results (Figure 1) demonstrated that increasing concentrations of *M. thermophila* cellobiose dehydrogenase led to reduced hydrolysis. Under these specific hydrolysis conditions, the addition of 10% cellobiose dehydrogenase resulted in approximately 5% loss of hydrolysis. At 4% cellobiose dehydrogenase, where the *Trichoderma reesei* cellulase composition was modestly inhibited, the extent of hydrolysis was completely restored at the lowest concentration of 1 unit of horseradish peroxidase per ml.

10 **Example 5: The effect of horseradish peroxidase on hydrolysis of pretreated corn stover (PCS)**

The effect of horseradish peroxidase (HRP) on PCS hydrolysis by the *Trichoderma reesei* cellulase composition was determined in the absence of *Myceliophthora thermophila* CBS 117.65 cellobiose dehydrogenase.

15 The hydrolysis of PCS was conducted using 2.2 ml deep-well plates in a total reaction volume of 1.0 ml. The hydrolysis was performed with 50 mg of washed PCS per ml of 50 mM sodium acetate pH 5.0 buffer containing 1 mM manganese sulfate and the *Trichoderma reesei* cellulase composition at 4 mg per g of PCS. Horseradish peroxidase was added at 0-4 units per ml. The plate was then sealed using an ALPS-300™ plate heat sealer, mixed thoroughly, and incubated at 50°C for 72 hours with shaking at 150 rpm. All experiments were performed in triplicate.

HPLC analysis of the extent of hydrolysis was performed according to the procedure described in Example 4.

25 The results (Figure 2) demonstrated that the extent of hydrolysis increased linearly with horseradish peroxidase activity over the range of peroxidase activity tested, and the dependence of the extent of hydrolysis on horseradish peroxidase activity was approximately 2-fold higher at 3 days of incubation. Addition of horseradish peroxidase at 4 units per ml increased hydrolysis approximately 10% over reactions performed without horseradish peroxidase, both at day 1 and day 3 of hydrolysis.

30

Example 6: Preparation of *Coprinus cinereus* peroxidase

Coprinus cinereus peroxidase was purified as described by WO 1992/016634, and Xu *et al.*, 2003, "Fusion proteins containing *Coprinus cinereus* peroxidase and the cellulose-binding domain of *Humicola insolens* family 45 endoglucanase" in Application of Enzymes to Lignocellulosics (Mansfield, S. D. and Saddler, J. N. eds.) pp. 382-402, American Chemical Society, Washington, DC. The purification scheme comprised ultrafiltration and anion-exchange chromatography. Cell-free broth of a *Coprinus cinereus* peroxidase (pH 7.7, 11

mS conductivity) was filtered with Whatman #2 paper and ultrafiltered with a polyethersulfone membrane (30 kD molecular weight cutoff). The washed and concentrated broth (pH 7.7, 1 mS) was then loaded onto a Q-SEPHAROSE BIG BEAD™ column pre-equilibrated with 5 mM CaCl₂-10 mM Tris-HCl pH 7.6 (Buffer A). The active fraction eluted
5 by 5% Buffer B (Buffer A plus 2 M NaCl) was washed (with 5 mM CaCl₂) to 1 mS, then applied to a MONO-Q™ column (GE Healthcare, Piscataway, NJ, USA) equilibrated with Buffer A. Buffer B was used again for the elution. Fractions were analyzed for peroxidase activity and by SDS-PAGE. Specific peroxidase activity was assayed at 30°C with 0.1 M sodium phosphate pH 7, 0.9 mM H₂O₂, and 1.7 mM 2,2'-azinobis(3-ethylbenzothiazoline-6-
10 sulfonic acid) (ABTS), by monitoring the absorption increase at 418 nm. A stock concentration of 630 μM peroxidase was used.

Example 7: The effect of various peroxidases on hydrolysis of pretreated corn stover (PCS)

15 The effect of various peroxidases on PCS hydrolysis by the *Trichoderma reesei* cellulase composition was determined. The peroxidases included manganese peroxidase (Sigma Chemical Co., St. Louis, MO, USA), bovine milk lactoperoxidase (Sigma Chemical Co., St. Louis, MO, USA), lignin peroxidase (Sigma Chemical Co., St. Louis, MO, USA), and
20 *Coprinus cinereus* peroxidase (Example 6). One unit of manganese peroxidase is defined as the amount of enzyme necessary to oxidize 1 μmole of Mn²⁺ per minute to Mn³⁺ at pH 4.5 and 25°C. One unit of lactoperoxidase is defined as that quantity of enzyme necessary to form 1.0 mg of purpurogallin from pyrogallol in 20 seconds at pH 6.0 and 20°C. One unit of lignin peroxidase is defined as the amount of enzyme necessary to oxidize 1 μmole 3,4-
25 dimethoxybenzyl alcohol per minute at pH 3.0 and 30°C. One unit of *Coprinus cinereus* peroxidase is defined as the quantity of enzyme necessary to consume 1 μmole of H₂O₂ per minute.

The hydrolysis of PCS was conducted using 2.2 ml deep-well plates in a total reaction volume of 1.0 ml. The hydrolysis was performed with 50 mg of washed PCS per ml of 50 mM sodium acetate pH 5.0 containing 1 mM manganese sulfate and the *Trichoderma*
30 *reesei* cellulase composition at 4 mg per g of PCS. Manganese peroxidase, lactoperoxidase, lignin peroxidase, and *Coprinus cinereus* peroxidase were added at volumes of 0-100 μl, to give the final concentrations indicated in Figure 3, from the following stock solutions: Mn peroxidase: 0.005 unit per μl, 50 μg per μl; lignin peroxidase: 0.04 unit per μl, 20 μg per μl; lactoperoxidase: 0.2 unit per μl, 5 μg per μl; and *Coprinus cinereus*
35 peroxidase: 630 μM. The plate was then sealed using an ALPS-300™ plate heat sealer, mixed thoroughly, and incubated at 50°C for 72 hours with shaking at 150 rpm. All experiments were performed in triplicate.

HPLC analysis of the extent of hydrolysis was performed according to the procedure described in Example 5.

The results (Figure 3) demonstrated that each of the peroxidases, with the exception of manganese peroxidase, enhanced PCS hydrolysis in a concentration-dependent manner.

5 The effect did not saturate under the concentrations utilized.

The present invention is further described by the following paragraphs:

[1] A method for degrading or converting a cellulosic material, comprising: treating the cellulosic material with an enzyme composition in the presence of a polypeptide having
10 peroxidase activity.

[2] The method of paragraph 1, wherein the enzyme composition comprises one or more (several) cellulolytic enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

[3] The method of paragraph 1 or 2, wherein the enzyme composition further
15 comprises a polypeptide having cellulolytic enhancing activity.

[4] The method of any of paragraphs 1-3, wherein the enzyme composition further comprises one or more (several) enzymes selected from the group consisting of a hemicellulase, an esterase, a protease, and a laccase.

[5] The method of any of paragraphs 1-4, wherein the enzyme composition further
20 comprises one or more (several) enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, a glucuronidase, and a combination thereof.

[6] The method of any of paragraphs 1-5, wherein the cellulosic material is corn
stover.

[7] The method of any of paragraphs 1-6, wherein the cellulosic material is
25 pretreated.

[8] The method of any of paragraphs 1-7, further comprising recovering the degraded
cellulosic material.

[9] The method of paragraph 8, wherein the degraded cellulosic material is a sugar.

[10] The method of paragraph 9, wherein the sugar is selected from the group
30 consisting of glucose, xylose, mannose, galactose, and arabinose.

[11] The method of any of paragraphs 1-10, wherein the K_m of the polypeptide having
peroxidase activity is in the range of preferably 0.0001 to 50 mM, more preferably 0.001 to
10 mM, even more preferably 0.005 to 1 mM, and most preferably 0.01 to 0.1 mM.

[12] The method of any of paragraphs 1-11, wherein the presence of the polypeptide
35 having peroxidase activity increases the hydrolysis of the cellulosic material compared to the
absence of the polypeptide having peroxidase activity.

[13] A method for producing a fermentation product, comprising: (a) saccharifying a cellulosic material with an enzyme composition in the presence of a polypeptide having peroxidase activity; (b) fermenting the saccharified cellulosic material with one or more (several) fermenting microorganisms to produce the fermentation product; and (c) recovering the fermentation product from the fermentation.

[14] The method of paragraph 13, wherein the enzyme composition comprises one or more (several) cellulolytic enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

[15] The method of paragraph 13 or 14, wherein the enzyme composition further comprises a polypeptide having cellulolytic enhancing activity.

[16] The method of any of paragraphs 13-15, wherein the enzyme composition further comprises one or more (several) enzymes selected from the group consisting of a hemicellulase, an esterase, a protease, and a laccase.

[17] The method of any of paragraphs 13-16, wherein the enzyme composition further comprises one or more (several) enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, a glucuronidase, and a combination thereof.

[18] The method of any of paragraphs 13-17, wherein the cellulosic material is corn stover.

[19] The method of any of paragraphs 13-18, wherein the cellulosic material is pretreated.

[20] The method of any of paragraphs 13-19, wherein the fermentation product is an alcohol, an organic acid, a ketone, an amino acid, or a gas.

[21] The method of any of paragraphs 13-20, wherein the K_m of the polypeptide having peroxidase activity is in the range of preferably 0.0001 to 50 mM, more preferably 0.001 to 10 mM, even more preferably 0.005 to 1 mM, and most preferably 0.01 to 0.1 mM.

[22] The method of any of paragraphs 13-21, wherein the presence of the polypeptide having peroxidase activity increases the hydrolysis of the cellulosic material compared to the absence of the polypeptide having peroxidase activity.

[23] A method of fermenting a cellulosic material, comprising: fermenting the cellulosic material with one or more (several) fermenting microorganisms, wherein the cellulosic material is hydrolyzed with an enzyme composition in the presence of a polypeptide having peroxidase activity.

[24] The method of paragraph 23, wherein the enzyme composition comprises one or more (several) cellulolytic enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

[25] The method of paragraph 23 or 24, wherein the enzyme composition further

comprises a polypeptide having cellulolytic enhancing activity.

[26] The method of any of paragraphs 23-25, wherein the enzyme composition further comprises one or more (several) enzymes selected from the group consisting of a hemicellulase, an esterase, a protease, and a laccase.

5 [27] The method of any of paragraphs 23-26, wherein the enzyme composition further comprises one or more (several) enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, a glucuronidase, and a combination thereof.

10 [28] The method of any of paragraphs 23-27, wherein the cellulosic material is corn stover.

[29] The method of any of paragraphs 23-28, wherein the cellulosic material is pretreated.

[30] The method of any of paragraphs 23-29, wherein the fermentation produces a fermentation product.

15 [31] The method of paragraph 30, further comprising recovering the fermentation product.

[32] The method of paragraph 31, wherein the fermentation product is an alcohol, an organic acid, a ketone, an amino acid, or a gas.

20 [33] The method of any of paragraphs 23-32, wherein the K_m of the polypeptide having peroxidase activity is in the range of preferably 0.0001 to 50 mM, more preferably 0.001 to 10 mM, even more preferably 0.005 to 1 mM, and most preferably 0.01 to 0.1 mM.

[34] The method of any of paragraphs 23-33, wherein the presence of the polypeptide having peroxidase activity increases the hydrolysis of the cellulosic material compared to the absence of the polypeptide having peroxidase activity.

25

The invention described and claimed herein is not to be limited in scope by the specific aspects herein disclosed, since these aspects are intended as illustrations of several aspects of the invention. Any equivalent aspects are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

30

Claims

What is claimed is:

- 5 1. A method for degrading or converting a cellulosic material, comprising: treating the cellulosic material with an enzyme composition in the presence of a polypeptide having peroxidase activity.
- 10 2. The method of claim 1, wherein the enzyme composition comprises one or more (several) cellulolytic enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.
- 15 3. The method of claim 1 or 2, wherein the enzyme composition further comprises a polypeptide having cellulolytic enhancing activity.
4. The method of any of claims 1-3, wherein the enzyme composition further comprises one or more (several) enzymes selected from the group consisting of a hemicellulase, an esterase, a protease, and a laccase.
- 20 5. The method of any of claims 1-4, wherein the enzyme composition further comprises one or more (several) enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, a glucuronidase, and a combination thereof.
- 25 6. The method of any of claims 1-5, wherein the cellulosic material is pretreated.
7. The method of any of claims 1-6, further comprising recovering the degraded cellulosic material.
- 30 8. The method of claim 7, wherein the degraded cellulosic material is a sugar.
9. The method of any of claims 1-8, wherein the K_m of the polypeptide having peroxidase activity is in the range of preferably 0.0001 to 50 mM, more preferably 0.001 to 10 mM, even more preferably 0.005 to 1 mM, and most preferably 0.01 to 0.1 mM.
- 35 10. A method for producing a fermentation product, comprising:
(a) saccharifying a cellulosic material with an enzyme composition in the presence of a polypeptide having peroxidase activity;

- (b) fermenting the saccharified cellulosic material with one or more (several) fermenting microorganisms to produce the fermentation product; and
- (c) recovering the fermentation product from the fermentation.

- 5 11. The method of claim 10, wherein the enzyme composition comprises one or more (several) cellulolytic enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.
- 10 12. The method of claim 10 or 11, wherein the enzyme composition further comprises a polypeptide having cellulolytic enhancing activity.
13. The method of any of claims 10-12, wherein the enzyme composition further comprises one or more (several) enzymes selected from the group consisting of a hemicellulase, an esterase, a protease, and a laccase.
- 15 14. The method of any of claims 10-13, wherein the enzyme composition further comprises one or more (several) enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, a glucuronidase, and a combination thereof.
- 20 15. The method of any of claims 10-14, wherein the cellulosic material is pretreated.
16. The method of any of claims 10-15, wherein the K_m of the polypeptide having peroxidase activity is in the range of preferably 0.0001 to 50 mM, more preferably 0.001 to 25 10 mM, even more preferably 0.005 to 1 mM, and most preferably 0.01 to 0.1 mM.
17. A method of fermenting a cellulosic material, comprising: fermenting the cellulosic material with one or more (several) fermenting microorganisms, wherein the cellulosic material is hydrolyzed with an enzyme composition in the presence of a polypeptide having 30 peroxidase activity.
18. The method of claim 17, wherein the enzyme composition comprises one or more (several) cellulolytic enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.
- 35 19. The method of claim 17 or 18, wherein the enzyme composition further comprises a polypeptide having cellulolytic enhancing activity.

20. The method of any of claims 17-19, wherein the enzyme composition further comprises one or more (several) enzymes selected from the group consisting of a hemicellulase, an esterase, a protease, and a laccase.
- 5 21. The method of any of claims 17-20, wherein the enzyme composition further comprises one or more (several) enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, a glucuronidase, and a combination thereof.
- 10 22. The method of any of claims 17-21, wherein the cellulosic material is pretreated.
23. The method of any of claims 17-22, wherein the fermentation produces a fermentation product.
- 15 24. The method of any of claims 17-23, further comprising recovering the fermentation product.
25. The method of any of claims 17-24, wherein the K_m of the polypeptide having peroxidase activity is in the range of preferably 0.0001 to 50 mM, more preferably 0.001 to
20 10 mM, even more preferably 0.005 to 1 mM, and most preferably 0.01 to 0.1 mM.

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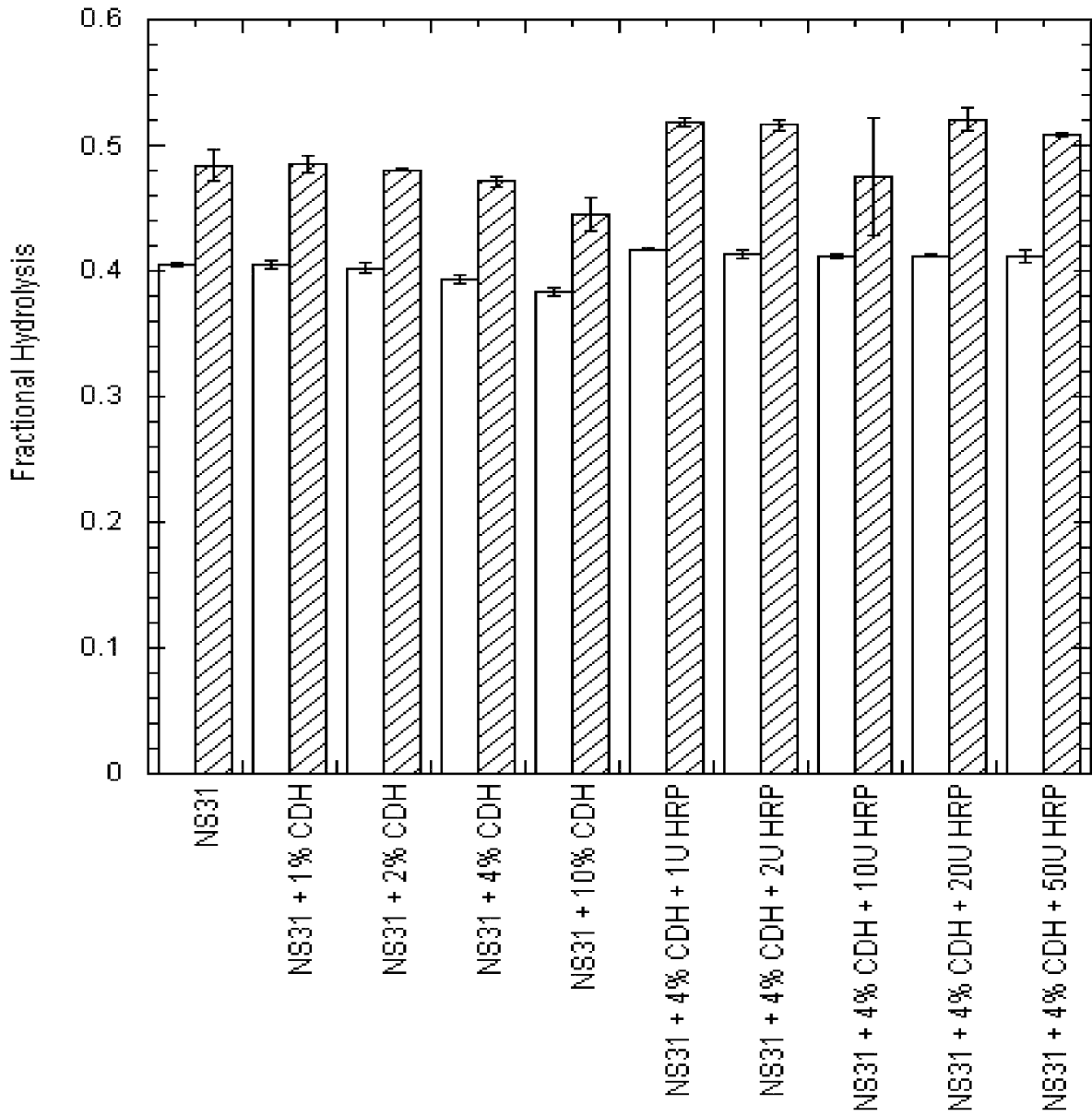


Fig. 1

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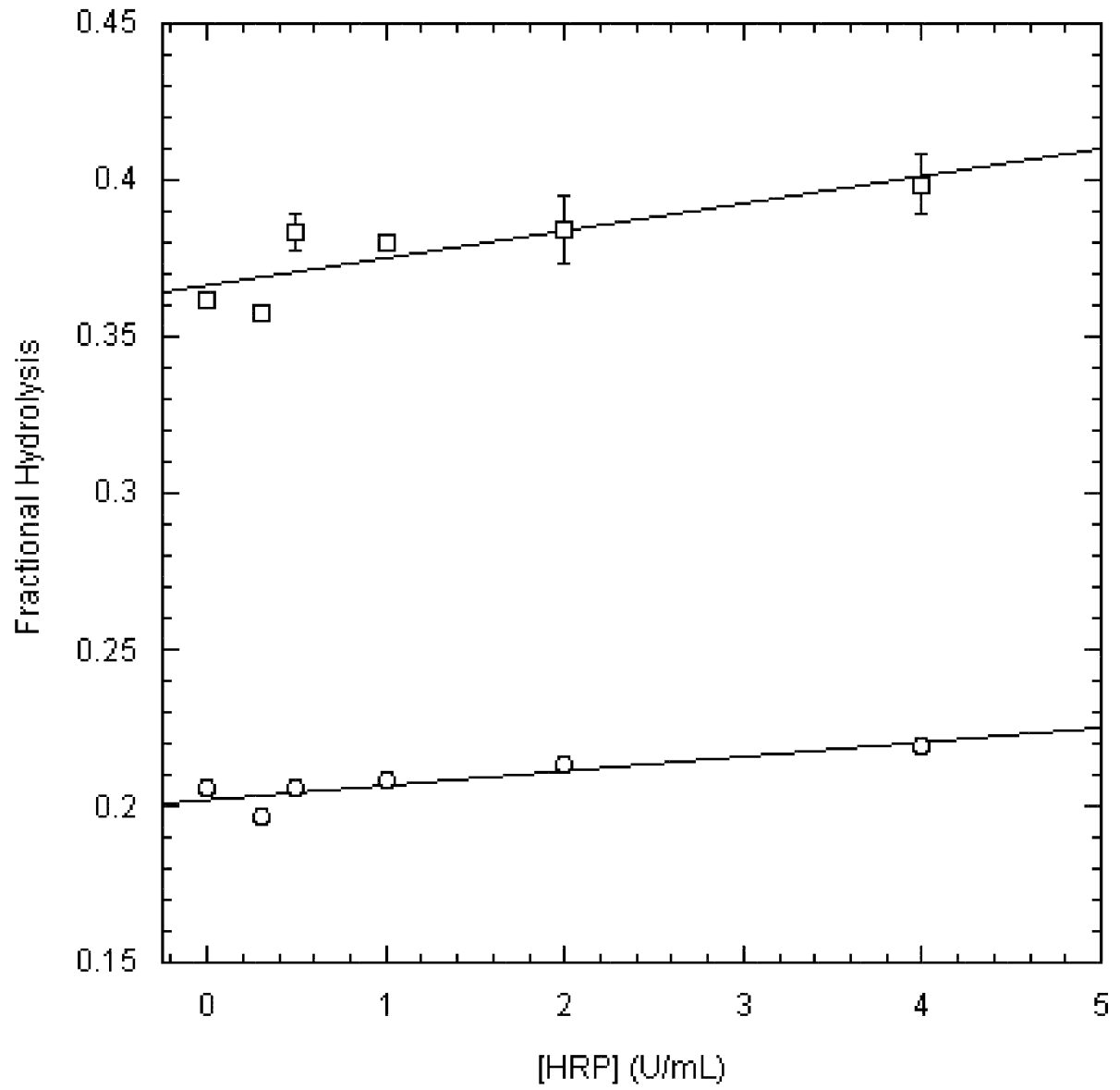


Fig. 2

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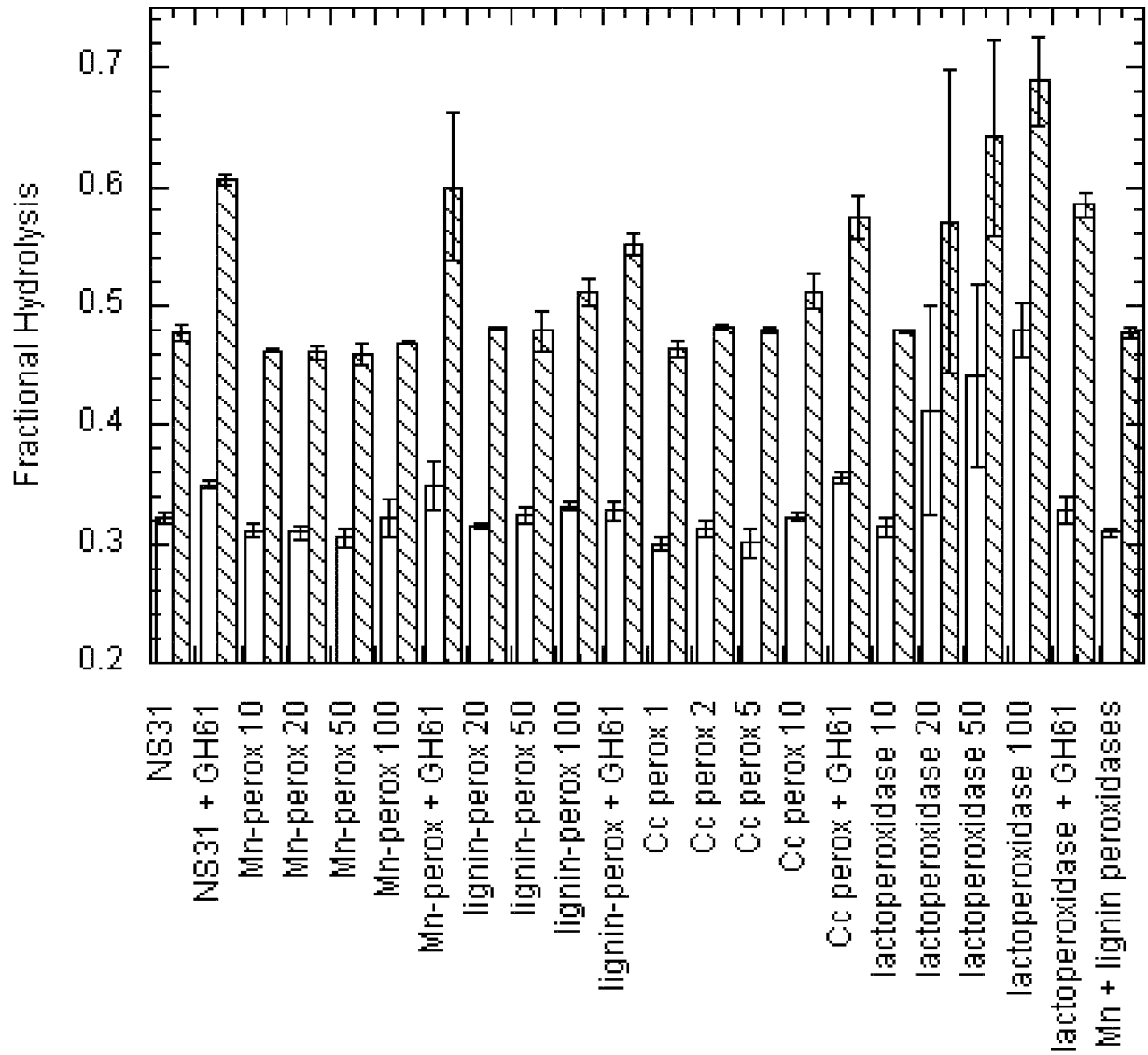


Fig. 3