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(54) Title: METHODS FOR ENHANCING THE DEGRADATION OR CONVERSION OF CELLULOSIC MATERIAL

(57) Abstract: 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 cellulolytic enhancing activity.



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**METHODS FOR ENHANCING THE DEGRADATION OR CONVERSION
OF CELLULOSIC MATERIAL**

5 **Statement as to Rights to Inventions Made Under
 Federally Sponsored Research and Development**

This invention was made with Government support under Cooperative Agreement DE-FC36-08GO18080 awarded by the Department of Energy. The government has certain rights in this invention.

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Reference to a Sequence Listing

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

15

Background of the Invention

Field of the Invention

The present invention relates to methods for enhancing the degradation or conversion of cellulosic material with enzyme compositions.

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Description of the Related Art

25 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.

30 The conversion of lignocellulosic feedstocks into ethanol has the advantages of the ready availability of large amounts of feedstock, the desirability of avoiding burning or land filling the materials, and the cleanliness of the ethanol fuel. Wood, agricultural residues, herbaceous crops, and municipal solid wastes have been considered as feedstocks for ethanol production. These materials primarily consist of cellulose, hemicellulose, and lignin. Once the cellulose is converted to glucose, the glucose is easily fermented by yeast into ethanol.

35

It would be advantageous in the art to improve the ability to convert cellulosic feedstocks.

Nierman *et al.*, 2005, *Nature* 438: 1151-1156 disclose the genome sequence of *Aspergillus fumigatus*.

The present invention relates to improved enzyme compositions for degrading or converting cellulosic material.

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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 cellulolytic enhancing activity, wherein the polypeptide having cellulolytic enhancing activity is selected from the group consisting of:

- (a) a polypeptide comprising an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2;
- (b) a polypeptide encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii);
- (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof; and
- (d) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.

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 cellulolytic enhancing activity, wherein the polypeptide having cellulolytic enhancing activity is selected from the group consisting of:
 - (a) a polypeptide comprising an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2;
 - (b) a polypeptide encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii);
 - (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof; and
 - (d) a variant comprising a substitution, deletion, and/or insertion of one or

more (several) amino acids of the mature polypeptide of SEQ ID NO: 2;

(B) fermenting the saccharified cellulosic material with one or more (several) fermenting microorganisms; and

(C) recovering the fermentation product from the fermentation.

5 The present invention also 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 saccharified with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity, wherein the polypeptide having cellulolytic enhancing activity is selected from the group consisting of:

10 (a) a polypeptide comprising an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2;

(b) a polypeptide encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii);

15 (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof; and

20 (d) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.

The present invention further relates to enzyme compositions comprising a polypeptide having cellulolytic enhancing activity and one or more (several) cellulolytic enzymes, wherein the polypeptide having cellulolytic enhancing activity is selected from the group consisting of:

25 (a) a polypeptide comprising an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2;

(b) a polypeptide encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii);

30 (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof; and

35 (d) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.

Brief Description of the Figures

Figure 1 shows the genomic DNA sequence and the deduced amino acid sequence of an *Aspergillus fumigatus* gene encoding a GH61B polypeptide having cellulolytic enhancing activity (SEQ ID NOs: 1 and 2, respectively).

Figure 2 shows a restriction map of pAG43.

Figure 3 shows hydrolysis vs. concentration of added *Aspergillus fumigatus* GH61B polypeptide having cellulolytic enhancing activity to a *Trichoderma reesei* cellulase composition in the hydrolysis of washed pretreated corn stover (PCS). Open circles: 3-day extent of hydrolysis; closed circles: 7-day extent of hydrolysis. Data were not corrected for sugars present in the PCS liquor. Data were fitted with a modified non-cooperative saturation-binding model.

Definitions

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

The GH61 polypeptides having cellulolytic enhancing activity enhance the hydrolysis of a cellulosic material catalyzed by enzyme having cellulolytic activity by reducing the amount of cellulolytic enzyme required to reach the same degree of hydrolysis preferably at least 1.01-fold, more preferably at least 1.05-fold, more preferably at least 1.10-fold, more preferably at least 1.25-fold, more preferably at least 1.5-fold, more preferably at least 2-fold, more preferably at least 3-fold, more preferably at least 4-fold, more preferably at least 5-

fold, even more preferably at least 10-fold, and most preferably at least 20-fold.

The polypeptides having cellulolytic enhancing activity have at least 20%, preferably at least 40%, more preferably at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 100% of the cellulolytic enhancing activity of the polypeptide of the mature polypeptide of SEQ ID NO: 2.

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

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

For purposes of the present invention, cellulolytic enzyme activity is determined by measuring the increase in hydrolysis of a cellulosic material by cellulolytic enzyme(s) under the following conditions: 1-20 mg of cellulolytic enzyme protein/g of cellulose in PCS for 3-7 days at 50°C compared to a control hydrolysis without addition of cellulolytic enzyme protein. Typical conditions are 1 ml reactions, washed or unwashed PCS, 5% insoluble solids, 50 mM sodium acetate pH 5, 1 mM MnSO₄, 50-65°C, 72 hours, sugar analysis by AMINEX® HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Endoglucanase: The term "endoglucanase" means an endo-1,4-(1,3;1,4)-beta-D-glucan 4-glucanohydrolase (E.C. 3.2.1.4), which catalyses endohydrolysis of 1,4-beta-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxymethyl cellulose and hydroxyethyl cellulose), lichenin, beta-1,4 bonds in mixed beta-1,3 glucans such as cereal beta-D-glucans or xyloglucans, and other plant material containing cellulosic components.

Endoglucanase activity can be determined by measuring reduction in substrate viscosity or increase in reducing ends determined by a reducing sugar assay (Zhang *et al.*, 2006, *Biotechnology Advances* 24: 452-481). For purposes of the present invention, endoglucanase activity is determined using carboxymethyl cellulose (CMC) as substrate according to the procedure of Ghose, 1987, *Pure and Appl. Chem.* 59: 257-268, at pH 5, 40°C.

Cellobiohydrolase: The term "cellobiohydrolase" means a 1,4-beta-D-glucan cellobiohydrolase (E.C. 3.2.1.91), which catalyzes the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, celooligosaccharides, or any beta-1,4-linked glucose containing polymer, releasing cellobiose from the reducing or non-reducing ends of the chain (Teeri, 1997, Crystalline cellulose degradation: New insight into the function of cellobiohydrolases, *Trends in Biotechnology* 15: 160-167; Teeri *et al.*, 1998, *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose?, *Biochem. Soc. Trans.* 26: 173-178). For purposes of the present invention, cellobiohydrolase activity is determined according to the procedures described by Lever *et al.*, 1972, *Anal. Biochem.* 47: 273-279; van Tilbeurgh *et al.*, 1982, *FEBS Letters*, 149: 152-156; van Tilbeurgh and Claeysens, 1985, *FEBS Letters*, 187: 283-288; and Tomme *et al.*, 1988, *Eur. J. Biochem.* 170: 575-581. In the present invention, the Lever *et al.* method can be employed to assess hydrolysis of cellulose in corn stover, while the methods of van Tilbeurgh *et al.* and Tomme *et al.* can be used to determine the cellobiohydrolase activity on a fluorescent disaccharide derivative, 4-methylumbelliferyl- β -D-lactoside.

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

Hemicellulolytic enzyme or hemicellulase: The term "hemicellulolytic enzyme" or "hemicellulase" means one or more (several) enzymes that hydrolyze a hemicellulosic material. See, for example, Shallom, D. and Shoham, Y. Microbial hemicellulases. *Current Opinion In Microbiology*, 2003, 6(3): 219-228). Hemicellulases are key components in the degradation of plant biomass. Examples of hemicellulases include, but are not limited to, an acetylmannan esterase, an acetyxylan esterase, an arabinanase, an arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl

esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase. The substrates of these enzymes, the hemicelluloses, are a heterogeneous group of branched and linear polysaccharides that are bound via hydrogen bonds to the cellulose microfibrils in the plant cell wall, crosslinking them into a robust network. Hemicelluloses are also covalently attached to lignin, forming together with cellulose a highly complex structure. The variable structure and organization of hemicelluloses require the concerted action of many enzymes for its complete degradation. The catalytic modules of hemicellulases are either glycoside hydrolases (GHs) that hydrolyze glycosidic bonds, or carbohydrate esterases (CEs), which hydrolyze ester linkages of acetate or ferulic acid side groups. These catalytic modules, based on homology of their primary sequence, can be assigned into GH and CE families marked by numbers. Some families, with overall similar fold, can be further grouped into clans, marked alphabetically (e.g., GH-A). A most informative and updated classification of these and other carbohydrate active enzymes is available on the Carbohydrate-Active Enzymes (CAZy) database. Hemicellulolytic enzyme activities can be measured according to Ghose and Bisaria, 1987, *Pure & Appl. Chem.* 59: 1739-1752.

Xylan degrading activity or xylanolytic activity: The term “xylan degrading activity” or “xylanolytic activity” means a biological activity that hydrolyzes xylan-containing material. The two basic approaches for measuring xylanolytic activity include: (1) measuring the total xylanolytic activity, and (2) measuring the individual xylanolytic activities (e.g., endoxylanases, beta-xylosidases, arabinofuranosidases, alpha-glucuronidases, acetylxylan esterases, feruloyl esterases, and alpha-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, for example, oat spelt, beechwood, and larchwood xylans, or by photometric determination of dyed xylan fragments released from various covalently dyed xylans. The most common total xylanolytic activity assay is based on production of reducing sugars from polymeric 4-O-methyl glucuronoxylan as described in Bailey, Biely, Poutanen, 1992, Interlaboratory testing of methods for assay of xylanase activity, *Journal of Biotechnology* 23(3): 257-270. Xylanase activity can also be determined with 0.2% AZCL-arabinoxylan as substrate in 0.01% Triton X-100 and 200 mM sodium phosphate buffer pH 6 at 37°C. One unit of xylanase activity is defined as 1.0 μ mole of

azurine produced per minute at 37°C, pH 6 from 0.2% AZCL-arabinoxylan as substrate in 200 mM sodium phosphate pH 6 buffer.

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

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

Beta-xylosidase: The term "beta-xylosidase" means a beta-D-xyloside xylohydrolase (E.C. 3.2.1.37) that catalyzes the exo-hydrolysis of short beta-(4) - xylooligosaccharides, to remove successive D-xylose residues from the non-reducing termini. For purposes of the present invention, one unit of beta-xylosidase is defined as 1.0 μmole of *p*-nitrophenolate anion produced per minute at 40°C, pH 5 from 1 mM *p*-nitrophenyl-beta-D-xyloside as substrate in 100 mM sodium citrate containing 0.01% TWEEN® 20.

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

Feruloyl esterase: The term "feruloyl esterase" means a 4-hydroxy-3-methoxycinnamoyl-sugar hydrolase (EC 3.1.1.73) that catalyzes the hydrolysis of the 4-hydroxy-3-methoxycinnamoyl (feruloyl) group from an esterified sugar, which is usually arabinose in "natural" substrates, to produce ferulate (4-hydroxy-3-methoxycinnamate). Feruloyl esterase is also known as ferulic acid esterase, hydroxycinnamoyl esterase, FAE-III, cinnamoyl ester hydrolase, FAEA, cinnAE, FAE-I, or FAE-II. For purposes of the present invention, feruloyl esterase activity is determined using 0.5 mM *p*-nitrophenylferulate as

substrate in 50 mM sodium acetate pH 5.0. One unit of feruloyl esterase equals the amount of enzyme capable of releasing 1 μ mole of *p*-nitrophenolate anion per minute at pH 5, 25°C.

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

Alpha-L-arabinofuranosidase: The term "alpha-L-arabinofuranosidase" means an alpha-L-arabinofuranoside arabinofuranohydrolase (EC 3.2.1.55) that catalyzes the hydrolysis of terminal non-reducing alpha-L-arabinofuranoside residues in alpha-L-arabinosides. The enzyme acts on alpha-L-arabinofuranosides, alpha-L-arabinans containing (1,3)- and/or (1,5)-linkages, arabinoxylans, and arabinogalactans. Alpha-L-arabinofuranosidase is also known as arabinosidase, alpha-arabinosidase, alpha-L-arabinosidase, alpha-arabinofuranosidase, polysaccharide alpha-L-arabinofuranosidase, alpha-L-arabinofuranoside hydrolase, L-arabinosidase, or alpha-L-arabinanase. For purposes of the present invention, alpha-L-arabinofuranosidase activity is determined using 5 mg of medium viscosity wheat arabinoxylan (Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland) per ml of 100 mM sodium acetate pH 5 in a total volume of 200 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 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.*,

1995, in Handbook on Bioethanol (Charles E. Wyman, editor), pp.105-118, Taylor & Francis, Washington D.C.; Wyman, 1994, *Bioresource Technology* 50: 3-16; Lynd, 1990, *Applied Biochemistry and Biotechnology* 24/25: 695-719; Mosier *et al.*, 1999, Recent Progress in Bioconversion of Lignocellulosics, in *Advances in Biochemical Engineering/Biotechnology*, T. Scheper, managing editor, Volume 65, pp.23-40, Springer-Verlag, New York). It is understood herein that the cellulose may be in the form of lignocellulose, a plant cell wall material containing lignin, cellulose, and hemicellulose in a mixed matrix. In a preferred aspect, the cellulosic material is lignocelluloses, which comprises cellulose, hemicellulose, and lignin.

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

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

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

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

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

Isolated or Purified: The term "isolated" or "purified" means a polypeptide or polynucleotide that is removed from at least one component with which it is naturally associated. For example, a polypeptide may be at least 1% pure, *e.g.*, at least 5% pure, at least 10% pure, at least 20% pure, at least 40% pure, at least 60% pure, at least 80% pure, at least 90% pure, or at least 95% pure, as determined by SDS-PAGE and a polynucleotide may be at least 1% pure, *e.g.*, at least 5% pure, at least 10% pure, at least 20% pure, at least 40% pure, at least 60% pure, at least 80% pure, at least 90% pure, or at least 95%

pure, as determined by agarose electrophoresis.

Mature polypeptide: The term “mature polypeptide” means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. It is known in the art that a host cell may produce a mixture of two or more different mature polypeptides (*i.e.*, with a different C-terminal and/or N-terminal amino acid) expressed by the same polynucleotide. In one aspect, the mature polypeptide is amino acids 22 to 250 of SEQ ID NO: 2 based on the SignalP program (Nielsen *et al.*, 1997, *Protein Engineering* 10: 1-6) that predicts amino acids 1 to 21 of SEQ ID NO: 2 are a signal peptide.

Mature polypeptide coding sequence: The term “mature polypeptide coding sequence” is defined herein as a nucleotide sequence that encodes a mature polypeptide having biological activity. In one aspect, the mature polypeptide coding sequence is nucleotides 64 to 859 of SEQ ID NO: 1 based on the SignalP program (Nielsen *et al.*, 1997, *supra*) that predicts nucleotides 1 to 63 of SEQ ID NO: 1 encode a signal peptide. In another aspect, the mature polypeptide coding sequence is the cDNA sequence contained in nucleotides 64 to 859 of SEQ ID NO: 1.

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

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

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

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

(Identical Deoxyribonucleotides x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

Polypeptide fragment: The term “fragment” means a polypeptide having one or more (several) amino acids deleted from the amino and/or carboxyl terminus of a mature polypeptide; wherein the fragment has biological activity. In one aspect, a fragment contains at least 200 amino acid residues, e.g., at least 210 amino acid residues or at least 220 amino acid residues of the mature polypeptide of SEQ ID NO: 2.

Subsequence: The term “subsequence” means a polynucleotide having one or more (several) nucleotides deleted from the 5' and/or 3' end of a mature polypeptide coding sequence; wherein the subsequence encodes a fragment having biological activity. In one aspect, a subsequence contains at least 600 nucleotides, e.g., at least 630 nucleotides or at least 660 nucleotides of the mature polypeptide coding sequence of SEQ ID NO: 1.

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

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

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

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

Control sequences: The term “control sequences” means all components necessary for the expression of a polynucleotide encoding a polypeptide of the present

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

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

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

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

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

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

Detailed Description 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 cellulolytic enhancing activity, wherein the polypeptide having cellulolytic enhancing activity is selected from the group consisting of: (a) a

polypeptide comprising an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii); (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof; and (d) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.

In one aspect, the method above further comprises recovering the degraded or converted cellulosic material. Soluble products of degradation or conversion of the cellulosic material can be separated from the insoluble cellulosic material using technology well known in the art such as, for example, centrifugation, filtration, and gravity settling.

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 cellulolytic enhancing activity, wherein the polypeptide having cellulolytic enhancing activity is selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii); (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof; and (d) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2; (B) fermenting the saccharified cellulosic material with one or more (several) fermenting microorganisms; and (C) recovering the fermentation product from the fermentation.

The present invention also 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 saccharified with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity, wherein the polypeptide having cellulolytic enhancing activity is selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature

polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii); (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof; and (d) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2. In a preferred aspect, the fermenting of the cellulosic material produces a fermentation product. In another preferred aspect, the method further comprises recovering the fermentation product from the fermentation.

The present invention further relates to enzyme compositions comprising a polypeptide having cellulolytic enhancing activity and one or more (several) cellulolytic enzymes, wherein the polypeptide having cellulolytic enhancing activity is selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii); (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof; and (d) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.

Enzyme Compositions

In the methods of the present invention, the enzyme composition can comprise any protein that is useful in degrading or converting a cellulosic material.

In one aspect, the enzyme composition comprises one or more (several) cellulolytic enzymes. In another aspect, the enzyme composition comprises or further comprises one or more (several) hemicellulolytic enzymes. In another aspect, the enzyme composition comprises one or more (several) cellulolytic enzymes and one or more (several) hemicellulolytic enzymes. In another aspect, the enzyme composition comprises one or more (several) enzymes selected from the group of cellulolytic enzymes and hemicellulolytic enzymes.

In another aspect, the enzyme composition comprises one or more (several) cellulolytic enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase. In another aspect, the enzyme composition comprises or further comprises one or more (several) proteins selected from the group consisting of a hemicellulase, an expansin, an esterase, a ligninolytic enzyme, a pectinase, a

peroxidase, a protease, and a swollenin. The hemicellulase is preferably one or more (several) enzymes selected from the group consisting of an acetylmannan esterase, an acetyxylan esterase, an arabinanase, an arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase.

In another aspect, the enzyme composition comprises an endoglucanase. In another aspect, the enzyme composition comprises a cellobiohydrolase. In another aspect, the enzyme composition comprises a beta-glucosidase. In another aspect, the enzyme composition comprises an acetylmannan esterase. In another aspect, the enzyme composition comprises an acetyxylan esterase. In another aspect, the enzyme composition comprises an arabinanase (*e.g.*, alpha-L-arabinanase). In another aspect, the enzyme composition comprises an arabinofuranosidase (*e.g.*, alpha-L-arabinofuranosidase). In another aspect, the enzyme composition comprises a coumaric acid esterase. In another aspect, the enzyme composition comprises a feruloyl esterase. In another aspect, the enzyme composition comprises a galactosidase (*e.g.*, alpha-galactosidase and/or beta-galactosidase). In another aspect, the enzyme composition comprises a glucuronidase (*e.g.*, alpha-D-glucuronidase). In another aspect, the enzyme composition comprises a glucuronoyl esterase. In another aspect, the enzyme composition comprises a mannanase. In another aspect, the enzyme composition comprises a mannosidase (*e.g.*, beta-mannosidase). In another aspect, the enzyme composition comprises a xylanase. In a preferred aspect, the xylanase is a Family 10 xylanase. In another aspect, the enzyme composition comprises a xylosidase.

In another aspect, the enzyme composition comprises an expansin. In another aspect, the enzyme composition comprises an esterase. In another aspect, the enzyme composition comprises a ligninolytic enzyme. In a preferred aspect, the ligninolytic enzyme is a laccase. In another preferred aspect, the ligninolytic enzyme is a manganese peroxidase. In another preferred aspect, the ligninolytic enzyme is a lignin peroxidase. In another preferred aspect, the ligninolytic enzyme is a H₂O₂-producing enzyme. In another aspect, the enzyme composition comprises a pectinase. In another aspect, the enzyme composition comprises a peroxidase. In another aspect, the enzyme composition comprises a protease. In another aspect, the enzyme composition comprises a swollenin.

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

One or more (several) components of the enzyme composition may be wild-type proteins, recombinant proteins, or a combination of wild-type proteins and recombinant proteins. For example, one or more (several) components may be native proteins of a cell,

which is used as a host cell to express recombinantly one or more (several) other components of the enzyme composition. One or more (several) components of the enzyme composition may be produced as monocomponents, which are then combined to form the enzyme composition. The enzyme composition may be a combination of multicomponent and monocomponent protein preparations.

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

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

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

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

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

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

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

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

In another preferred aspect, the polypeptide is an *Acremonium cellulolyticus*, *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus fumigatus*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium tropicum*, *Chrysosporium merdarium*, *Chrysosporium inops*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium zonatum*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium 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 enzyme activity.

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

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

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

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

Examples of fungal endoglucanases that can be used in the present invention include, but are not limited to, a *Trichoderma reesei* endoglucanase I (Penttila *et al.*, 1986, *Gene* 45: 253-263; *Trichoderma reesei* Cel7B endoglucanase I; GENBANK™ accession no. M15665; SEQ ID NO: 4); *Trichoderma reesei* endoglucanase II (Saloheimo, *et al.*, 1988,

Gene 63:11-22; *Trichoderma reesei* Cel5A endoglucanase II; GENBANK™ accession no. M19373; SEQ ID NO: 6); *Trichoderma reesei* endoglucanase III (Okada *et al.*, 1988, *Appl. Environ. Microbiol.* 64: 555-563; GENBANK™ accession no. AB003694; SEQ ID NO: 8); *Trichoderma reesei* endoglucanase IV (Saloheimo *et al.*, 1997, *Eur. J. Biochem.* 249: 584-591; GENBANK™ accession no. Y11113; SEQ ID NO: 10); *Trichoderma reesei* endoglucanase V (Saloheimo *et al.*, 1994, *Molecular Microbiology* 13: 219-228; GENBANK™ accession no. Z33381; SEQ ID NO: 12); *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: 14); *Myceliophthora thermophila* CBS 117.65 endoglucanase (SEQ ID NO: 16); basidiomycete CBS 495.95 endoglucanase (SEQ ID NO: 18); basidiomycete CBS 494.95 endoglucanase (SEQ ID NO: 20); *Thielavia terrestris* NRRL 8126 CEL6B endoglucanase (SEQ ID NO: 22); *Thielavia terrestris* NRRL 8126 CEL6C endoglucanase (SEQ ID NO: 24); *Thielavia terrestris* NRRL 8126 CEL7C endoglucanase (SEQ ID NO: 26); *Thielavia terrestris* NRRL 8126 CEL7E endoglucanase (SEQ ID NO: 28); *Thielavia terrestris* NRRL 8126 CEL7F endoglucanase (SEQ ID NO: 30); *Cladorrhinum foecundissimum* ATCC 62373 CEL7A endoglucanase (SEQ ID NO: 32); and *Trichoderma reesei* strain No. VTT-D-80133 endoglucanase (SEQ ID NO: 34; GENBANK™ accession no. M15665). The endoglucanases of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, and SEQ ID NO: 34 described above are encoded by the mature polypeptide coding sequence of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, and SEQ ID NO: 33, respectively.

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

cellobiohydrolase II (SEQ ID NO: 50). The cellobiohydrolases of SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, and SEQ ID NO: 50 described above are encoded by the mature polypeptide coding sequence of SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, and SEQ ID NO: 49, respectively.

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

The *Aspergillus oryzae* polypeptide having beta-glucosidase activity can be obtained 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 aculeatus* polypeptide having beta-glucosidase activity can be obtained according to Kawaguchi *et al.*, 1996, *Gene* 173: 287-288.

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

In one aspect, the one or more (several) cellulolytic enzymes comprise endoglucanase. In another aspect, the one or more (several) cellulolytic enzymes comprise endoglucanase I. In another aspect, the one or more (several) cellulolytic enzymes comprise endoglucanase II. In another aspect, the one or more (several) cellulolytic enzymes comprise endoglucanase III. In another aspect, the one or more (several) cellulolytic enzymes comprise endoglucanase IV. In another aspect, the one or more (several) cellulolytic enzymes comprise endoglucanase V. In another aspect, the one or more (several) cellulolytic enzymes comprise cellobiohydrolase. In another aspect, the one or more (several) cellulolytic enzymes comprise cellobiohydrolase I. In another aspect, the one or more (several) cellulolytic enzymes comprise beta-glucosidase. In another aspect, the one or more (several) cellulolytic enzymes comprise a beta-glucosidase fusion protein. In

another aspect, the one or more (several) cellulolytic enzymes comprise endoglucanase and beta-glucosidase. In another aspect, the one or more (several) cellulolytic enzymes comprise endoglucanase and cellobiohydrolase I. In another aspect, the one or more (several) cellulolytic enzymes comprise endoglucanase, cellobiohydrolase I, and beta-glucosidase.

In another aspect, the beta-glucosidase is *Aspergillus oryzae* beta-glucosidase (SEQ ID NO: 52). In another aspect, the beta-glucosidase is *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 54). In another aspect, the beta-glucosidase is *Penicillium brasilianum* IBT 20888 beta-glucosidase (SEQ ID NO: 56). In another aspect, the beta-glucosidase is *Aspergillus niger* beta-glucosidase (SEQ ID NO: 58). In another aspect, the beta-glucosidase is *Aspergillus aculeatus* beta-glucosidase (SEQ ID NO: 60). In another aspect, the beta-glucosidase is the *Aspergillus oryzae* beta-glucosidase variant fusion protein of SEQ ID NO: 62 or the *Aspergillus oryzae* beta-glucosidase fusion protein of SEQ ID NO: 64. In another aspect, the *Aspergillus oryzae* beta-glucosidase variant fusion protein is encoded by the polynucleotide of SEQ ID NO: 61 or the *Aspergillus oryzae* beta-glucosidase fusion protein is encoded by the polynucleotide of SEQ ID NO: 63.

In another aspect, the one or more (several) cellulolytic enzymes comprise a beta-glucosidase; a *Trichoderma reesei* cellobiohydrolase I (CEL7A), a *Trichoderma reesei* cellobiohydrolase II (CEL6A), and a *Trichoderma reesei* endoglucanase I (CEL7B). In another aspect, the one or more (several) cellulolytic enzymes comprise an *Aspergillus oryzae* beta-glucosidase; a *Trichoderma reesei* cellobiohydrolase I (CEL7A), a *Trichoderma reesei* cellobiohydrolase II (CEL6A), and a *Trichoderma reesei* endoglucanase I (CEL7B). In another aspect, the one or more (several) cellulolytic enzymes comprise an *Aspergillus niger* beta-glucosidase; a *Trichoderma reesei* cellobiohydrolase I (CEL7A), a *Trichoderma reesei* cellobiohydrolase II (CEL6A), and a *Trichoderma reesei* endoglucanase I (CEL7B). In another aspect, the one or more (several) cellulolytic enzymes comprise an *Aspergillus fumigatus* beta-glucosidase; a *Trichoderma reesei* cellobiohydrolase I (CEL7A), a *Trichoderma reesei* cellobiohydrolase II (CEL6A), and a *Trichoderma reesei* endoglucanase I (CEL7B). In another aspect, the one or more (several) cellulolytic enzymes comprise a *Penicillium brasilianum* beta-glucosidase; a *Trichoderma reesei* cellobiohydrolase I (CEL7A), a *Trichoderma reesei* cellobiohydrolase II (CEL6A), and a *Trichoderma reesei* endoglucanase I (CEL7B). In another aspect, the one or more (several) cellulolytic enzymes comprise an *Aspergillus oryzae* beta-glucosidase variant BG fusion protein, a *Trichoderma reesei* cellobiohydrolase I (CEL7A), a *Trichoderma reesei* cellobiohydrolase II (CEL6A), and a *Trichoderma reesei* endoglucanase I (CEL7B). In another aspect, the one or more (several) cellulolytic enzymes comprise an *Aspergillus oryzae* beta-glucosidase fusion protein, a *Trichoderma reesei* cellobiohydrolase I (CEL7A), a *Trichoderma reesei*

cellobiohydrolase II (CEL6A), and a *Trichoderma reesei* endoglucanase I (CEL7B).

In another aspect, the one or more (several) cellulolytic enzymes above further comprise one or more (several) enzymes selected from the group consisting of a *Trichoderma reesei* endoglucanase II (CEL5A), a *Trichoderma reesei* endoglucanase V (CEL45A), and a *Trichoderma reesei* endoglucanase III (CEL12A).

Other cellulolytic enzymes that may be useful in the present invention are described in EP 495,257, EP 531,315, EP 531,372, WO 89/09259, WO 94/07998, WO 95/24471, WO 96/11262, WO 96/29397, WO 96/034108, WO 97/14804, WO 98/08940, WO 98/012307, WO 98/13465, WO 98/015619, WO 98/015633, WO 98/028411, WO 99/06574, WO 99/10481, WO 99/025846, WO 99/025847, WO 99/031255, WO 2000/009707, WO 2002/050245, WO 2002/0076792, WO 2002/101078, WO 2003/027306, WO 2003/052054, WO 2003/052055, WO 2003/052056, WO 2003/052057, WO 2003/052118, WO 2004/016760, WO 2004/043980, WO 2004/048592, WO 2005/001065, WO 2005/028636, WO 2005/093050, WO 2005/093073, WO 2006/074005, WO 2006/117432, WO 2007/071818, WO 2007/071820, WO 2008/008070, WO 2008/008793, U.S. Patent No. 4,435,307, U.S. Patent No. 5,457,046, U.S. Patent No. 5,648,263, U.S. Patent No. 5,686,593, U.S. Patent No. 5,691,178, U.S. Patent No. 5,763,254, and U.S. Patent No. 5,776,757.

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

Examples of xylanases useful in the 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).

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).

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*

5 *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 esterase (Uniprot accession number Q0UJH1), and *Humicola insolens* DSM 1800 acetylxylan esterase (WO 2009/073709).

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).

10 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).

15 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*
20 *fumigatus* alpha-glucuronidase (SwissProt accession number Q4WW45).

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

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

Polypeptides Having Cellulolytic Enhancing Activity and Polynucleotides Thereof

In a first aspect, the isolated polypeptides having cellulolytic enhancing activity have a sequence identity to the mature polypeptide of SEQ ID NO: 2 of at least 75%, e.g., at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, which have cellulolytic enhancing activity. In one aspect, the polypeptides differ by no more than ten amino acids, e.g., by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 2.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 2 or an allelic variant thereof; or is a fragment thereof having cellulolytic enhancing activity. In another aspect, the polypeptide comprises or consists of SEQ ID NO: 2. In another aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 2. In another preferred aspect, the polypeptide comprises or consists of amino acids 22 to 250 of SEQ ID NO: 2.

In a second aspect, the isolated polypeptides having cellulolytic enhancing activity are encoded by polynucleotides that hybridize under preferably medium-high stringency conditions, more preferably high stringency conditions, and most preferably very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii) (J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York).

The polynucleotide of SEQ ID NO: 1 or a subsequence thereof, as well as the amino acid sequence of SEQ ID NO: 2 or a fragment thereof, may be used to design nucleic acid probes to identify and 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 sequence, but should be at least 14, e.g., at least 25, at least 35, or at least 70 nucleotides in length. Preferably, the nucleic acid probe is at least 100 nucleotides in length, e.g., at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500 nucleotides, at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, or 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 ^{32}P , ^3H , ^{35}S , biotin, or avidin). Such probes are encompassed by the present invention.

A genomic DNA or cDNA library prepared from such other strains may 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 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 polynucleotide hybridizes to a labeled nucleic acid probe corresponding to SEQ ID NO: 1; the mature polypeptide coding sequence of SEQ ID NO: 1; the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1; its full-length complementary strand; or a subsequence thereof; under very low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions can be detected using, for example, X-ray film.

In one aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof. In another aspect, the nucleic acid probe is nucleotides 64 to 859 of SEQ ID NO: 1 or the cDNA sequence thereof. In another aspect, the nucleic acid probe is a polynucleotide that encodes the polypeptide of SEQ ID NO: 2 or the mature polypeptide thereof; or a fragment thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 1 or the cDNA sequence thereof.

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 micrograms/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% formamide for high and very high stringencies, following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 45°C (very low stringency), at 50°C (low stringency), at 55°C (medium stringency), at 60°C (medium-high stringency), at 65°C (high stringency), and 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 and hybridization at about 5°C to about 10°C below the calculated T_m using the calculation according to Bolton and McCarthy (1962, *Proc. Natl. Acad. Sci. USA* 48:1390) in 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. The carrier material is finally washed once

in 6X SCC 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 third aspect, the isolated polypeptides having cellulolytic enhancing activity are encoded by polynucleotides having a sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof of at least 75%, e.g., at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, which encode a polypeptide having cellulolytic enhancing activity.

In a fourth aspect, the isolated polypeptides having cellulolytic enhancing activity are variants comprising a substitution, deletion, and/or insertion of one or more (or several) amino acids of the mature polypeptide of SEQ ID NO: 2, or a homologous sequence thereof. Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

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

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

Essential amino acids in a parent polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for cellulolytic enhancing activity to identify amino acid

residues that are critical to the activity of the molecule. See also, Hilton *et al.*, 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Lett.* 309: 59-64. The identities of essential amino acids can also be inferred from analysis of identities with polypeptides that are related to the parent polypeptide.

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

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

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

The polypeptide having cellulolytic enhancing activity may be hybrid polypeptide in which a portion of one polypeptide is fused at the N-terminus or the C-terminus of a portion of another polypeptide.

The polypeptide having cellulolytic enhancing activity may be a fused polypeptide or cleavable fusion polypeptide in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide. A fused polypeptide is produced by fusing a polynucleotide encoding another polypeptide to a polynucleotide of the present invention. Techniques for producing fusion polypeptides are known in the art, and include ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fused polypeptide is under control of the same promoter(s) and terminator. Fusion proteins may also be constructed using intein technology in which fusions are created post-translationally (Cooper *et al.*, 1993, *EMBO J.* 12: 2575-2583; Dawson *et al.*, 1994, *Science* 266: 776-779).

A fusion polypeptide can further comprise a cleavage site between the two polypeptides. Upon secretion of the fusion protein, the site is cleaved releasing the two polypeptides. Examples of cleavage sites include, but are not limited to, the sites disclosed in Martin *et al.*, 2003, *J. Ind. Microbiol. Biotechnol.* 3: 568-576; Svetina *et al.*, 2000, *J. Biotechnol.* 76: 245-251; Rasmussen-Wilson *et al.*, 1997, *Appl. Environ. Microbiol.* 63: 3488-3493; Ward *et al.*, 1995, *Biotechnology* 13: 498-503; and Contreras *et al.*, 1991, *Biotechnology* 9: 378-381; Eaton *et al.*, 1986, *Biochemistry* 25: 505-512; Collins-Racie *et al.*, 1995, *Biotechnology* 13: 982-987; Carter *et al.*, 1989, *Proteins: Structure, Function, and Genetics* 6: 240-248; and Stevens, 2003, *Drug Discovery World* 4: 35-48.

A polypeptide having cellulolytic enhancing activity may be obtained from microorganisms of any genus. For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the polypeptide encoded by a nucleotide sequence is produced by the source or by a strain in which the nucleotide sequence from the source has been inserted. In a preferred aspect, the polypeptide obtained from a given source is secreted extracellularly.

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

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.

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

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

The polypeptide may also be a fungal polypeptide. For example, the polypeptide may be a yeast polypeptide such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* polypeptide; or 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*,
5 or *Xylaria* polypeptide.

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

10 In another aspect, the polypeptide is an *Acremonium cellulolyticus*, *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*,
15 *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*
20 *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 setosa*, *Thielavia*
25 *spededonium*, *Thielavia subthermophila*, *Thielavia terrestris*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* polypeptide.

In another aspect, the polypeptide is an *Aspergillus fumigatus* polypeptide having cellulolytic enhancing activity, e.g., the polypeptide comprising the mature polypeptide of
30 SEQ ID NO: 2.

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.

35 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).

5 The polypeptide 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 encoding the polypeptide may then be obtained by similarly screening a genomic or cDNA library of another microorganism or mixed DNA sample. 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 that encode polypeptides having cellulolytic enhancing activity can be isolated and utilized to practice the methods of the present invention, as described herein.

15 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. The cloning of the polynucleotides 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 procedures such as ligase chain reaction (LCR), ligation activated transcription (LAT) and polynucleotide-based amplification (NASBA) may be used. The polynucleotides may be cloned from a strain of *Aspergillus*, or another or related organism and thus, for example, may be an allelic or species variant of the polypeptide encoding region of the polynucleotide.

25 In the methods of the present invention, the isolated polynucleotides comprise or consist of nucleotide sequences that have a sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof of at least 75%, e.g., at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which encode a polypeptide having cellulolytic enhancing activity.

30 Modification of a polynucleotide encoding a polypeptide may be necessary for the synthesis of polypeptides substantially similar to the polypeptide. The term "substantially similar" to the polypeptide refers to non-naturally occurring forms of the polypeptide. These polypeptides may differ in some engineered way from the polypeptide isolated from its native source, e.g., variants that differ in specific activity, thermostability, pH optimum, or the like. The variant may be constructed on the basis of the polynucleotide presented as the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof, e.g., a

subsequence thereof, and/or by introduction of nucleotide substitutions that do not result in a change in the amino acid sequence of the polypeptide, but which correspond to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions that may give rise to a different amino acid sequence. For a general description of nucleotide substitution, see, *e.g.*, Ford *et al.*, 1991, *Protein Expression and Purification* 2: 95-107.

In the methods of the present invention, the isolated polynucleotides hybridize under preferably medium-high stringency conditions, more preferably high stringency conditions, and most preferably very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii); or allelic variants and subsequences thereof (Sambrook *et al.*, 1989, *supra*), as defined herein.

In one aspect, the polynucleotide comprises or consists of SEQ ID NO: 1, the mature polypeptide coding sequence of SEQ ID NO: 1; or the cDNA sequence thereof; or a subsequence of SEQ ID NO: 1 that encodes a fragment of SEQ ID NO: 2 having cellulolytic enhancing activity, such as the polynucleotide of nucleotides 64 to 859 of SEQ ID NO: 1.

Nucleic Acid Constructs

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

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

Examples of suitable promoters for directing the transcription of the nucleic acid constructs in the present invention in a bacterial host cell are the promoters obtained from

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

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

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

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

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

10 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*.

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

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

20 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).

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

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

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

35 The control sequence may also be a signal peptide coding region that encodes a signal peptide linked to the N-terminus of a polypeptide and directs the polypeptide into the cell's secretory pathway. The 5'-end of the coding sequence of the polynucleotide may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the polypeptide. Alternatively,

the 5'-end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. The foreign signal peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding sequence. Alternatively, the foreign signal peptide coding sequence may simply replace the natural signal peptide coding sequence in order to enhance secretion of the polypeptide. However, any signal peptide coding sequence that directs the expressed polypeptide into the secretory pathway of a host cell of choice may be used.

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

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

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

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

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

It may also be desirable to add regulatory sequences that allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those that cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound.

Regulatory systems in prokaryotic systems include the *lac*, *tac*, and *trp* operator systems. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the *Aspergillus niger* glucoamylase promoter, *Aspergillus oryzae* TAKA alpha-amylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used. Other examples of regulatory sequences are those that allow for gene amplification. In eukaryotic systems, these regulatory sequences include the dihydrofolate reductase gene that is amplified in the presence of methotrexate, and the metallothionein genes that are amplified with heavy metals. In these cases, the polynucleotide encoding the polypeptide would be operably linked with the regulatory sequence.

Expression Vectors

The various nucleotide and control sequences 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 polynucleotide encoding a polypeptide, e.g., a polypeptide having cellulolytic enhancing activity, a cellulolytic enzyme, a hemicellulolytic enzyme, etc., at such sites. Alternatively, the polynucleotide may be expressed by inserting the polynucleotide or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

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

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

The vector preferably contains one or more (several) selectable markers that permit easy selection of transformed, transfected, transduced, or the like cells. A selectable marker

is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

5 Examples of bacterial selectable markers are the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers that confer antibiotic resistance such as ampicillin, chloramphenicol, kanamycin, or tetracycline resistance. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for use in a filamentous fungal host cell include, but are not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in an *Aspergillus* cell are the *amdS* and *pyrG* genes of *Aspergillus nidulans* or *Aspergillus oryzae* and the *bar* gene of *Streptomyces hygroscopicus*.
10

The vector preferably contains an element(s) that permits integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome.
15

For integration into the host cell genome, the vector may rely on the polynucleotide's sequence encoding the polypeptide or any other element of the vector for integration into the genome by homologous or non-homologous recombination. Alternatively, the vector may contain additional polynucleotides for directing integration by homologous recombination into the genome of the host cell at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, 400 to 10,000 base pairs, and 800 to 10,000 base pairs, which have a high degree of sequence identity to the corresponding target sequence to enhance the probability of homologous recombination.
20
25 The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding polynucleotides. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in a cell. The term "origin of replication" or "plasmid replicator" means a polynucleotide that enables a plasmid or vector to replicate *in vivo*.
30

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*.
35

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.

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

10 More than one copy of a polynucleotide may be inserted into a host cell to increase production of a polypeptide. An increase in the copy number of the polynucleotide can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the polynucleotide where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the polynucleotide, can be selected for by cultivating the cells in the presence of
15 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*).

20 **Host Cells**

Recombinant host cells comprising a polynucleotide encoding a polypeptide, *e.g.*, a polypeptide having cellulolytic enhancing activity, a cellulolytic enzyme, a hemicellulolytic enzyme, etc., can be advantageously used in the recombinant production of the polypeptide. A construct or vector comprising such a polynucleotide is introduced into a host cell so that
25 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.

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

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

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

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

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

The introduction of DNA into a *Bacillus* cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Mol. Gen. Genet.* 168: 111-115), by using competent cells (see, e.g., Young and Spizizen, 1961, *J. Bacteriol.* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *J. Mol. Biol.* 56: 209-221), by electroporation
15 (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or by conjugation (see, e.g., Koehler and Thorne, 1987, *J. Bacteriol.* 169: 5271-5278). The introduction of DNA into an *E. coli* cell may, for instance, be effected by protoplast transformation (see, e.g., Hanahan, 1983, *J. Mol. Biol.* 166: 557-580) or electroporation (see, e.g., Dower et al., 1988, *Nucleic Acids Res.* 16: 6127-6145). The introduction of DNA into a *Streptomyces* cell may,
20 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
25 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).
30 However, any method known in the art for introducing DNA into a host cell can be used.

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

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

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

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

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

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

For example, the filamentous fungal host cell may be an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*,
30 *Aspergillus niger*, *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*,
35 *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*,

Fusarium reticulatum, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochromum*,
Fusarium sporotrichioides, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium*
trichothecioides, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor*
miehei, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*,
5 *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*
10 *se*. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are
described in EP 238023 and Yelton *et al.*, 1984, *Proc. Natl. Acad. Sci. 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*
15 *Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187,
Academic Press, Inc., New York; Ito *et al.*, 1983, *J. Bacteriol.* 153: 163; and Hinnen *et al.*,
1978, *Proc. Natl. Acad. Sci. USA* 75: 1920.

Methods of Production

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

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

30 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
35 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, it can be recovered from cell lysates.

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

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

15 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.

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

Methods for Processing Cellulosic Material

25 The compositions and 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.

30 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.

35 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 co-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.

5 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).

10 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.

15 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.

20 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.

25 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.

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

35 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 %, and most preferably between 30-60 wt %, and most preferably between 30-60 wt %, and most preferably between 30-60 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).

5 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.

10 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
15 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
20 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.

25 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,
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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).

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

Enzymatic hydrolysis is preferably carried out in a suitable aqueous environment under conditions that can be readily determined by one skilled in the art. In a preferred aspect, hydrolysis is performed under conditions suitable for the activity of the enzyme(s), *i.e.*, optimal for the enzyme(s). The hydrolysis can be carried out as a fed batch or continuous process
15 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
20 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
25 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
30 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 one aspect, an effective amount of cellulolytic enzyme protein to cellulosic material
35 is about 0.5 to about 50 mg, preferably at about 0.5 to about 40 mg, more preferably at about 0.5 to about 25 mg, more preferably at about 0.75 to about 20 mg, more preferably at about 0.75 to about 15 mg, even more preferably at about 0.5 to about 10 mg, and most

preferably at about 2.5 to about 10 mg per g of cellulosic material.

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

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

Fermentation. The fermentable sugars obtained from the hydrolyzed cellulosic material can be fermented by one or more (several) fermenting microorganisms capable of fermenting the sugars directly or indirectly into a desired fermentation product. "Fermentation" or "fermentation process" refers to any fermentation process or any process comprising a fermentation step. Fermentation processes also include fermentation processes used in the consumable alcohol industry (e.g., beer and wine), dairy industry (e.g., fermented dairy products), leather industry, and tobacco industry. The fermentation conditions depend on the desired fermentation product and fermenting organism and can easily be determined by one skilled in the art.

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

5 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.

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

10 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*.

15 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*.

20 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.

25 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*.
30 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*:
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Production and Utilization, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212).

Bacteria that can efficiently ferment hexose and pentose to ethanol include, for example, *Zymomonas mobilis* 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
5 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
10 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
15 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
20 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-
25 124; 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
30 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
35 more preferred aspect, the organic acid is glucaric acid. In another more preferred aspect, the organic acid is gluconic acid. In another more preferred aspect, the organic acid is glucuronic acid. In another more preferred aspect, the organic acid is glutaric acid. In

another preferred aspect, the organic acid is 3-hydroxypropionic acid. In another more preferred aspect, the organic acid is itaconic acid. In another more preferred aspect, the organic acid is lactic acid. In another more preferred aspect, the organic acid is malic acid. In another more preferred aspect, the organic acid is malonic acid. In another more preferred aspect, the organic acid is oxalic acid. In another more preferred aspect, the organic acid is propionic acid. In another more preferred aspect, the organic acid is succinic acid. In another more preferred aspect, the organic acid is xylonic acid. See, for example, Chen, R., and Lee, Y. Y., 1997, Membrane-mediated extractive fermentation for lactic acid production from cellulosic biomass, *Appl. Biochem. Biotechnol.* 63-65: 435-448.

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

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

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

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. 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.

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

Examples

5

Materials

Chemicals used as buffers and substrates were commercial products of at least reagent grade.

10

Strains

Aspergillus fumigatus (NN051616) was used as the source of a gene encoding a Family 61 polypeptide having cellulolytic enhancing activity. *Aspergillus oryzae* JaL355 strain (WO 2002/40694) was used for expression of the *Aspergillus fumigatus* Family 61 polypeptide having cellulolytic enhancing activity.

15

Media

PDA plates were composed of 39 g of potato dextrose agar and deionized water to 1 liter.

20

YPG medium was composed of 10 g of yeast extract, 10 g of Bacto peptone, 20 g of glucose, and deionized water to 1 liter.

YPM medium was composed of 10 g of yeast extract, 10 g of Bacto peptone, 20 g of maltose, and deionized water to 1 liter.

25

M410 medium was composed of 50 g of maltose, 50 g of glucose, 2 g of $MgSO_4 \cdot 7H_2O$, 2 g of KH_2PO_4 , 4 g of citric acid anhydrous powder, 8 g of yeast extract, 2 g of urea, 0.5 g of AMG trace metals solution, 0.5 g of $CaCl_2$, and deionized water to 1 liter (pH 6.0).

AMG trace metals solution was composed of 14.3 g of $ZnSO_4 \cdot 7H_2O$, 2.5 g of $CuSO_4 \cdot 5H_2O$, 0.5 g of $NiCl_2 \cdot 6H_2O$, 13.8 g of $FeSO_4 \cdot 7H_2O$, 8.5 g of $MnSO_4 \cdot H_2O$, 3 g of citric acid, and deionized water to 1 liter.

30

Example 1: Identification of a glycosyl hydrolase Family GH61 gene in the genomic sequence of *Aspergillus fumigatus*

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A tblastn search (Altschul *et al.*, 1997, *Nucleic Acids Res.* 25: 3389-3402) of the *Aspergillus fumigatus* partial genome sequence (The Institute for Genomic Research, Rockville, MD) was carried out using as query several known GH61 proteins including GH61A from *Thermoascus aurantiacus* (GeneSeqP Accession Number AEC05922). Several genes were identified as putative Family GH61 homologs based upon a high degree of similarity to the query sequences at the amino acid level. One genomic region of

approximately 850 bp with greater than 70% sequence identity to the *Thermoascus aurantiacus* GH61A sequence at the amino acid level was chosen for further study.

Example 2: *Aspergillus fumigatus* genomic DNA extraction

5 *Aspergillus fumigatus* NN051616 was grown and harvested as described in US Patent No. 7,244,605. Frozen mycelia were ground, by mortar and pestle, to a fine powder and genomic DNA was isolated using a DNEASY® Plant Kit (QIAGEN Inc., Valencia, CA, USA) according to manufacturer's instructions.

10 Example 3: Construction of an *Aspergillus oryzae* expression vector for the *Aspergillus fumigatus* Family GH61B gene

Two synthetic oligonucleotide primers shown below were designed to PCR amplify the *Aspergillus fumigatus* Family GH61B protein gene from the genomic DNA. An IN-FUSION® Cloning Kit (BD Biosciences, Palo Alto, CA, USA) was used to clone the fragment
15 directly into the expression vector pAllo2 (WO 2005/074647), without the need for restriction digestion and ligation.

Forward primer:

5'-ACTGGATTTACCAT**GACTTTGTCCAAGATCACTTCCA**-3' (SEQ ID NO: 65)

Reverse primer:

20 5'-TCACCTCTAGTTAATTA**AGCGTTGAACAGTGCAGGACCAG**-3' (SEQ ID NO: 66)

Bold letters represent coding sequence. The remaining sequences are homologous to the insertion sites of pAllo2.

Fifty picomoles of each of the primers above were used in an amplification reaction containing 204 ng of *Aspergillus fumigatus* genomic DNA (prepared as described in Example
25 2), 1X Pfx Amplification Buffer (Invitrogen, Carlsbad, CA, USA), 1.5 µl of a 10 mM blend of dATP, dTTP, dGTP, and dCTP, 2.5 units of PLATINUM® Pfx DNA Polymerase (Invitrogen, Carlsbad, CA, USA), and 1 µl of 50 mM MgSO₄ in a final volume of 50 µl. The amplification was performed using an EPPENDORF® MASTERCYCLER® 5333 eppgradient S (Eppendorf Scientific, Inc., Westbury, NY, USA) programmed for one cycle at 94°C for 3 minutes; and 30
30 cycles each at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minutes. The heat block was then held at 72°C for 15 minutes followed by a 4°C soak cycle.

The reaction products were isolated by 1.0% agarose gel electrophoresis using 40 mM Tris base-20 mM sodium acetate-1 mM disodium EDTA (TAE) buffer where an approximately 850 bp product band was excised from the gel and purified using a
35 MINELUTE® Gel Extraction Kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's instructions.

The fragment was then cloned into pAlLo2 using an IN-FUSION® Cloning Kit. The vector was digested with *Nco* I and *Pac* I. The fragment was purified by gel electrophoresis as above and a QIAQUICK® Gel Purification Kit (QIAGEN Inc., Valencia, CA, USA). The gene fragment and the digested vector were combined together in a reaction resulting in the expression plasmid pAG43 (Figure 2), in which transcription of the Family GH61B protein gene was under the control of the NA2-tpi promoter (a hybrid of the promoters from the genes for *Aspergillus niger* neutral alpha-amylase and *Aspergillus nidulans* triose phosphate isomerase). The recombination reaction (20 µl) was composed of 1X IN-FUSION® Buffer (BD Biosciences, Palo Alto, CA, USA), 1X BSA (BD Biosciences, Palo Alto, CA, USA), 1 µl of IN-FUSION® enzyme (diluted 1:10) (BD Biosciences, Palo Alto, CA, USA), 166 ng of pAlLo2 digested with *Nco* I and *Pac* I, and 110 ng of the *Aspergillus fumigatus* GH61B protein purified PCR product. The reaction was incubated at 37°C for 15 minutes followed by 15 minutes at 50°C. The reaction was diluted with 40 µl of 10 mM Tris-0.1 M EDTA buffer and 2.5 µl of the diluted reaction was used to transform *E. coli* SOLOPACK® Gold Competent cells (Stratagene, La Jolla, CA, USA). An *E. coli* transformant containing pAG43 (GH61B protein gene) was identified by restriction enzyme digestion and plasmid DNA was prepared using a BIOROBOT® 9600 (QIAGEN Inc., Valencia, CA, USA).

Example 4: Characterization of the *Aspergillus fumigatus* genomic sequence encoding a Family 61 polypeptide having cellulolytic enhancing activity

DNA sequencing of the 862 bp PCR fragment was performed with a Perkin-Elmer Applied Biosystems Model 377 XL Automated DNA Sequencer using dye-terminator chemistry (Giesecke *et al.*, 1992, *supra*) and primer walking strategy. The following vector specific primers were used for sequencing:

pAllo2 5 Seq:

5' TGTCCCTTGTTCGATGCG 3' (SEQ ID NO: 67)

pAllo2 3 Seq:

5' CACATGACTTGGCTTCC 3' (SEQ ID NO: 68)

Nucleotide sequence data were scrutinized for quality and all sequences were compared to each other with assistance of PHRED/PHRAP software (University of Washington, Seattle, WA, USA).

A gene model for the *Aspergillus fumigatus* sequence was constructed based on similarity of the encoded protein to a *Thermoascus aurantiacus* GH61A polypeptide having cellulolytic enhancing activity (GeneSeqP Accession Number AEC05922). The nucleotide sequence and deduced amino acid sequence, SEQ ID NO: 1 and SEQ ID NO: 2, respectively, of the *Aspergillus fumigatus* GH61B gene are shown in Figure 1. The genomic

fragment encodes a polypeptide of 250 amino acids, interrupted by 2 introns of 53 and 56 bp. The % G+C content of the gene and the mature coding sequence are 53.9% and 57%, respectively. Using the SignalP software program (Nielsen *et al.*, 1997, *supra*), a signal peptide of 21 residues was predicted. The predicted mature protein contains 229 amino acids with a predicted molecular mass of 23.39 kDa.

A comparative pairwise global alignment of amino acid sequences was determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of EMBOSS with gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 matrix. The alignment showed that the deduced amino acid sequence of the *Aspergillus fumigatus* gene encoding the GH61 mature polypeptide having cellulolytic enhancing activity shares 72.6% sequence identity (excluding gaps) to the deduced amino acid sequence of a *Thermoascus aurantiacus* GH61A polypeptide having cellulolytic enhancing activity (GeneSeqP Accession Number AEC05922).

Example 5: Expression of the *Aspergillus fumigatus* genomic DNA encoding a GH61B polypeptide having cellulolytic enhancing activity in *Aspergillus oryzae* JaL355

Aspergillus oryzae JaL355 protoplasts were prepared according to the method of Christensen *et al.*, 1988, *Bio/Technology* 6: 1419-1422 and transformed with 6 µg of pAG43. Twenty-six transformants were isolated to individual PDA plates.

Confluent PDA plates of 24 transformants were each washed with 5 ml of 0.01% TWEEN® 20 and the spores were each collected. Eight µl of each spore stock was added to 1 ml of YPG, YPM, and M410 media separately in 24 well plates and incubated at 34°C. After 3 days of incubation, 7.5 µl of supernatant from four transformants were analyzed using a CRITERION® stain-free, 8-16% gradient SDS-PAGE gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. Based on this gel, M410 was chosen as the best medium. Five days after incubation, 7.5 µl of supernatant from each M410 culture was analyzed using a CRITERION® stain-free, 8-16% gradient SDS-PAGE gel. SDS-PAGE profiles of the cultures showed that several transformants had a new major band of approximately 25 kDa.

A confluent plate of one transformant (grown on PDA) was washed with 5 ml of 0.01% TWEEN® 20 and inoculated into four 500 ml Erlenmeyer flasks containing 100 ml of M410 medium to generate broth for characterization of the enzyme. The flasks were harvested on day 5 (300 ml), filtered using a 0.22 µm stericup suction filter (Millipore, Bedford, MA, USA), and stored at 4°C.

Example 6: Purification of an *Aspergillus fumigatus* GH61B polypeptide having

cellulolytic enhancing activity

The filtered shake flask broth (Example 5) containing *Aspergillus fumigatus* GH61B polypeptide having cellulolytic enhancing activity was concentrated using a 10 kDa MWCO AMICON® Ultra centrifugal concentrator (Millipore, Bedford, MA, USA) to an approximately 10-fold smaller volume. The concentrated filtrate was buffer-exchanged and desalted using a BIO-GEL® P-6 desalting column (Bio-Rad Laboratories, Inc., Hercules, CA, USA) pre-equilibrated in 20 mM Tris-(hydroxymethyl)aminomethane (Sigma, St. Louis, MO, USA) pH 8.0, according to the manufacturer's instructions with the following exception: 3 ml of sample was loaded and eluted with 3 ml of buffer. Concentrated, desalted GH61B protein was quantified using a BCA assay (Pierce, Rockford, IL, USA) using bovine serum albumin (Pierce, Rockford, IL, USA) as a protein concentration standard. Quantification was performed in triplicate. Enzyme purity was confirmed using 8-16% gradient SDS-PAGE at 200 volts for 1 hour and staining with Coomassie Bio-Safe Stain (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Example 7: 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 contained 57.5% 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 pretreated corn stover was milled and washed with water prior to use. Milled, washed pretreated corn stover (initial dry weight 32.35%) was prepared by milling in a Cosmos ICMG 40 wet multi-utility grinder (EssEmm Corporation, Tamil Nadu, India), and subsequently washing repeatedly with deionized water and decanting off the supernatant fraction. The dry weight of the milled, water-washed pretreated corn stover was found to be 7.114%.

Example 8: Hydrolysis of pretreated corn stover is enhanced by *Aspergillus fumigatus* GH61B polypeptide having cellulolytic enhancing activity

The hydrolysis of pretreated corn stover was conducted using 2.2 ml, 96-deep well plates (Axygen, Union City, CA) containing a total reaction mass of 1 g. The hydrolysis was performed with 5% total solids of washed, pretreated corn stover, equivalent to 28.75 mg of

cellulose per ml, in 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 cellulose. *Aspergillus fumigatus* GH61B polypeptide was added at concentrations between 0 and 93% (w/w) of total protein. Plates were sealed using an ALPS-300™ plate heat sealer (Abgene, Epsom, United Kingdom) and incubated at 50°C for 0-168 hours with shaking at 150 rpm. All experiments were performed in duplicate or triplicate.

At various time points between 24 and 168 hours of incubation, 100 µl aliquots were removed and the extent of hydrolysis was assayed by high-performance liquid chromatography (HPLC) using the protocol described below.

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. 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 pretreated corn stover 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 is reported as the overall mass conversion to [glucose+cellobiose]/[total cellulose]. Triplicate data points were averaged and standard deviation was calculated.

Fractional hydrolysis was plotted as a function of *Aspergillus fumigatus* GH61B protein concentration, and was fitted with a modified saturation-binding model using Kaleidagraph (Synergy Software). The results shown in Figure 3 indicated enhancement of hydrolysis by the *Trichoderma reesei* cellulase composition in the presence of the *Aspergillus fumigatus* GH61B polypeptide.

The present invention is further described by the following numbered 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 cellulolytic enhancing activity, wherein the polypeptide having cellulolytic enhancing activity is selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii); (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof; and (d) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.

[2] The method of paragraph 1, wherein the cellulosic material is pretreated.

[3] The method of paragraph 1 or 2, 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.

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

[5] The method of paragraph 4, wherein the hemicellulase is one or more (several) enzymes selected from the group consisting of an acetylmannan esterase, an acetyxylan esterase, an arabinanase, an arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase.

[6] The method of any of paragraphs 1-5, further comprising recovering the degraded cellulosic material.

[7] The method of paragraph 6, wherein the degraded cellulosic material is a sugar.

[8] The method of paragraph 7, wherein the sugar is selected from the group consisting of glucose, xylose, mannose, galactose, and arabinose.

[9] The method of any of paragraphs 1-8, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2.

[10] The method of paragraph 9, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 80% sequence identity

with the mature polypeptide of SEQ ID NO: 2.

[11] The method of paragraph 10, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 85% sequence identity with the mature polypeptide of SEQ ID NO: 2.

5 [12] The method of paragraph 11, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 90% sequence identity with the mature polypeptide of SEQ ID NO: 2.

10 [13] The method of paragraph 12, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 95% sequence identity with the mature polypeptide of SEQ ID NO: 2.

[14] The method of paragraph 13, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 97% sequence identity with the mature polypeptide of SEQ ID NO: 2.

15 [15] The method of any of paragraphs 1-14, wherein the polypeptide having cellulolytic enhancing activity comprises or consists of the amino acid sequence of SEQ ID NO: 2 or the mature polypeptide thereof; or a fragment thereof having cellulolytic enhancing activity.

20 [16] The method of any of paragraphs 1-8, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii).

25 [17] The method of paragraph 16, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide that hybridizes under high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii).

30 [18] The method of paragraph 17, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide that hybridizes under very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii).

35 [19] The method of any of paragraphs 1-8, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

[20] The method of paragraph 19, wherein the polypeptide having cellulolytic

enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 80% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

5 [21] The method of paragraph 20, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 85% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

10 [22] The method of paragraph 21, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 90% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

15 [23] The method of paragraph 22, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 95% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

[24] The method of paragraph 23, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 97% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

20 [25] The method of any of paragraphs 1-24, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising or consisting of the nucleotide sequence of SEQ ID NO: 1 or the mature polypeptide coding sequence thereof; or a subsequence thereof encoding a polypeptide fragment having cellulolytic enhancing activity.

25 [26] The method of any of paragraphs 1-8, wherein the polypeptide having cellulolytic enhancing activity is a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.

[27] The method of any of paragraphs 1-26, wherein the mature polypeptide coding sequence of SEQ ID NO: 1 is nucleotides 64 to 859 of SEQ ID NO: 1.

30 [28] The method of any of paragraphs 1-27, wherein the mature polypeptide of SEQ ID NO: 2 is amino acids 22 to 250 of SEQ ID NO: 2.

35 [29] A method for producing a fermentation product, comprising: (A) saccharifying a cellulosic material with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity, wherein the polypeptide having cellulolytic enhancing activity is selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide that hybridizes under medium-high

stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii); (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof; and (d) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2; (B) fermenting the saccharified cellulosic material with one or more (several) fermenting microorganisms; and (C) recovering the fermentation product from the fermentation.

10 [30] The method of paragraph 29, wherein the cellulosic material is pretreated.

[31] The method of paragraph 29 or 30, 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 [32] The method of any of paragraphs 29-31, wherein the enzyme composition comprises or further comprises one or more (several) proteins selected from the group consisting of a hemicellulase, an expansin, an esterase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.

20 [33] The method of paragraph 32, wherein the hemicellulase is one or more (several) enzymes selected from the group consisting of an acetylmannan esterase, an acetyxylan esterase, an arabinanase, an arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase.

[34] The method of any of paragraphs 29-33, wherein steps (A) and (B) are performed simultaneously in a simultaneous saccharification and fermentation.

25 [35] The method of any of paragraphs 29-34, wherein the fermentation product is an alcohol, organic acid, ketone, amino acid, or gas.

[36] The method of any of paragraphs 29-35, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2.

30 [37] The method of paragraph 36, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 2.

35 [38] The method of paragraph 37, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 85% sequence identity with the mature polypeptide of SEQ ID NO: 2.

[39] The method of paragraph 38, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 90% sequence identity

with the mature polypeptide of SEQ ID NO: 2.

[40] The method of paragraph 39, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 95% sequence identity with the mature polypeptide of SEQ ID NO: 2.

5 [41] The method of paragraph 40, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 97% sequence identity with the mature polypeptide of SEQ ID NO: 2.

[42] The method of any of paragraphs 29-41, wherein the polypeptide having cellulolytic enhancing activity comprises or consists of the amino acid sequence of SEQ ID
10 NO: 2 or the mature polypeptide thereof; or a fragment thereof having cellulolytic enhancing activity.

[43] The method of any of paragraphs 29-35, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1,
15 (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii).

[44] The method of paragraph 43, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide that hybridizes under high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA
20 sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii).

[45] The method of paragraph 44, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide that hybridizes under very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA
25 sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii).

[46] The method of any of paragraphs 29-35, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding
30 sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

[47] The method of paragraph 46, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 80% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

35 [48] The method of paragraph 47, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 85% sequence identity with the mature polypeptide coding sequence of SEQ ID NO:

1 or the cDNA sequence thereof.

[49] The method of paragraph 48, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 90% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

[50] The method of paragraph 49, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 95% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

[51] The method of paragraph 50, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 97% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

[52] The method of any of paragraphs 29-35, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising or consisting of the nucleotide sequence of SEQ ID NO: 1 or the mature polypeptide coding sequence thereof; or a subsequence thereof encoding a polypeptide fragment having cellulolytic enhancing activity.

[53] The method of any of paragraphs 29-35, wherein the polypeptide having cellulolytic enhancing activity is a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.

[54] The method of any of paragraphs 29-53, wherein the mature polypeptide coding sequence of SEQ ID NO: 1 is nucleotides 64 to 859 of SEQ ID NO: 1.

[55] The method of any of paragraphs 29-54, wherein the mature polypeptide of SEQ ID NO: 2 is amino acids 22 to 250 of SEQ ID NO: 2.

[56] A method of fermenting a cellulosic material, comprising: fermenting the cellulosic material with one or more (several) fermenting microorganisms, wherein the cellulosic material is saccharified with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity, wherein the polypeptide having cellulolytic enhancing activity is selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii); (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence

thereof; and (d) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.

[57] The method of paragraph 56, wherein the fermenting of the cellulosic material produces a fermentation product.

5 [58] The method of paragraph 57, further comprising recovering the fermentation product from the fermentation.

[59] The method of any of paragraphs 56-58, wherein the cellulosic material is pretreated before saccharification.

10 [60] The method of any of paragraphs 56-59, 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.

[61] The method of any of paragraphs 56-60, wherein the enzyme composition comprises or further comprises one or more (several) proteins selected from the group consisting of a hemicellulase, an expansin, an esterase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.

15 [62] The method of paragraph 61, wherein the hemicellulase is one or more (several) enzymes selected from the group consisting of an acetylmannan esterase, an acetyxylan esterase, an arabinanase, an arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase.

[63] The method of any of paragraphs 56-62, wherein the fermentation product is an alcohol, organic acid, ketone, amino acid, or gas.

20 [64] The method of any of paragraphs 56-63, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2.

[65] The method of paragraph 64, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 2.

25 [66] The method of paragraph 65, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 85% sequence identity with the mature polypeptide of SEQ ID NO: 2.

[67] The method of paragraph 66, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 90% sequence identity with the mature polypeptide of SEQ ID NO: 2.

30 [68] The method of paragraph 67, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 95% sequence identity with the mature polypeptide of SEQ ID NO: 2.

[69] The method of paragraph 68, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 97% sequence identity with the mature polypeptide of SEQ ID NO: 2.

5 [70] The method of any of paragraphs 56-69, wherein the polypeptide having cellulolytic enhancing activity comprises or consists of the amino acid sequence of SEQ ID NO: 2 or the mature polypeptide thereof; or a fragment thereof having cellulolytic enhancing activity.

10 [71] The method of any of paragraphs 56-63, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii).

15 [72] The method of paragraph 71, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide that hybridizes under high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii).

20 [73] The method of paragraph 72, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide that hybridizes under very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii).

25 [74] The method of any of paragraphs 56-63, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

30 [75] The method of paragraph 74, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 80% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

[76] The method of paragraph 75, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 85% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

35 [77] The method of paragraph 76, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 90% sequence identity with the mature polypeptide coding sequence of SEQ ID NO:

1 or the cDNA sequence thereof.

[78] The method of paragraph 77, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 95% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

[79] The method of paragraph 78, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 97% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

[80] The method of any of paragraphs 56-79, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising or consisting of the nucleotide sequence of SEQ ID NO: 1 or the mature polypeptide coding sequence thereof; or a subsequence thereof encoding a polypeptide fragment having cellulolytic enhancing activity.

[81] The method of any of paragraphs 56-63, wherein the polypeptide having cellulolytic enhancing activity is a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.

[82] The method of any of paragraphs 56-81, wherein the mature polypeptide coding sequence of SEQ ID NO: 1 is nucleotides 64 to 859 of SEQ ID NO: 1.

[83] The method of any of paragraphs 56-82, wherein the mature polypeptide of SEQ ID NO: 2 is amino acids 22 to 250 of SEQ ID NO: 2.

[84] An enzyme composition comprising a polypeptide having cellulolytic enhancing activity and one or more (several) cellulolytic enzymes, wherein the polypeptide having cellulolytic enhancing activity is selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii); (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof; and (d) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.

[85] The enzyme composition of paragraph 84, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2.

[86] The enzyme composition of paragraph 85, wherein the polypeptide having

cellulolytic enhancing activity comprises an amino acid sequence having at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 2.

[87] The enzyme composition of paragraph 86, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 85% sequence identity with the mature polypeptide of SEQ ID NO: 2.

[88] The enzyme composition of paragraph 87, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 90% sequence identity with the mature polypeptide of SEQ ID NO: 2.

[89] The enzyme composition of paragraph 88, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 95% sequence identity with the mature polypeptide of SEQ ID NO: 2.

[90] The enzyme composition of paragraph 89, wherein the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence having at least 97% sequence identity with the mature polypeptide of SEQ ID NO: 2.

[91] The enzyme composition of any of paragraphs 84-90, wherein the polypeptide having cellulolytic enhancing activity comprises or consists of the amino acid sequence of SEQ ID NO: 2 or the mature polypeptide thereof; or a fragment thereof having cellulolytic enhancing activity.

[92] The enzyme composition of paragraph 84, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii).

[93] The enzyme composition of paragraph 92, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide that hybridizes under high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii).

[94] The enzyme composition of paragraph 93, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide that hybridizes under very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii).

[95] The enzyme composition of paragraph 84, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

[96] The enzyme composition of paragraph 95, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 80% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

5 [97] The enzyme composition of paragraph 96, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 85% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

10 [98] The enzyme composition of paragraph 97, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 90% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

15 [99] The enzyme composition of paragraph 98, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 95% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

20 [100] The enzyme composition of paragraph 99, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising a nucleotide sequence having at least 97% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof.

25 [101] The enzyme composition of any of paragraphs 84-100, wherein the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising or consisting of the nucleotide sequence of SEQ ID NO: 1 or the mature polypeptide coding sequence thereof; or a subsequence thereof encoding a polypeptide fragment having cellulolytic enhancing activity.

[102] The enzyme composition of paragraph 84, wherein the polypeptide having cellulolytic enhancing activity is a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.

30 [103] The enzyme composition of any of paragraphs 84-102, wherein the mature polypeptide coding sequence of SEQ ID NO: 1 is nucleotides 77 to 766 of SEQ ID NO: 1.

[104] The enzyme composition of any of paragraphs 84-103, wherein the mature polypeptide of SEQ ID NO: 2 is amino acids 20 to 249 of SEQ ID NO: 2.

35 [105] The enzyme composition of any of paragraphs 84-104, wherein the one or more (several) cellulolytic enzymes are selected from the group consisting of an endoglucanase, a celobiohydrolase, and a beta-glucosidase.

[106] The enzyme composition of any of paragraphs 84-105, wherein the one or more (several) cellulolytic enzymes comprise an endoglucanase.

[107] The enzyme composition of any of paragraphs 84-106, wherein the one or more (several) cellulolytic enzymes comprise a cellobiohydrolase.

[108] The enzyme composition of any of paragraphs 84-107, wherein the one or more (several) cellulolytic enzymes comprise a beta-glucosidase.

5 [109] The enzyme composition of any of paragraphs 84-108, which further comprises one or more (several) proteins selected from the group consisting of a hemicellulase, an expansin, an esterase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin

10 [110] The enzyme composition of paragraph 109, wherein the hemicellulase is one or more (several) enzymes selected from the group consisting of an acetylmannan esterase, an acetyxylan esterase, an arabinanase, an arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase.

15 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
20 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.

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 cellulolytic enhancing activity, wherein the polypeptide having cellulolytic enhancing activity is selected from the group consisting of:
- 10 (a) a polypeptide comprising an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2;
- (b) a polypeptide encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii);
- 15 (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof; and
- (d) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.
- 20 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.
- 25 3. The method of claim 1 or 2, wherein the enzyme composition comprises or further comprises one or more (several) proteins selected from the group consisting of a hemicellulase, an expansin, an esterase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.
- 30 4. The method of any of claims 1-3, further comprising recovering the degraded cellulosic material.
5. The method of claim 4, wherein the degraded cellulosic material is a sugar.
- 35 6. The method of any of claims 1-5, wherein the polypeptide having cellulolytic enhancing activity comprises or consists of the amino acid sequence of SEQ ID NO: 2 or the mature polypeptide thereof; or a fragment thereof having cellulolytic enhancing activity.

7. A method for producing a fermentation product, comprising:

(A) saccharifying a cellulosic material with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity, wherein the polypeptide having cellulolytic enhancing activity is selected from the group consisting of:

(a) a polypeptide comprising an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2;

(b) a polypeptide encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii);

(c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof; and

(d) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2;

(B) fermenting the saccharified cellulosic material with one or more (several) fermenting microorganisms; and

(C) recovering the fermentation product from the fermentation.

8. The method of claim 7, 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.

9. The method of claim 7 or 8, wherein the enzyme composition comprises or further comprises one or more (several) proteins selected from the group consisting of a hemicellulase, an expansin, an esterase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.

10. The method of any of claims 7-9, wherein the polypeptide having cellulolytic enhancing activity comprises or consists of the amino acid sequence of SEQ ID NO: 2 or the mature polypeptide thereof; or a fragment thereof having cellulolytic enhancing activity.

11. A method of fermenting a cellulosic material, comprising: fermenting the cellulosic material with one or more (several) fermenting microorganisms, wherein the cellulosic material is saccharified with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity, wherein the polypeptide having cellulolytic enhancing activity

is selected from the group consisting of:

(a) a polypeptide comprising an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2;

5 (b) a polypeptide encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii);

10 (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof; and

(d) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.

12. The method of claim 11, wherein the fermenting of the cellulosic material produces a fermentation product.

13. The method of claim 12, further comprising recovering the fermentation product from the fermentation.

20 14. The method of any of claims 11-13, 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.

25 15. The method of any of claims 11-14, wherein the enzyme composition comprises or further comprises one or more (several) proteins selected from the group consisting of a hemicellulase, an expansin, an esterase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.

30 16. The method of any of claims 11-15, wherein the polypeptide having cellulolytic enhancing activity comprises or consists of the amino acid sequence of SEQ ID NO: 2 or the mature polypeptide thereof; or a fragment thereof having cellulolytic enhancing activity.

35 17. An enzyme composition comprising a polypeptide having cellulolytic enhancing activity and one or more (several) cellulolytic enzymes, wherein the polypeptide having cellulolytic enhancing activity is selected from the group consisting of:

(a) a polypeptide comprising an amino acid sequence having at least 75% sequence identity with the mature polypeptide of SEQ ID NO: 2;

(b) a polypeptide encoded by a polynucleotide that hybridizes under medium-high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence contained in the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii);

5 (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 75% sequence identity with the mature polypeptide coding sequence of SEQ ID NO: 1 or the cDNA sequence thereof; and

(d) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.

10 18. The enzyme composition of claim 17, wherein the polypeptide having cellulolytic enhancing activity comprises or consists of the amino acid sequence of SEQ ID NO: 2 or the mature polypeptide thereof; or a fragment thereof having cellulolytic enhancing activity.

15 19. The enzyme composition of claim 17 or 18, wherein the one or more (several) cellulolytic enzymes are selected from the group consisting of an endoglucanase, a celobiohydrolase, and a beta-glucosidase.

20 20. The enzyme composition of any of claims 17-19, which comprises or further comprises one or more (several) proteins selected from the group consisting of a hemicellulase, an expansin, an esterase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin

M T L S K I T S I A G L L A S A S L V A G H G F V S G
 1 ATGACTTTGT CCAAGATCAC TTCCATTGCT GGCCTTCTGG CCTCAGCGTC TCTCGTGGCT GGCCACGGCT TTGTTTCTGG
 I V A D G K Y Y G
 81 CATTGTTGCT GATGGGAAAT AGTATGTGCT TGAACCACAC AAATGACAGC TGCAACAGCT AACTTCTATT CCAGTTACGG
 G Y L V N Q Y P Y M S N P P D T I A W S T T A T D L
 161 AGGGTACCTT GTTAACCAAT ACCCCTACAT GAGCAACCCT CCCGACACCA TTGCCTGGTC CACCACCGCC ACCGACCTCG
 G F V D G T G Y Q S P D I I C H R D A K N G K L T A T
 241 GCTTTGTGGA CGGCACCGGC TACCAGTCTC CGGATATTAT CTGCCACAGA GACGCAAAGA ATGGCAAGTT GACCGCAACC
 V A A G S Q I E F Q W T T W P E S H H G P
 321 GTTGCAGCCG GTTCACAGAT CGAATTCCAG TGGACGACGT GGCCAGAGTC TCACCATGGA CCGGTACGAC GCCGAAGAGA
 L I T Y L A P C N G D C A T
 401 AGAGAACATA TTGTGACCAG ATAGGCTAAC ATAGCATAGT TGATTACTTA CCTCGCTCCA TGCAACGGCG ACTGTGCCAC
 V D K T T L K F V K I A A Q G L I D G S N P P G V W
 481 CGTGGACAAG ACCACCCTGA AGTTTGTCAA GATCGCCGCT CAAGGCTTGA TCGACGGCTC CAACCCACCT GGTGTTTGGG
 A D D E M I A N N N T A T V T I P A S Y A P G N Y V L
 561 CTGATGATGA AATGATCGCC AACAAACA CGGCCACAGT GACCATTCTT GCCTCCTATG CCCC CGAAA CTACGTCCTT
 R H E I I A L H S A G N L N G A Q N Y P Q C F N I Q I
 641 CGCCACGAGA TCATCGCCCT TCACTCTGCG GGTAACCTGA ACGGCGCGCA GAACTACCC CAGTGTTC AATCCAAAT
 T G G G S A Q G S G T A G T S L Y K N T D P G I K F
 721 CACCGGTGGC GGCAGTGCTC AGGGATCTGG CACCGCTGGC ACGTCCCTGT ACAAGAATAC TGATCCTGGC ATCAAGTTTG
 D I Y S D L S G G Y P I P G P A L F N A *
 801 ACATCTACTC GGATCTGAGC GGTGGATACC CTATTCTGG TCCTGCACTG TTCACGCTT AA

FIG. 1

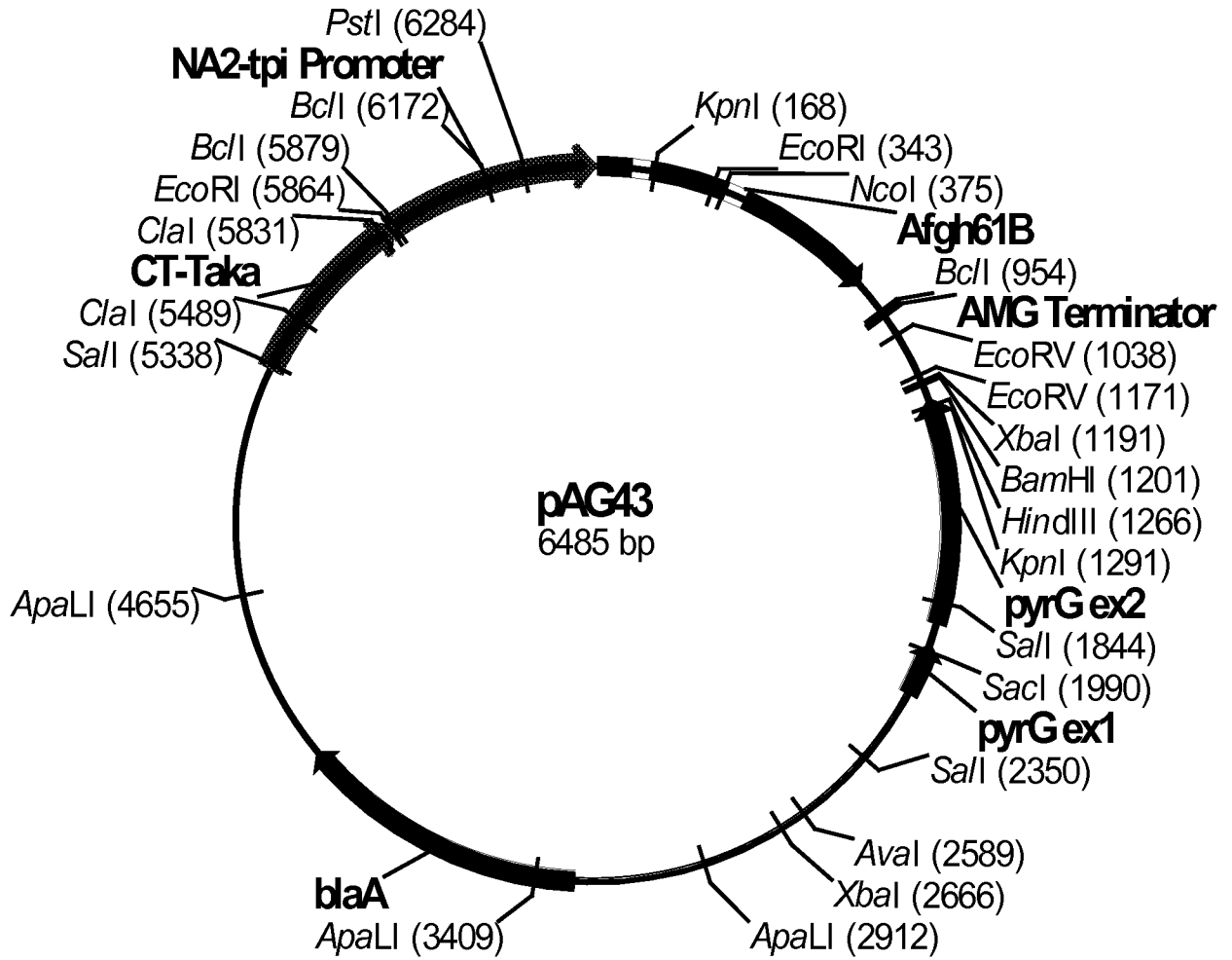


FIG. 2

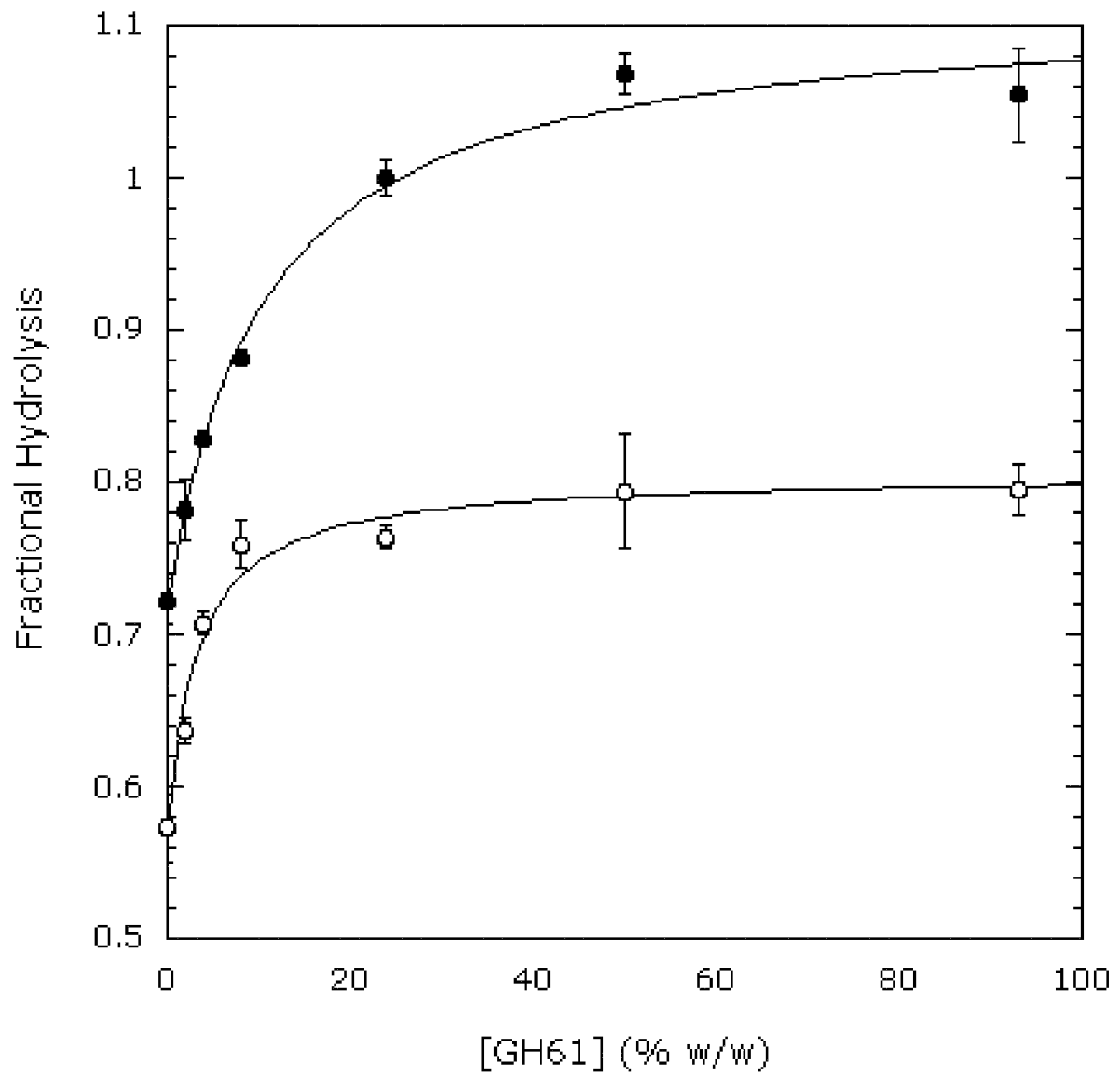


FIG. 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/036461

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/42
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, Sequence Search, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005/074656 A2 (NOVOZYMES INC [US]) 18 August 2005 (2005-08-18) the whole document	1-20
X	WO 2008/148131 A1 (NOVOZYMES INC [US]; LOPEZ DE LEON ALFREDO [US]; DING HANSHU [US]; BROW) 4 December 2008 (2008-12-04) the whole document	1-20

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

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

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *8* document member of the same patent family

Date of the actual completion of the international search

20 July 2010

Date of mailing of the international search report

03/08/2010

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Authorized officer

Pilat, Daniel

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2010/036461

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	DATABASE UniProt [Online] 8 April 2008 (2008-04-08), "SubName: Full=Endoglucanase, putative;" XP002592673 retrieved from EBI accession no. UNIPROT:BOY5X9 Database accession no. BOY5X9 * abstract; compound -----	6,10,16
A	WO 2008/140749 A2 (NOVOZYMES INC [US]; MCFARLAND KEITH [US]) 20 November 2008 (2008-11-20) the whole document -----	1-20

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2010/036461

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