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(54) **Title:** POLYPEPTIDES HAVING ACETYL XYLAN ESTERASE ACTIVITY AND POLYNUCLEOTIDES ENCODING SAME

(57) **Abstract:** The present invention relates to isolated polypeptides having acetyl xylan esterase activity and isolated polynucleotides encoding the polypeptides. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the polynucleotides as well as methods of producing and using the polypeptides.

**POLYPEPTIDES HAVING ACETYL XYLAN ESTERASE ACTIVITY
AND POLYNUCLEOTIDES ENCODING SAME**

Reference to a Sequence Listing

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

Background of the Invention

Field of the Invention

10 The present invention relates to isolated polypeptides having acetyl xylan esterase activity and isolated polynucleotides encoding the polypeptides. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the polynucleotides as well as methods of producing and using the polypeptides.

15 **Description of the Related Art**

 Plant cell wall polysaccharides constitute 90% of the plant cell wall and can be divided into three groups: cellulose, hemicellulose, and pectin. Cellulose represents the major constituent of call wall polysaccharides. Hemicelluloses are the second most abundant constituent of plant cell walls. The major hemicellulose polymer is xylan. The structure of
20 xylans found in cell walls of plants can differ significantly depending on their origin, but they always contain a beta-1,4-linked D-xylose backbone. The beta-1,4-linked D-xylose backbone can be substituted by various side groups, such as L-arabinose, D-galactose, acetyl, feruloyl, *p*-coumaroyl, and glucuronic acid residues.

 The biodegradation of the xylan backbone depends on two classes of enzymes:
25 endoxylanases and beta-xylosidases. Endoxylanases (EC 3.2.1.8) cleave the xylan backbone into smaller oligosaccharides, which can be further degraded to xylose by beta-xylosidases (EC 3.2.1.37). Other enzymes involved in the degradation of xylan include, for example, acetyl xylan esterase, arabinase, alpha-glucuronidase, ferulic acid esterase, and *p*-coumaric acid esterase.

30 Acetyl xylan esterase (EC 3.1.1.6) removes the *O*-acetyl groups from positions 2 and/or 3 on the beta-D-xylopyranosyl residues of acetyl xylan. Acetyl xylan plays an important role in the hydrolysis of xylan because the acetyl side groups can interfere sterically with the approach of enzymes that cleave the backbone. Removal of the acetyl side groups facilitates the action of endoxylanases. A classification system for carbohydrate esterases, based on
35 sequence similarity, has led to the definition of 13 families, seven of which contain acetyl xylan esterases (Henrissat B., 1991, *Biochem. J.* 280: 309-316, and Henrissat and Bairoch, 1996,

Biochem. J. 316: 695-696).

The present invention relates to an acetyl xylan esterase from *Aspergillus aculeatus* and polynucleotides encoding the polypeptide.

5 An acetyl xylan esterase from *Aspergillus aculeatus* is known from WO 1995/002689 (GENESEQP:AAR63066); however, this enzyme has no significant homology to the acetyl xylan esterase of the present invention.

An acetyl xylan esterase from *Aspergillus niger* is known from EP507369 (GENESEQP:AAR25291). This enzyme is 80% identical to the acetyl xylan esterase of the present invention.

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Summary of the Invention

The present invention relates to acetyl xylan esterases, said acetyl xylan esterase having the amino acid sequence shown in SEQ ID NO:2 has a pI of 5.0 and a MW of 32.7 Da and in particular to acetyl xylan esterases having an amino acid sequence homologous with or identical to the an acetyl xylan esterase of *Aspergillus aculeatus* disclosed in SEQ ID NO:2.

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In a first aspect the present invention relates to an isolated polypeptide having acetyl xylan esterase activity, selected from the group consisting of:

(a) a polypeptide comprising an amino acid sequence having at least 85% identity to the mature polypeptide of SEQ ID NO:2;

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

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(c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 85% identity to the mature polypeptide coding sequence of SEQ ID NO:1; 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 second aspect the present invention relates to an isolated polynucleotide comprising a nucleotide sequence that encodes the polypeptide of the first aspect.

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In a third aspect the present invention relates to a nucleic acid construct comprising the polynucleotide operably linked to one or more control sequences that direct the production of the polypeptide in an expression host.

In a fourth aspect the present invention relates to a recombinant expression vector comprising the nucleic acid construct of the third aspect.

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In a fifth aspect the present invention relates to a recombinant host cell comprising the nucleic acid construct of the third aspect.

In a sixth aspect the present invention relates to a method of producing the polypeptide of the first aspect, comprising: (a) cultivating a host cell comprising a nucleic acid construct comprising a nucleotide sequence encoding the polypeptide under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

5 In an seventh aspect the present invention relates to a method of producing the polypeptide of the first aspect, comprising: (a) cultivating a transgenic plant or a plant cell comprising a polynucleotide encoding the polypeptide under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

10 In a eighth aspect the present invention relates to a transgenic plant, plant part or plant cell transformed with a polynucleotide encoding the polypeptide of the first aspect.

In a ninth aspect the present invention relates to a method for degrading an acetylated xylan, comprising treating a material comprising an acetylated xylan with the polypeptide having acetyl xylan esterase activity of the first aspect.

15 In an 10th aspect the present invention relates to a composition comprising the polypeptide of the first aspect and one or more additional enzyme(s) selected from xylanase, arabinofuranosidase, glucanase, pectinase, protease, acetyl xylan esterase, ferulic acid esterase, rhamnogalacturonas and xylanase.

In an 11th aspect the present invention relates to a use of the polypeptide of the first aspect or of the composition of the 10th aspect in a process of producing a fermentation product from lignocellulose-containing material, comprising the steps of:

- 20 (a) pre-treating lignocellulose-containing material;
(b) hydrolyzing the material in the presence of a cellulolytic enzyme and an enzyme of the invention;
(c) fermenting using a fermenting organism.

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Definitions

Acetyl xylan esterase activity: The term "acetyl xylan esterase activity" is defined herein as a carboxylesterase activity (EC 3.1.1.72) that catalyses the hydrolysis of acetyl groups from polymeric xylan, acetylated xylose, acetylated glucose, alpha-naphthyl acetate, *p*-nitrophenyl acetate. For purposes of the present invention, acetyl xylan esterase activity is determined according to the procedure described in section titled "Determination of acetyl xylan esterase activity".

30 The polypeptides of the present invention 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 85%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 100% of the acetyl xylan esterase activity of the

mature polypeptide of SEQ ID NO:2.

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

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

Mature polypeptide: The term “mature polypeptide” is defined herein as a polypeptide having acetyl xylan esterase activity that is in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. In a preferred aspect, the mature polypeptide is amino acids 34 to 308 of SEQ ID NO:2.

Mature polypeptide coding sequence: The term “mature polypeptide coding sequence” is defined herein as a nucleotide sequence that encodes a mature polypeptide having acetyl xylan esterase activity. In a preferred aspect, the mature polypeptide coding sequence is nucleotides 136 to 963 of SEQ ID NO:1.

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

For purposes of the present invention, the degree of identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS

package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends in Genetics* 16: 276-277), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the `-nobrief` option) is used as the percent identity and is calculated as follows:

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

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

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

Homologous sequence: The term "homologous sequence" is defined herein as a predicted protein that gives an E value (or expectancy score) of less than 0.001 in a tfasty search (Pearson, W.R., 1999, in *Bioinformatics Methods and Protocols*, S. Misener and S. A. Krawetz, ed., pp. 185-219) with the *Aspergillus aculeatus* acetyl xylan esterase of SEQ ID NO:2, the mature peptide thereof, or a fragment thereof.

Alternatively, the term "homologous sequence" is defined as an amino acid sequence having a degree of identity to the mature polypeptide of SEQ ID NO:2 of preferably at least 85%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 96%, at least 97%, at least 98%, or at least 99%, which have acetyl xylan esterase activity.

The term "homologous sequence" when used to characterize a polynucleotide sequence indicates that the polynucleotide sequence encodes an amino acid sequence which is a "homologous sequence".

Polypeptide fragment: The term "polypeptide fragment" is defined herein as a polypeptide having one or more (several) amino acids deleted from the amino and/or carboxyl terminus of the mature polypeptide of SEQ ID NO:2; or a homologous sequence thereof; wherein the fragment has acetyl xylan esterase activity. In a preferred aspect, a fragment contains at least 200 amino acid residues, more preferably at least 215 amino acid residues, and most preferably at least 230 amino acid residues, of the mature polypeptide of SEQ ID

NO:2 or a homologous sequence thereof.

Subsequence: The term "subsequence" is defined herein as a nucleotide sequence having one or more (several) nucleotides deleted from the 5' and/or 3' end of the mature polypeptide coding sequence of SEQ ID NO:1; or a homologous sequence thereof; wherein the subsequence encodes a polypeptide fragment having acetyl xylan esterase activity. In a preferred aspect, a subsequence contains at least 600 nucleotides, more preferably at least 650 nucleotides, and most preferably at least 700 nucleotides of the mature polypeptide coding sequence of SEQ ID NO:1 or a homologous sequence thereof.

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

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

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

cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

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

cDNA: The term "cDNA" is defined herein as a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic cell. cDNA lacks intron sequences that are usually present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps before appearing as mature spliced mRNA. These steps include the removal of intron sequences by a process called splicing. cDNA derived from mRNA lacks, therefore, any intron sequences.

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

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

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

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

translation, post-translational modification, and secretion.

Expression vector: The term "expression vector" is defined herein as a linear or circular DNA molecule that comprises a polynucleotide encoding a polypeptide of the present invention and is operably linked to additional nucleotides that provide for its expression.

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

10 **Modification:** The term "modification" means herein any chemical modification of the polypeptide consisting of the mature polypeptide of SEQ ID NO:2; or a homologous sequence thereof; as well as genetic manipulation of the DNA encoding such a polypeptide. The modification can be a substitution, a deletion and/or an insertion of one or more (several) amino acids as well as replacements of one or more (several) amino acid side chains.

15 **Artificial variant:** When used herein, the term "artificial variant" means a polypeptide having acetyl xylan esterase activity produced by an organism expressing a modified polynucleotide sequence of the mature polypeptide coding sequence of SEQ ID NO:1; or a homologous sequence thereof. The modified nucleotide sequence is obtained through human intervention by modification of the polynucleotide sequence disclosed in SEQ ID NO:1; or a homologous sequence thereof.

20

Detailed Description of the Invention

Polypeptides Having Acetyl xylan Esterase Activity

25 In a preferred aspect, the present invention relates to isolated polypeptides comprising an amino acid sequence having a degree of identity to the mature polypeptide of SEQ ID NO:2 of at least 85%, more preferably at least 90%, most preferably at least 95%, and even most preferably at least 96%, at least 97%, at least 98%, or at least 99%, which have acetyl xylan esterase activity (hereinafter "homologous polypeptides"). In a preferred aspect, the homologous polypeptides have an amino acid sequence that differs by ten amino acids, preferably by five amino acids, more preferably by four amino acids, even more preferably by 30 three amino acids, most preferably by two amino acids, and even most preferably by one amino acid from the mature polypeptide of SEQ ID NO:2.

35 A polypeptide of the present invention preferably comprises the amino acid sequence of SEQ ID NO:2 or an allelic variant thereof; or a fragment thereof having acetyl xylan esterase activity. In a preferred aspect, the polypeptide comprises the amino acid sequence of SEQ ID NO:2. In another preferred aspect, the polypeptide comprises the mature polypeptide of SEQ ID NO:2. In another preferred aspect, the polypeptide comprises amino acids 1 to 308 of SEQ

ID NO:2, or an allelic variant thereof; or a fragment thereof having acetyl xylan esterase activity. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO:2 or an allelic variant thereof; or a fragment thereof having acetyl xylan esterase activity. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO:2. In another preferred aspect, the polypeptide consists of the mature polypeptide of SEQ ID NO:2. In another preferred aspect, the polypeptide consists of amino acids 1 to 308 of SEQ ID NO:2 or an allelic variant thereof; or a fragment thereof having acetyl xylan esterase activity.

In a preferred aspect, the present invention relates to isolated polypeptides having acetyl xylan esterase activity that are encoded by polynucleotides that hybridize under preferably very low stringency conditions, more preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even 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 genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO:1, (iii) a subsequence of (i) or (ii), or (iv) a full-length complementary strand of (i), (ii), or (iii) (J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York). A subsequence of the mature polypeptide coding sequence of SEQ ID NO:1 contains at least 100 contiguous nucleotides or preferably at least 200 contiguous nucleotides. Moreover, the subsequence may encode a polypeptide fragment having acetyl xylan esterase activity. In a preferred aspect, the complementary strand is the full-length complementary strand of the mature polypeptide coding sequence of SEQ ID NO:1.

The nucleotide sequence 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 acetyl xylan esterase 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, preferably at least 25, more preferably at least 35, and most preferably at least 70 nucleotides in length. It is, however, preferred that the nucleic acid probe is at least 100 nucleotides in length. For example, the nucleic acid probe may be at least 200 nucleotides, preferably at least 300 nucleotides, more preferably at least 400 nucleotides, or most preferably at least 500 nucleotides in length. Even longer probes may be used, e.g., nucleic acid probes that are preferably at least 550 nucleotides, more preferably at least 600 nucleotides, even more preferably at least 650 nucleotides, or most preferably at

least 700 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.

5 A genomic DNA or cDNA library prepared from such other strains may, therefore, be screened for DNA that hybridizes with the probes described above and encodes a polypeptide having acetyl xylan esterase 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
10 homologous with SEQ ID NO:1; or a subsequence thereof; the carrier material is preferably used in a Southern blot.

For purposes of the present invention, hybridization indicates that the nucleotide sequence hybridizes to a labeled nucleic acid probe corresponding to the mature polypeptide coding sequence of SEQ ID NO:1; the genomic DNA sequence comprising the mature
15 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 a preferred aspect, the nucleic acid probe is the mature polypeptide coding
20 sequence of SEQ ID NO:1. In another preferred aspect, the nucleic acid probe is nucleotides 136 to 963 of SEQ ID NO:1. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO:2, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO:1.

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

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

35 For short probes that are about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as prehybridization, hybridization, and washing post-

hybridization at about 5°C to about 10°C below the calculated T_m using the calculation according to Bolton and McCarthy (1962, *Proceedings of the National Academy of Sciences USA* 48:1390) in 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.

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

In a preferred aspect, the present invention relates to isolated polypeptides having acetyl xylan esterase activity encoded by polynucleotides comprising or consisting of nucleotide sequences that have a degree of identity to the mature polypeptide coding sequence of SEQ ID NO:1 of at least 85%, preferably at least 90%, more preferably at least 95%, and even most preferably at least 96%, at least 97%, at least 98%, or at least 99%, which encode an active polypeptide. See polynucleotide section herein.

In a preferred aspect, the present invention relates to artificial 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.

In addition to the 20 standard amino acids, non-standard amino acids (such as 4-hydroxyproline, 6-N-methyl lysine, 2-aminoisobutyric acid, isovaline, and alpha-methyl serine) may be substituted for amino acid residues of a wild-type polypeptide. A limited number of

non-conservative amino acids, amino acids that are not encoded by the genetic code, and unnatural amino acids may be substituted for amino acid residues. "Unnatural amino acids" have been modified after protein synthesis, and/or have a chemical structure in their side chain(s) different from that of the standard amino acids. Unnatural amino acids can be chemically synthesized, and preferably, are commercially available, and include pipercolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, and 3,3-dimethylproline.

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 the 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 biological activity (*i.e.*, acetyl xylan esterase 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 a polypeptide according to the invention.

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, *Biochem.* 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 of interest, and can be applied to polypeptides of unknown structure.

5 The total number of amino acid substitutions, deletions and/or insertions of the mature polypeptide of SEQ ID NO:2, such as amino acids 34 to 308 of SEQ ID NO:2, is 10, preferably 9, more preferably 8, more preferably 7, more preferably at most 6, more preferably 5, more preferably 4, even more preferably 3, most preferably 2, and even most preferably 1.

Sources of Polypeptides Having Acetyl xylan Esterase Activity

10 A polypeptide of the present invention 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
15 is secreted extracellularly.

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

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

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

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

A polypeptide having acetyl xylan esterase activity of the present invention may also be
35 a fungal polypeptide, and more preferably a yeast polypeptide such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* polypeptide

having acetyl xylan esterase 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*,
5 *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*
10 polypeptide having acetyl xylan esterase activity.

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

15 In another preferred aspect, the polypeptide is an *Acremonium cellulolyticus*, *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus fumigatus*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium tropicum*, *Chrysosporium merdarium*, *Chrysosporium inops*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium zonatum*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*,
20 *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*,
25 *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* polypeptide having acetyl xylan esterase activity.

In a more preferred aspect, the polypeptide is a *Aspergillus aculeatus* polypeptide having acetyl xylan esterase activity, e.g., the polypeptide comprising the mature polypeptide
35 of SEQ ID NO:2 or a homologous sequence.

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.

5 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).

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

Polypeptides of the present invention also include fused polypeptides or cleavable fusion polypeptides in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by fusing a nucleotide
20 sequence (or a portion thereof) encoding another polypeptide to a nucleotide sequence (or a portion thereof) 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.

25 A fusion polypeptide can further comprise a cleavage site. Upon secretion of the fusion protein, the site is cleaved releasing the polypeptide having acetylxylin esterase activity from the fusion protein. Examples of cleavage sites include, but are not limited to, a Kex2 site that encodes the dipeptide Lys-Arg (Martin *et al.*, 2003, *J. Ind. Microbiol. Biotechnol.* 3: 568-76; 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
30 Contreras *et al.*, 1991, *Biotechnology* 9: 378-381), an Ile-(Glu or Asp)-Gly-Arg site, which is cleaved by a Factor Xa protease after the arginine residue (Eaton *et al.*, 1986, *Biochem.* 25: 505-512); a Asp-Asp-Asp-Asp-Lys site, which is cleaved by an enterokinase after the lysine (Collins-Racie *et al.*, 1995, *Biotechnology* 13: 982-987); a His-Tyr-Glu site or His-Tyr-Asp site,
35 which is cleaved by Genenase I (Carter *et al.*, 1989, *Proteins: Structure, Function, and Genetics* 6: 240-248); a Leu-Val-Pro-Arg-Gly-Ser site, which is cleaved by thrombin after the

Arg (Stevens, 2003, *Drug Discovery World* 4: 35-48); a Glu-Asn-Leu-Tyr-Phe-Gln-Gly site, which is cleaved by TEV protease after the Gln (Stevens, 2003, *supra*); and a Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro site, which is cleaved by a genetically engineered form of human rhinovirus 3C protease after the Gln (Stevens, 2003, *supra*).

5

Polynucleotides

The present invention also relates to isolated polynucleotides comprising or consisting of nucleotide sequences that encode polypeptides having acetylxylylan esterase activity of the present invention.

10 In a preferred aspect, the nucleotide sequence comprises or consists of SEQ ID NO:1. In another preferred aspect, the nucleotide sequence comprises or consists of the mature polypeptide coding sequence of SEQ ID NO:1. In another preferred aspect, the nucleotide sequence comprises or consists of nucleotides 37 to 963 of SEQ ID NO:1. The present invention also encompasses nucleotide sequences that encode polypeptides comprising or
15 consisting of the amino acid sequence of SEQ ID NO:2 or the mature polypeptide thereof, which differ from SEQ ID NO:1 or the mature polypeptide coding sequence thereof by virtue of the degeneracy of the genetic code. The present invention also relates to subsequences of SEQ ID NO:1 that encode fragments of SEQ ID NO:2 that have acetylxylylan esterase activity.

The present invention also relates to mutant polynucleotides comprising or consisting of
20 at least one mutation in the mature polypeptide coding sequence of SEQ ID NO:1, in which the mutant nucleotide sequence encodes the mature polypeptide of SEQ ID NO:2.

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 of the present invention from such
25 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), ligated activated transcription (LAT) and nucleotide sequence-based
30 amplification (NASBA) may be used. The polynucleotides may be cloned from a strain of *Thielavia*, or another or related organism and thus, for example, may be an allelic or species variant of the polypeptide encoding region of the nucleotide sequence.

The present invention also relates to isolated polynucleotides comprising or consisting of nucleotide sequences that have a degree of identity to the mature polypeptide coding
35 sequence of SEQ ID NO:1 of at least 85%, more preferably at least 90%, even more preferably at least 95%, and most preferably at least 96%, at least 97%, at least 98%, or at

least 99% identity, which encode an active polypeptide.

Modification of a nucleotide sequence encoding a polypeptide of the present invention 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.*, artificial variants that differ in specific activity, thermostability, pH optimum, or the like. The variant sequence may be constructed on the basis of the nucleotide sequence presented as the mature polypeptide coding sequence of SEQ ID NO:1, *e.g.*, a subsequence thereof, and/or by introduction of nucleotide substitutions that do not give rise to another amino acid sequence of the polypeptide encoded by the nucleotide sequence, 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.

It will be apparent to those skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acid residues essential to the activity of the polypeptide encoded by an isolated polynucleotide of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (see, *e.g.*, Cunningham and Wells, 1989, *supra*). In the latter technique, mutations are introduced at every positively charged residue in the molecule, and the resultant mutant molecules are tested for acetylxylnan esterase activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of the three-dimensional structure as determined by such techniques as nuclear magnetic resonance analysis, crystallography or photoaffinity labeling (see, *e.g.*, de Vos *et al.*, 1992, *supra*; Smith *et al.*, 1992, *supra*; Wlodaver *et al.*, 1992, *supra*).

The present invention also relates to isolated polynucleotides encoding polypeptides of the present invention, which hybridize under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even 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 genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO:1, or (iii) a full-length complementary strand of (i) or (ii); or allelic variants and subsequences thereof (Sambrook *et al.*, 1989, *supra*), as defined herein. In a preferred aspect, the complementary strand is the full-length complementary strand of the

mature polypeptide coding sequence of SEQ ID NO:1.

The present invention also relates to isolated polynucleotides obtained by (a) hybridizing a population of DNA under very low, low, medium, medium-high, high, or very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO:1, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO:1, or (iii) a full-length complementary strand of (i) or (ii); and (b) isolating the hybridizing polynucleotide, which encodes a polypeptide having acetylxylan esterase activity. In a preferred aspect, the complementary strand is the full-length complementary strand of the mature polypeptide coding sequence of SEQ ID NO:1.

10

Nucleic Acid Constructs

The present invention also relates to nucleic acid constructs comprising an isolated polynucleotide of the present invention 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.

15

An isolated polynucleotide encoding a polypeptide of the present invention may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the polynucleotide's sequence prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotide sequences utilizing recombinant DNA methods are well known in the art.

20

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

25

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

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25). Further promoters are described in "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242: 74-94; and in Sambrook *et al.*, 1989, *supra*.

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

In a yeast host, useful promoters are obtained from the genes for *Saccharomyces*
20 *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
25 Romanos *et al.*, 1992, *Yeast* 8: 423-488.

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

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

Preferred terminators for yeast host cells are obtained from the genes for
35 *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful

terminators for yeast host cells are described by Romanos *et al.*, 1992, *supra*.

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

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

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

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

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
20 *Aspergillus niger* alpha-glucosidase.

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

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

Effective signal peptide coding sequences for bacterial host cells are the signal peptide

coding sequences obtained from the genes for *Bacillus* NCIB 11837 maltogenic amylase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* neutral proteases (*nprT*, *nprS*, *nprM*), and *Bacillus subtilis* *prsA*. Further signal peptides are described by Simonen and Palva, 5 1993, *Microbiological Reviews* 57:109-137.

Effective signal peptide coding sequences for filamentous fungal host cells are the signal peptide coding sequences obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Rhizomucor miehei* aspartic proteinase, *Humicola insolens* cellulase, *Humicola insolens* endoglucanase V, 10 and *Humicola lanuginosa* lipase.

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

The control sequence may also be a propeptide coding sequence that codes for an amino acid sequence positioned at the amino terminus of a polypeptide. The resultant 15 polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propeptide is generally inactive and can be converted to a mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding sequence may be obtained from the genes for *Bacillus subtilis* alkaline protease (*aprE*), 20 *Bacillus subtilis* neutral protease (*nprT*), *Saccharomyces cerevisiae* alpha-factor, *Rhizomucor miehei* aspartic proteinase, and *Myceliophthora thermophila* laccase (WO 95/33836).

Where both signal peptide and propeptide sequences are present at the amino terminus of a polypeptide, the propeptide sequence is positioned next to the amino terminus of a polypeptide and the signal peptide sequence is positioned next to the amino terminus of the 25 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 30 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 TAKA alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used as regulatory sequences. Other examples of regulatory sequences are those that allow for gene amplification. In eukaryotic systems, these regulatory sequences 35 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 nucleotide

sequence encoding the polypeptide would be operably linked with the regulatory sequence.

Expression Vectors

5 The present invention also relates to recombinant expression vectors comprising a polynucleotide of the present invention, a promoter, and transcriptional and translational stop signals. The various nucleic acids and control sequences described herein may be joined together to produce a recombinant expression vector that may include one or more (several) convenient restriction sites to allow for insertion or substitution of the nucleotide sequence encoding the polypeptide at such sites. Alternatively, a polynucleotide sequence of the present
10 invention may be expressed by inserting the nucleotide sequence 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
15 can be conveniently subjected to recombinant DNA procedures and can bring about expression of the nucleotide sequence. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids.

The vector may be an autonomously replicating vector, *i.e.*, a vector that exists as an
20 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.
25 Furthermore, a single vector or plasmid or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

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

Examples of bacterial selectable markers are the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers that confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol, or tetracycline resistance. Suitable markers for yeast host cells
35 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*
5 *nidulans* or *Aspergillus oryzae* and the *bar* gene of *Streptomyces hygroscopicus*.

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

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

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
25 cell. The term "origin of replication" or "plasmid replicator" is defined herein as a nucleotide sequence that enables a plasmid or vector to replicate *in vivo*.

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 pAMB1 permitting replication in *Bacillus*.

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

Examples of origins of replication useful in a filamentous fungal cell are AMA1 and ANS1 (Gems *et al.*, 1991, *Gene* 98: 61-67; Cullen *et al.*, 1987, *Nucleic Acids Research* 15:
35 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.

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

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

Host Cells

The present invention also relates to recombinant host cells, comprising an isolated polynucleotide of the present invention, which are advantageously used in the recombinant production of the polypeptides. A vector comprising a polynucleotide of the present invention is introduced into a host cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source.

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

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

The bacterial host cell may be any *Bacillus* cell. *Bacillus* cells useful in the practice of the present invention include, but are 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 *Bacillus thuringiensis* cells.

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

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

5 The bacterial host cell may also be any *Streptococcus* cell. *Streptococcus* cells useful in the practice of the present invention include, but are not limited to, *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, and *Streptococcus equi* subsp. *Zooepidemicus* cells.

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

15 The bacterial host cell may also be any *Streptomyces* cell. *Streptomyces* cells useful in the practice of the present invention include, but are not limited to, *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, and *Streptomyces lividans* cells.

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

25 The introduction of DNA into a *Bacillus* cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Molecular General Genetics* 168:111-115), by using competent cells (see, e.g., Young and Spizizen, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), by electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or by conjugation (see, e.g., Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5271-5278). The introduction of DNA into an *E coli* cell may, for instance, be effected by protoplast transformation (see, e.g., Hanahan, 1983, *J. Mol. Biol.* 166: 557-580) or electroporation (see, 30 e.g., Dower *et al.*, 1988, *Nucleic Acids Res.* 16: 6127-6145). The introduction of DNA into a *Streptomyces* cell may, for instance, be effected by protoplast transformation and electroporation (see, e.g., Gong *et al.*, 2004, *Folia Microbiol. (Praha)* 49: 399-405), by conjugation (see, e.g., Mazodier *et al.*, 1989, *J. Bacteriol.* 171: 3583-3585), or by transduction (see, e.g., Burke *et al.*, 2001, *Proc. Natl. Acad. Sci. USA* 98: 6289-6294). The introduction of 35 DNA into a *Pseudomonas* cell may, for instance, be effected by electroporation (see, e.g.,

Choi *et al.*, 2006, *J. Microbiol. Methods* 64: 391-397) or by conjugation (see, *e.g.*, Pinedo and Smets, 2005, *Appl. Environ. Microbiol.* 71: 51-57). The introduction of DNA into a *Streptococcus* cell may, for instance, be effected by natural competence (see, *e.g.*, Perry and Kuramitsu, 1981, *Infect. Immun.* 32:1295-1297), by protoplast transformation (see, *e.g.*, Catt and Jollick, 1991, *Microbios.* 68:189-2070, by electroporation (see, *e.g.*, Buckley *et al.*, 1999, *Appl. Environ. Microbiol.* 65: 3800-3804) or by conjugation (see, *e.g.*, Clewell, 1981, *Microbiol. Rev.* 45: 409-436). However, any method known in the art for introducing DNA into a host cell can be used.

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

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

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

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

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

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

fermentative.

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

In a most preferred aspect, the filamentous fungal host cell is an *Aspergillus awamori*, *Aspergillus fumigatus*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*,
10 *Aspergillus niger* or *Aspergillus oryzae* cell. In another most preferred aspect, the filamentous fungal host cell is a *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochromum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*,
15 *Fusarium torulosum*, *Fusarium trichothecioides*, or *Fusarium venenatum* cell. In another most preferred aspect, the filamentous fungal host cell is a *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium tropicum*,
20 *Chrysosporium merdarium*, *Chrysosporium inops*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma harzianum*,
25 *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described
30 in EP 238 023 and Yelton *et al.*, 1984, *Proceedings of the National Academy of Sciences USA* 81:1470-1474. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78:147-156, and WO 96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-
35 187, Academic Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153:163; and

Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75:1920.

Methods of Production

5 The present invention also relates to methods of producing a polypeptide of the present invention, comprising: (a) cultivating a cell, which in its wild-type form produces the polypeptide, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide. In a preferred aspect, the cell is of the genus *Penicillium*. In a more preferred aspect, the cell is *Penicillium aurantiogriseum*.

10 The present invention also relates to methods of producing a polypeptide of the present invention, comprising: (a) cultivating a recombinant host cell, as described herein, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

15 The present invention also relates to methods of producing a polypeptide of the present invention, comprising: (a) cultivating a recombinant host cell under conditions conducive for production of the polypeptide, wherein the host cell comprises a mutant nucleotide sequence having at least one mutation in the mature polypeptide coding sequence of SEQ ID NO:1, wherein the mutant nucleotide sequence encodes a polypeptide that comprises or consists of the mature polypeptide of SEQ ID NO:2, and (b) recovering the polypeptide.

20 In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods well known in the art. For example, the cell may be cultivated by shake flask cultivation, and small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known
25 in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted into the medium, it can be recovered from cell lysates.

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

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

evaporation, or precipitation.

The polypeptides of the present invention 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.

Plants

The present invention also relates to plants, e.g., a transgenic plant, plant part, or plant cell, comprising an isolated polynucleotide encoding a polypeptide having acetyl xylan esterase activity of the present invention so as to express and produce the polypeptide in recoverable quantities. The polypeptide may be recovered from the plant or plant part. Alternatively, the plant or plant part containing the recombinant polypeptide may be used as such for improving the quality of a food or feed, e.g., improving nutritional value, palatability, and rheological properties, or to destroy an antinutritive factor.

The transgenic plant can be dicotyledonous (a dicot) or monocotyledonous (a monocot). Examples of monocot plants are grasses, such as meadow grass (blue grass, *Poa*), forage grass such as *Festuca*, *Lolium*, temperate grass, such as *Agrostis*, and cereals, e.g., wheat, oats, rye, barley, rice, sorghum, and maize (corn).

Examples of dicot plants are tobacco, legumes, such as lupins, potato, sugar beet, pea, bean and soybean, and cruciferous plants (family *Brassicaceae*), such as cauliflower, rape seed, and the closely related model organism *Arabidopsis thaliana*.

Examples of plant parts are stem, callus, leaves, root, fruits, seeds, and tubers as well as the individual tissues comprising these parts, e.g., epidermis, mesophyll, parenchyme, vascular tissues, meristems. Specific plant cell compartments, such as chloroplasts, apoplasts, mitochondria, vacuoles, peroxisomes and cytoplasm are also considered to be a plant part. Furthermore, any plant cell, whatever the tissue origin, is considered to be a plant part. Likewise, plant parts such as specific tissues and cells isolated to facilitate the utilisation of the invention are also considered plant parts, e.g., embryos, endosperms, aleurone and seeds coats.

Also included within the scope of the present invention are the progeny of such plants, plant parts, and plant cells.

The transgenic plant or plant cell expressing a polypeptide of the present invention may be constructed in accordance with methods known in the art. In short, the plant or plant cell is constructed by incorporating one or more (several) expression constructs encoding a

polypeptide of the present invention into the plant host genome or chloroplast genome and propagating the resulting modified plant or plant cell into a transgenic plant or plant cell.

The expression construct is conveniently a nucleic acid construct that comprises a polynucleotide encoding a polypeptide of the present invention operably linked with appropriate regulatory sequences required for expression of the nucleotide sequence in the plant or plant part of choice. Furthermore, the expression construct may comprise a selectable marker useful for identifying host cells into which the expression construct has been integrated and DNA sequences necessary for introduction of the construct into the plant in question (the latter depends on the DNA introduction method to be used).

The choice of regulatory sequences, such as promoter and terminator sequences and optionally signal or transit sequences, is determined, for example, on the basis of when, where, and how the polypeptide is desired to be expressed. For instance, the expression of the gene encoding a polypeptide of the present invention may be constitutive or inducible, or may be developmental, stage or tissue specific, and the gene product may be targeted to a specific tissue or plant part such as seeds or leaves. Regulatory sequences are, for example, described by Tague *et al.*, 1988, *Plant Physiology* 86: 506.

For constitutive expression, the 35S-CaMV, the maize ubiquitin 1, and the rice actin 1 promoter may be used (Franck *et al.*, 1980, *Cell* 21: 285-294, Christensen *et al.*, 1992, *Plant Mo. Biol.* 18: 675-689; Zhang *et al.*, 1991, *Plant Cell* 3:1155-1165). organ-specific promoters may be, for example, a promoter from storage sink tissues such as seeds, potato tubers, and fruits (Edwards & Coruzzi, 1990, *Ann. Rev. Genet.* 24: 275-303), or from metabolic sink tissues such as meristems (Ito *et al.*, 1994, *Plant Mol. Biol.* 24: 863-878), a seed specific promoter such as the glutelin, prolamin, globulin, or albumin promoter from rice (Wu *et al.*, 1998, *Plant and Cell Physiology* 39: 885-889), a *Vicia faba* promoter from the legumin B4 and the unknown seed protein gene from *Vicia faba* (Conrad *et al.*, 1998, *Journal of Plant Physiology* 152: 708-711), a promoter from a seed oil body protein (Chen *et al.*, 1998, *Plant and Cell Physiology* 39: 935-941), the storage protein *napA* promoter from *Brassica napus*, or any other seed specific promoter known in the art, *e.g.*, as described in WO 91/14772. Furthermore, the promoter may be a leaf specific promoter such as the *rbcS* promoter from rice or tomato (Kyoizuka *et al.*, 1993, *Plant Physiology* 102: 991-1000, the chlorella virus adenine methyltransferase gene promoter (Mittra and Higgins, 1994, *Plant Molecular Biology* 26: 85-93), or the *aldP* gene promoter from rice (Kagaya *et al.*, 1995, *Molecular and General Genetics* 248: 668-674), or a wound inducible promoter such as the potato *pin2* promoter (Xu *et al.*, 1993, *Plant Molecular Biology* 22: 573-588). Likewise, the promoter may be inducible by abiotic treatments such as temperature, drought, or alterations in salinity or induced by exogenously applied substances that activate the promoter, *e.g.*, ethanol, oestrogens, plant hormones such as ethylene,

abscisic acid, and gibberellic acid, and heavy metals.

A promoter enhancer element may also be used to achieve higher expression of a polypeptide of the present invention in the plant. For instance, the promoter enhancer element may be an intron that is placed between the promoter and the nucleotide sequence encoding a polypeptide of the present invention. For instance, Xu *et al.*, 1993, *supra*, disclose the use of the first intron of the rice actin 1 gene to enhance expression.

The selectable marker gene and any other parts of the expression construct may be chosen from those available in the art.

The nucleic acid construct is incorporated into the plant genome according to conventional techniques known in the art, including *Agrobacterium*-mediated transformation, virus-mediated transformation, microinjection, particle bombardment, biolistic transformation, and electroporation (Gasser *et al.*, 1990, *Science* 244:1293; Potrykus, 1990, *Bio/Technology* 8: 535; Shimamoto *et al.*, 1989, *Nature* 338: 274).

Presently, *Agrobacterium tumefaciens*-mediated gene transfer is the method of choice for generating transgenic dicots (for a review, see Hooykas and Schilperoort, 1992, *Plant Molecular Biology* 19:15-38) and can also be used for transforming monocots, although other transformation methods are often used for these plants. Presently, the method of choice for generating transgenic monocots is particle bombardment (microscopic gold or tungsten particles coated with the transforming DNA) of embryonic calli or developing embryos (Christou, 1992, *Plant Journal* 2: 275-281; Shimamoto, 1994, *Current Opinion Biotechnology* 5:158-162; Vasil *et al.*, 1992, *Bio/Technology* 10: 667-674). An alternative method for transformation of monocots is based on protoplast transformation as described by Omirulleh *et al.*, 1993, *Plant Molecular Biology* 21: 415-428.

Following transformation, the transformants having incorporated the expression construct are selected and regenerated into whole plants according to methods well-known in the art. Often the transformation procedure is designed for the selective elimination of selection genes either during regeneration or in the following generations by using, for example, co-transformation with two separate T-DNA constructs or site specific excision of the selection gene by a specific recombinase.

The present invention also relates to methods of producing a polypeptide of the present invention comprising: (a) cultivating a transgenic plant or a plant cell comprising a polynucleotide encoding the polypeptide having acetyl xylan esterase activity of the present invention under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

Compositions

The present invention also relates to compositions comprising a polypeptide of the present invention. Preferably, the compositions are enriched in such a polypeptide. The term "enriched" indicates that the acetyl xylan esterase activity of the composition has been increased, e.g., with an enrichment factor of at least 1.1.

5 The composition may comprise a polypeptide of the present invention as the major enzymatic component, e.g., a mono-component composition. Alternatively, the composition may comprise multiple enzymatic activities, such as an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, 10 glucoamylase, alpha-glucosidase, beta-glucosidase, haloperoxidase, invertase, laccase, lipase, mannosidase, oxidase, pectinolytic enzyme, peptidoglutaminase, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, or xylanase. The additional enzyme(s) may be produced, for example, by a microorganism belonging to the genus *Aspergillus*, preferably *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus fumigatus*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, 15 or *Aspergillus oryzae*; *Fusarium*, preferably *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sulphureum*, 20 *Fusarium toruloseum*, *Fusarium trichothecioides*, or *Fusarium venenatum*; *Humicola*, preferably *Humicola insolens* or *Humicola lanuginosa*; or *Trichoderma*, preferably *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride*.

The polypeptide compositions may be prepared in accordance with methods known in 25 the art and may be in the form of a liquid or a dry composition. For instance, the polypeptide composition may be in the form of a granulate or a microgranulate. The polypeptide to be included in the composition may be stabilized in accordance with methods known in the art.

Examples are given below of preferred uses of the polypeptide compositions of the invention. The dosage of the polypeptide composition of the invention and other conditions 30 under which the composition is used may be determined on the basis of methods known in the art.

Uses

The present invention is also directed to methods for using the polypeptides having 35 acetyl xylan esterase activity, or compositions thereof.

A polypeptide having acetyl xylan esterase activity of the present invention may be

used in several applications to degrade or convert an acetyl xylan-containing material by treating the material with an effective amount of the polypeptide (see, for example, WO 2002/18561). The polypeptides of the present invention are preferably used in conjunction with other xylan degrading enzymes such as xylanases, arabinofuranosidases, xylosidases, and glucuronidases in processes wherein xylan has to be degraded. As a consequence of the deacylating reaction the xylan becomes better accessible for xylanases and other xylan-degrading enzymes.

The polypeptides having acetyl xylan esterase activity are useful in a number of applications: *in vivo* modification of xylan containing animal feeds to improve digestability; general applications resulting from biomass degradation or conversion to fermentable sugars in the production of, for example, fuel and/or potable ethanol; processing aids used in pulp and paper de-lignification; component of enzymatic scouring systems for textiles; food applications, e.g., baking, in combination with other enzymatic functionalities to improve the physical properties of baked goods; and laundry detergent applications in combination with other enzyme functionalities.

The polypeptides may be used in methods for the treatment of Kraft pulp according to U.S. Patent No. 5,658,765. Generally Kraft pulp is treated with xylanase in order to remove lignin in the preparation of paper products. Due to the high degree of acetylation of xylan, the effectiveness of xylanase is greatly increased when pulp is treated with acetyl xylan esterase either before or at the same time as the xylanase treatment.

The polypeptides may also be used in processes for producing xylose or xylo-oligosaccharide according to U.S. Patent No. 5,658,765.

The polypeptides may also be used as feed enhancing enzymes that improve feed digestibility to increase the efficiency of its utilization according to U.S. Patent No. 6,245,546. The use of acetyl xylan esterase in feed can decrease the solubility of the feed components thereby diminishing the viscosity and reducing anti-nutritional effect of pentosanes.

The polypeptides may also be used in baking according to U.S. Patent No. 5,693,518.

The polypeptides may further be used in brewing according to WO 2002/24926, where combinations of this enzyme with other enzymes can be used to degrade biological cell-wall material to increase digestibility or flow characteristics in applications relating to the preparation of fruit juices or beer.

Consequently, the present invention also relates to methods for degrading an acetylated xylan, comprising treating a composition comprising the acetylated xylan with such a polypeptide having acetyl xylan esterase activity. In a preferred aspect, the material comprising the acetylated xylan is further treated with a xylan degrading enzyme. The xylan degrading enzyme can be selected from the group consisting of a xylanase, an

arabinofuranosidase, a xylosidase, a glucuronidase, and a combination thereof.

Processing of Cellulosic Material

5 The present invention also relates to methods for degrading or converting a cellulosic material, comprising: treating the cellulosic material with an enzyme composition in the presence of a cellulolytic enzyme and an enzyme of the invention. In a preferred aspect, the method further comprises recovering the degraded or converted cellulosic material.

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

15 The present invention also relates to methods of fermenting a cellulosic material, comprising: fermenting the cellulosic material with one or more fermenting microorganisms, wherein the cellulosic material is saccharified with a cellulolytic enzyme composition in the presence of an enzyme of the invention. 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.

20 The enzyme of the invention can be in the form of a crude fermentation broth with or without the cells removed or in the form of a semi-purified or purified enzyme preparation or the composition can comprise a host cell of the present invention as a source of the enzyme of the invention in a fermentation process with the biomass.

25 The methods of the present invention can be used to saccharify a cellulosic material to fermentable sugars and convert the fermentable sugars to many useful substances, e.g., chemicals and fuels. 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 fermentation (HHCF), and direct microbial conversion (DMC). SHF uses separate process steps to first enzymatically hydrolyze lignocellulose 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 lignocellulose 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, lignocellulose hydrolysis, and fermentation) in one or more steps where the same organism is used to produce the enzymes for conversion of the lignocellulose 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 the plant cell wall components (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 hydrolysis, such as simultaneously with treatment of the cellulosic material with an enzyme composition of the present invention 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, the 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. The lignocellulose 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 the 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
5 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.*,
10 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),
15 ammonia percolation (APR), and organosolv pretreatments.

In dilute acid pretreatment, the 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
20 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).
25

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
30 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,
35 *J. Chem. Technol. Biotechnol.* 81: 1669-1677). The pretreatment is performed at preferably 1-40% dry matter, more preferably 2-30% dry matter, and most preferably 5-20% dry matter, and

often the initial pH is increased by the addition of alkali such as sodium carbonate.

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

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

Organosolv pretreatment delignifies cellulosic material by extraction using aqueous ethanol (40-60% ethanol) at 160-200°C for 30-60 minutes (Pan *et al.*, 2005, *Biotechnol. Bioeng.* 90: 473-481; Pan *et al.*, 2006, *Biotechnol. Bioeng.* 94: 851-861; Kurabi *et al.*, 2005, *Appl. Biochem. Biotechnol.* 121:219-230). Sulphuric acid is usually added as a catalyst. In organosolv pretreatment, the majority of the hemicellulose is removed.

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

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

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

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

known in the art, e.g., washed with water.

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

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

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

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

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

Biological Pretreatment: The term "biological pretreatment" refers to any biological pretreatment that promotes the separation and/or release of cellulose, hemicellulose, and/or lignin from the cellulosic material. Biological pretreatment techniques can involve applying lignin-solubilizing microorganisms (see, for example, Hsu, T.-A., 1996, Pretreatment of biomass, in *Handbook on Bioethanol: Production and Utilization*, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212; Ghosh and Singh, 1993, Physicochemical and biological treatments for enzymatic/microbial conversion of cellulosic biomass, *Adv. Appl. Microbiol.* 39: 295-333; McMillan, J. D., 1994, Pretreating lignocellulosic biomass: a review, in *Enzymatic Conversion of Biomass for Fuels Production*, Himmel, M. E., Baker, J. O., and Overend, R. P., eds., ACS Symposium Series 566, American Chemical Society, Washington, DC, chapter 15; Gong, C. S., Cao, N. J., Du, J., and Tsao, G. T., 1999, Ethanol production from renewable resources, in *Advances in Biochemical Engineering/Biotechnology*, Scheper, T., ed., Springer-Verlag Berlin Heidelberg, Germany, 65: 207-241; Olsson and Hahn-Hagerdal, 1996,

Fermentation of lignocellulosic hydrolysates for ethanol production, *Enz. Microb. Tech.* 18: 312-331; and Vallander and Eriksson, 1990, Production of ethanol from lignocellulosic materials: State of the art, *Adv. Biochem. Eng./Biotechnol.* 42: 63-95).

Saccharification. In the hydrolysis step, also known as saccharification, the pretreated
5 cellulose material is hydrolyzed to break down cellulose and alternatively also hemicellulose to fermentable sugars, such as glucose, cellobiose, xylose, xylulose, arabinose, mannose, galactose, or soluble oligosaccharides. The hydrolysis is performed enzymatically by an enzyme composition in the presence of an enzyme of the invention. The composition can further comprise one or more hemicellulolytic enzymes. The enzymes of the compositions can
10 also be added sequentially.

Enzymatic hydrolysis is preferably carried out in a suitable aqueous environment under conditions that can be readily determined by one skilled in the art. In a preferred aspect, hydrolysis is performed under conditions suitable for the activity of the enzyme(s), *i.e.*, optimal for the enzyme(s). The hydrolysis can be carried out as a fed batch or continuous process
15 where the pretreated cellulose 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 168 hours, more preferably about 24 to about 120 hours, and most preferably about 48 to about 72 hours. The temperature is in the range of preferably about 40°C to about 70°C, more preferably about 45°C to about 65°C, and more preferably about 50°C to 60°C, in particular about 55°C. The pH is in the range of preferably about 3 to about 9, more preferably
25 about 3.5 to about 8, more preferably about 4 to about 7, and most preferably about 4.5 to about 6, in particular about pH 5. The dry solids content is in the range of preferably about 1 to about 50 wt %, more preferably about 5 to about 40 wt %, more preferably about 10 to about 30 wt %, and most preferably about 15 to about 25 wt %.

In addition to an enzyme of the present invention, the cellulolytic enzyme components of the
30 composition are preferably enzymes having endoglucanase, cellobiohydrolase, and beta-glucosidase activities. In a preferred aspect, the enzyme composition comprises one or more (several) cellulolytic enzymes selected from the group consisting of a cellulase, an endoglucanase, a cellobiohydrolase, and a beta-glucosidase. In another preferred aspect, the cellulolytic enzyme preparation further or even further comprises one or more additional
35 enzyme activities selected from the group consisting of hemicellulases, esterases (*e.g.*, lipases, phospholipases, and/or cutinases), proteases, laccases, peroxidases, or mixtures

thereof. In the methods of the present invention, the additional enzyme(s) can be added prior to or during fermentation, including during or after propagation of the fermenting microorganism(s).

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 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 enzymes used in the present invention can be in any form suitable for use in the methods described herein, such as a crude fermentation broth with or without cells or substantially pure polypeptides. The enzyme(s) can be a dry powder or granulate, a liquid, a stabilized liquid, or a protected enzyme(s). Liquid enzyme preparations can, 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 process.

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

In a preferred aspect, an effective amount of cellulolytic enzyme(s) to cellulosic material is about 0.5 to about 50 mg, preferably at about 0.5 to about 40 mg, more preferably at about 0.5 to about 25 mg, more preferably at about 0.75 to about 20 mg, more preferably at about 0.75 to about 15 mg, even more preferably at about 0.5 to about 10 mg, and most preferably at about 2.5 to about 10 mg per g of cellulosic material.

In another preferred aspect, an effective amount of polypeptide(s) having cellulolytic enhancing activity to cellulosic material is about 0.01 to about 50.0 mg, preferably about 0.01 to about 40 mg, more preferably about 0.01 to about 30 mg, more preferably about 0.01 to about 20 mg, more preferably about 0.01 to about 10 mg, more preferably about 0.01 to about 5 mg, more preferably at about 0.025 to about 1.5 mg, more preferably at about 0.05 to about

1.25 mg, more preferably at about 0.075 to about 1.25 mg, more preferably at about 0.1 to about 1.25 mg, even more preferably at about 0.15 to about 1.25 mg, and most preferably at about 0.25 to about 1.0 mg per g of cellulosic material.

5 In the methods of the present invention, the enzyme composition may comprise any protein involved in the processing of a cellulose-containing material to glucose, or hemicellulose to xylose, mannose, galactose, and arabinose, their polymers, or products derived from them as described below. In one aspect, the enzyme composition comprises one or more enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-
10 glucosidase. In another aspect, the enzyme composition further or even further comprises one or more additional enzyme activities to improve the degradation of the cellulose-containing material. Preferred additional enzymes are xylanases, hemicellulases, esterases (e.g., lipases, phospholipases, and/or cutinases), proteases, laccases, peroxidases, or mixtures thereof.

15 The enzyme composition may be a monocomponent preparation, e.g., an endoglucanase, a multicomponent preparation, e.g., endoglucanase, cellobiohydrolase, beta-glucosidase, or a combination of multicomponent and monocomponent protein preparations. The cellulolytic proteins may have activity, i.e., hydrolyze cellulose, either in the acid, neutral, or alkaline pH range.

20 One or more 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
25 host). Monocomponent cellulolytic proteins may also be prepared by purifying such a protein from a fermentation broth.

The enzymes used in the present invention may be in any form suitable for use in the processes described herein, such as, for example, a crude fermentation broth with or without cells, a dry powder or granulate, a liquid, a stabilized liquid, or a protected enzyme. Liquid
30 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 process.

A polypeptide having cellulolytic enzyme activity may be a bacterial polypeptide. For example, the polypeptide may be a gram positive bacterial polypeptide such as a *Bacillus*,
35 *Streptococcus*, *Streptomyces*, *Staphylococcus*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Clostridium*, *Geobacillus*, or *Oceanobacillus* polypeptide having cellulolytic 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 cellulolytic enzyme activity.

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

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

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

15 The polypeptide having cellulolytic 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 cellulolytic enzyme activity; or more preferably a filamentous fungal polypeptide such as an *Acremonium*, *Agaricus*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Botryosphaeria*, *Ceriporiopsis*, *Chaetomidium*, *Chrysosporium*,
20 *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*,
25 *Scytalidium*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trichoderma*, *Trichophaea*, *Verticillium*, *Volvariella*, or *Xylaria* polypeptide having cellulolytic enzyme activity.

In a preferred aspect, the polypeptide is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, or *Saccharomyces oviformis* polypeptide having cellulolytic enzyme
30 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*,
35

Chrysosporium zonatum, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*,
Fusarium culmorum, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*,
Fusarium negundi, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium*
sambucinum, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*,
5 *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*,
10 *Thielavia peruviana*, *Thielavia spededonium*, *Thielavia setosa*, *Thielavia subthermophila*,
Thielavia terrestris, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma*
longibrachiatum, *Trichoderma reesei*, *Trichoderma viride*, or *Trichophaea saccata* polypeptide
having cellulolytic enzyme activity.

Chemically modified or protein engineered mutants of cellulolytic proteins may also be used.

15 Examples of commercial cellulolytic protein preparations suitable for use in the present
invention include, for example, CELLIC™ Ctec2 (Novozymes A/S), CELLUCLAST™
(Novozymes A/S), and NOVOZYM™ 188 (Novozymes A/S). Other commercially available
preparations comprising cellulase that may be used include CELLUZYME™, CEREFLO™ and
ULTRAFLO™ (Novozymes A/S), LAMINEX™ and SPEZYME™ CP (Genencor Int.),
20 ROHAMENT™ 7069 W (Röhm GmbH), and FIBREZYME® LDI, FIBREZYME® LBR, or
VISCOSTAR® 150L (Dyadic International, Inc., Jupiter, FL, USA). 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.

25 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).

30 Examples of fungal endoglucanases that can be used in the methods of the present invention,
include, but are not limited to, a *Trichoderma reesei* endoglucanase I (Penttila *et al.*, 1986,
Gene 45: 253-263; GENBANK™ accession no. M15665); *Trichoderma reesei* endoglucanase
II (Saloheimo, *et al.*, 1988, *Gene* 63:11-22; GENBANK™ accession no. M19373); *Trichoderma*
reesei endoglucanase III (Okada *et al.*, 1988, *Appl. Environ. Microbiol.* 64: 555-563;
35 GENBANK™ accession no. AB003694); *Trichoderma reesei* endoglucanase IV (Saloheimo *et*
al., 1997, *Eur. J. Biochem.* 249: 584-591; GENBANK™ accession no. Y11113); and

Trichoderma reesei endoglucanase V (Saloheimo *et al.*, 1994, *Molecular Microbiology* 13: 219-228; GENBANK™ accession no. Z33381); *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.*, 5 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; *Myceliophthora thermophila* CBS 117.65 endoglucanase; 10 basidiomycete CBS 495.95 endoglucanase; basidiomycete CBS 494.95 endoglucanase; *Thielavia terrestris* NRRL 8126 CEL6B endoglucanase; *Thielavia terrestris* NRRL 8126 CEL6C endoglucanase); *Thielavia terrestris* NRRL 8126 CEL7C endoglucanase; *Thielavia terrestris* NRRL 8126 CEL7E endoglucanase; *Thielavia terrestris* NRRL 8126 CEL7F endoglucanase; *Cladorrhinum foecundissimum* ATCC 62373 CEL7A endoglucanase; and *Trichoderma reesei* 15 strain No. VTT-D-80133 endoglucanase (GENBANK™ accession no. M15665).

Examples of cellobiohydrolases useful in the methods of the present invention include, but are not limited to, *Trichoderma reesei* cellobiohydrolase I; *Trichoderma reesei* cellobiohydrolase II; *Humicola insolens* cellobiohydrolase I, *Myceliophthora thermophila* cellobiohydrolase II, *Thielavia terrestris* cellobiohydrolase II (CEL6A), *Chaetomium thermophilum* cellobiohydrolase 20 I, and *Chaetomium thermophilum* cellobiohydrolase II.

Examples of beta-glucosidases useful in the methods of the present invention include, but are not limited to, *Aspergillus oryzae* beta-glucosidase; *Aspergillus fumigatus* beta-glucosidase; *Penicillium brasilianum* IBT 20888 beta-glucosidase; *Aspergillus niger* beta-glucosidase; and *Aspergillus aculeatus* beta-glucosidase.

25 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 30 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.

The beta-glucosidase may be a fusion protein. In one aspect, the beta-glucosidase is the *Aspergillus oryzae* beta-glucosidase variant BG fusion protein or the *Aspergillus oryzae* beta- 35 glucosidase fusion protein obtained according to WO 2008/057637.

Other 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
5 classification of glycosyl hydrolases, *Biochem. J.* 316: 695-696.

Other cellulolytic enzymes that may be used in the present invention are described in EP 495,257, EP 531,315, EP 531,372, WO 89/09259, WO 94/07998, WO 95/24471, WO 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,
10 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
15 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.

Examples of commercial xylan degrading enzyme preparations suitable for use in the present invention include, for example, SHEARZYME™ (Novozymes A/S), CELLIC™ Htec
20 (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).

25 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
30 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 terrestris* NRRL
35 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 Q0UHJ1), and *Humicola insolens* DSM 1800 acetylxylan esterase (WO 2009/073709).

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

20 The cellulolytic enzymes and proteins used in the methods of the present invention may be produced by fermentation of the above-noted microbial strains on a nutrient medium containing suitable carbon and nitrogen sources and inorganic salts, using procedures known in the art (see, e.g., Bennett, J.W. and LaSure, L. (eds.), *More Gene Manipulations in Fungi*, Academic Press, CA, 1991). Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type

25 Culture Collection). Temperature ranges and other conditions suitable for growth and cellulolytic 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 a cellulolytic 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 cellulolytic enzyme to be expressed or isolated. The resulting cellulolytic enzymes produced by the methods described above may be recovered from the fermentation medium and purified by conventional procedures.

35 Fermentation. The fermentable sugars obtained from the pretreated and hydrolyzed cellulosic material can be fermented by one or more 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 the 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. Such methods 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); SHCF (separate hydrolysis and co-fermentation), HHCF (hybrid hydrolysis and fermentation), and direct microbial conversion (DMC).

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. Examples of substrates suitable for use in the methods of present invention, include cellulosic materials, such as wood or plant residues or low molecular sugars DP1-3 obtained from processed cellulosic material that can be metabolized by the fermenting microorganism, and which can be supplied by direct addition to the fermentation medium.

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

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

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

Examples of fermenting microorganisms that can ferment C₆ sugars include bacterial and

fungal organisms, such as yeast. Preferred yeast includes strains of the *Saccharomyces* spp., preferably *Saccharomyces cerevisiae*.

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
5 *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*.

Other fermenting organisms include strains of *Zymomonas*, such as *Zymomonas mobilis*; *Hansenula*, such as *Hansenula anomala*; *Kluyveromyces*, such as *K. fragilis*;
10 *Schizosaccharomyces*, such as *S. pombe*; and *E. coli*, especially *E. coli* strains that have been genetically modified to improve the yield of ethanol.

In a preferred aspect, the yeast is a *Saccharomyces* spp. In a more preferred aspect, the yeast is *Saccharomyces cerevisiae*. In another more preferred aspect, the yeast is *Saccharomyces distaticus*. In another more preferred aspect, the yeast is *Saccharomyces*
15 *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
20 another more preferred aspect, the yeast is *Candida pseudotropicalis*. In another more preferred aspect, the yeast is *Candida utilis*. In another preferred aspect, the yeast is a *Clavispora*. In another more preferred aspect, the yeast is *Clavispora lusitaniae*. In another more preferred aspect, the yeast is *Clavispora opuntiae*. In another preferred aspect, the yeast is a *Pachysolen*. In another more preferred aspect, the yeast is *Pachysolen tannophilus*.
25 In another preferred aspect, the yeast is a *Pichia*. In another more preferred aspect, the yeast is a *Pichia stipitis*. In another preferred aspect, the yeast is a *Bretannomyces*. In another more preferred aspect, the yeast is *Bretannomyces clausenii* (Philippidis, G. P., 1996, Cellulose bioconversion technology, in *Handbook on Bioethanol: Production and Utilization*, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212).

30 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*.

35 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).

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*.

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 lignocellulose or hydrolysate 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
5 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, e.g., bacterial fermenting organisms have higher fermentation temperature optima. Yeast or another microorganism is preferably applied in amounts of approximately 10^5 to 10^{12} , preferably from
10 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.

The most widely used process in the art is the simultaneous saccharification and fermentation
15 (SSF) process where there is no holding stage for the saccharification, meaning that yeast and enzyme are added together.

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 enzymatic 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
25 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
30 minerals and mineral salts that can supply nutrients comprising P, K, Mg, S, Ca, Fe, Zn, Mn, and Cu.

Fermentation products: A fermentation product can be any substance derived from the fermentation. The fermentation product can be, without limitation, an alcohol (e.g., arabinitol, butanol, ethanol, glycerol, methanol, 1,3-propanediol, sorbitol, and xylitol); an organic acid
35 (e.g., acetic acid, acetonc 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, propionic acid, succinic acid, and xylonic acid); a ketone (e.g., acetone); an amino acid (e.g., aspartic acid, glutamic acid, glycine, lysine, serine, and threonine); and a gas (e.g., methane, hydrogen (H₂), carbon dioxide (CO₂), and carbon monoxide (CO)). The fermentation product can also be protein as a high value product.

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

In another preferred aspect, the fermentation product is an organic acid. In another more preferred aspect, the organic acid is acetic acid. In another more preferred aspect, the organic acid is acetic acid. In another more preferred aspect, the organic acid is adipic acid. In another more preferred aspect, the organic acid is ascorbic acid. In another more preferred aspect, the organic acid is citric acid. In another more preferred aspect, the organic acid is 2,5-diketo-D-gluconic acid. In another more preferred aspect, the organic acid is formic acid. In another more preferred aspect, the organic acid is fumaric acid. In another more preferred aspect, the organic acid is glucaric acid. In another more preferred aspect, the organic acid is gluconic acid. In another more preferred aspect, the organic acid is glucuronic acid. In another more preferred aspect, the organic acid is glutaric acid. In another preferred aspect, the organic acid is 3-hydroxypropionic acid. In another more preferred aspect, the organic acid is itaconic acid. In another more preferred aspect, the organic acid is lactic acid. In another more preferred aspect, the organic acid is malic acid. In another more preferred aspect, the organic acid is malonic acid. In another more preferred aspect, the organic acid is oxalic acid. In another more preferred aspect, the organic acid is propionic acid. In another more preferred aspect, the organic acid is succinic acid. In another more preferred aspect, the

organic acid is xylonic acid. See, for example, Chen, R., and Lee, Y. Y., 1997, Membrane-mediated extractive fermentation for lactic acid production from cellulosic biomass, *Appl. Biochem. Biotechnol.* 63-65: 435-448.

5 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*.

10 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*
15 *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
20 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,
25 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.

30 **Determination of acetyl xylan esterase activity**

Acetyl xylan esterase activity is determined using p-nitrophenylacetate (Sigma Chemical Co., St. Louis, MO, USA) as substrate. The sample enzyme preparation is diluted to provide less than 15% conversion of p-nitrophenylacetate by making an initial dilution of 550-fold in a 1.5 ml microcentrifuge tube with 50 mM sodium acetate pH 5.0 followed by 2-fold serial dilutions
35 with 50 mM sodium acetate pH 5.0. Then 100 µl aliquots of the diluted enzyme are transferred to wells of a 96-well plate.

A *p*-nitrophenylacetate stock solution is made by dissolving *p*-nitrophenylacetate in dimethylsulfoxide (DMSO) to constitute a 0.1 M solution. Before assay, a sample of the stock solution is diluted 100-fold in 50 mM sodium acetate pH 5.0 to make a 1 mM solution. A 100 μ l volume of 1 mM *p*-nitrophenylacetate is mixed with each dilution of the enzyme and then
 5 incubated at 25°C for 10 minutes. Substrate alone, enzyme alone, and buffer alone are run as controls. *p*-Nitrophenol standard solutions of 0.25, 0.2, 0.1, 0.05, and 0.02 mM are prepared by diluting a 10 mM stock solution in 50 mM sodium acetate pH 5.0. At 10 minutes, 50 μ l of 1.0 M Tris-HCl pH 8.0 buffer is added to each well (including samples, substrate control, enzyme control, reagent control, and standards), mixed, and the absorbance at 405 nm immediately
 10 measured on e.g. a SPECTRAMAX™ 340 PC plate reader (Molecular Devices, Sunnyvale, CA, USA). One unit of acetyl xylan esterase activity is defined as the amount of enzyme capable of releasing 1 μ mole of *p*-nitrophenolate anion per minute at pH 5, 25°C.

EXAMPLES

15

Assays

Acetylxylan esterase assay

To assay for AXE activity, 20 μ l of culture broth was added to 1mM *p*-nitrophenyl acetate (Sigma-Aldrich, St. Louis, MO, USA) in assay buffer and incubated for 15 minutes at 37°C.
 20 The reaction was stopped by the addition of 2M TRIS buffer pH 8.0, and release of *p*-nitrophenyl monitored immediately by measuring the absorbance at 405 nm.

Media and buffers

FG4P medium

25	Ingredient	amount /liter
	Soy flour, degreased	30 g
	Maltodextrin	15 g
	Bacto™ Peptone (Difco Laboratories)	5 g
	KH ₂ PO ₄	15 g
30	Dowfax™ 63N10 (Dow Chemical Co.)	0.2 ml

Assay buffer

50mM phosphoric acid
 50mM acetic acid
 35 50mM boric acid
 50mM KCl

1mM CaCl₂

0.01% Triton X-100

Adjust pH to 6.0

5 p-nitrophenyl acetate stock

100mM pNP-acetate dissolved in DMSO

Stored at 4°C and diluted immediately before use

Example 1. Expression of the Acetylxylan esterase from *Aspergillus aculeatus*

10 In order to obtain material for testing the acetylxylan esterase from *Aspergillus aculeatus* the DNA sequence from SEQ ID NO: 1 is cloned and expressed in *Aspergillus oryzae*.

A synthetic DNA construct can be ordered from GENEART AG (Regensburg, Germany), or a similar provider of DNA synthesis, that includes the coding portion of SEQ ID NO: 1 with the
15 Kozak sequence "TCACC" added immediately 5' of the start codon, and appropriate restriction sites, for example BamHI and XhoI, added to the 5' and 3' ends respectively to facilitate subcloning to an *Aspergillus* expression vector. The synthetic gene fragment is subcloned to an appropriate *Aspergillus* expression vector, for example pMStr57 (WO 04/032648) and the resulting *Aspergillus* expression construct can be sequenced to confirm that the sequence
20 agrees completely with the coding sequence of SEQ ID NO: 1.

An appropriate *Aspergillus oryzae* expression host, for example BECh2 (WO 00/39322), is transformed with the expression construct according to the protocol described by Christensen et al., 1988, Biotechnology 6, 1419-1422 and WO 04/032648. To identify transformants producing the recombinant AXE, the transformants and BECh2 is cultured in a suitable
25 medium, for example, 100 ml of FG4P medium in 500 ml shake flasks 4 days at 30°C and 200RPM, and is assayed for recombinant acetylxylan esterase production.

To assay for AXE activity, 20 µl of culture broth is added to 1mM p-nitrophenyl acetate (Sigma-Aldrich, St. Louis, MO, USA) in assay buffer and incubated for 15 minutes at 37°C. The
30 reaction is stopped by the addition of 2M TRIS buffer pH 8.0, and release of p-nitrophenyl is monitored immediately by measuring the absorbance at 405 nm.

Based on the results, one of these transformants are selected and fermented in a suitable medium to provide material for purification.

35

Example 2. Expression of an Acetylxylan esterase from *Aspergillus niger*

The closest prior art acetylxylan esterase from *Aspergillus niger*, described in US 6,010,892 as SEQ ID NO: 7, was cloned and expressed in *Aspergillus niger*.

To obtain the gene, a synthetic DNA construct was ordered from GENEART AG (Regensburg, Germany) that included the coding portion of Seq ID NO: 7 from US 6,010,892 with the Kozak sequence "TCACC" appended immediately 5' of the start codon, and restriction sites BamHI and XhoI added to the 5' and 3' ends respectively to facilitate subcloning to an *Aspergillus* expression vector. The BamHI-XhoI synthetic gene fragment was subcloned to the *Aspergillus* expression vector pMStr57 (WO 04/032648) and the resulting *Aspergillus* expression construct, pMStr201, was sequenced. The sequence agreed completely with the coding sequence of SEQ ID NO: 7 from US 6,010,892.

The *Aspergillus niger* strain MBin118 (WO 04/090155) was transformed with pMStr201 according to the protocol described by Christensen et al., 1988, Biotechnology 6, 1419-1422 and WO 04/032648. To identify transformants producing the recombinant AXE, the transformants and MBin118 were cultured in 100 ml of FG4P medium in 500 ml shake flasks 4 days at 30°C and 200RPM, and assayed for recombinant acetylxylan esterase production. To assay for AXE activity, 20 µl of culture broth was added to 1mM p-nitrophenyl acetate (Sigma-Aldrich, St. Louis, MO, USA) in assay buffer and incubated for 15 minutes at 37°C. The reaction was stopped by the addition of 2M TRIS buffer pH 8.0, and release of p-nitrophenyl monitored immediately by measuring the absorbance at 405 nm.

Based on the results, one of these transformants, designated MStr362, was selected and fermented in FG4P medium as described above to provide material for purification.

Example 3. Purification of Acetylxylan esterase from *Aspergillus aculeatus*

The Acetyl-Xylan esterase from *A. aculeatus* expressed in *A. oryzae* as e.g described in example 1 was purified from the fermentation supernatant. The fermentation supernatant was sterile filtered and adjusted to a final concentration of 1 Molar ammonium sulphate and pH was adjusted to 7.

A 50ml column was packed with Phenyl sepharose (Pharmacia/ Now GE-health care) and equilibrated with 50 mM phosphate containing 1 M ammonium sulphate and pH was 7. The fermentation supernatant was applied on the Phenyl sepharose column and unbound material was washed with the Phosphate buffer containing 1 M Ammonium sulphate. The column was washed till UV absorbance at 280nm was below 0.05.

The bound proteins were eluted with 50 % ethanol. The eluted proteins were dialyzed and conductivity was adjusted below 2 MSi and pH was 7.

Anion exchange on 50 ml Q Sepharose column (Pharmacia/ now GE Health care) was equilibrated with 50 mM Tris acetate pH 7 buffer. The dialyzed eluate from Phenyl Sepharose was applied on the Q Sepharose column. The unbound material was washed with 50 mM Tris acetate buffer pH 7.

The bound protein was eluted using 50 mM Tris acetate buffer pH 7 containing 0.5 M NaCl using linear salt gradient with 10 column volum.

SDS-PAGE was used to check the purity and molecular weight was determined by Mass spectra . N-terminal was determined by Edman degradation.

Example 4. Purification of Acetylxylnan esterase from *Aspergillus niger*

In order to decrease the colour, the fermentation supernatant, obtained as described in Example 2, was filtered/buffer exchanged using a Sartorius UF 10 kDa membrane prior to purification. The final volume was 800 mL and the pH was adjusted to 4.4 using HOAc.

Step 1

The sample was purified using a 70 mL column with a stationary phase of XpressLine ProA (Upfront, Copenhagen, Denmark). The sample was washed using 50 mM HOAc pH 4.4 until the absorbance were below 0.05, thereafter the protein was eluted using 50 mM HEPES, pH 7.5.

An activity assay (as described in Example 2) was used to identify the fractions that contained AXE activity, which were then pooled for further purification.

Step 2

The sample from step 1 was purified using a 20 mL column with a stationary phase of Source 15Q (GE Healthcare, Uppsala, Sweden). The sample was washed using 20 mM HEPES pH 7.5 until the absorbance were below 0.05, thereafter the protein was eluted with a linear salt gradient over 400 mL using 50 mM HEPES + 1 M NaCl, pH 7.

Again the activity assay above was used to identify the fractions that contained AXE activity, which then were pooled for further purification.

Step 3

The sample was purified using a 60 mL column with a stationary phase of Butyl Toyopearl

(Tosoh Bioscience, Stuttgart, Germany). 1.8 M NH₄OAc, pH 7.5 was added to the sample. The sample was washed using 1.8 M NH₄OAc, pH 7.5 until the absorbance were below 0.05, thereafter the protein was eluted with a step gradient using 20 mM HEPES, pH 7.5 followed by another step gradient using 20 mM HEPES, pH 7.5 + 50% EtOH.

5

Example 5. Effect of *Aspergillus aculeatus* acetylxyylan esterase on hydrolysis of pretreated corn fiber

The effect of *Aspergillus aculeatus* acetylxyylan esterase and *Aspergillus niger* acetylxyylan esterase on hydrolysis of pretreated corn fiber was compared and evaluated. Corn fiber is a fraction from the wet milling of corn kernels. Corn fiber is the seed coat and residual endosperm left after starch is removed and further processed. Corn fiber was pretreated by autoclaving at 140°C for 150 minutes. The amount of theoretical arabinose, glucose and xylose in the substrate was determined to be 114, 302, and 204 g per kg dry matter using the following methods.

Arabinose and xylose were determined by carbohydrate hydrolysis using dilute hydrochloric acid. The pretreated corn fiber was transferred to 125 ml conical flasks and diluted to contain approximately 10% dry matter. The corn fiber sample was preheated at 100°C in an oil bath. Hydrolysis was started by adding 5 ml of 2 M hydrochloric acid for 2 hours at 100°C. After incubation the flasks were cooled on ice and neutralized with 4 M sodium hydroxide. Samples were filtered with a MINISART® 0.2 micron syringe filter (Sartorius AG, Goettingen, Germany) and analyzed for arabinose and xylose on a DIONEX BIOLC® System (Dionex Corporation, Sunnyvale, CA, USA). Glucose was determined by subjecting the pretreated sample of corn fiber to a two step sulfuric acid hydrolysis. Three ml of 72% sulfuric acid was added to approximately 300 mg of dried corn fiber in pressure tubes (Ace Glass, Inc., Vineland, NJ, USA). Samples were mixed and placed in a water bath at 30°C for 60 minutes. Samples were stirred every 5 to 10 minutes. After 60 minutes the samples were removed and 84 ml of deionized water was added. Samples were placed in an autoclave and heated for 1 hour at 121°C. After cooling the samples were filtered to remove remaining solids and neutralized by addition of calcium carbonate.

Glucose concentration was determined with a DIONEX® BIOLC® System according to the following method. Samples (10 µl) were loaded onto a DIONEX BIOLC® System equipped with a DIONEX® CARBOPAC™ PA1 analytical column (4 x 250 mm) (Dionex Corporation, Sunnyvale, CA, USA) combined with a CARBOPAC™ PA1 guard column (4 x 50 mm) (Dionex Corporation, Sunnyvale, CA, USA). The monosaccharides were separated isocratically with 10 mM potassium hydroxide at a flow rate of 1 ml per minute and detected by a pulsed electrochemical detector in the pulsed amperometric detection mode. The potential of the

35

electrode was programmed for +0.1 volt (t=0-0.4 second) to -2.0 volt (t=0.41-0.42 second) to 0.6 volt (t=0.43 second) and finally -0.1 volt (t=0.44-0.50 second), while integrating the resulting signal from t=0.2-0.4 second. A mixture of arabinose, galactose, glucose, and xylose (concentration of each component: 0.0050-0.075 g per liter) was used as a standard.

5 The hydrolysis of the pretreated corn fiber was conducted with a *Trichoderma reesei* cellulolytic protein composition (*Trichoderma reesei* broth comprising *Thermoascus aurantiacus* GH61A polypeptide having cellulolytic enhancing activity and *Aspergillus oryzae* beta-glucosidase fusion; WO2008/151079 (PCT/US2008/065417)) and a *Trichoderma reesei* beta-xylosidase. The *Trichoderma reesei* beta-xylosidase was obtained recombinantly by
10 expression in *Aspergillus oryzae* as described in Rasmussen *et al.*, 2006, *Biotechnology and Bioengineering* 94: 869-876 using standard cultivation methods for *Aspergillus oryzae*. The *Aspergillus aculeatus* acetylxylan esterase can be obtained as described in Example 1. The *Aspergillus niger* acetylxylan esterase was obtained as described in Example 2.

The hydrolysis of the pretreated corn fiber was performed in 2 ml EPPENDORF® tubes
15 (Eppendorf AG, Germany) at a temperature of 50°C and a pH of 5.0 in 50 mM succinic acid. Samples were incubated in a THERMOMIXER® Comfort (Eppendorf AG, Germany) that subjected each sample with constant heating and mixing. The substrate amount used was 2.5 w/w % in a total sample volume of 2 ml. The *Aspergillus aculeatus* acetylxylan esterase and the *Aspergillus niger* acetylxylan esterase was added at an enzyme loading of 1 mg enzyme
20 per g of dry matter on top of both the *Trichoderma reesei* cellulolytic protein composition and the *Trichoderma reesei* beta-xylosidase. The *Trichoderma reesei* cellulolytic protein composition was added at a loading of 5 mg enzyme per g of dry matter and the *Trichoderma reesei* beta-xylosidase at a loading of 1 mg enzyme per g of dry matter. Hydrolysis was
25 terminated after 24 hours by heating the samples for 10 minutes at 100°C in a heat block (Techne Inc., Burlington NJ, USA).

Conversion was calculated by determining the amount of sugars released from the substrate as a percentage of what was added from the start using the formula below. T-tests were performed with a two tailed distribution and equal variance of sample data.

Conversion (%) = (Sugar amount in hydrolysate / Sugar amount in added substrate) x
30 100

Comparing the conversion of pretreated corn fiber when adding the *Aspergillus aculeatus* acetylxylan esterase at an enzyme loading of 1 mg of enzyme per gram dry matter together with 1 mg enzyme per g of dry matter of *Trichoderma reesei* beta-xylosidase and 5 mg enzyme per g of dry matter of *Trichoderma reesei* cellulolytic protein composition to just
35 adding 1 mg enzyme per g of dry matter of beta-xylosidase from *Trichoderma reesei* and 5 mg enzyme per g of dry matter of *Trichoderma reesei* cellulolytic protein composition

demonstrated a significant ($P \leq 0,000007$) increase in relative conversion from 100.0 to 140.5 (Table 1).

Table 1

Samples	Relative total conversion	Standard deviation	T-test
Trichoderma reesei cellulolytic protein composition and Trichoderma reesei beta-xylosidase	100,0	1,4	0,000007
Trichoderma reesei cellulolytic protein composition, Trichoderma reesei beta-xylosidase, and <i>Aspergillus aculeatus</i> acetylxyylan esterase	140,5	1,8	

5

Comparing the conversion of pretreated corn fiber when adding the *Aspergillus niger* acetylxyylan esterase at an enzyme loading of 1 mg of enzyme per gram dry matter together with 1 mg enzyme per g of dry matter of *Trichoderma reesei* beta-xylosidase and 5 mg enzyme per g of dry matter of *Trichoderma reesei* cellulolytic protein composition to just adding 1 mg enzyme per g of dry matter of beta-xylosidase from *Trichoderma reesei* and 5 mg enzyme per g of dry matter of *Trichoderma reesei* cellulolytic protein composition demonstrated a significant ($P \leq 0,012$) increase in relative conversion from 100.0 to 104.5 (Table 2).

10

Table 2

Samples	Relative total conversion	Standard deviation	T-test
Trichoderma reesei cellulolytic protein composition and Trichoderma reesei beta-xylosidase	100,0	1,4	0,012
Trichoderma reesei cellulolytic protein composition, Trichoderma reesei beta-xylosidase, and <i>Aspergillus niger</i> acetylxyylan esterase	104,5	1,1	

15

Example 6. Effect of *Aspergillus aculeatus* acetylxyylan esterase on the hydrolysis of D-xylose tetraacetate

The effect of *Aspergillus aculeatus* acetylxylan esterase on the hydrolysis of D-xylose tetraacetate was evaluated. The *Aspergillus aculeatus* acetylxylan esterase can be obtained as described in Example 1.

5 Hydrolysis of D-xylose tetraacetate (*Benn Chemicals*, Dielsdorf, Switzerland) was performed in 1.5 ml EPPENDORF® tubes at a temperature of 50°C and a pH of 5.0 in 50 mM succinic acid for 48 hours. Samples were incubated in a THERMOMIXER® Comfort that subjected each sample with constant heating and mixing. The substrate amount used was 1 ml at a concentration of 1 w/w % of D-xylose tetraacetate. The control sample (1 ml of
10 substrate) was compared with the *Aspergillus aculeatus* acetylxylan esterase sample (1 ml of substrate + 6 µl of enzyme). The *Aspergillus aculeatus* acetylxylan esterase was added at an enzyme loading of 0.5 mg *Aspergillus aculeatus* acetylxylan esterase/g dry solids. Hydrolysis was terminated after 48 hours by heating the samples for 10 minutes at 100°C in a heat block.

Quantification of acetic acid was performed by high pressure liquid chromatography
15 using two AMINEX® HPX-87H columns (Bio-Rad Laboratories, Hercules, CA, USA) coupled in series with a pre-column (Micro-Guard Cation H Refill Cartridges, Bio-Rad Laboratories, Hercules, CA, USA) with a WATERS® 515 Pump, WATERS® MPSA Millipore, WATERS® 717 Plus Autosampler, WATERS® Column Heater Module and WATERS® 2410 RI detector (Waters Corporation, Milford, MA, USA). The chromatography was performed at 60°C with a
20 flow of 0.4 ml/minute of 0.005 M sulfuric acid.

Addition of 0.5 mg of *Aspergillus aculeatus* acetylxylan esterase to 1 ml of substrate (1 w/w % of D-xylose tetraacetate) resulted in a calculated release of 76.1 µmol/ml acetate (Table 3). The release of acetate by *Aspergillus aculeatus* acetylxylan esterase was calculated from the concentrations of the control sample (1.9 µmol/ml) and the *Aspergillus*
25 *aculeatus* acetylxylan esterase sample (78.0 µmol/ml).

Table 3

Samples	Concentration of acetate (micromol/ml)	Enzyme released acetate (micromol/ml)
Control	1.9	
<i>A. aculeatus</i> acetylxylan esterase	78.0	76.1

30

Claims

1. An isolated polypeptide having acetyl xylan esterase activity, selected from the group consisting of:
- 5 (a) a polypeptide comprising an amino acid sequence having at least 85% identity to the mature polypeptide of SEQ ID NO:2;
- (b) a polypeptide encoded by a polynucleotide that hybridizes under at least high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO:1, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO:1, 10 or (iii) a full-length complementary strand of (i) or (ii);
- (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 85% identity to the mature polypeptide coding sequence of SEQ ID NO:1; 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.
- 15
2. The polypeptide of claim 1, which is encoded by a polynucleotide that hybridizes under at least high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO:1, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO:1, or (iii) a full-length complementary strand of (i) or (ii).
- 20
3. The polypeptide of any of claims 1 or 2, which is encoded by a polynucleotide comprising or consisting of the nucleotide sequence of SEQ ID NO:1; or a subsequence thereof encoding a fragment having acetyl xylan esterase activity.
- 25
4. The polypeptide of any of claims 1 to 3, which is encoded by a polynucleotide comprising or consisting of nucleotides 136 to 963 of SEQ ID NO:1.
5. The polypeptide of any of claims 1 to 4, which is encoded by a polynucleotide comprising or consisting of the mature polypeptide coding sequence of SEQ ID NO:1.
- 30
6. The polypeptide of any of claims 1 to 5, wherein the polypeptide 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.
- 35
7. The polypeptide of any of claims 1 to 6, wherein the mature polypeptide is amino acids 34 to 308 of SEQ ID NO:2.

8. The polypeptide of any of claims 1 to 7, wherein the mature polypeptide coding sequence is nucleotides 136 to 963 of SEQ ID NO:1.
9. An isolated polynucleotide comprising a nucleotide sequence that encodes the polypeptide of any of claims 1 to 8.
10. A nucleic acid construct comprising the polynucleotide of claim 9 operably linked to one or more control sequences that direct the production of the polypeptide in an expression host.
11. A recombinant expression vector comprising the nucleic acid construct of claim 10.
12. A recombinant host cell comprising the nucleic acid construct of claim 10.
13. A method of producing the polypeptide of any of claims 1 to 8, comprising: (a) cultivating a host cell comprising a nucleic acid construct comprising a nucleotide sequence encoding the polypeptide under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.
14. The isolated polynucleotide of claim 9, wherein the mature polypeptide coding sequence is nucleotides 136 to 963 of SEQ ID NO:1, or a homologous sequence.
15. A method of producing the polypeptide of any of claims 1 to 8, comprising: (a) cultivating a transgenic plant or a plant cell comprising a polynucleotide encoding the polypeptide under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.
16. A transgenic plant, plant part or plant cell transformed with a polynucleotide encoding the polypeptide of any of claims 1 to 8.
17. A method for degrading an acetylated xylan, comprising treating a material comprising an acetylated xylan with the polypeptide having acetyl xylan esterase activity of any of claims 1 to 8.
18. The method of claim 17, further comprising treating the material comprising the acetylated xylan with a xylan degrading enzyme.

19. The method of claim 17 or 18, wherein the xylan degrading enzyme is selected from the group consisting of a xylanase, an arabinofuranosidase, a xylosidase, a glucuronidase, and a combination thereof.
- 5 20. The method of any of claims 17 to 19, wherein the material comprising the acetylated xylan is an animal feed.
21. The method of any of claims 17 to 20, wherein the material comprising the acetylated xylan is a Kraft pulp.
- 10 22. The method of any of claims 17 to 21, wherein the material comprising the acetylated xylan is a cellulosic or lignocellulosic biomass.
23. A composition comprising the polypeptide of any of claims 1 to 8 and one or more additional enzyme(s) selected from xylanase, arabinofuranosidase, glucanase, pectinase, 15 protease, acetyl xylan esterase, ferulic acid esterase, rhamnogalacturonas and xylanase.
24. A use of the polypeptide of any of claims 1 to 8 or of the composition of claim 23 in a process of producing a fermentation product from lignocellulose-containing material, 20 comprising the steps of:
- (a) pre-treating lignocellulose-containing material;
 - (b) hydrolyzing the material in the presence of a cellulolytic enzyme and an enzyme of the invention;
 - (c) fermenting using a fermenting organism.

25

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/053770

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/18 C12N15/82
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, BIOSIS, Sequence Search, EMBASE, FSTA, 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	<p>KOSEKI ET AL: "An Aspergillus oryzae acetyl xylan esterase: Molecular cloning and characteristics of recombinant enzyme expressed in Pichia pastoris" JOURNAL OF BIOTECHNOLOGY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL LNKD- DOI:10.1016/J.JBIOTEC.2005.07.015, vol. 121, no. 3, 10 February 2006 (2006-02-10), pages 381-389, XP005256610 ISSN: 0168-1656</p>	<p>1,3, 9-14,23</p>
Y	<p>the whole document</p> <p style="text-align: center;">----- -/--</p>	<p>4-8, 15-22,24</p>

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search

22 June 2010

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/053770

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>SORENSEN HANNE R ET AL: "Characterization of solubilized arabinoxylo-oligosaccharides by MALDI-TOF MS analysis to unravel and direct enzyme catalyzed hydrolysis of insoluble wheat arabinoxylan" ENZYME AND MICROBIAL TECHNOLOGY, vol. 41, no. 1-2, July 2007 (2007-07), pages 103-110, XP022069888 ISSN: 0141-0229 page 104, column 2, paragraph 2 - paragraph 3</p>	<p>4-8, 15-22,24</p>
X	<p>EP 0 507 369 A2 (GIST BROCADES NV [NL]) 7 October 1992 (1992-10-07) examples 4,5; sequence 7</p>	<p>1-3, 9-14,23</p>
A	<p>HOWARD R L ET AL: "Lignocellulose biotechnology: Issues of bioconversion and enzyme production" AFRICAN JOURNAL OF BIOTECHNOLOGY, ACADEMIC PRESS, US, vol. 2, no. 12, 1 December 2003 (2003-12-01), pages 602-619, XP009130695 ISSN: 1684-5315 figure 1</p>	<p>15-24</p>

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2010/053770

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0507369	A2	07-10-1992	
		AT 166386 T	15-06-1998
		CA 2063352 A1	19-09-1992
		DE 69225522 D1	25-06-1998
		DE 69225522 T2	10-09-1998
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		ES 2120428 T3	01-11-1998
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