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(54) Title: POLYPEPTIDES HAVING ACETYLXYLAN ESTERASE ACTIVITY AND POLYNUCLEOTIDES ENCODING SAME

(57) Abstract: The present invention relates to isolated polypeptides having acetylxylylan 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.



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**POLYPEPTIDES HAVING ACETYLYLAN ESTERASE ACTIVITY
AND POLYNUCLEOTIDES ENCODING SAME**

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Reference to a Sequence Listing

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

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Reference to a Deposit of Biological Material

This application contains a reference to a deposit of biological material, which deposit is incorporated herein by reference.

Background of the Invention

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Field of the Invention

The present invention relates to isolated polypeptides having acetylxylin 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.

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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 cell wall polysaccharides. Hemicelluloses are the second most abundant constituent of plant cell walls. The major hemicellulose polymer is xylan. The structure of 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.

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The biodegradation of the xylan backbone depends on two classes of enzymes: 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, acetylxylin esterase, arabinase, alpha-glucuronidase, ferulic acid esterase, and *p*-coumaric acid esterase.

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Acetylxylan esterase (EC 3.1.1.6) removes O-acetyl groups from positions 2 and/or 3 on the beta-D-xylopyranosyl residues of acetylxylan. Acetylxylan 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 sequence similarity, has led to the definition of 13 families, seven of which contain acetylxylan esterases (Henrissat B., 1991, *Biochem. J.* 280: 309-316, and Henrissat and Bairoch, 1996, *Biochem. J.* 316: 695-696).

Margolles-Clark *et al.*, 1996, *Eur. J. Biochem.* 237:553-560, disclose an acetylxylan esterase from *Trichoderma reesei*. Sundberg and Poutanen, 1991, *Biotechnol. Appl. Biochem.* 13:1-11, disclose the purification and properties of two acetylxylan esterases of *Trichoderma reesei*. WO 2005/001036 discloses an acetylxylan esterase gene from *Trichoderma reesei*. U.S. Patent No. 5,681,732 discloses an acetylxylan esterase gene from *Aspergillus niger*. U.S. Patent No. 5,763,260 discloses methods to alter the properties of acetylated xylan.

The present invention relates to polypeptides having acetylxylan esterase activity and polynucleotides encoding the polypeptides.

Summary of the Invention

The present invention relates to isolated polypeptides having acetylxylan 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;

(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);

(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.

The present invention also relates to isolated polynucleotides encoding polypeptides having acetylxylan esterase activity, selected from the group consisting of:

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

(b) 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);

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

The present invention also relates to nucleic acid constructs, recombinant expression vectors, recombinant host cells comprising the polynucleotides, and methods of producing a polypeptide having acetylxylin esterase activity.

The present invention also relates to methods of inhibiting the expression of a polypeptide in a cell, comprising administering to the cell or expressing in the cell a double-stranded RNA (dsRNA) molecule, wherein the dsRNA comprises a subsequence of a polynucleotide of the present invention. The present also relates to such a double-stranded inhibitory RNA (dsRNA) molecule, wherein optionally the dsRNA is a siRNA or a miRNA molecule.

The present invention also relates to methods for degrading a material comprising an acetylated xylan.

The present invention also relates to plants comprising an isolated polynucleotide encoding such a polypeptide having acetylxylin esterase activity.

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

The present invention further relates to nucleic acid constructs comprising a gene encoding a protein, wherein the gene is operably linked to a nucleotide sequence encoding a signal peptide comprising or consisting of amino acids 1 to 16 of SEQ ID NO: 2, wherein the gene is foreign to the nucleotide sequence.

Brief Description of the Figures

Figure 1 shows the cDNA sequence and the deduced amino acid sequence of a *Thielavia terrestris* NRRL 8126 acetylxylan esterase (SEQ ID NOs: 1 and 2, respectively).

Figure 2 shows a restriction map of pTterAxe2A.

Figure 3 shows a restriction map of pAilLo39.

Definitions

Acetylxylan esterase activity: The term "acetylxylan esterase activity" is defined herein as a carboxylesterase activity (EC 3.1.1.72) that catalyses the hydrolysis of acetyl groups from polymeric xylan, acetylated xylose, acetylated glucose, alpha-naphthyl acetate, *p*-nitrophenyl acetate. For purposes of the present invention, acetylxylan esterase activity is determined according to the procedure described in Example 8. One unit of acetylxylan esterase activity is defined as the amount of enzyme capable of releasing 1 μ mole of *p*-nitrophenolate anion per minute at pH 5, 25°C.

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 80%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 100% of the acetylxylan esterase activity of the mature polypeptide of SEQ ID NO: 2.

Family 2 or Family CE2 or CE2: The term "Family 2" or "Family CE2" or "CE2" is defined herein as a polypeptide falling into Family 2 of the carbohydrate esterases according to the classification of Coutinho, P.M. and Henrissat, B. (1999) Carbohydrate-active enzymes: an integrated database approach. In "Recent Advances in Carbohydrate Bioengineering", H.J. Gilbert, G. Davies, B. Henrissat and B. Svensson eds., The Royal Society of Chemistry, Cambridge, pp. 3-12.

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 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 acetylxylan 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 17 to 413 of SEQ ID NO: 2 based on the SignalP software program (Nielsen *et al.*, 1997, *Protein Engineering* 10: 1-6) that predicts amino acids 1 to 16 of SEQ ID NO: 2 are a signal peptide.

Mature polypeptide coding sequence: The term "mature polypeptide coding sequence" is defined herein as a nucleotide sequence that encodes a mature polypeptide having acetylxylan esterase activity. In a preferred aspect, the mature polypeptide coding sequence is nucleotides 49 to 1239 of SEQ ID NO: 1 based on the SignalP software program (Nielsen *et al.*, 1997, *Protein Engineering* 10: 1-6) that predicts nucleotides 1 to 48 encode a signal peptide.

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
5 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
10 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
15 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
20 a tfasty search (Pearson, W.R., 1999, in *Bioinformatics Methods and Protocols*, S. Misener and S. A. Krawetz, ed., pp. 185-219) with the *Thielavia terrestris* acetylxylian esterase of SEQ ID NO: 2 or the mature polypeptide thereof.

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
25 carboxyl terminus of the mature polypeptide of SEQ ID NO: 2; or a homologous sequence thereof; wherein the fragment has acetylxylian esterase activity. In a preferred aspect, a fragment contains at least 340 amino acid residues, more preferably at least 360 amino acid residues, and most preferably at least 380 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
30 the mature polypeptide coding sequence of SEQ ID NO: 1; or a homologous sequence thereof; wherein the subsequence encodes a polypeptide fragment having acetylxylian esterase activity. In a preferred aspect, a subsequence contains at least 1020
35 nucleotides, more preferably at least 1080 nucleotides, and most preferably at least

1140 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.

5 **cDNA:** The term "cDNA" is defined herein as a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic cell. cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps before appearing as mature spliced
10 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
15 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
20 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
25 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.

30 **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
35 of a polypeptide including, but not limited to, transcription, post-transcriptional

modification, translation, post-translational modification, and secretion.

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

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

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

Artificial variant: When used herein, the term "artificial variant" means a polypeptide having acetylxylan 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.

Detailed Description of the Invention

Polypeptides Having Acetylxylan Esterase Activity

In a first 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 preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 96%, at least 97%, at least 98%, or at least 99%, which have acetylxylan 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 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.

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 acetylxylin 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 17 to 413 of SEQ ID NO: 2, or an allelic variant thereof; or a fragment thereof having acetylxylin esterase activity. In another preferred aspect, the polypeptide comprises amino acids 17 to 413 of SEQ ID NO: 2. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 2 or an allelic variant thereof; or a fragment thereof having acetylxylin 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 17 to 413 of SEQ ID NO: 2 or an allelic variant thereof; or a fragment thereof having acetylxylin esterase activity. In another preferred aspect, the polypeptide consists of amino acids 17 to 413 of SEQ ID NO: 2.

In a second aspect, the present invention relates to isolated polypeptides having acetylxylin 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 acetylxylin 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 acetylxylin 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
5 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
10 200 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 600 nucleotides, more preferably at least 700 nucleotides, even more preferably at least 800 nucleotides, or most preferably at least 900 nucleotides in length. Both DNA and RNA probes can
15 be used. The probes are typically labeled for detecting the corresponding gene (for example, with ^{32}P , ^3H , ^{35}S , biotin, or avidin). Such probes are encompassed by the present invention.

A genomic DNA or cDNA library prepared from such other strains may, therefore, be screened for DNA that hybridizes with the probes described above and
20 encodes a polypeptide having acetylxylin 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 homologous with
25 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
30 the mature polypeptide coding sequence of SEQ ID NO: 1; its full-length complementary strand; or a subsequence thereof; under very low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions can be detected using, for example, X-ray film.

In a preferred aspect, the nucleic acid probe is the mature polypeptide coding
35 sequence of SEQ ID NO: 1. In another preferred aspect, the nucleic acid probe is

nucleotides 49 to 1239 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. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pTterAxe2A which is contained in *E. coli* NRRL B-50067, wherein the polynucleotide sequence thereof encodes a polypeptide having acetylxylin esterase activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding region contained in plasmid pTterAxe2A which is contained in *E. coli* NRRL B-50067.

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

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

For short probes of about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as prehybridization, hybridization, and washing post-hybridization at about 5°C to about 10°C below the calculated T_m using the calculation according to Bolton and McCarthy (1962, *Proceedings of the National Academy of Sciences USA* 48:1390) in 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 of about 15 nucleotides to about 70 nucleotides in length, the carrier material is washed once in 6X SSC plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6X SSC at 5°C to 10°C below the calculated T_m .

In a third aspect, the present invention relates to isolated polypeptides having acetylxylin 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 preferably at least 60%, more preferably at least 65%,

more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 96%, at least 97%, at least 98%, or at least 99%, which encode an active polypeptide. See polynucleotide section herein.

5 In a fourth 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;
10 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.

15 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
20 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.

25 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.
30 "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 pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, and 3,3-dimethylproline.

35 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
5 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.*, acetylcholinesterase activity) to identify amino acid residues that are critical to the activity of the
10 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.*,
15 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
20 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-
25 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
30 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
35 structure.

The total number of amino acid substitutions, deletions and/or insertions of the mature polypeptide of SEQ ID NO: 2, such as amino acids 17 to 413 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 Acetylxylyl Esterase Activity

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 is secreted extracellularly.

A polypeptide having acetylxylyl 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*, *Lactobacillus*, *Lactococcus*, *Clostridium*, *Geobacillus*, or *Oceanobacillus* polypeptide having acetylxylyl 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 acetylxylyl esterase activity.

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

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

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

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

polypeptide having acetylxylan 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*,
 5 *Filibasidium*, *Fusarium*, *Gibberella*, *Holomastigotoides*, *Humicola*, *Irpex*, *Lentinula*, *Leptosphaeria*, *Magnaporthe*, *Melanocarpus*, *Meripilus*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Piromyces*, *Poitrasia*, *Pseudoplectania*, *Pseudotrichonympha*, *Rhizomucor*, *Schizophyllum*, *Scytalidium*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trichoderma*,
 10 *Trichophaea*, *Verticillium*, *Volvariella*, or *Xylaria* polypeptide having acetylxylan 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*
 15 polypeptide having acetylxylan esterase 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*,
 20 *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*,
 25 *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola grisea*, *Humicola insolens*, *Humicola lanuginosa*, *Irpex lacteus*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium funiculosum*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Trichoderma harzianum*, *Trichoderma koningii*,
 30 *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* polypeptide having acetylxylan esterase activity.

In another preferred aspect, the polypeptide is a *Thielavia achromatica*, *Thielavia albomyces*, *Thielavia albopilosa*, *Thielavia australeinsis*, *Thielavia fimeti*, *Thielavia microspora*, *Thielavia ovispora*, *Thielavia peruviana*, *Thielavia spededonium*,
 35 *Thielavia setosa*, *Thielavia subthermophila*, or *Thielavia terrestris* polypeptide.

In a more preferred aspect, the polypeptide is a *Thielavia terrestris* polypeptide having acetylxylan esterase activity. In a most preferred aspect, the polypeptide is a *Thielavia terrestris* NRRL 8126 polypeptide having acetylxylan esterase activity, e.g., the polypeptide comprising the mature polypeptide of SEQ ID NO: 2.

5 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.

10 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 (DSM), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

15 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 encoding a polypeptide has been detected with the probe(s), the
20 polynucleotide can be isolated or cloned by utilizing techniques that are well known to those of ordinary skill in the art (see, e.g., Sambrook *et al.*, 1989, *supra*).

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
25 by fusing a nucleotide 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.

30 A fusion polypeptide can further comprise a cleavage site. Upon secretion of the fusion protein, the site is cleaved releasing the polypeptide having acetylxylan 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-
35 Wilson *et al.*, 1997, *Appl. Environ. Microbiol.* 63: 3488-3493; Ward *et al.*, 1995,

Biotechnology 13: 498-503; and Contreras *et al.*, 1991, *Biotechnology* 9: 378-381), 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, 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*).

Polynucleotides

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

In a preferred aspect, the nucleotide sequence comprises or consists of SEQ ID NO: 1. In another more preferred aspect, the nucleotide sequence comprises or consists of the sequence contained in plasmid pTterAxe2A which is contained in *E. coli* NRRL B-50067. 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 49 to 1239 of SEQ ID NO: 1. In another more preferred aspect, the nucleotide sequence comprises or consists of the mature polypeptide coding sequence contained in plasmid pTterAxe2A which is contained in *E. coli* NRRL B-50067. The present invention also encompasses nucleotide sequences that encode polypeptides comprising or 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 acetylxyylan esterase activity.

The present invention also relates to mutant polynucleotides comprising or consisting of 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 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 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 sequence of SEQ ID NO: 1 of preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 96%, at least 97%, at least 98%, or at least 99%, which encode 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 acetylxylin 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 acetylxylin 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.

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.

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.

5 Manipulation of the polynucleotide's sequence prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotide sequences utilizing recombinant DNA methods are well known in the art.

The control sequence may be an appropriate promoter sequence, a nucleotide
10 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
15 encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs 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*),
20 *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:
25 3727-3731), as well as the *tac* promoter (DeBoer *et al.*, 1983, *Proceedings of the National Academy of Sciences USA* 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242: 74-94; and in Sambrook *et al.*, 1989, *supra*.

Examples of suitable promoters for directing the transcription of the nucleic acid
30 constructs of the present invention in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae*
35 triose phosphate isomerase, *Aspergillus nidulans* acetamidase, *Fusarium venenatum*

amyloglucosidase (WO 00/56900), *Fusarium venenatum* Daria (WO 00/56900),
Fusarium venenatum Quinn (WO 00/56900), *Fusarium oxysporum* trypsin-like protease
(WO 96/00787), *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei*
cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei*
5 endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei*
endoglucanase III, *Trichoderma reesei* endoglucanase IV, *Trichoderma reesei*
endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II,
Trichoderma reesei beta-xylosidase, as well as the NA2-tpi promoter (a hybrid of the
promoters from the genes for *Aspergillus niger* neutral alpha-amylase and *Aspergillus*
10 *oryzae* triose phosphate isomerase); and mutant, truncated, and hybrid promoters
thereof.

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

The control sequence may also be a suitable transcription terminator sequence,
20 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.

Preferred terminators for filamentous fungal host cells are obtained from the
25 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
Saccharomyces cerevisiae enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1),
30 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
35 polypeptide. Any leader sequence that is functional in the host cell of choice may be

used in the present invention.

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

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

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

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

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

25 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 polypeptide into the cell's secretory pathway. The 5' end of the coding sequence of the nucleotide sequence may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. The foreign signal peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding sequence.

30 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 medium, may be used in the present invention.

35 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
5 Simonen and Palva, 1993, *Microbiological Reviews* 57: 109-137.

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

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

15 In a preferred aspect, the signal peptide comprises or consists of amino acids 1 to 16 of SEQ ID NO: 2. In another preferred aspect, the signal peptide coding sequence comprises or consists of nucleotides 1 to 48 of SEQ ID NO: 1.

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
20 resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propeptide is generally inactive and can be converted to a mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding sequence may be obtained from the genes for *Bacillus subtilis* alkaline protease (*aprE*), *Bacillus subtilis* neutral protease (*nprT*),
25 *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
30 terminus of a polypeptide and the signal peptide sequence is positioned next to the amino terminus of the propeptide sequence.

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

and *trp* operator systems. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the 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
5 amplification. In eukaryotic systems, these regulatory sequences include the dihydrofolate reductase gene that is amplified in the presence of methotrexate, and the metallothionein genes that are amplified with heavy metals. In these cases, the nucleotide sequence encoding the polypeptide would be operably linked with the regulatory sequence.

Expression Vectors

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
15 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 invention may be expressed by inserting the nucleotide sequence or a nucleic acid construct comprising the sequence
20 into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

The recombinant expression vector may be any vector (e.g., a plasmid or virus) that can be conveniently subjected to recombinant DNA procedures and can bring
25 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 extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, an extrachromosomal element, a minichromosome, or an
30 artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one that, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or
35 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 like cells. A selectable marker is a gene the product of which provides for biocide
5 or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

Examples of bacterial selectable markers are the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers that confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol, or tetracycline resistance. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for
10 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
15 *Aspergillus* cell are the *amdS* and *pyrG* genes of *Aspergillus 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
20 polynucleotide's sequence encoding the polypeptide or any other element of the vector for integration into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleotide sequences for directing integration by homologous recombination into the genome of the host cell at a precise
25 location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, preferably 400 to 10,000 base pairs, and most preferably 800 to 10,000 base pairs, which have a high degree of identity to the corresponding target sequence to enhance the probability of homologous
30 recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding nucleotide sequences. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of
35

replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in a cell. The term "origin of replication" or "plasmid replicator" is defined herein as a nucleotide sequence that enables a plasmid or vector to replicate
5 *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*.

10 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: 9163-9175; WO 00/24883). Isolation of the AMA1 gene and construction
15 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
20 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
25 the art (see, e.g., Sambrook *et al.*, 1989, *supra*).

Host Cells

The present invention also relates to recombinant host cells, comprising an
30 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
35 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.

5 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*,
10 *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*,
15 *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
20 another more preferred aspect, the bacterial host cell is a *Bacillus licheniformis* cell. In another more preferred aspect, the bacterial host cell is a *Bacillus subtilis* cell.

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

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

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

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.

5 In another preferred aspect, the bacterial host cell is a *Streptomyces lividans* cell.

The introduction of DNA into a *Bacillus* cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), by using competent cells (see, e.g., Young and Spizizen, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of*
10 *Molecular Biology* 56: 209-221), by electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or by conjugation (see, e.g., Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5271-5278). The introduction of DNA into an *E coli* cell may, for instance, be effected by protoplast transformation (see, e.g., Hanahan, 1983, *J. Mol. Biol.* 166: 557-580) or electroporation (see, e.g., Dower et al., 1988,
15 *Nucleic Acids Res.* 16: 6127-6145). The introduction of DNA into a *Streptomyces* cell may, for instance, be effected by protoplast transformation and electroporation (see, e.g., Gong et al., 2004, *Folia Microbiol. (Praha)* 49: 399-405), by conjugation (see, e.g., Mazodier et al., 1989, *J. Bacteriol.* 171: 3583-3585), or by transduction (see, e.g., Burke et al., 2001, *Proc. Natl. Acad. Sci. USA* 98: 6289-6294). The introduction of DNA into a
20 *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
25 (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
30 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
35 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 ascosporeogenous yeast (Endomycetales), basidiosporeogenous 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*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, or *Trichoderma* cell.

In a most preferred aspect, the filamentous fungal host cell is an *Aspergillus awamori*, *Aspergillus fumigatus*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or *Aspergillus oryzae* cell. In another most preferred aspect, the filamentous fungal host cell is a *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium gramineum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium*

- reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, or *Fusarium venenatum* cell. In another most preferred aspect, the filamentous fungal host cell is a *Bjerkandera adusta*, *Ceriporiopsis aneirina*,
 5 *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvessens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium tropicum*, *Chrysosporium merdarium*, *Chrysosporium inops*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus*
 10 *hirsutus*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.
- 15 Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described in EP 238 023 and Yelton *et al.*, 1984, *Proceedings of the National Academy of Sciences USA* 81: 1470-1474. Suitable methods for transforming
 20 *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156, and WO 96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153: 163; and Hinnen *et al.*,
 25 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920.

Methods of Production

- 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
 30 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 *Thielavia*. In a more preferred aspect, the cell is *Thielavia terrestris*. In a most preferred aspect, the cell is *Thielavia terrestris* NRRL 8126.

- The present invention also relates to methods of producing a polypeptide of the
 35 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.

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.

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 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.

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.

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.

5 **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 acetylxyylan 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
10 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
15 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
20 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
25 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
30 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
35 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 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 acetylxylan esterase activity of the present invention under conditions conducive for production of the polypeptide; and (b)
5 recovering the polypeptide.

Removal or Reduction of Acetylxylan Esterase Activity

The present invention also relates to methods of producing a mutant of a parent cell, which comprises disrupting or deleting a polynucleotide sequence, or a portion
10 thereof, encoding a polypeptide of the present invention, which results in the mutant cell producing less of the polypeptide than the parent cell when cultivated under the same conditions.

The mutant cell may be constructed by reducing or eliminating expression of a nucleotide sequence encoding a polypeptide of the present invention using methods
15 well known in the art, for example, insertions, disruptions, replacements, or deletions. In a preferred aspect, the nucleotide sequence is inactivated. The nucleotide sequence to be modified or inactivated may be, for example, the coding region or a part thereof essential for activity, or a regulatory element required for the expression of the coding region. An example of such a regulatory or control sequence may be a promoter
20 sequence or a functional part thereof, *i.e.*, a part that is sufficient for affecting expression of the nucleotide sequence. Other control sequences for possible modification include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, signal peptide sequence, transcription terminator, and transcriptional activator.

25 Modification or inactivation of the nucleotide sequence may be performed by subjecting the parent cell to mutagenesis and selecting for mutant cells in which expression of the nucleotide sequence has been reduced or eliminated. The mutagenesis, which may be specific or random, may be performed, for example, by use of a suitable physical or chemical mutagenizing agent, by use of a suitable
30 oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the mutagenesis may be performed by use of any combination of these mutagenizing agents.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-
35 nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane

sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues.

When such agents are used, the mutagenesis is typically performed by incubating the parent cell to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions, and screening and/or selecting for mutant cells exhibiting reduced or no expression of the gene.

Modification or inactivation of the nucleotide sequence may be accomplished by introduction, substitution, or removal of one or more (several) nucleotides in the gene or a regulatory element required for the transcription or translation thereof. For example, nucleotides may be inserted or removed so as to result in the introduction of a stop codon, the removal of the start codon, or a change in the open reading frame. Such modification or inactivation may be accomplished by site-directed mutagenesis or PCR generated mutagenesis in accordance with methods known in the art. Although, in principle, the modification may be performed *in vivo*, i.e., directly on the cell expressing the nucleotide sequence to be modified, it is preferred that the modification be performed *in vitro* as exemplified below.

An example of a convenient way to eliminate or reduce expression of a nucleotide sequence by a cell is based on techniques of gene replacement, gene deletion, or gene disruption. For example, in the gene disruption method, a nucleic acid sequence corresponding to the endogenous nucleotide sequence is mutagenized *in vitro* to produce a defective nucleic acid sequence that is then transformed into the parent cell to produce a defective gene. By homologous recombination, the defective nucleic acid sequence replaces the endogenous nucleotide sequence. It may be desirable that the defective nucleotide sequence also encodes a marker that may be used for selection of transformants in which the nucleotide sequence has been modified or destroyed. In a particularly preferred aspect, the nucleotide sequence is disrupted with a selectable marker such as those described herein.

Alternatively, modification or inactivation of the nucleotide sequence may be performed by established anti-sense or RNAi techniques using a sequence complementary to the nucleotide sequence. More specifically, expression of the nucleotide sequence by a cell may be reduced or eliminated by introducing a sequence complementary to the nucleotide sequence of the gene that may be transcribed in the cell and is capable of hybridizing to the mRNA produced in the cell. Under conditions allowing the complementary anti-sense nucleotide sequence to hybridize to the mRNA, the amount of protein translated is thus reduced or eliminated.

The present invention further relates to a mutant cell of a parent cell that

comprises a disruption or deletion of a nucleotide sequence encoding the polypeptide or a control sequence thereof, which results in the mutant cell producing less of the polypeptide or no polypeptide compared to the parent cell.

5 The polypeptide-deficient mutant cells so created are particularly useful as host cells for the expression of native and/or heterologous polypeptides. Therefore, the present invention further relates to methods of producing a native or heterologous polypeptide comprising: (a) cultivating the mutant cell under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide. The term "heterologous polypeptides" is defined herein as polypeptides that are not native to the
10 host cell, a native protein in which modifications have been made to alter the native sequence, or a native protein whose expression is quantitatively altered as a result of a manipulation of the host cell by recombinant DNA techniques.

In a further aspect, the present invention relates to a method of producing a protein product essentially free of acetylxylyl esterase activity by fermentation of a cell
15 that produces both a polypeptide of the present invention as well as the protein product of interest by adding an effective amount of an agent capable of inhibiting acetylxylyl esterase activity to the fermentation broth before, during, or after the fermentation has been completed, recovering the product of interest from the fermentation broth, and optionally subjecting the recovered product to further purification.

20 In a further aspect, the present invention relates to a method of producing a protein product essentially free of acetylxylyl esterase activity by cultivating the cell under conditions permitting the expression of the product, subjecting the resultant culture broth to a combined pH and temperature treatment so as to reduce the acetylxylyl esterase activity substantially, and recovering the product from the culture
25 broth. Alternatively, the combined pH and temperature treatment may be performed on an enzyme preparation recovered from the culture broth. The combined pH and temperature treatment may optionally be used in combination with a treatment with an acetylxylyl esterase inhibitor.

In accordance with this aspect of the invention, it is possible to remove at least
30 60%, preferably at least 75%, more preferably at least 85%, still more preferably at least 95%, and most preferably at least 99% of the acetylxylyl esterase activity. Complete removal of acetylxylyl esterase activity may be obtained by use of this method.

The combined pH and temperature treatment is preferably carried out at a pH in the range of 2-4 or 9-11 and a temperature in the range of at least 60-70°C for a
35 sufficient period of time to attain the desired effect, where typically, 30 to 60 minutes is

sufficient.

The methods used for cultivation and purification of the product of interest may be performed by methods known in the art.

The methods of the present invention for producing an essentially acetylxylan
5 esterase-free product is of particular interest in the production of eukaryotic
polypeptides, in particular fungal proteins such as enzymes. The enzyme may be
selected from, e.g., an amylolytic enzyme, lipolytic enzyme, proteolytic enzyme,
cellulolytic enzyme, oxidoreductase, or plant cell-wall degrading enzyme. Examples of
such enzymes include an aminopeptidase, amylase, amyloglucosidase, carbohydrase,
10 carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase,
cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase,
galactosidase, beta-galactosidase, glucoamylase, glucose oxidase, glucosidase,
haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, lyase,
mannosidase, oxidase, pectinolytic enzyme, peroxidase, phytase, phenoloxidase,
15 polyphenoloxidase, proteolytic enzyme, ribonuclease, transferase, transglutaminase, or
xylanase. The acetylxylan esterase-deficient cells may also be used to express
heterologous proteins of pharmaceutical interest such as hormones, growth factors,
receptors, and the like.

It will be understood that the term "eukaryotic polypeptides" includes not only
20 native polypeptides, but also those polypeptides, e.g., enzymes, which have been
modified by amino acid substitutions, deletions or additions, or other such modifications
to enhance activity, thermostability, pH tolerance and the like.

In a further aspect, the present invention relates to a protein product essentially
free from acetylxylan esterase activity that is produced by a method of the present
25 invention.

Methods of Inhibiting Expression of a Polypeptide Having Acetylxylan Esterase Activity

The present invention also relates to methods of inhibiting the expression of a
30 polypeptide having acetylxylan esterase activity in a cell, comprising administering to
the cell or expressing in the cell a double-stranded RNA (dsRNA) molecule, wherein the
dsRNA comprises a subsequence of a polynucleotide of the present invention. In a
preferred aspect, the dsRNA is about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more
duplex nucleotides in length.

The dsRNA is preferably a small interfering RNA (siRNA) or a micro RNA (miRNA). In a preferred aspect, the dsRNA is small interfering RNA (siRNAs) for inhibiting transcription. In another preferred aspect, the dsRNA is micro RNA (miRNAs) for inhibiting translation.

5 The present invention also relates to such double-stranded RNA (dsRNA) molecules, comprising a portion of the mature polypeptide coding sequence of SEQ ID NO: 1 for inhibiting expression of a polypeptide in a cell. While the present invention is not limited by any particular mechanism of action, the dsRNA can enter a cell and cause the degradation of a single-stranded RNA (ssRNA) of similar or identical sequences,
10 including endogenous mRNAs. When a cell is exposed to dsRNA, mRNA from the homologous gene is selectively degraded by a process called RNA interference (RNAi).

 The dsRNAs of the present invention can be used in gene-silencing therapeutics. In one aspect, the invention provides methods to selectively degrade RNA using the dsRNAs of the present invention. The process may be practiced *in vitro*, *ex vivo* or *in vivo*. In one aspect, the dsRNA molecules can be used to generate a loss-of-
15 function mutation in a cell, an organ or an animal. Methods for making and using dsRNA molecules to selectively degrade RNA are well known in the art, see, for example, U.S. Patent No. 6,506,559; U.S. Patent No. 6,511,824; U.S. Patent No. 6,515,109; and U.S. Patent No. 6,489,127.

20

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 acetylxylan esterase activity of the composition
25 has been increased, e.g., with an enrichment factor of at least 1.1.

 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,
30 cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, 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
35 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*, 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*, *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 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 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 acetylxylan esterase activity, or compositions thereof.

A polypeptide having acetylxylan esterase activity of the present invention may be used in several applications to degrade or convert an acetylxylan-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 more accessible for xylanases and other xylan-degrading enzymes.

The polypeptides having acetylxylan 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.

5 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 generally 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 acetylxylan esterase either before or at the same time as the
10 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.
15 6,245,546. The use of acetylxylan 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.

20 The polypeptides may further be used in brewing according to WO 2002/24926, where combinations 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
25 acetylated xylan, comprising treating a material comprising an acetylated xylan with a composition comprising an effective amount of a polypeptide having acetylxylan esterase activity of the present invention. In a preferred aspect, the composition further comprises one or more (several) xylan degrading enzymes. The one or more (several) xylan degrading enzymes can be selected from the group consisting of a xylanase, an
30 arabinofuranosidase, a xylosidase, a glucuronidase, and combinations thereof. The composition may be further used in conjunction with a composition comprising one or more cellulolytic proteins for the degradation or conversion of a cellulosic material, e.g., lignocellulose.

A composition comprising the polypeptide having acetylxylan esterase activity
35 may 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 may comprise a host cell of the present invention as a source of the polypeptide having acetylxylnan esterase activity.

5 **Signal Peptide**

The present invention also relates to nucleic acid constructs comprising a gene encoding a protein, wherein the gene is operably linked to a nucleotide sequence encoding a signal peptide comprising or consisting of amino acids 1 to 16 of SEQ ID NO: 2, wherein the gene is foreign to the nucleotide sequence.

10 In a preferred aspect, the nucleotide sequence comprises or consists of nucleotides 1 to 48 of SEQ ID NO: 1.

The present invention also relates to recombinant expression vectors and recombinant host cells comprising such nucleic acid constructs.

The present invention also relates to methods of producing a protein comprising
15 (a) cultivating such a recombinant host cell under conditions suitable for production of the protein; and (b) recovering the protein.

The protein may be native or heterologous to a host cell. The term "protein" is not meant herein to refer to a specific length of the encoded product and, therefore, encompasses peptides, oligopeptides, and proteins. The term "protein" also
20 encompasses two or more polypeptides combined to form the encoded product. The proteins also include hybrid polypeptides that comprise a combination of partial or complete polypeptide sequences obtained from at least two different proteins wherein one or more (several) may be heterologous or native to the host cell. Proteins further include naturally occurring allelic and engineered variations of the above mentioned
25 proteins and hybrid proteins.

Preferably, the protein is a hormone or variant thereof, enzyme, receptor or portion thereof, antibody or portion thereof, or reporter. In a more preferred aspect, the protein is an oxidoreductase, transferase, hydrolase, lyase, isomerase, or ligase. In an even more preferred aspect, the protein is an aminopeptidase, amylase, carbohydrase,
30 carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, another lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase or
35 xylanase.

The gene may be obtained from any prokaryotic, eukaryotic, or other source.

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

5

Examples

Materials

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

Strains

Thielavia terrestris NRRL 8126 was used as the source of a gene encoding a
Family 7 polypeptide having acetylxytan esterase activity. *Aspergillus oryzae* JAL250
15 strain (WO 99/61651) was used for expression of the *Thielavia terrestris* NRRL 8126
acetylxytan esterase.

Media

PDA plates were composed per liter of 39 grams of potato dextrose agar.
20 NNCYP medium was composed per liter of 5.0 g of NH_4NO_3 , 0.5 g of
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g of CaCl_2 , 2.5 g of citric acid, 1.0 g of Bacto Peptone, 5.0 g of yeast
extract, 1 ml of COVE trace metals solution, and sufficient K_2HPO_4 to achieve a final pH
of approximately 5.4.

NNCYPmod medium was composed per liter of 1.0 g of NaCl, 5.0 g of NH_4NO_3 ,
25 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g of CaCl_2 , 2.0 g of citric acid, 1.0 g of Bacto Peptone, 5.0 g
of yeast extract, 1 ml of COVE trace metals solution, and sufficient K_2HPO_4 to achieve
the final pH of approximately 5.4.

COVE trace metals solution was composed per liter of 0.04 g of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$,
0.4 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.2 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.7 g of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.8 g of
30 $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 10 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$.

LB plates were composed per liter of 10 g of tryptone, 5 g of yeast extract, 5 g of
sodium chloride, and 15 g of Bacto Agar.

MDU2BP medium was composed per liter of 45 g of maltose, 1 g of
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g of NaCl, 2 g of K_2HSO_4 , 12 g of KH_2PO_4 , 2 g of urea, and 500 μl of
35 AMG trace metals solution, the pH was adjusted to 5.0 and then filter sterilized with a

0.22 µm filtering unit.

AMG trace metals was composed per liter of 14.3 g of ZnSO₄·7H₂O, 2.5 g of CuSO₄·5H₂O, 0.5 g of NiCl₂·6H₂O, 13.8 g of FeSO₄·7H₂O, 8.5 g of MnSO₄·7H₂O, and 3 g of citric acid.

5 SOC medium was composed of 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, and 10 mM MgSO₄, sterilized by autoclaving and then filter-sterilized glucose was added to 20 mM.

Freezing medium was composed of 60% SOC and 40% glycerol.

2X YT medium was composed per liter of 16 g of tryptone, 10 g of yeast extract,
10 5 g of NaCl, and 15 g of Bacto agar.

Example 1: Expressed sequence tags (EST) cDNA library construction

Thielavia terrestris NRRL 8126 was cultivated in 50 ml of NNCYPmod medium supplemented with 1% glucose in a 250 ml flask at 45°C, 200 rpm for 24 hours. A two
15 ml aliquot from a 24-hour liquid culture of *Thielavia terrestris* NRRL 8126 grown in 50 ml of NNCYPmod supplemented with 1% glucose in a 250 ml flask at 45°C and 200 rpm was used to seed a 500 ml flask containing 100 ml of NNCYPmod medium supplemented with 2% SIGMACELL® 20. The culture was incubated at 45°C, 200 rpm for 3 days. The mycelia were harvested by filtration through a funnel with a glass fiber
20 prefilter (Nalgene, Rochester, NY, USA), washed twice with 10 mM Tris-HCl-1 mM EDTA pH 8 (TE), and quick frozen in liquid nitrogen.

To isolate total RNA frozen mycelia of *Thielavia terrestris* NRRL 8126 were ground in an electric coffee grinder. The ground material was mixed 1:1 v/v with 20 ml of FENAZOL™ (Ambion, Inc., Austin, TX, USA) in a 50 ml FALCON® tube. Once the
25 mycelia were suspended, they were extracted with chloroform and three times with a mixture of phenol-chloroform-isoamyl alcohol 25:24:1 v/v/v. From the resulting aqueous phase, the RNA was precipitated by adding 1/10 volume of 3 M sodium acetate pH 5.2 and 1.25 volumes of isopropanol. The precipitated RNA was recovered by centrifugation at 12,000 x g for 30 minutes at 4°C. The final pellet was washed with
30 cold 70% ethanol, air dried, and resuspended in 500 µl of diethylpyrocarbonate treated water (DEPC-water).

The quality and quantity of the purified RNA was assessed with an AGILENT® 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA, USA). Polyadenylated mRNA was isolated from 360 µg of total RNA with the aid of a POLY(A)PURIST™
35 Magnetic Kit (Ambion, Inc., Austin, TX, USA) according to the manufacturer's

instructions.

To create the cDNA library, a CLONEMINER™ Kit (Invitrogen Corp., Carlsbad, CA, USA) was employed to construct a directional library that does not require the use of restriction enzyme cloning, thereby reducing the number of chimeric clones and size bias.

To insure the successful synthesis of the cDNA, two reactions were performed in parallel with two different concentrations of mRNA (2.2 and 4.4 µg of poly (A)⁺ mRNA). The mRNA samples were mixed with a Biotin-attB2-Oligo(dt) primer (Invitrogen Corp., Carlsbad, CA, USA), 1X first strand buffer (Invitrogen Corp., Carlsbad, CA, USA), 2 µl of 0.1 M dithiothreitol (DTT), 10 mM of each dNTP, and water to a final volume of 18 and 16 µl, respectively.

The reaction mixtures were mixed and then 2 and 4 µl of SUPERSCRIPT™ reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA) were added. The reaction mixtures were incubated at 45°C for 60 minutes to synthesize the first complementary strand. For second strand synthesis, to each first strand reaction was added 30 µl of 5X second strand buffer (Invitrogen Corp., Carlsbad, CA, USA), 3 µl of 10 mM of each dNTP, 10 units of *E. coli* DNA ligase (Invitrogen Corp., Carlsbad, CA, USA), 40 units of *E. coli* DNA polymerase I (Invitrogen Corp., Carlsbad, CA, USA), and 2 units of *E. coli* RNase H (Invitrogen Corp., Carlsbad, CA, USA) in a total volume of 150 µl. The mixtures were then incubated at 16°C for two hours. After the two-hour incubation 2 µl of T4 DNA polymerase (Invitrogen Corp., Carlsbad, CA, USA) were added to each reaction and incubated at 16°C for 5 minutes to create a blunt-ended cDNA. The cDNA reactions were extracted with a mixture of phenol-chloroform-isoamyl alcohol 25:24:1 v/v/v and precipitated in the presence of 20 µg of glycogen, 120 µl of 5 M ammonium acetate, and 660 µl of ethanol. After centrifugation at 12,000 x g for 30 minutes at 4°C, the cDNA pellets were washed with cold 70% ethanol, dried under vacuum for 2-3 minutes, and resuspended in 18 µl of DEPC-water. To each resuspended cDNA sample were added 10 µl of 5X adapted buffer (Invitrogen, Carlsbad, CA), 10 µg of each attB1 adapter (Invitrogen, Carlsbad, CA), 7 µl of 0.1 M DTT, and 5 units of T4 DNA ligase (Invitrogen, Carlsbad, CA).

Ligation reactions were incubated overnight at 16°C. Excess adapters were removed by size-exclusion chromatography in 1 ml of SEPHACRYL™ S-500 HR resin (Amersham Biosciences, Piscataway, NJ, USA). Column fractions were collected according to the CLONEMINER™ Kit's instructions and fractions 3 to 14 were analyzed with an AGILENT® 2100 Bioanalyzer to determine the fraction at which the attB1

adapters started to elute. Analysis showed that the adapters started eluting around fraction 10 or 11. For the first library fractions 6 to 11 were pooled and for the second library fractions 4-11 were pooled.

Cloning of the cDNA was performed by homologous DNA recombination according to the GATEWAY® System protocol (Invitrogen Corp., Carlsbad, CA, USA) using BP CLONASE™ (Invitrogen Corp., Carlsbad, CA, USA) as the recombinase. Each BP CLONASE™ recombination reaction contained approximately 70 ng of attB-flanked-cDNA, 250 ng of pDONR™222, 2 µl of 5X BP CLONASE™ buffer, 2 µl of TE, and 3 µl of BP CLONASE™. All reagents were obtained from Invitrogen, Carlsbad, CA, USA. Recombination reactions were incubated at 25°C overnight.

Heat-inactivated BP recombination reactions were then divided into 6 aliquots and electroporated into ELECTROMAX™ DH10B electrocompetent cells (Invitrogen Corp., Carlsbad, CA, USA) using a GENE PULSER™ (Bio-Rad, Hercules, CA, USA) with the following parameters: Voltage: 2.0 kV; Resistance: 200 Ω; and Capacity: 25 µF. Electroporated cells were resuspended in 1 ml of SOC medium and incubated at 37°C for 60 minutes with constant shaking at 200 rpm. After the incubation period, the transformed cells were pooled and mixed 1:1 with freezing medium. A 200 µl aliquot was removed for library titration and then the rest of each library was aliquoted into 1.8 ml cryovials (Wheaton Science Products, Millville, NJ, USA) and stored frozen at -80°C.

Four serial dilutions of each library were prepared: 1/100, 1/1000, 1/10⁴, 1/10⁵. From each dilution 100 µl were plated onto 150 mm LB plates supplemented with 50 µg of kanamycin per ml and incubated at 37°C overnight. The number of colonies on each dilution plate was counted and used to calculate the total number of transformants in each library.

The first library contained approximately 5.4 million independent clones and the second library contained approximately 9 million independent clones.

Example 2: Template preparation and nucleotide sequencing of cDNA clones

Aliquots from both libraries described in Example 1 were mixed and plated onto 25 x 25 cm LB plates supplemented with 50 µg of kanamycin per ml. Individual colonies were arrayed onto 96-well plates containing 100 µl of LB plate medium supplemented with 50 µg of kanamycin per ml with the aid of a QPix Robot (Genetix Inc., Boston, MA, USA). Forty-five 96-well plates were obtained for a total of 4320 individual clones. The plates were incubated overnight at 37°C with shaking at 200 rpm. After incubation, 100 µl of sterile 50% glycerol were added to each well. The transformants were replicated

with the aid of a 96-pin tool (Boekel, Feasterville, PA, USA) into secondary, deep-dish 96-well microculture plates (Advanced Genetic Technologies Corporation, Gaithersburg, MD, USA) containing 1 ml of MAGNIFICENT BROTH™ (MacConnell Research, San Diego, CA, USA) supplemented with 50 µg of kanamycin per ml in each well. The primary microtiter plates were stored frozen at -80°C. The secondary deep-dish plates were incubated at 37°C overnight with vigorous agitation at 300 rpm on a rotary shaker. To prevent spilling and cross-contamination, and to allow sufficient aeration, each secondary culture plate was covered with a polypropylene pad (Advanced Genetic Technologies Corporation, Gaithersburg, MD, USA) and a plastic microtiter dish cover. Plasmid DNA was prepared with a Robot-Smart 384 (MWG Biotech Inc., High Point, NC, USA) and a MONTAGE™ Plasmid Miniprep Kit (Millipore, Billerica, MA, USA).

Sequencing reactions were performed using BIGDYE® (Applied Biosystems, Inc., Foster City, CA, USA) terminator chemistry (Giesecke *et al.*, 1992, *Journal of Virology Methods* 38: 47-60) and a M13 Forward (-20) sequencing primer:

5'-GTAAAACGACGGCCAG-3' (SEQ ID NO: 3)

The sequencing reactions were performed in a 384-well format with a Robot-Smart 384 (MWG Biotech Inc., High Point, NC, USA). Terminator removal was performed with a MULTISCREEN® Seq384 Sequencing Clean-up Kit (Millipore, Billerica, MA, USA). Reactions contained 6 µl of plasmid DNA and 4 µl of sequencing master-mix (Applied Biosystems, Foster City, CA, USA) containing 1 µl of 5X sequencing buffer (Millipore, Billerica, MA, USA), 1 µl of BIGDYE® terminator (Applied Biosystems, Inc., Foster City, CA, USA), 1.6 pmoles of M13 Forward primer, and 1 µl of water. Single-pass DNA sequencing was performed with an ABI PRISM Automated DNA Sequencer Model 3700 (Applied Biosystems, Foster City, CA, USA).

Example 3: Analysis of DNA sequence data of cDNA clones

Base calling, quality value assignment, and vector trimming were performed with the assistance of PHRED/PHRAP software (University of Washington, Seattle, WA, USA). Clustering analysis of the ESTs was performed with a Transcript Assembler v. 2.6.2. (Paracel, Inc., Pasadena, CA, USA). Analysis of the EST clustering indicated the presence of 395 independent clusters.

Sequence homology analysis of the assembled EST sequences against various databases was performed with the Blastx program (Altschul *et al.*, 1990, *J. Mol. Biol.* 215:403-410) on a 32-node Linux cluster (Paracel, Inc., Pasadena, CA, USA) using the BLOSUM 62 matrix (Henikoff, 1992, *Proc. Natl. Acad. Sci. USA* 89: 10915-10919)

From these, 246 had hits to known genes and 149 had no significant hits to known genes. Among these 246 genes, 13 had hits against well characterized homologues of glycosyl hydrolase genes.

5 **Example 4: Identification of cDNA clones encoding a *Thielavia terrestris* Family 2A acetylxylan esterase (Axe2A)**

A cDNA clone encoding a *Thielavia terrestris* Family 2 acetylxylan esterase (Axe2A) was initially identified by sequence homology to a cellulase from *Humicola insolens* GenBank accession number X76046.

10 After this initial identification, a clone designated Tter08G1 was retrieved from the original frozen stock plate and streaked onto a LB plate supplemented with 50 µg of kanamycin per ml. The plate was incubated overnight at 37°C and the next day a single colony from the plate was used to inoculate 3 ml of LB medium supplemented with 150 µg of kanamycin per ml. The liquid culture was incubated overnight at 37°C and
15 plasmid DNA was prepared with a BIOROBOT® 9600 (QIAGEN, Inc., Valencia, CA, USA). Clone Tter08G1 plasmid DNA was sequenced again with BIGDYE® terminator chemistry as described above, using the M13 forward and a Poly-T primer shown below to sequence the 3' end of the clone.

5'-TTTTTTTTTTTTTTTTTTTTTTTTTVN-3' (SEQ ID NO: 4)

20 where V=G, A, C and N=G, A,C,T

Analysis of the deduced protein sequence of clone Tter08G1 with the Interproscan program (Zdobnov and Apweiler, 2001, *Bioinformatics* 17: 847-8) showed that clone Tter08G1 contained the sequence signature of the esterase SGNH hydrolase type G3DSA. This sequence signature known as InterPro: IPR013831 was found 185
25 amino acids from the starting amino acid methionine confirming that clone Tter08G1 encoded a Family 2 acetylxylan esterase.

The cDNA sequence (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) are shown in Figure 1. The cDNA clone encodes a polypeptide of 413 amino acids. The %G+C content of the cDNA clone is 67.5% and of the mature protein coding region (nucleotides 50 to 1239 of SEQ ID NO: 1) is 67.6%. Using the SignalP software program (Nielsen *et al.*, 1997, *Protein Engineering* 10: 1-6), a signal peptide of 16
30 residues was predicted. The predicted mature protein contains 397 amino acids with a molecular mass of 40.9 kDa.

A comparative pairwise global alignment of amino acid sequences was
35 determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J.*

Mol. Biol. 48: 443-453) as implemented in the Needle program of EMBOSS with gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 matrix. The alignment showed that the deduced amino acid sequence of the *Thielavia terrestris* acetylxylin esterase gene shared 81.1% and 56.8% identity (excluding gaps) to the deduced amino acid sequences of two Family 2 acetylxylin esterases from *Chaetomium globosum* and *Phaeosphaeria nodorum*, respectively (Uniprot accession numbers Q2GWX4 and Q0UJH1, respectively).

Once the identity of clone Tter08G1 was confirmed a 0.5 µl aliquot of plasmid DNA from this clone designated pTterAxe2A (Figure 2) was transferred into a vial of *E. coli* TOP10 cells (Invitrogen Corp., Carlsbad, CA, USA), gently mixed, and incubated on ice for 10 minutes. The cells were then heat-shocked at 42°C for 30 seconds and incubated again on ice for 2 minutes. The cells were resuspended in 250 µl of SOC and incubated at 37°C for 60 minutes with constant shaking (200 rpm). After the incubation period, two 30 µl aliquots were plated onto LB plates supplemented with 50 µg of kanamycin per ml and incubated overnight at 37°C. The next day a single colony was picked and streaked onto three 1.8 ml cryovials containing about 1.5 ml of LB agarose supplemented with 50 µg of kanamycin per ml. The vials were sealed with PETRISEAL™ (Diversified Biotech, Boston MA, USA) and deposited with the Agricultural Research Service Patent Culture Collection, Northern Regional Research Center, Peoria, IL, USA, as NRRL B-50067, with a deposit date of September 20, 2007.

Example 5: Cloning of the Family 2A acetylxylin esterase gene into an *Aspergillus oryzae* expression vector

Two synthetic oligonucleotide primers, shown below, were designed to PCR amplify the full-length open reading frame from *Thielavia terrestris* EST Tter08G1 encoding the Family 2A acetylxylin esterase. An IN-FUSION™ Cloning Kit (BD Biosciences, Palo Alto, CA, USA) was used to clone the fragment directly into pAILo2 (WO 2004/099228).

Forward primer:

5'-ACTGGATTTACCATGAAGCCGTCTGTCGTCGCCGGGCTCTT-3' (SEQ ID NO: 5)

Reverse primer:

5'-TCACCTCTAGTTAATTAATCACCATCCCAGCGCAGACC-3' (SEQ ID NO: 6)

Bold letters represent coding sequence. The remaining sequence contains sequence identity compared with the insertion sites of pAILo2.

Fifty picomoles of each of the primers above were used in a PCR reaction

containing 50 ng of pTter08G1, 1X *Pfx* Amplification Buffer (Invitrogen Corp., Carlsbad, CA, USA), 6 µl of a 10 mM blend of dATP, dTTP, dGTP, and dCTP, 2.5 units of PLATINUM® *Pfx* DNA Polymerase (Invitrogen Corp., Carlsbad, CA, USA), 1 µl of 50 mM MgSO₄, and 5 µl of 10X pCRx Enhancer Solution (Invitrogen Corp., Carlsbad, CA, USA) in a final volume of 50 µl. The amplification was performed in an EPPENDORF® MASTERCYCLER® 5333 (Eppendorf Scientific, Inc., Westbury, NY, USA) programmed for one cycle at 98°C for 2 minutes; and 35 cycles each at 96°C for 30 seconds, 64°C for 30 seconds, and 68°C for 1.5 minutes. After the 35 cycles, the reaction was incubated at 68°C for 10 minutes and then cooled at 10°C until further processed. A 1.2 kb PCR reaction product was isolated by 0.8% GTG® agarose gel electrophoresis (Cambrex Bioproducts One Meadowlands Plaza East Rutherford, NJ, USA) using 40 mM Tris base-20 mM sodium acetate-1 mM disodium EDTA (TAE) buffer and 0.1 µg of ethidium bromide per ml. The DNA band was visualized with the aid of a DARKREADER™ (Clare Chemical Research, Dolores, CO, USA). The 1.2 kb DNA band was excised with a disposable razor blade and purified with an ULTRAFREE® DA spin cup (Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

Plasmid pAlLo2 was linearized by digestion with *Nco* I and *Pac* I using standard conditions. The fragment was purified by gel electrophoresis and ultrafiltration as described above. Cloning of the purified PCR fragment into the linearized and purified pAlLo2 vector was performed with an IN-FUSION™ Cloning Kit (BD Biosciences, Palo Alto, CA, USA). The reaction (20 µl) contained of 2 µl of 1X IN-FUSION™ Buffer (BD Biosciences, Palo Alto, CA, USA), 1X BSA (BD Biosciences, Palo Alto, CA, USA), 1 µl of IN-FUSION™ enzyme (diluted 1:10) (BD Biosciences, Palo Alto, CA, USA), 100 ng of pAlLo2 digested with *Nco* I and *Pac* I, and 100 ng of the *Thielavia terrestris* Axe2A purified PCR product. The reaction was incubated at room temperature for 30 minutes.

A 2 µl sample of the reaction were used to transform *E. coli* XL10 SOLOPACK® Gold cells (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. After the recovery period, two 100 µl aliquots from the transformation reaction were plated onto 150 mm 2X YT plates supplemented with 100 µg of ampicillin per ml. The plates were incubated overnight at 37°C. A set of eight putative recombinant clones was selected at random from the selection plates and plasmid DNA was prepared from each one using a BIOROBOT® 9600. Clones were analyzed by *Xho* I restriction digestion. Two clones that had the expected restriction digestion pattern were then sequenced to confirm that there were no mutations in the cloned insert. Clone #1 was selected and designated pAlLo39 (Figure 3).

Example 6: Expression of the *Thielavia terrestris* Family 2A acetylxylan esterase gene in *Aspergillus oryzae* JaL250

Aspergillus oryzae JaL250 (WO 99/61651) protoplasts were prepared according to the method of Christensen *et al.*, 1988, *Bio/Technology* 6: 1419-1422. Five micrograms of pAILo39 (as well as pAILo2 as a control) were used to transform *Aspergillus oryzae* JaL250 protoplasts.

The transformation of *Aspergillus oryzae* JaL250 with pAILo39 yielded about 50 transformants. Eight transformants were isolated to individual PDA plates and incubated for five days at 34°C.

Confluent spore plates were washed with 3 ml of 0.01% TWEEN® 80 and the spore suspension was used to inoculate 25 ml of MDU2BP medium in 125 ml glass shake flasks. Transformant cultures were incubated at 34°C with constant shaking at 200 rpm. At day five post-inoculation, cultures were centrifuged at 6000 x *g* and their supernatants collected. Five microliters of each supernatant were mixed with an equal volume of 2X loading buffer (10% beta-mercaptoethanol) and loaded onto a 1.5 mm 8%-16% Tris-Glycine SDS-PAGE gel, run using 1X NOVEX® buffer (Invitrogen Corp., Carlsbad, CA, USA), and stained with SIMPLYBLUE™ SafeStain (Invitrogen Corp., Carlsbad, CA, USA). SDS-PAGE profiles of the culture broths showed that eight out of eight transformants had a new protein band of approximately 50 kDa. Transformant number 5 was selected for further studies and designated *Aspergillus oryzae* JaL250AILo39.

Example 7: Large shake flask cultures of *Aspergillus oryzae* JaL250AILo39

Aspergillus oryzae JaL250AILo39 spores were spread onto a PDA plate and incubated for five days at 34°C. The confluent spore plate was washed twice with 5 ml of 0.01% TWEEN® 80 to maximize the number of spores collected. The spore suspension was then used to inoculate 500 ml of MDU2BP in a two-liter Fernbach flask. The transformant culture was incubated at 34°C with constant shaking at 200 rpm. At day five post-inoculation, the culture broth was collected by filtration on a 500 ml 75 mm Nylon filter unit (Nalge Nunc International, Rochester, NY, USA) with a pore size of 0.45 µm with a glass-fiber pre-filter. A 5 µl sample of the broth was analyzed by SDS-PAGE as described above, which showed that the broth contained approximately 50 kDa protein band.

Example 8: Biochemical characterization of the *Thielavia terrestris* Family 2 acetylxyylan esterase

The *Thielavia terrestris* Family 2 acetylxyylan esterase was obtained as described in Example 7. The filtered broth was desalted using an ECONO-PAC® 10DG column (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Protein in the desalted sample was measured using a BCA reagent Kit (Pierce, Rockford, IL, USA).

SDS-PAGE analysis of the desalted acetylxyylan esterase was performed using a 8-16% Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA, USA) with Tris/glycine/SDS buffer (Bio-Rad Laboratories, Hercules, CA, USA). The sample (approximately 4 µg) was diluted at a 1:1 (v:v) ratio with Laemmli sample buffer with 5% 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO, USA), and boiled for 5 minutes before loading onto the gel. The gel was developed using BIO-SAFE™ Coomassie Blue (Bio-Rad Laboratories, Hercules, CA, USA). The results indicated that the enzyme was at least 90% pure. The observed molecular weight was approximately 50 kDa. The desalted acetylxyylan esterase was then characterized biochemically.

Substrate Specificity. A substrate panel (S1-S21, Table 1) was used to determine the *Thielavia terrestris* Family 2 acetylxyylan esterase's substrate specificity at 40°C (S1-S19) or 25°C (S20-S21), pH 5. Enzyme loadings were based on loadings of model enzymes (positive controls) for each substrate. For S1-S14, reducing sugars were measured using a *p*-hydroxybenzoic acid hydrazide (PHBAH) assay (Lever, 1972, *Anal. Biochem.*, 47: 273-279) to calculate the substrate conversion; for S15-S21, *p*-nitrophenolate anion production was determined at 405 nm. The results, as shown in Table 2, demonstrated that the *Thielavia terrestris* Family 2 acetylxyylan esterase possessed activity toward *p*-nitrophenylacetate. No significant activity toward other substrates was observed.

Table 1. Cellulosic/hemicellulosic substrates for substrate specificity assay

#	Name	From	Sub. mg/ml
S1	PCS	NREL, ABC050621-22	5
S2	Avicel	FMC	5
S3	CMC	Hercules	5
S4	Arabinan (sugar beet)	Megazyme	5
S5	β-glucan	Megazyme	5
S6	Xyloglucan	Megazyme	5
S7	Arabinoxylan (wheat)	Megazyme	5
S8	Mannan	Megazyme	5
S9	Galactomannan (Carob)	Megazyme	5

S10	Galactan (potato)	Megazyme	5
S11	PASC	prep from S2	2
S12	pectic galactan	Megazyme	5
S13	pectin	Sigma	5
S14	Chitin	Sigma	5
S15	p-nitrophenyl- β -D-glucopyranoside	Sigma	1 mM
S16	p-nitrophenyl- β -D-xylopyranoside	Sigma	1 mM
S17	p-nitrophenyl- β -D-grabinopyranoside	Sigma	1 mM
S18	p-nitrophenyl- β -D-galactopyranoside	Sigma	1 mM
S19	p-nitrophenyl- β -D-mannopyranoside	Sigma	1 mM
S20	p-nitrophenylacetate	Sigma	1 mM
S21	p-nitrophenylferulate	Slovak Academy of Sci.	1 mM

Table 2. Substrate specificity of *Thielavia terrestris* Family 2 acetylxyylan esterase

#	Name	Loading, E in rxn, μ g/ml	Incubation time	Conversion
S1	PCS	50.00	24 h	0.00%
S2	Avicel	62.50	24 h	0.00%
S3	CMC	6.25	24 h	0.00%
S4	Arabinan (sugar beet)	12.50	24 h	0.00%
S5	β -glucan	0.78	4 h	0.00%
S6	Xyloglucan	0.78	4 h	0.00%
S7	Arabinoxylan (wheat)	0.78	4 h	0.00%
S8	Mannan	5.50	24 h	0.00%
S9	Galactomannan (Carob)	5.50	24 h	0.00%
S10	Galactan (potato)	22.00	24 h	0.89%
S11	PASC	10.00	24 h	0.00%
S12	pectic galactan	50.00	24 h	3.10%
S13	pectin	50.00	24 h	1.04%
S14	Chitin	50.00	24 h	0.35%
S15	p-nitrophenyl- β -D-glucopyranoside	0.017	30 min	0.10%
S16	p-nitrophenyl- β -D-xylopyranoside	0.333	30 min	0.00%
S17	p-nitrophenyl- β -D-grabinopyranoside	0.033	30 min	0.05%
S18	p-nitrophenyl- β -D-galactopyranoside	0.333	30 min	0.18%
S19	p-nitrophenyl- β -D-mannopyranoside	0.333	30 min	0.01%
S20	p-nitrophenylacetate	0.097	10 min	19.00%
S21	p-nitrophenylferulate	0.625	10 min	0.00%

Specific activity on p-nitrophenylacetate. The desalted acetylxyylan esterase was
 5 assayed for esterase activity using p-nitrophenylacetate as substrate. The enzyme

preparation was diluted to provide less than 15% conversion of *p*-nitrophenylacetate by making an initial dilution in a 1.5 ml microcentrifuge tube with 50 mM sodium acetate pH 5.0 buffer with 0.01% TWEEN® 20 to protein content of 2 µg/ml, followed by 2-fold serial dilutions with 50 mM sodium acetate pH 5.0 buffer with 0.01% TWEEN® 20. Then
 5 100 µl aliquots of the diluted enzyme were transferred to wells of a 96-well plate.

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

20 The results indicated that the *Thielavia terrestris* Family 2 acetylxylyl esterase had a specific activity of approximately 70.5 IU per mg of protein.

Thermostability. The *Thielavia terrestris* Family 2 acetylxylyl esterase was diluted in 50 mM sodium acetate pH 5 buffer with 0.01% TWEEN® 20 to 1 mg per ml, and then incubated at 50°C for 3 days, or 60°C for 3 hours or 1 day, or was stored at
 25 4°C to serve as a control. After incubation, the activity of the samples toward *p*-nitrophenylacetate as substrate was measured using the same assay protocol described above for determining the specific activity on *p*-nitrophenylacetate. The activity of the sample at 4°C was normalized to 100%, and the activities of the other samples at other incubation conditions were compared to the 4°C activity. The results,
 30 as shown in Table 3, demonstrated that the *Thielavia terrestris* Family 2 acetylxylyl esterase was relatively stable after the 3 day 50°C incubation, or 3 hour 60°C incubation, and retained 46.6% activity after the 1 day 60°C incubation.

Table 3. Thermostability of *Thielavia terrestris* Family 2 acetylxylyl esterase

E Name	4°C	50°C, 3 day	60°C, 3 hours	60°C, 1 day
<i>Thielavia terrestris</i> Family 2 AXE	100.0%	94.0%	88.5%	46.6%

Temperature profile. Activity of the *Thielavia terrestris* Family 2 acetylxylian esterase versus temperature at 34, 50, 60, and 70°C was determined using the same assay protocol described above for determining the specific activity on *p*-nitrophenylacetate. The highest activity was normalized to 100%, and activities at the other temperatures were compared to the highest activity and expressed in % activity. The results are shown in Table 4.

Table 4. Temperature profile of *Thielavia terrestris* Family 2 acetylxylian esterase

E Name	34°C	50°C	60°C	70°C
<i>Thielavia terrestris</i> Family 2 AXE	84.9%	96.4%	94.7%	100.0%

pH profile. The pH activity profile of the *Thielavia terrestris* Family 2 acetylxylian esterase at pHs 4, 5, 6, 7, and 8 was determined using the same assay protocol described above for determining the specific activity on *p*-nitrophenylacetate, except Britton Robinson buffer was used as the buffer system. A 100 mM stock solution of Britton Robinson buffer was adjusted to pHs 4, 5, 6, 7, and 8 using 5 N NaOH and then diluted to 40 mM. The substrate was prepared into the corresponding buffer. The highest activity was normalized to be 100%, and activities at other pHs were compared to the highest activity and expressed in % activity. The results, as shown in Table 5, demonstrated that the optimal pH of the *Thielavia terrestris* Family 2 acetylxylian esterase was pH 6.

Table 5. pH profile of *Thielavia terrestris* Family 2 acetylxylian esterase

E Name	pH 4	pH 5	pH 6	pH 7	pH 8
<i>Thielavia terrestris</i> Family 2 AXE	22.8%	76.4%	100.0%	33.1%	45.9%

Deposit of Biological Material

The following biological material has been deposited under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA, and given the following accession number:

Deposit	Accession Number	Date of Deposit
<i>E. coli</i> pTterAxe2A	NRRL B-50067	September 20, 2007

The strain has been deposited under conditions that assure that access to the culture will be available during the pendency of this patent application to one determined

by foreign patent laws to be entitled thereto. The deposit represents a substantially pure culture of the deposited strain. The deposit is available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny are filed. However, it should be understood that the availability of a deposit does not
5 constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

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

Claims

What is claimed is:

- 5 1. An isolated polypeptide having acetylxylan esterase activity, selected from the group consisting of:
- (a) a polypeptide comprising an amino acid sequence having preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, and most preferably at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%
10 identity to the mature polypeptide of SEQ ID NO: 2;
- (b) a polypeptide encoded by a polynucleotide that hybridizes under preferably 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
15 strand of (i) or (ii);
- (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having preferably at least 85%, more preferably at least 90%, and most preferably at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the mature polypeptide coding sequence of SEQ ID NO: 1; and
20 (d) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.
2. The polypeptide of claim 1, comprising or consisting of the amino acid sequence of SEQ ID NO: 2; or a fragment thereof having acetylxylan esterase activity.
25
3. The polypeptide of claim 1, 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 acetylxylan esterase activity.
- 30 4. The polypeptide of claim 1, which is encoded by the polynucleotide contained in plasmid pTterAxe2A which is contained in *E. coli* NRRL B-50067.
5. An isolated polynucleotide comprising a nucleotide sequence that encodes the polypeptide of any of claims 1-4.
35

6. A nucleic acid construct comprising the polynucleotide of claim 5 operably linked to one or more control sequences that direct the production of the polypeptide in an expression host.

5 7. A recombinant host cell comprising the nucleic acid construct of claim 6.

8. A method of producing the polypeptide of any of claims 1-4, 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.

10

9. A method of producing the polypeptide of any of claims 1-4, 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.

15

10. A method of producing a mutant of a parent cell, comprising disrupting or deleting a nucleotide sequence encoding the polypeptide of any of claims 1-4, which results in the mutant producing less of the polypeptide than the parent cell.

20

11. A method of producing the polypeptide of any of claims 1-4, 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.

25

12. A transgenic plant, plant part or plant cell transformed with a polynucleotide encoding the polypeptide of any of claims 1-4.

30

13. A double-stranded inhibitory RNA (dsRNA) molecule comprising a subsequence of the polynucleotide of claim 5, wherein optionally the dsRNA is an siRNA or an miRNA molecule.

14. A method of inhibiting the expression of a polypeptide in a cell, comprising administering to the cell or expressing in the cell the double-stranded RNA (dsRNA) molecule of claim 13.

35

15. A nucleic acid construct comprising a gene encoding a protein operably linked to a nucleotide sequence encoding a signal peptide comprising or consisting of amino acids 1 to 16 of SEQ ID NO: 2, wherein the gene is foreign to the nucleotide sequence.

5 16. A recombinant host cell comprising the nucleic acid construct of claim 15.

17. A method of producing a protein, comprising: (a) cultivating the recombinant host cell of claim 16 under conditions conducive for production of the protein; and (b) recovering the protein.

10

18. A method for degrading an acetylated xylan, comprising treating a material comprising an acetylated xylan with a composition comprising an effective amount of the polypeptide having acetylxylan esterase activity of any of claims 1-4.

15 19. The method of claim 18, wherein the composition further comprises one or more (several) xylan degrading enzymes.

20 20. The method of claim 19, wherein the one or more (several) xylan degrading enzymes are selected from the group consisting of a xylanase, an arabinofuranosidase, a xylosidase, and a glucuronidase.

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      M K P S V V A G L F A S G A A A Q S G A
1   ATGAAGCCGCTCTGTCTGTCGCCCGGGCTCTTTGCATCGGGTGTGTCGCCGCTCAGAGCGGTGCC
      W G Q C G G I G Y T G P T S C V S G Y R
61  TGGGGGCAGTGCAGCGCCGTCATTGGCTATAACCGGTCCCAACAGCTGCGTGTCTGGGTATAGG
      C V Y V N D W Y S Q C Q P G A A T T T T
121 TGTGTCTACGTGAATGACTGGTACAGCCAGTGCAGAGCCCGGCGCTGCCACAACCAACCACC
      S P P A S S T S T P P T S T G T A G V R
181 TCGCCGCGCTGCCTCGAGCAGCTCCACCCCGCCACCTCGACCGGGCACTGCTGGCGTTCGC
      Y V G R V N P S T K E L S W P G T G I S
241 TATGTTGGCCCGCTCAACCCGAGCAGCAAGGAGCTGAGCTGGCCAGGCACCGGCATCTCA
      F T F T G T S A T I G I A S V S G T N S
301 TTCACCTTCACGGGAACCTCCGCCACCATCGGSCATCGGCTCCGTCAGCGGCACCAACAGC
      V D L V V D D G D P I V I T S F G S S I
361 GTSGACCTCGTCTGTCGAGCAGCGCGACCCCATCGTCATCACCAGCTTCGGCTCCAGCATT
      T T P A G L S Q G T H T V T L R K R S E
421 ACCACGCCCCCGGCTCTCTCCAGGGCAGGCACACCGTCAACCTCCGCAAGCGGTCCGAG
      A L Y S S I F L G S V T T D G A F V A G
481 GCCCTCTACGGCTCCATCTTCTCCGCTCTGTGTCAGGACCGGCGCGCTTCGTTGGCGGGC
      T V P T R Q I E I I G D S I T V G Y G L
541 ACCGTCCCCACCCCGCCAGATCGAATTCATCGGCGGACTCCATCACCGTGGCTACGGCCTC
      D G T N P C T N N A T V E D N P K T Y G
601 GACGGCACCAACCCGTGCACCAACAACGGGACCGTTCGAGGACAAACCCCAAACCTACGGC
      A L A A A A L G A D Y N V I A W S G K G
661 GCCCTGGCCGCGCGCGCTCGGCGCAGACTACAACGTGATCGCGTGGAGCGGCAAGGGC
      V V R N V A T G S P D T S P L M P E L Y
721 GTAGTGGCGCAACGTGCGACCGGCGAGCCAGACACCAGCCCGCTGATGCCAGAGCTCTAC
      T R Y G A N D P D N S Y P Y P P T W S P
781 ACCCGCTACGGCGCCCAACGACCCCGACAAACAGCTACCCCTACCCGCGCCACCTGGTCCCCG
      D A V V I N L G T N D F S Y I A W D A S
841 GACGCGGTGGTTCATCAACCTCGGCACCAACGACTTCAGCTACATCGGCTGGGACGCATCG
      G N A Y A A R P P L N A T T Y T D G M V
901 GGCAACGCGTACGCGCGCGCGCCCGCGCTGAACGCGGACGACGCTACACCGACGGCATGGTG
      A F A Q S I R A H Y P A A H V F L V G S
961 GCCTTCGCGCAGAGCATCCGGGCGCACTACCCGGCGCGCACGCTCTCCTGGTCCGGCTCG
      P M L S D S Y P T A A D A Q K T T Q T N
1021 CCCATGCTGAGCGACTCGTACCCGACCGCGCGGACGCGCAGAAAGACCACGAGAGCAAT
      A L K S A V A Q L G A N A H F V D W P T
1081 GCGCTGAAGAGCGCTGTTGCGCAGCTCGGCGCGAACGCGCACTTTGTGCACTGGGCGACC
      Q G S D V G C D Y H P N A A T H A A E A
1141 CAGGGCAGCGATGTGGGCTGCGATTATCATCCCAATGCGGCGCACGCATGCGGCTGAGGCT
      A V L A D A I R S A L G W *
1201 GCGGTGCTCGCGGATGCGATCAGCTCTGCGCTGGGATGGTGA

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Fig. 1

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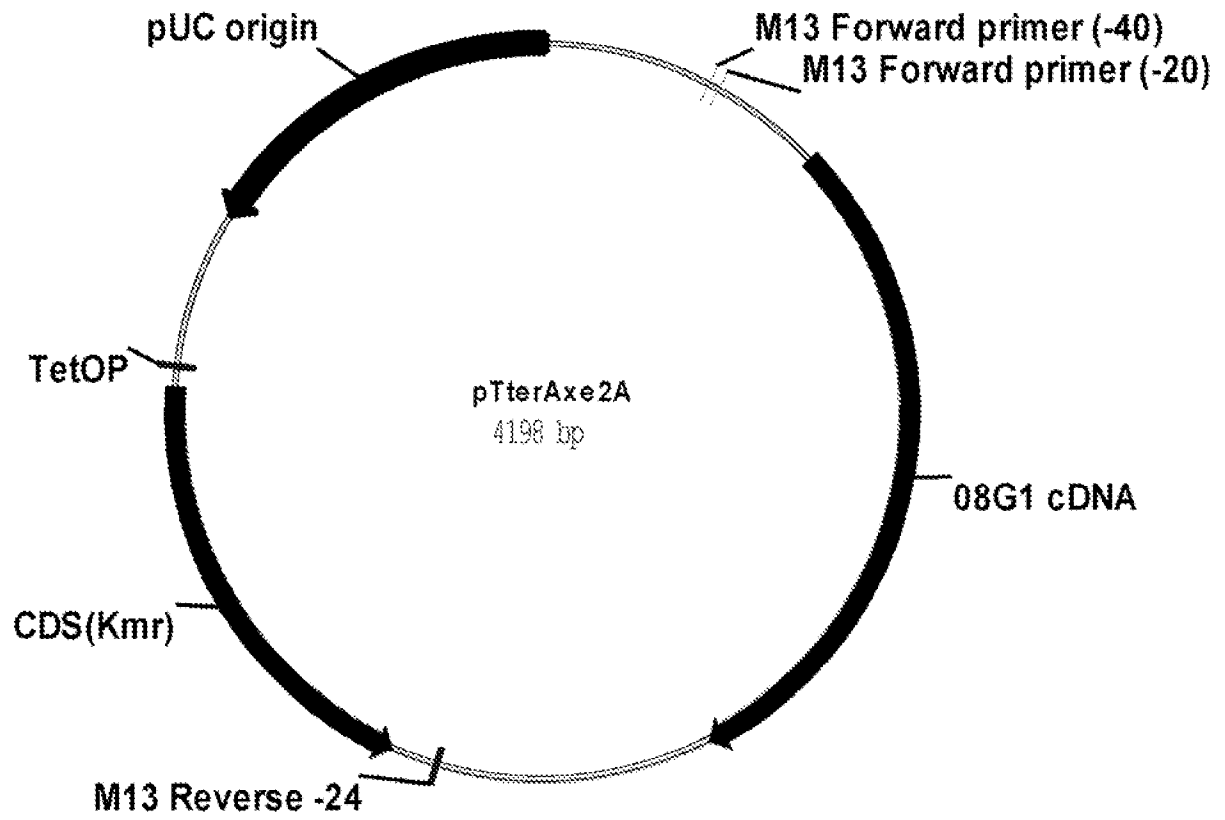


Fig. 2

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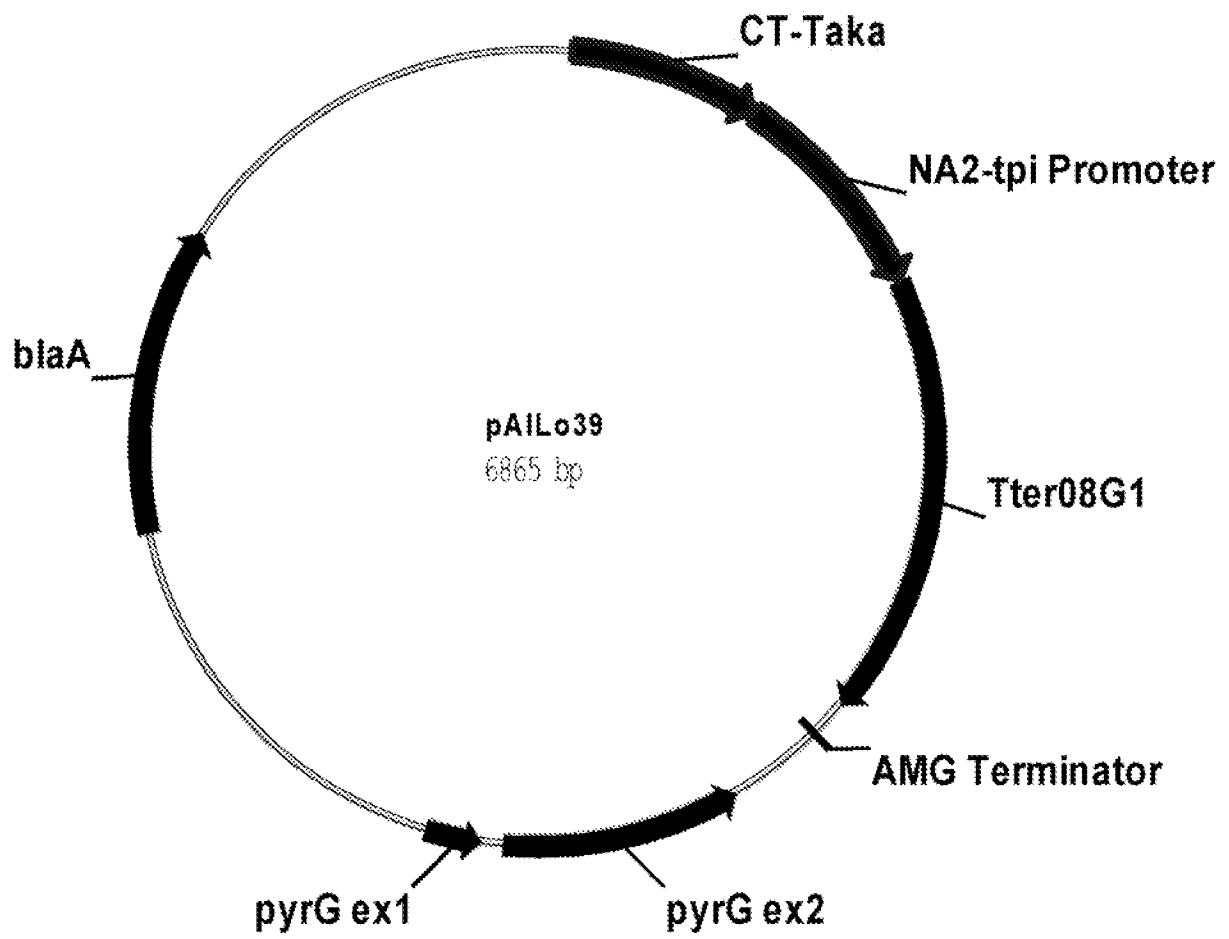


Fig. 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/077823

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N9/18 C12N15/55 C12N1/15 C12P19/04

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, WPI Data, EMBASE, BIOSIS, CHEM ABS Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/125068 A (NOVOZYMES NORTH AMERICA INC [US]; NOVOZYMES INC [US]; SMITH MADS PETER) 23 November 2006 (2006-11-23) see especially page 7 the whole document	1-20
X	EP 0 507 369 A (GIST BROCADES NV [NL]) 7 October 1992 (1992-10-07) the whole document	1-20



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
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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- *&* document member of the same patent family

Date of the actual completion of the international search

27 November 2008

Date of mailing of the international search report

08/12/2008

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Authorized officer

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INTERNATIONAL SEARCH REPORT

International application No

PCT/US2008/077823

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE EMBL [Online] 2 May 1994 (1994-05-02), "H.insolens CMC3 mRNA for endoglucanase" XP002505808 retrieved from EBI accession no. EMBL:X76046 Database accession no. X76046 cited in the application abstract</p>	1-3,5-7
X	<p>----- DATABASE UniProt [Online] 1 October 2001 (2001-10-01), "SubName: Full=Acetylxy lan esterase, acyl-CoA esterase or GDSL lipase family, strong similarity to C-terminal region of endoglucanase E;" XP002505807 retrieved from EBI accession no. UNIPROT:Q977Y6 Database accession no. Q977Y6 abstract</p>	1-3,7
X	<p>----- WO 2007/100897 A (EDENSPACE SYSTEM CORP [US]; BLAYLOCK MICHAEL J [US]; FERGUSON BRUCE W) 7 September 2007 (2007-09-07) page 20 - page 21</p>	11,12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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			US	2007250961 A1	25-10-2007

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2008/077823

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
- a. type of material
- ☒ a sequence listing
- ☐ table(s) related to the sequence listing
- b. format of material
- ☒ on paper
- ☒ in electronic form
- c. time of filing/furnishing
- ☒ contained in the international application as filed
- ☒ filed together with the international application in electronic form
- ☐ furnished subsequently to this Authority for the purpose of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments: