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DESCRIPTION

Reference to a Sequence Listing

[0001] This application contains a Sequence Listing in computer readable form.

Reference to a Deposit of Biological Material

[0002] This application contains a reference to a deposit of biological material.

Background of the Invention

Field of the Invention

[0003] The present invention relates to isolated polypeptides having alpha-glucuronidase activity as defined in claim 1 and isolated polynucleotides encoding the polypeptides as defined in claim 1. 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.

Description of the Related Art

[0004] Plant cell wall polysaccharides constitute 90% of the plant cell wall and can be divided into three groups: cellulose, hemicellulose, and pectin. Cellulose represents the major constituent of call wall polysaccharides. Hemicelluloses are the second most abundant constituent of plant cell walls. The major hemicellulose polymer is xylan. The structure of 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-arabinosyl, D-galactosyl, acetyl, feruloyl, p-coumaroyl, and glucuronoyl residues.

[0005] The biodegradation of xylan-containing material depends on two classes of enzymes: endoxylanases and beta-xylosidases that degrade the xylan backbone. 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). Biodegradation of xylan-containing material also requires accessory enzyme activities that remove non-xylose substituents, e.g., glucuronic acid substituents, from the xylan backbone. Such accessory enzyme activities include, for example, acetylxylan esterase, arabinase, alpha-glucuronidase, ferulic acid esterase, and p-coumaric acid esterase.

[0006] Alpha-glucuronidases are produced naturally by several microorganisms including *Trichoderma reesei* RutC30 (Siika-aho et al., 1994, Enzyme Microb. Technol. 16: 813-819), *Trichoderma viride* (Ishihara et al., 1990, Bull. For. & For. Prod. Res. Inst. 359: 141-157), *Aspergillus niger* (Uchida et al., 1992, Biosci. Biotech. Biochem. 56: 1608-1615), *Thermoascus aurantiacus* (Khandke et al., 1989, Arch. Biochem. Biophys. 274: 511-517), and *Thermoanaerobacterium* sp. strain JW/SL-YS485 (Shao et al., 1995, Appl. Environ. Microbiol. 61: 1077-1081), *Talaromyces emetsorün* (Heneglan et al., 2007, Enzyme and Microbial Technology, vol.41 no.6-7, 677-682). WO 1997/043423 discloses alpha-glucuronidases from *Aspergillus*.

[0007] Several reports have shown a synergistic effect between xylanase, beta-xylosidase and alpha-glucuronidase in the breakdown of glucuronoarabinoxylans (Siika-aho et al., 1994, *supra*). Alpha-glucuronidase therefore has application in the total hydrolysis of hemicellulose to produce xylose (Uchida et al., 1992, J. Ferment. Bioeng. 74: 153-158).

[0008] The present invention relates to polypeptides having alpha-glucuronidase activity and polynucleotides encoding the polypeptides.

Summary of the Invention

[0009] The claims define the matter for which protection is sought, any statement going beyond of that which is defined in the claims is provided for information only.

[0010] The present invention relates to isolated polypeptides having alpha-glucuronidase activity selected from the group consisting of:

1. (a) a polypeptide comprising an amino acid sequence having at least 65% identity to the mature polypeptide of SEQ ID NO: 2;
2. (b) a polypeptide encoded by a polynucleotide that hybridizes under at least medium stringency conditions with the mature polypeptide coding sequence of SEQ ID NO: 1 or its full-length complementary strand;
3. (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 90% identity to the mature polypeptide coding sequence of SEQ ID NO: 1; and
4. (d) a variant comprising a substitution, deletion, and/or insertion of one or more to a total number of 1-10 amino acids of the mature polypeptide of SEQ ID NO: 2.

[0011] The present invention also relates to isolated polynucleotides encoding polypeptides having alpha-glucuronidase activity, selected from the group consisting of:

1. (a) a polynucleotide encoding a polypeptide comprising an amino acid sequence having at least 90% identity to the mature polypeptide of SEQ ID NO: 2;
2. (b) a polynucleotide that hybridizes under at least high stringency conditions with the mature polypeptide coding sequence of SEQ ID NO: 1 or its full-length complementary strand;
3. (c) a polynucleotide comprising a nucleotide sequence having at least 90% identity to the mature polypeptide coding sequence of SEQ ID NO: 1; and
4. (d) a polynucleotide encoding a variant comprising a substitution, deletion, and/or insertion of one or more to a total number of 1-10 amino acids of the mature polypeptide of SEQ ID NO: 2.

[0012] The present invention also relates to nucleic acid constructs, recombinant expression vectors, and, recombinant host cells comprising said polynucleotides, and to methods of producing said polypeptides having alpha-glucuronidase activity.

[0013] The present invention also relates to methods of inhibiting the expression of a polypeptide having alpha-glucuronidase 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. The present also relates to such a double-stranded inhibitory RNA (dsRNA) molecule, wherein optionally the dsRNA is a siRNA or a miRNA molecule.

[0014] The present invention also relates to plants comprising an isolated polynucleotide as defined above encoding a polypeptide having alpha-glucuronidase activity as defined above.

[0015] The present invention also relates to methods of producing a polypeptide having alpha-glucuronidase activity as defined above, comprising: (a) cultivating a transgenic plant or a plant cell comprising a polynucleotide encoding the polypeptide having alpha-glucuronidase activity under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

[0016] The present invention also relates to methods of using the polypeptides having alpha-glucuronidase activity as defined above in the degradation or conversion of xylan-containing material or cellulosic material.

[0017] The present invention also relates to an isolated polynucleotide encoding a signal peptide comprising or consisting of amino acids 1 to 15 of SEQ ID NO: 2; to nucleic acid construct expression vectors, and recombinant host cells comprising said isolated polynucleotide; and to methods of producing a protein.

Brief Description of the Figures

[0018]

Figures 1A and 1B show the genomic DNA sequence and the deduced amino acid sequence of a *Humicola insolens* DSM 1800 Family 67 alpha-glucuronidase gene (SEQ ID NOs: 1 and 2, respectively).

Figure 2 shows a restriction map of pHinsGH67A.

Definitions

[0019] Alpha-glucuronidase activity: The term "alpha-glucuronidase activity" is defined herein as an alpha-D-glucosiduronate glucuronohydrolase activity (EC 3.2.1.139) that catalyzes the hydrolysis of an alpha-D-glucuronoside to D-glucuronate and an alcohol. For purposes of the present invention, alpha-glucuronidase activity is determined according to de Vries, 1998, J. Bacteriol. 180: 243-249. One unit of alpha-glucuronidase activity equals the amount of enzyme capable of releasing 1 μ mole of glucuronic or 4-O-methylglucuronic acid per minute at pH 5, 40°C.

[0020] The polypeptides of the present invention have at least 90%, most preferably at least 95%, and even most preferably at least 100% of the alpha-glucuronidase activity of the mature polypeptide of SEQ ID NO: 2.

[0021] Family 67 glycoside hydrolase: The term "Family 67 glycoside hydrolase" or "Family GH67" or "GH67" is defined herein as a polypeptide falling into the glycoside hydrolase Family 67 according to Henrissat, 1991, A classification of glycosyl hydrolases based on amino-acid sequence similarities, Biochem. J. 280: 309-316, and Henrissat and Bairoch, 1996, Updating the sequence-based classification of glycosyl hydrolases, Biochem. J. 316: 695-696.

[0022] Xylan degrading activity: The terms "xylan degrading activity" or "xylanolytic activity" are defined herein as a biological activity that hydrolyzes xylan-containing material. The two basic approaches for measuring xylanolytic activity include: (1) measuring the total xylanolytic activity, and (2) measuring the individual xylanolytic activities (endoxylanases, beta-xylosidases, arabinofuranosidases, alpha-glucuronidases, acetyl xylan esterases, ferulic acid esterases, and alpha-glucuronyl esterases). Recent progress in assays of xylanolytic enzymes was summarized in several publications including Biely and Puchard, Recent progress in the assays of xylanolytic enzymes, 2006, Journal of the Science of Food and Agriculture 86(11): 1636-1647; Spanikova and Biely, 2006, Glucuronoyl esterase - Novel carbohydrate esterase produced by *Schizophyllum commune*, FEBS Letters 580(19): 4597-4601; Herrmann, Vrsanska, Jurickova, Hirsch, Biely, and Kubicek, 1997, The beta-D-xylosidase of *Trichoderma reesei* is a multifunctional beta-D-xylan xylohydrolase, Biochemical Journal 321: 375-381.

[0023] Total xylan degrading activity can be measured by determining the reducing sugars formed from various types of xylan, including oat spelt, beechwood, and larchwood xylans, or by photometric determination of dyed xylan fragments released from various covalently dyed xylans. The most common total xylanolytic activity assay is based on production of reducing sugars from polymeric 4-O-methyl glucuronoxylan as described in Bailey, Biely, Poutanen, 1992, Interlaboratory testing of methods for assay of xylanase activity, Journal of Biotechnology 23(3): 257-270.

[0024] Xylan degrading activity is determined by measuring the increase in hydrolysis of birchwood xylan (Sigma Chemical Co., Inc., St. Louis, MO, USA) by xylan-degrading enzyme(s) under the following typical conditions: 1 ml reactions, 5 mg/ml substrate (total solids), 5 mg of xylanolytic protein/g of substrate, 50 mM sodium acetate pH 5, 50°C, 24 hours, sugar analysis using p-hydroxybenzoic acid hydrazide (PHBAH) assay as described by Lever, 1972, A new reaction for colorimetric determination of carbohydrates, Anal. Biochem 47: 273-279.

[0025] Xylanase activity: The term "xylanase activity" is defined herein as a 1,4-beta-D-xylan-xylohydrolase activity (E.C. 3.2.1.8) that catalyzes the endo-hydrolysis of 1,4-beta-D-xylosidic linkages in xylans. For purposes of the present invention, xylanase activity is determined using birchwood xylan as substrate. One unit of xylanase activity is defined as 1.0 μ mole of reducing sugar (measured in glucose equivalents as described by Lever, 1972, A new reaction for colorimetric determination of carbohydrates, Anal. Biochem 47: 273-279) produced per minute during the initial period of hydrolysis at 50°C, pH 5 from 2 g of birchwood xylan per liter as substrate in 50 mM sodium acetate, 0.01% TWEEN® 20.

[0026] Beta-xylosidase activity: The term "beta-xylosidase activity" is defined herein as a beta-D-xyloside xylohydrolase (E.C. 3.2.1.37) that catalyzes the exo-hydrolysis of short beta (1→4) -xylooligosaccharides, to remove successive D-xylose residues from the non-reducing termini. For purposes of the present invention, one unit of beta-xylosidase activity is defined as 1.0 μ mole of p-nitrophenol produced per minute at 40°C, pH 5 from 1 mM p-nitrophenyl-beta-D-xyloside as substrate in 100 mM sodium citrate, 0.01% TWEEN® 20.

[0027] Cellulolytic activity: The term "cellulolytic activity" is defined herein as a biological activity that hydrolyzes a cellulosic material. The two basic approaches for measuring cellulolytic activity include: (1) measuring the total cellulolytic activity, and (2) measuring the individual cellulolytic activities (endoglucanases, cellobiohydrolases, and beta-glucosidases) as reviewed in Zhang et al., Outlook for cellulase improvement: Screening and selection strategies, 2006, *Biotechnology Advances* 24: 452-481. Total cellulolytic activity is usually measured using insoluble substrates, including Whatman N°1 filter paper, microcrystalline cellulose, bacterial cellulose, algal cellulose, cotton, pretreated lignocellulose, etc. The most common total cellulolytic activity assay is the filter paper assay using Whatman N°1 filter paper as the substrate. The assay was established by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987, Measurement of cellulase activities, *Pure Appl. Chem.* 59: 257-68).

[0028] Cellulolytic activity is determined by measuring the increase in hydrolysis of a cellulosic material by cellulolytic enzyme(s) under the following conditions: 1-20 mg of cellulolytic protein/g of cellulose in PCS for 3-7 days at 50-65°C compared to a control hydrolysis without addition of cellulolytic protein. Typical conditions are: 1-ml reactions, washed or unwashed PCS, 5% insoluble solids, 50 mM sodium acetate pH 5, 1 mM MnSO₄, 50-65°C, 72 hours, sugar analysis by AMINEX® HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

[0029] Endoglucanase: The term "endoglucanase" is defined herein as an endo-1,4-(1,3;1,4)-beta-D-glucan 4-glucanohydrolase (E.C. 3.2.1.4), which catalyses endohydrolysis of 1,4-beta-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxymethyl cellulose and hydroxyethyl cellulose), lichenin, beta-1,4 bonds in mixed beta-1,3 glucans such as cereal beta-D-glucans or xyloglucans, and other plant material containing cellulosic components. Endoglucanase activity can be determined based on a reduction in substrate viscosity or increase in reducing ends determined by a reducing sugar assay (Zhang et al., 2006, *Biotechnology Advances* 24: 452-481). Endoglucanase activity is determined using carboxymethyl cellulose (CMC) hydrolysis according to the procedure of Ghose, 1987, *Pure and Appl. Chem.* 59: 257-268.

[0030] Cellobiohydrolase: The term "cellobiohydrolase" is defined herein as a 1,4-beta-D-glucan cellobiohydrolase (E.C. 3.2.1.91), which catalyzes the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, cellobiosaccharides, or any beta-1,4-linked glucose containing polymer, releasing cellobiose from the reducing or non-reducing ends of the chain (Teeri, 1997, Crystalline cellulose degradation: New insight into the function of cellobiohydrolases, *Trends in Biotechnology* 15: 160-167; Teeri et al., 1998, Trichoderma reesei cellobiohydrolases: why so efficient on crystalline cellulose?, *Biochem. Soc. Trans.* 26: 173-178). Cellobiohydrolase activity is determined on a fluorescent disaccharide derivative 4-methylumbelliferyl-β-D-lactoside according to the procedures described by van Tilbeurgh et al., 1982, *FEBS Letters* 149: 152-156 and van Tilbeurgh and Claeysens, 1985, *FEBS Letters* 187: 283-288.

[0031] Beta-glucosidase: The term "beta-glucosidase" is defined herein as a beta-D-glucoside glucohydrolase (E.C. 3.2.1.21), which catalyzes the hydrolysis of terminal non-reducing beta-D-glucose residues with the release of beta-D-glucose. Beta-glucosidase activity is determined according to the basic procedure described by Venturi et al., 2002, Extracellular beta-D-glucosidase from *Chaetomium thermophilum* var. *coprophilum*: production, purification and some biochemical properties, *J. Basic Microbiol.* 42: 55-66, except different conditions were employed as described herein. One unit of beta-glucosidase activity is defined as 1.0 μmole of p-nitrophenol produced per minute at 40°C, pH 5 from 1 mM p-nitrophenyl-beta-D-glucopyranoside as substrate in 100 mM sodium citrate, 0.01% TWEEN® 20.

[0032] Cellulolytic enhancing activity: The term "cellulolytic enhancing activity" is defined herein as a biological activity that enhances the hydrolysis of a cellulosic material by polypeptides having cellulolytic activity. Cellulolytic enhancing activity is determined by measuring the increase in reducing sugars or in the increase of the total of cellobiose and glucose from the hydrolysis of a cellulosic material by cellulolytic protein under the following conditions: 1-50 mg of total protein/g of cellulose in PCS, wherein total protein is comprised of 50-99.5% w/w cellulolytic protein and 0.5-50% w/w protein of cellulolytic enhancing activity for 1-7 day at 50-65°C compared to a control hydrolysis with equal total protein loading without cellulolytic enhancing activity (1-50 mg of cellulolytic protein/g of cellulose in PCS). In a preferred aspect, a mixture of CELLULAST® 1.5L (Novozymes A/S, Bagsværd, Denmark) in the presence of 3% of total protein weight *Aspergillus oryzae* beta-glucosidase (recombinantly produced in *Aspergillus oryzae* according to WO 02/095014) or 3% of total protein weight *Aspergillus fumigatus* beta-glucosidase (recombinantly produced in *Aspergillus oryzae* as described in WO 2002/095014) of cellulase protein loading is used as the source of the cellulolytic activity.

[0033] The polypeptides having cellulolytic enhancing activity have at least 20%, preferably at least 40%, more preferably at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 100% of the cellulolytic enhancing activity of the mature polypeptide of a GH61 polypeptide.

[0034] The polypeptides having cellulolytic enhancing activity enhance the hydrolysis of a cellulosic material catalyzed by proteins having cellulolytic activity by reducing the amount of cellulolytic enzyme required to reach the same degree of hydrolysis preferably at least 1.01-fold, more preferably at least 1.05-fold, more preferably at least 1.10-fold, more preferably at least 1.25-fold, more preferably at least 1.5-fold, more preferably at least 2-fold, more preferably at least 3-fold, more preferably at least 4-fold, more preferably at least 5-fold, even more preferably at least 10-fold, and most preferably at least 20-fold.

[0035] Xylan-containing material: The term "xylan-containing material" is defined herein as any material comprising a plant cell wall polysaccharide containing a backbone of beta-(1-4)-linked xylose residues. Xylans of terrestrial plants are heteropolymers possessing a beta-(1-4)-D-xylopyranose backbone, which is branched by short carbohydrate chains. They comprise D-glucuronic acid or its 4-O-methyl ether, L-arabinose, and/or various oligosaccharides, composed of D-xylose, L-arabinose, D- or L-galactose, and D-glucose. Xylan-type polysaccharides can be divided into homoxylans and heteroxylans, which include glucuronoxylans, (arabino)glucuronoxylans, (glucurono)arabinoxylans, arabinoxylans, and complex heteroxylans. See, for example, Ebringerova et al., 2005, Adv. Polym. Sci. 186: 1-67.

[0036] In the methods of the present invention, any material containing xylan may be used. In a preferred aspect, the xylan-containing material is lignocellulose.

[0037] Cellulosic material: The cellulosic material can be any material containing cellulose. The predominant polysaccharide in the primary cell wall of biomass is cellulose, the second most abundant is hemicellulose, and the third is pectin. The secondary cell wall, produced after the cell has stopped growing, also contains polysaccharides and is strengthened by polymeric lignin covalently cross-linked to hemicellulose. Cellulose is a homopolymer of anhydrocellobiose and thus a linear beta-(1-4)-D-glucan, while hemicelluloses include a variety of compounds, such as xylans, xyloglucans, arabinoxylans, and mannans in complex branched structures with a spectrum of substituents. Although generally polymorphous, cellulose is found in plant tissue primarily as an insoluble crystalline matrix of parallel glucan chains. Hemicelluloses usually hydrogen bond to cellulose, as well as to other hemicelluloses, which help stabilize the cell wall matrix.

[0038] Cellulose is generally found, for example, in the stems, leaves, hulls, husks, and cobs of plants or leaves, branches, and wood of trees. The cellulosic material can be, but is not limited to, herbaceous material, agricultural residue, forestry residue, municipal solid waste, waste paper, and pulp and paper mill residue (see, for example, Wiseloge et al., 1995, in Handbook on Bioethanol (Charles E. Wyman, editor), pp.105-118, Taylor & Francis, Washington D.C.; Wyman, 1994, Bioresource Technology 50: 3-16; Lynd, 1990, Applied Biochemistry and Biotechnology 24/25: 695-719; Mosier et al., 1999, Recent Progress in Bioconversion of Lignocellulosics, in Advances in Biochemical Engineering/Biotechnology, T. Scheper, managing editor, Volume 65, pp.23-40, Springer-Verlag, New York). It is understood herein that the cellulose may be in the form of lignocellulose, a plant cell wall material containing lignin, cellulose, and hemicellulose in a mixed matrix. In a preferred aspect, the cellulosic material is lignocellulose.

[0039] In one aspect, the cellulosic material is herbaceous material. In another aspect, the cellulosic material is agricultural residue. In another aspect, the cellulosic material is forestry residue. In another aspect, the cellulosic material is municipal solid waste. In another aspect, the cellulosic material is waste paper. In another aspect, the cellulosic material is pulp and paper mill residue.

[0040] In another aspect, the cellulosic material is corn stover. In another aspect, the cellulosic material is corn fiber. In another aspect, the cellulosic material is corn cob. In another aspect, the cellulosic material is orange peel. In another aspect, the cellulosic material is rice straw. In another aspect, the cellulosic material is wheat straw. In another aspect, the cellulosic material is switch grass. In another aspect, the cellulosic material is miscanthus. In another aspect, the cellulosic material is bagasse.

[0041] In another aspect, the cellulosic material is microcrystalline cellulose. In another aspect, the cellulosic material is bacterial cellulose. In another aspect, the cellulosic material is algal cellulose. In another aspect, the cellulosic material is cotton linter. In another aspect, the cellulosic material is amorphous phosphoric-acid treated cellulose. In another aspect, the cellulosic material is filter paper.

[0042] The cellulosic material may be used as is or may be subjected to pretreatment, using conventional methods known in the art, as described herein. In a preferred aspect, the cellulosic material is pretreated.

[0043] Pretreated corn stover: The term "PCS" or "Pretreated Corn Stover" is defined herein as a cellulosic material derived from corn stover by treatment with heat and dilute sulfuric acid.

[0044] 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.

[0045] Substantially pure polypeptide: The term "substantially pure polypeptide" denotes herein a polypeptide preparation that contains at most 10%, preferably at most 8%, more preferably at most 6%, more preferably at most 5%, more preferably at most 4%, more preferably at most 3%, even more preferably at most 2%, most preferably at most 1%, and even most preferably at most 0.5% by weight of other polypeptide material with which it is natively or recombinantly associated. It is, therefore, preferred that the substantially pure polypeptide is at least 92% pure, preferably at least 94% pure, more preferably at least 95% pure, more preferably at least 96% pure, more preferably at least 97% pure, more preferably at least 98% pure, even more preferably at least 99% pure, most preferably at least 99.5% pure, and even most preferably 100% pure by weight of the total polypeptide material present in the preparation. The polypeptides 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.

[0046] Mature polypeptide: The term "mature polypeptide" is defined herein as a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. In one aspect, the mature polypeptide is amino acids 16 to 861 of SEQ ID NO: 2 based on the SignalP program (Nielsen et al., 1997, Protein Engineering 10: 1-6) that predicts amino acids 1 to 15 of SEQ ID NO: 2 are a signal peptide.

[0047] Mature polypeptide coding sequence: The term "mature polypeptide coding sequence" is defined herein as a nucleotide sequence that encodes a mature polypeptide having alpha-glucuronidase activity. In one aspect, the mature polypeptide coding sequence is nucleotides 46 to 2583 of SEQ ID NO: 1 based on the SignalP program (Nielsen *et al.*, 1997, *supra*) that predicts nucleotides 1 to 45 of SEQ ID NO: 1 encode a signal peptide.

[0048] Identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "identity".

[0049] The degree of identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, J. Mol. Biol. 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, Trends in Genetics 16: 276-277), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:

(Identical Residues x 100)/(Length of Alignment - Total Number of Gaps in Alignment)

[0050] The degree of identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, *supra*), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:

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

[0051] Homologous sequence: The term "homologous sequence" is defined herein as a predicted protein having an E value (or expectancy score) of less than 0.001 in a tfasty search (Pearson, W.R., 1999, in Bioinformatics Methods and Protocols, S. Misener and S. A. Krawetz, ed., pp. 185-219) with the *Humicola insolens* alpha-glucuronidase of SEQ ID NO: 2 or the mature polypeptide thereof.

[0052] Polypeptide fragment: The term "polypeptide fragment" is defined herein as a polypeptide having one or more (several)

amino acids deleted from the amino and/or carboxyl terminus of the mature polypeptide of SEQ ID NO: 2; wherein the fragment has alpha-glucuronidase activity. In a preferred aspect, a fragment contains at least 730 amino acid residues, more preferably at least 770 amino acid residues, and most preferably at least 810 amino acid residues of the mature polypeptide of SEQ ID NO: 2.

[0053] Subsequence: The term "subsequence" is defined herein as a nucleotide sequence having one or more (several) nucleotides deleted from the 5' and/or 3' end of the mature polypeptide coding sequence of SEQ ID NO: 1; wherein the subsequence encodes a polypeptide fragment having alpha-glucuronidase activity. In a preferred aspect, a subsequence contains at least 2190 nucleotides, more preferably at least 2310 nucleotides, and most preferably at least 2430 nucleotides of the mature polypeptide coding sequence of SEQ ID NO: 1.

[0054] 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.

[0055] 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.

[0056] Substantially pure polynucleotide: The term "substantially pure polynucleotide" as used herein refers to a polynucleotide preparation free of other extraneous or unwanted nucleotides and in a form suitable for use within genetically engineered protein production systems. Thus, a substantially pure polynucleotide contains at most 10%, preferably at most 8%, more preferably at most 6%, more preferably at most 5%, more preferably at most 4%, more preferably at most 3%, even more preferably at most 2%, most preferably at most 1%, and even most preferably at most 0.5% by weight of other polynucleotide material with which it is natively or recombinantly associated. A substantially pure polynucleotide may, however, include naturally occurring 5' and 3' untranslated regions, such as promoters and terminators. It is preferred that the substantially pure polynucleotide is at least 90% pure, preferably at least 92% pure, more preferably at least 94% pure, more preferably at least 95% pure, more preferably at least 96% pure, more preferably at least 97% pure, even more preferably at least 98% pure, most preferably at least 99% pure, and even most preferably at least 99.5% pure by weight. The polynucleotides 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.

[0057] 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.

[0058] cDNA: The term "cDNA" is defined herein as a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic cell. cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps before appearing as mature spliced mRNA. These steps include the removal of intron sequences by a process called splicing. cDNA derived from mRNA lacks, therefore, any intron sequences.

[0059] Nucleic acid construct: The term "nucleic acid construct" as used herein refers to a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or which is modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature or which is synthetic. The term nucleic acid construct is synonymous with the term "expression cassette" when the nucleic acid construct contains the control sequences required for expression of a coding sequence of the present invention.

[0060] Control sequences: The term "control sequences" is defined herein to include all components necessary for the expression of a polynucleotide encoding a polypeptide of the present invention. Each control sequence may be native or foreign to the nucleotide sequence encoding the polypeptide or native or foreign to each other. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the

control sequences with the coding region of the nucleotide sequence encoding a polypeptide.

[0061] 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 a polynucleotide sequence such that the control sequence directs the expression of the coding sequence of a polypeptide.

[0062] Expression: The term "expression" includes any step involved in the production of a polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

[0063] 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.

[0064] 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.

[0065] Modification: The term "modification" means herein any chemical modification of the polypeptide comprising or 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.

[0066] Artificial variant: When used herein, the term "artificial variant" means a polypeptide having alpha-glucuronidase activity produced by a micro organism expressing a modified polynucleotide sequence of the mature polypeptide coding sequence of SEQ ID NO: 1. The modified nucleotide sequence is obtained through human intervention by modification of the polynucleotide sequence disclosed in SEQ ID NO: 1.

Detailed Description of the Invention

[0067] The claims define the matter for which protection is sought, any statement going beyond of that which is defined in the claims is provided for information only.

Polypeptides Having Alpha-Glucuronidase Activity

[0068] In a first aspect, the present invention relates to isolated polypeptides comprising amino acid sequences having a degree of identity to the mature polypeptide of SEQ ID NO: 2 of 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 alpha-glucuronidase activity (hereinafter "homologous polypeptides"). In a preferred aspect, the homologous polypeptides comprise amino acid sequences that differ 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.

[0069] 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 alpha-glucuronidase 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 16 to 861 of SEQ ID NO: 2, or an allelic variant thereof; or a fragment thereof having alpha-glucuronidase activity. In another preferred aspect, the polypeptide comprises amino acids 16 to 861 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 alpha-glucuronidase 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 16 to 861 of SEQ ID NO: 2 or an allelic variant thereof; or a fragment thereof having alpha-glucuronidase activity. In another preferred aspect, the polypeptide consists of amino acids 16 to 861 of SEQ ID NO: 2.

[0070] In a second aspect, the present invention relates to isolated polypeptides having alpha-glucuronidase activity that are encoded by polynucleotides that hybridize under high stringency conditions, and most preferably very high stringency conditions with the mature polypeptide coding sequence of SEQ ID NO: 1 or its full-length complementary strand (J. Sambrook, E.F. Fritsch,

and T. Maniatis, 1989, Molecular Cloning, A Laboratory Manual, 2d edition, Cold Spring Harbor, New York).

[0071] 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 alpha-glucuronidase activity from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 14, preferably at least 25, more preferably at least 35, and most preferably at least 70 nucleotides in length. It is, however, preferred that the nucleic acid probe is at least 100 nucleotides in length. For example, the nucleic acid probe may be at least 200 nucleotides, preferably at least 300 nucleotides, more preferably at least 400 nucleotides, or most preferably at least 500 nucleotides in length. Even longer probes may be used, e.g., nucleic acid probes that are preferably at least 600 nucleotides, more preferably at least 700 nucleotides, even more preferably at least 800 nucleotides, or most preferably at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with ^{32}P , ^3H , ^{35}S , biotin, or avidin). Such probes are encompassed by the present invention.

[0072] A genomic DNA or cDNA library prepared from such other strains may, therefore, be screened for DNA that hybridizes with the probes described above and encodes a polypeptide having alpha-glucuronidase activity. Genomic or other DNA from such other strains may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA that is homologous with SEQ ID NO: 1, or a subsequence thereof, the carrier material is preferably used in a Southern blot.

[0073] 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 or 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.

[0074] In a preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 1. In another preferred aspect, the nucleic acid probe is nucleotides 46 to 2583 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 pHinsGH67A which is contained in *E. coli* NRRL B-50155, wherein the polynucleotide sequence thereof encodes a polypeptide having alpha-glucuronidase activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding region contained in plasmid pHinsGH67A which is contained in *E. coli* NRRL B-50155.

[0075] 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.

[0076] 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).

[0077] 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.

[0078] 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 .

[0079] In a third aspect, the present invention relates to isolated polypeptides having alpha-glucuronidase activity encoded by polynucleotides comprising or consisting of nucleotide sequences that have a degree of identity to the mature polypeptide coding sequence of SEQ ID NO: 1 of at least 90%, most preferably at least 95%, and even most preferably at least 96%, at least 97%, at least 98%, or at least 99%, which encode a polypeptide having alpha-glucuronidase activity. See polynucleotide section herein.

[0080] In a fourth aspect, the present invention relates to artificial variants comprising a substitution, deletion, and/or insertion of one or more to a total number of 1-10 amino acids of the mature polypeptide of SEQ ID NO: 2. Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 10 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 10 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.

[0081] Examples of conservative substitutions are within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, In, *The Proteins*, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

[0082] In addition to the 20 standard amino acids, non-standard amino acids (such as 4-hydroxyproline, 6-N-methyl lysine, 2-aminoisobutyric acid, isovaline, and alpha-methyl serine) may be substituted for amino acid residues of a wild-type polypeptide. A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, and unnatural amino acids may be substituted for amino acid residues. "Unnatural amino acids" have been modified after protein synthesis, and/or have a chemical structure in their side chain(s) different from that of the standard amino acids. Unnatural amino acids can be chemically synthesized, and preferably, are commercially available, and include pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, and 3,3-dimethylproline.

[0083] Alternatively, the amino acid changes are of such a nature that the physicochemical 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.

[0084] Essential amino acids in the parent polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (*i.e.*, alpha-glucuronidase activity) to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton et al., 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et al., 1992, *Science* 255: 306-312; Smith et al., 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver et al., 1992, *FEBS Lett.* 309: 59-64. The identities of essential amino acids can also be inferred from analysis of identities with polypeptides that are related to a polypeptide according to the invention.

[0085] Single or multiple amino acid substitutions, deletions, and/or insertions can be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR, phage display (*e.g.*, Lowman et al., 1991, *Biochem.* 30: 10832-10837; U.S. Patent No. 5,223,409; WO 92/06204), and region-directed mutagenesis (Derbyshire et al., 1986, *Gene* 46: 145; Ner et al., 1988, *DNA* 7: 127).

[0086] Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells (Ness et al., 1999, *Nature Biotechnology* 17: 893-896). Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

[0087] The total number of amino acid substitutions, deletions and/or insertions of the mature polypeptide 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 Alpha-Glucuronidase Activity

[0088] A polypeptide having alpha-glucuronidase activity as defined in claim 1 may be obtained from microorganisms of any genus. 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.

[0089] A polypeptide having alpha-glucuronidase 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 alpha-glucuronidase activity, or a Gram negative bacterial polypeptide such as an *E. coli*, *Pseudomonas*, *Salmonella*, *Campylobacter*, *Helicobacter*, *Flavobacterium*, *Fusobacterium*, *Ilyobacter*, *Neisseria*, or *Ureaplasma* polypeptide having alpha-glucuronidase activity.

[0090] 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 alpha-glucuronidase activity.

[0091] In another preferred aspect, the polypeptide is a *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, or *Streptococcus equi* subsp. *Zooepidemicus* polypeptide having alpha-glucuronidase activity.

[0092] In another preferred aspect, the polypeptide is a *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, or *Streptomyces lividans* polypeptide having alpha-glucuronidase activity.

[0093] A polypeptide having alpha-glucuronidase activity as defined in claim 1 may also be a fungal polypeptide, and more preferably a yeast polypeptide such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* polypeptide having alpha-glucuronidase activity; or more preferably a filamentous fungal polypeptide such as an *Acremonium*, *Agaricus*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Botryosphaeria*, *Ceriporiopsis*, *Chaetomidium*, *Chrysosporium*, *Claviceps*, *Cochliobolus*, *Coprinopsis*, *Coptotermes*, *Corynascus*, *Cryphonectria*, *Cryptococcus*, *Diplodia*, *Exidia*, *Filibasidium*, *Fusarium*, *Gibberella*, *Holomastigotoides*, *Humicola*, *Irpex*, *Lentinula*, *Leptosphaeria*, *Magnaporthe*, *Melanocarpus*, *Meripilus*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Piromyces*, *Poitrasia*, *Pseudoplectania*, *Pseudotriconympha*, *Rhizomucor*, *Schizophyllum*, *Scytalidium*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trichoderma*, *Trichophaea*, *Verticillium*, *Volvariella*, or *Xylaria* polypeptide.

[0094] In a preferred aspect, the polypeptide is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, or *Saccharomyces oviformis* polypeptide.

[0095] In another preferred aspect, the polypeptide is an *Acremonium cellulolyticus*, *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus fumigatus*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium tropicum*, *Chrysosporium merdarium*, *Chrysosporium inops*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium zonatum*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcocroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Irpex lacteus*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium funiculosum*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Thielavia achromatica*, *Thielavia albomyces*, *Thielavia albopilosa*, *Thielavia australeinsis*, *Thielavia fimeti*, *Thielavia microspora*, *Thielavia ovispora*, *Thielavia peruviana*, *Thielavia speditonum*, *Thielavia setosa*, *Thielavia subthermophila*, *Thielavia terrestris*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* polypeptide.

[0096] In another preferred aspect, the polypeptide is a *Humicola grisea*, *Humicola insolens*, or *Humicola lanuginosa* polypeptide.

[0097] In a more preferred aspect, the polypeptide is a *Humicola insolens* polypeptide. In a most preferred aspect, the polypeptide is a *Humicola insolens* DSM 1800 polypeptide, e.g., the polypeptide comprising the mature polypeptide of SEQ ID NO: 2.

[0098] 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.

[0099] 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).

[0100] 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 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*).

[0101] Polypeptides of the present invention also include fused polypeptides or cleavable fusion polypeptides in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by fusing a nucleotide 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.

[0102] A fusion polypeptide can further comprise a cleavage site. Upon secretion of the fusion protein, the site is cleaved releasing the polypeptide having alpha-glucuronidase 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. Microbial. Biotechnol.* 3: 568-576; Svetina *et al.*, 2000, *J. Biotechnol.* 76: 245-251; Rasmussen-Wilson *et al.*, 1997, *Appl. Environ. Microbiol.* 63: 3488-3493; Ward *et al.*, 1995, *Biotechnology* 13: 498-503; and Contreras *et al.*, 1991, *Biotechnology* 9: 378-381), 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

[0103] The present invention also relates to isolated polynucleotides comprising or consisting of nucleotide sequences that encode polypeptides having alpha-glucuronidase activity as defined in claim 1.

[0104] 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 pHinsGH67A which is contained in *E. coli* NRRL B-50155. 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 46 to 2583 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 pHinsGH67A which is contained in *E. coli* NRRL B-50155. 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 alpha-glucuronidase activity.

[0105] 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.

[0106] 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 *Humicola*, or another or related micro organism and thus, for example, may be an allelic or species variant of the polypeptide encoding region of the nucleotide sequence.

[0107] The present invention also relates to isolated polynucleotides comprising or consisting of nucleotide sequences having a degree of identity to the mature polypeptide coding sequence of SEQ ID NO: 1 of at least 90%, most preferably at least 95%, and even most preferably at least 96%, at least 97%, at least 98%, or at least 99%, which encode a polypeptide having alpha-glucuronidase activity.

[0108] 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.

[0109] 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 alpha-glucuronidase 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*).

[0110] The present invention also relates to isolated polynucleotides encoding polypeptides of the present invention, which hybridize under high stringency conditions, and most preferably very high stringency conditions with the mature polypeptide coding sequence of SEQ ID NO: 1 or its full-length complementary strand; or allelic variants and subsequences thereof (Sambrook *et al.*, 1989, *supra*), as defined herein.

[0111] 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 the mature polypeptide coding sequence of SEQ ID NO: 1 or its full-length complementary strand; and (b) isolating the hybridizing polynucleotide, which encodes a polypeptide having alpha-glucuronidase activity.

Nucleic Acid Constructs

[0112] 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.

[0113] An isolated polynucleotide encoding a polypeptide of the present invention may be manipulated in a variety of ways to

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

[0114] The control sequence may be an appropriate promoter sequence, a nucleotide sequence that is recognized by a host cell for expression of a polynucleotide encoding a polypeptide of the present invention. The promoter sequence contains transcriptional control sequences that mediate the expression of the polypeptide. The promoter may be any nucleotide sequence that shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

[0115] Examples of suitable promoters for directing the transcription of the nucleic acid constructs of the present invention, especially in a bacterial host cell, are the promoters obtained from the *E. coli lac* operon, *Streptomyces coelicolor* agarase gene (*dagA*), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus subtilis* *xylA* and *xylB* genes, and prokaryotic beta-lactamase gene (Villa-Kamaroff et al., 1978, Proceedings of the National Academy of Sciences USA 75: 3727-3731), as well as the *tac* promoter (DeBoer et al., 1983, Proceedings of the National Academy of Sciences USA 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242: 74-94; and in Sambrook et al., 1989, *supra*.

[0116] Examples of suitable promoters for directing the transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase, *Fusarium venenatum* amyloglucosidase (WO 00/56900), *Fusarium venenatum* Daria (WO 00/56900), *Fusarium venenatum* Quinn (WO 00/56900), *Fusarium oxysporum* trypsin-like protease (WO 96/00787), *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase IV, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* beta-xylosidase, as well as the NA2-tpi promoter (a hybrid of the promoters from the genes for *Aspergillus niger* neutral alpha-amylase and *Aspergillus oryzae* triose phosphate isomerase); and mutant, truncated, and hybrid promoters thereof.

[0117] In a yeast host, useful promoters are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* galactokinase (GAL1), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH1, ADH2/GAP), *Saccharomyces cerevisiae* triose phosphate isomerase (TPI), *Saccharomyces cerevisiae* metallothionein (CUP1), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase. Other useful promoters for yeast host cells are described by Romanos et al., 1992, Yeast 8: 423-488.

[0118] The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleotide sequence encoding the polypeptide. Any terminator that is functional in the host cell of choice may be used in the present invention.

[0119] Preferred terminators for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* alpha-glucosidase, and *Fusarium oxysporum* trypsin-like protease.

[0120] Preferred terminators for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are described by Romanos et al., 1992, *supra*.

[0121] The control sequence may also be a suitable leader sequence, a nontranslated region of an mRNA that is important for translation by the host cell. The leader sequence is operably linked to the 5' terminus of the nucleotide sequence encoding the polypeptide. Any leader sequence that is functional in the host cell of choice may be used in the present invention.

[0122] Preferred leaders for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase and *Aspergillus nidulans* triose phosphate isomerase.

[0123] 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).

[0124] 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.

[0125] 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.

[0126] Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, Molecular Cellular Biology 15: 5983-5990.

[0127] The control sequence may also be a signal peptide coding sequence that encodes a signal peptide linked to the amino terminus of a polypeptide and directs the encoded polypeptide into the cell's secretory pathway. The 5' end of the coding sequence of the nucleotide sequence may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. The foreign signal peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding sequence. Alternatively, the foreign signal peptide coding sequence may simply replace the 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.

[0128] Effective signal peptide coding sequences for bacterial host cells are the signal peptide coding sequences obtained from the genes for *Bacillus* NCIB 11837 maltogenic amylase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* neutral proteases (*nprT*, *nprS*, *nprM*), and *Bacillus subtilis* *prsA*. Further signal peptides are described by Simonen and Palva, 1993, Microbiological Reviews 57: 109-137.

[0129] Effective signal peptide coding sequences for filamentous fungal host cells are the signal peptide coding sequences obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Rhizomucor miehei* aspartic proteinase, *Humicola insolens* cellulase, *Humicola insolens* endoglucanase V, and *Humicola lanuginosa* lipase.

[0130] 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*.

[0131] In a preferred aspect, the signal peptide comprises or consists of amino acids 1 to 15 of SEQ ID NO: 2. In another preferred aspect, the signal peptide coding sequence comprises or consists of nucleotides 1 to 45 of SEQ ID NO: 1.

[0132] The control sequence may also be a propeptide coding sequence that encodes a propeptide positioned at the amino terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propeptide is generally inactive and can be converted to a mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding sequence may be obtained from the genes for *Bacillus subtilis* alkaline protease (*aprE*), *Bacillus subtilis* neutral protease (*nprT*), *Saccharomyces cerevisiae* alpha-factor, *Rhizomucor miehei* aspartic proteinase, and *Myceliophthora thermophila* laccase (WO 95/33836).

[0133] Where both signal peptide and propeptide sequences are present at the amino terminus of a polypeptide, the propeptide sequence is positioned next to the amino terminus of a polypeptide and the signal peptide sequence is positioned next to the amino terminus of the propeptide sequence.

[0134] It may also be desirable to add regulatory sequences that allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those that cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory

systems in prokaryotic systems include the *lac*, *tac*, and *trp* operator systems. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the TAKA alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used as regulatory sequences. Other examples of regulatory sequences are those that allow for gene amplification. In eukaryotic systems, these regulatory sequences include the dihydrofolate reductase gene that is amplified in the 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

[0135] The present invention also relates to recombinant expression vectors comprising a polynucleotide of the present invention, a promoter, and transcriptional and translational stop signals. The various nucleic acids and control sequences described herein may be joined together to produce a recombinant expression vector that may include one or more (several) convenient restriction sites to allow for insertion or substitution of the nucleotide sequence encoding the polypeptide at such sites. Alternatively, a polynucleotide sequence of the present invention may be expressed by inserting the nucleotide sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

[0136] The recombinant expression vector may be any vector (e.g., a plasmid or virus) that can be conveniently subjected to recombinant DNA procedures and can bring about expression of the 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.

[0137] The vector may be an autonomously replicating vector, *i.e.*, a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one that, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

[0138] 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 or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

[0139] Examples of bacterial selectable markers are the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers that confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol, or tetracycline resistance. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for use in a filamentous fungal host cell include, but are not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in an *Aspergillus* cell are the *amdS* and *pyrG* genes of *Aspergillus nidulans* or *Aspergillus oryzae* and the *bar* gene of *Streptomyces hygroscopicus*.

[0140] 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.

[0141] For integration into the host cell genome, the vector may rely on the polynucleotide's sequence encoding the polypeptide or any other element of the vector for integration into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleotide sequences for directing integration by homologous recombination into the genome of the host cell at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, preferably 400 to 10,000 base pairs, and most preferably 800 to 10,000 base pairs, which have a high degree of identity to the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding nucleotide sequences. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

[0142] For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in a cell. The term "origin of replication" or "plasmid replicator" is defined herein as a nucleotide sequence that enables a plasmid or vector to replicate *in vivo*.

[0143] Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*, and pUB110, pE194, pTA1060, and pAM β 1 permitting replication in *Bacillus*.

[0144] 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.

[0145] Examples of origins of replication useful in a filamentous fungal cell are AMA1 and ANS1 (Gems et al., 1991, Gene 98: 61-67; Cullen et al., 1987, Nucleic Acids Research 15: 9163-9175; WO 00/24883). Isolation of the AMA1 gene and construction of plasmids or vectors comprising the gene can be accomplished according to the methods disclosed in WO 00/24883.

[0146] More than one copy of a polynucleotide of the present invention may be inserted into a host cell to increase production of the gene product. An increase in the copy number of the polynucleotide can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the polynucleotide where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the polynucleotide, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

[0147] The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, e.g., Sambrook *et al.*, 1989, *supra*).

Host Cells

[0148] The present invention also relates to recombinant host cells, comprising an isolated polynucleotide of the present invention operably linked to one or more (several) control sequences that direct the production of a polypeptide having alpha-glucuronidase activity. A construct or vector comprising a polynucleotide of the present invention is introduced into a host cell so that the construct or vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source.

[0149] 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.

[0150] The prokaryotic host cell may be any Gram positive bacterium or a Gram negative bacterium. Gram positive bacteria include, but not limited to, *Bacillus*, *Streptococcus*, *Streptomyces*, *Staphylococcus*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Clostridium*, *Geobacillus*, and *Oceanobacillus*. Gram negative bacteria include, but not limited to, *E. coli*, *Pseudomonas*, *Salmonella*, *Campylobacter*, *Helicobacter*, *Flavobacterium*, *Fusobacterium*, *Ilyobacter*, *Neisseria*, and *Ureaplasma*.

[0151] The bacterial host cell may be any *Bacillus* cell. *Bacillus* cells useful in the practice of the present invention include, but are not limited to, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis* cells.

[0152] In a preferred aspect, the bacterial host cell is a *Bacillus amyloliquefaciens*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus stearothermophilus* or *Bacillus subtilis* cell. In a more preferred aspect, the bacterial host cell is a *Bacillus amyloliquefaciens* cell. In another more preferred aspect, the bacterial host cell is a *Bacillus clausii* cell. In another more preferred aspect, the bacterial host cell is a *Bacillus licheniformis* cell. In another more preferred aspect, the bacterial host cell is a *Bacillus subtilis* cell.

[0153] The bacterial host cell may also be any *Streptococcus* cell. *Streptococcus* cells useful in the practice of the present invention include, but are not limited to, *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, and *Streptococcus equi* subsp. *Zooepidemicus* cells.

[0154] In a preferred aspect, the bacterial host cell is a *Streptococcus equisimilis* cell. In another preferred aspect, the bacterial host cell is a *Streptococcus pyogenes* cell. In another preferred aspect, the bacterial host cell is a *Streptococcus uberis* cell. In another preferred aspect, the bacterial host cell is a *Streptococcus equi* subsp. *Zooepidemicus* cell.

[0155] The bacterial host cell may also be any *Streptomyces* cell. *Streptomyces* cells useful in the practice of the present invention include, but are not limited to, *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, and *Streptomyces lividans* cells.

[0156] In a preferred aspect, the bacterial host cell is a *Streptomyces achromogenes* cell. In another preferred aspect, the bacterial host cell is a *Streptomyces avermitilis* cell. In another preferred aspect, the bacterial host cell is a *Streptomyces coelicolor* cell. In another preferred aspect, the bacterial host cell is a *Streptomyces griseus* cell. In another preferred aspect, the bacterial host cell is a *Streptomyces lividans* cell.

[0157] The introduction of DNA into a *Bacillus* cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, Molecular General Genetics 168: 111-115), by using competent cells (see, e.g., Young and Spizizen, 1961, Journal of Bacteriology 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, Journal of Molecular Biology 56: 209-221), by electroporation (see, e.g., Shigekawa and Dower, 1988, Biotechniques 6: 742-751), or by conjugation (see, e.g., Koehler and Thorne, 1987, Journal of Bacteriology 169: 5271-5278). The introduction of DNA into an *E. coli* cell may, for instance, be effected by protoplast transformation (see, e.g., Hanahan, 1983, J. Mol. Biol. 166: 557-580) or electroporation (see, e.g., Dower et al., 1988, Nucleic Acids Res. 16: 6127-6145). The introduction of DNA into a *Streptomyces* cell may, for instance, be effected by protoplast transformation and electroporation (see, e.g., Gong et al., 2004, Folia Microbiol. (Praha) 49: 399-405), by conjugation (see, e.g., Mazodier et al., 1989, J. Bacteriol. 171: 3583-3585), or by transduction (see, e.g., Burke et al., 2001, Proc. Natl. Acad. Sci. USA 98: 6289-6294). The introduction of DNA into a *Pseudomonas* cell may, for instance, be effected by electroporation (see, e.g., Choi et al., 2006, J. Microbiol. Methods 64: 391-397) or by conjugation (see, e.g., Pinedo and Smets, 2005, Appl. Environ. Microbiol. 71: 51-57). The introduction of DNA into a *Streptococcus* cell may, for instance, be effected by natural competence (see, e.g., Perry and Kuramitsu, 1981, Infect. Immun. 32: 1295-1297), by protoplast transformation (see, e.g., Catt and Jollick, 1991, Microbios. 68: 189-207, by electroporation (see, e.g., Buckley et al., 1999, Appl. Environ. Microbiol. 65: 3800-3804) or by conjugation (see, e.g., Clewell, 1981, Microbiol. Rev. 45: 409-436). However, any method known in the art for introducing DNA into a host cell can be used.

[0158] The host cell may also be a eukaryote, such as a mammalian, insect, plant, or fungal cell.

[0159] In a preferred aspect, the host cell is a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth et al., In, Ainsworth and Bisby's Dictionary of The Fungi, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth et al., 1995, *supra*, page 171) and all mitosporic fungi (Hawksworth et al., 1995, *supra*).

[0160] In a more preferred aspect, the fungal host cell is a yeast cell. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in Biology and Activities of Yeast (Skinner, F.A., Passmore, S.M., and Davenport, R.R., eds, Soc. App. Bacteriol. Symposium Series No. 9, 1980).

[0161] In an even more preferred aspect, the yeast host cell is a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell.

[0162] 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.

[0163] 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.

[0164] 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.

[0165] 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 graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, or *Fusarium venenatum* cell. In another most preferred aspect, the filamentous fungal host cell is a *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium tropicum*, *Chrysosporium merdarium*, *Chrysosporium inops*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

[0166] Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described in EP 238 023 and Yelton et al., 1984, Proceedings of the National Academy of Sciences USA 81: 1470-1474. Suitable methods for transforming *Fusarium* species are described by Malardier et al., 1989, Gene 78: 147-156, and WO 96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J.N. and Simon, M.I., editors, Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito et al., 1983, Journal of Bacteriology 153: 163; and Hinnen et al., 1978, Proceedings of the National Academy of Sciences USA 75: 1920.

Methods of Production

[0167] The present invention also relates to methods of producing a polypeptide of the present invention, comprising: (a) cultivating a cell, which in its wild-type form produces the polypeptide, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide. In a preferred aspect, the cell is of the genus *Humicola*. In a more preferred aspect, the cell is *Humicola insolens*. In a most preferred aspect, the cell is *Humicola insolens* DSM 1800.

[0168] The present invention also relates to methods of producing a polypeptide of the present invention, comprising: (a) cultivating a recombinant host cell, as described herein, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

[0169] 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.

[0170] 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.

[0171] 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.

[0172] 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.

[0173] The polypeptides of the present invention may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction (see, e.g., Protein Purification, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989) to obtain substantially pure polypeptides.

Plants

[0174] 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 alpha-glucuronidase activity of the present invention so as to express and produce the polypeptide in recoverable quantities. The polypeptide may be recovered from the plant or plant part. Alternatively, the plant or plant part containing the recombinant polypeptide may be used as such for improving the quality of a food or feed, e.g., improving nutritional value, palatability, and rheological properties, or to destroy an antinutritive factor.

[0175] The transgenic plant can be dicotyledonous (a dicot) or monocotyledonous (a monocot). Examples of monocot plants are grasses, such as meadow grass (blue grass, *Poa*), forage grass such as *Festuca*, *Lolium*, temperate grass, such as *Agrostis*, and cereals, e.g., wheat, oats, rye, barley, rice, sorghum, and maize (corn).

[0176] Examples of dicot plants are tobacco, legumes, such as lupins, potato, sugar beet, pea, bean and soybean, and cruciferous plants (family Brassicaceae), such as cauliflower, rape seed, and the closely related model organism *Arabidopsis thaliana*.

[0177] Examples of plant parts are stem, callus, leaves, root, fruits, seeds, and tubers as well as the individual tissues comprising these parts, e.g., epidermis, mesophyll, parenchyme, vascular tissues, meristems. Specific plant cell compartments, such as chloroplasts, apoplasts, mitochondria, vacuoles, peroxisomes and cytoplasm are also considered to be a plant part. Furthermore, any plant cell, whatever the tissue origin, is considered to be a plant part. Likewise, plant parts such as specific tissues and cells isolated to facilitate the utilisation of the invention are also considered plant parts, e.g., embryos, endosperms, aleurone and seeds coats.

[0178] Also included within the scope of the present invention are the progeny of such plants, plant parts, and plant cells.

[0179] The transgenic plant or plant cell expressing a polypeptide of the present invention may be constructed in accordance with methods known in the art. In short, the plant or plant cell is constructed by incorporating one or more (several) expression constructs encoding a polypeptide of the present invention into the plant host genome or chloroplast genome and propagating the resulting modified plant or plant cell into a transgenic plant or plant cell.

[0180] 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).

[0181] 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.

[0182] 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 Mol. 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 and 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 *nap4* 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 chloroella virus adenine methyltransferase gene promoter (Mitra and Higgins, 1994, Plant Molecular Biology 26: 85-93), or the *aldP* gene promoter from rice (Kagaya et al., 1995, Molecular and General Genetics 248: 668-674), or a wound inducible promoter such as the potato *pin2* promoter (Xu et al., 1993, Plant Molecular Biology 22: 573-588). Likewise, the promoter may be inducible by abiotic treatments such as temperature, drought, or alterations in salinity or induced by exogenously applied substances that activate the promoter, e.g., ethanol, oestrogens, plant hormones such as ethylene, abscisic acid, and gibberellic acid, and heavy metals.

[0183] 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.

[0184] The selectable marker gene and any other parts of the expression construct may be chosen from those available in the art.

[0185] 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).

[0186] 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.

[0187] 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.

[0188] 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 alpha-glucuronidase activity of the present invention under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

Removal or Reduction of Alpha-Glucuronidase Activity

[0189] The present invention also relates to methods of producing a mutant of a parent cell, which comprises disrupting or deleting a polynucleotide, or a portion 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.

[0190] The mutant cell may be constructed by reducing or eliminating expression of a nucleotide sequence encoding a polypeptide of the present invention using methods 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 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.

[0191] 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 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.

[0192] Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues.

[0193] 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.

[0194] 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.

[0195] 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.

[0196] 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.

[0197] 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.

[0198] It is disclosed that the polypeptide-deficient mutant cells so created are particularly useful as host cells for the expression of native and/or heterologous polypeptides. Therefore, methods are disclosed for 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 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.

[0199] In a further aspect, a method is disclosed for producing a protein product essentially free of alpha-glucuronidase activity by fermentation of a cell 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 alpha-glucuronidase 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.

[0200] Further disclosed is a method of producing a protein product essentially free of alpha-glucuronidase 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 alpha-glucuronidase activity substantially, and recovering the product from the culture 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 alpha-glucuronidase inhibitor.

[0201] It is possible to remove at least 60%, preferably at least 75%, more preferably at least 85%, still more preferably at least 95%, and most preferably at least 99% of the alpha-glucuronidase activity. Complete removal of alpha-glucuronidase activity may be obtained by use of this method.

[0202] 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 sufficient period of time to attain the desired effect, where typically, 30 to 60 minutes is sufficient.

[0203] The methods used for cultivation and purification of the product of interest may be performed by methods known in the art.

[0204] The methods disclosed for producing an essentially alpha-glucuronidase-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, 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, polyphenoloxidase, proteolytic enzyme, ribonuclease, transferase, transglutaminase, or xylanase. The alpha-glucuronidase-deficient cells may also be used to express heterologous proteins of pharmaceutical interest such as hormones, growth factors, receptors, and the like.

[0205] It will be understood that the term "eukaryotic polypeptides" includes not only 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.

Methods of Inhibiting Expression of a Polypeptide Having Alpha-Glucuronidase Activity

[0206] The present invention also relates to methods of inhibiting the expression of a polypeptide having alpha-glucuronidase 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.

[0207] 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.

[0208] 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, 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).

[0209] The dsRNAs of the present invention can be used in gene-silencing. 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-function mutation in a cell. 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.

Compositions

[0210] 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 alpha-glucuronidase activity of the composition has been increased, e.g., with an enrichment factor of at least 1.1.

[0211] The composition may comprise a polypeptide of the present invention as the major enzymatic component, e.g., a mono-component composition. Alternatively, the composition may comprise multiple enzymatic activities, such as an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, haloperoxidase, invertase, laccase, lipase, mannosidase, oxidase, pectinolytic enzyme, peptidoglutaminase, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, or xylanase. The additional enzyme(s) may be produced, for example, by a microorganism belonging to the genus *Aspergillus*, preferably *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus fumigatus*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, 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*.

[0212] 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.

[0213] 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

[0214] The present invention is also directed to methods of using the polypeptides having alpha-glucuronidase activity as defined in claim 1, or compositions thereof. The polypeptides of the present invention can be used for degrading or converting plant cell walls or any xylan-containing material, e.g., lignocellulose, originating from plant cells walls (see, for example, WO 2002/18561). Examples of various uses are described below. The dosage of the polypeptides of the present invention and other conditions under which the polypeptides are used may be determined on the basis of methods known in the art.

[0215] The enzymatic degradation of a xylan-containing material is facilitated by full or partial removal of the side branches. The polypeptides of the present invention are preferably used in conjunction with other xylan degrading enzymes such as xylanases, acetyl xylan esterases, arabinofuranosidases, xylosidases, feruloyl esterases, glucuronidases, and a combination thereof, in processes wherein xylan-containing material is to be degraded. For example, acetyl groups can be removed by acetyl xylan esterases; arabinose groups by alpha-arabinosidases; feruloyl groups by feruloyl esterases, and glucuronic acid groups by alpha-glucuronidases. The oligomers released by the xylanases, or by a combination of xylanases and side branch-hydrolyzing enzymes, can be further degraded to free xylose by beta-xylosidases.

[0216] The present invention also relates to methods for degrading or converting a cellulosic or xylan-containing material, comprising: treating the cellulosic or xylan-containing material with an enzyme composition in the presence of a polypeptide having alpha-glucuronidase activity of the present invention. In a preferred aspect, the method further comprises recovering the degraded or converted cellulosic or xylan-containing material.

[0217] The present invention also relates to methods for producing a fermentation product, comprising: (a) saccharifying a cellulosic or xylan-containing material with an enzyme composition in the presence of a polypeptide having alpha-glucuronidase

activity of the present invention; (b) fermenting the saccharified cellulosic or xylan-containing material with one or more fermenting microorganisms to produce the fermentation product; and (c) recovering the fermentation product from the fermentation.

[0218] The present invention also relates to methods of fermenting a cellulosic or xylan-containing material, comprising: fermenting the cellulosic or xylan-containing material with one or more fermenting microorganisms, wherein the cellulosic or xylan-containing material is saccharified with an enzyme composition in the presence of a polypeptide having alpha-glucuronidase activity of the present invention. In a preferred aspect, the fermenting of the cellulosic or xylan-containing material produces a fermentation product. In another preferred aspect, the method further comprises recovering the fermentation product from the fermentation.

[0219] The methods of the present invention can be used to saccharify a cellulosic or xylan-containing material to fermentable sugars and convert the fermentable sugars to many useful substances, e.g., fuel, potable ethanol, and/or fermentation products (e.g., acids, alcohols, ketones, gases, and the like). The production of a desired fermentation product from cellulosic or xylan-containing material typically involves pretreatment, enzymatic hydrolysis (saccharification), and fermentation.

[0220] The processing of cellulosic or xylan-containing material according to the present invention can be accomplished using processes conventional in the art. Moreover, the methods of the present invention can be implemented using any conventional biomass processing apparatus configured to operate in accordance with the invention.

[0221] Hydrolysis (saccharification) and fermentation, separate or simultaneous, include, but are not limited to, separate hydrolysis and fermentation (SHF); simultaneous saccharification and fermentation (SSF); simultaneous saccharification and cofermentation (SSCF); hybrid hydrolysis and fermentation (HHF); separate hydrolysis and co-fermentation (SHCF); hybrid hydrolysis and fermentation (HHCF); and direct microbial conversion (DMC). SHF uses separate process steps to first enzymatically hydrolyze cellulosic or xylan-containing material to fermentable sugars, e.g., glucose, cellobiose, cellotriose, and pentose sugars, and then ferment the fermentable sugars to ethanol. In SSF, the enzymatic hydrolysis of cellulosic or xylan-containing material and the fermentation of sugars to ethanol are combined in one step (Philippidis, G. P., 1996, Cellulose bioconversion technology, in Handbook on Bioethanol: Production and Utilization, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212). SSCF involves the cofermentation of multiple sugars (Sheehan, J., and Himmel, M., 1999, Enzymes, energy and the environment: A strategic perspective on the U.S. Department of Energy's research and development activities for bioethanol, Biotechnol. Prog. 15: 817-827). HHF involves a separate hydrolysis step, and in addition a simultaneous saccharification and hydrolysis step, which can be carried out in the same reactor. The steps in an HHF process can be carried out at different temperatures, i.e., high temperature enzymatic saccharification followed by SSF at a lower temperature that the fermentation strain can tolerate. DMC combines all three processes (enzyme production, hydrolysis, and fermentation) in one or more steps where the same organism is used to produce the enzymes for conversion of the cellulosic or xylan-containing material to fermentable sugars and to convert the fermentable sugars into a final product (Lynd, L. R., Weimer, P. J., van Zyl, W. H., and Pretorius, I. S., 2002, Microbial cellulose utilization: Fundamentals and biotechnology, Microbiol. Mol. Biol. Reviews 66: 506-577). It is understood herein that any method known in the art comprising pretreatment, enzymatic hydrolysis (saccharification), fermentation, or a combination thereof can be used in the practicing the methods of the present invention.

[0222] A conventional apparatus can include a fed-batch stirred reactor, a batch stirred reactor, a continuous flow stirred reactor with ultrafiltration, and/or a continuous plug-flow column reactor (Fernanda de Castilhos Corazza, Flávio Faria de Moraes, Gisella Maria Zanin and Ivo Neitzel, 2003, Optimal control in fed-batch reactor for the cellobiose hydrolysis, Acta Scientiarum. Technology 25: 33-38; Gusakov, A. V., and Sinitsyn, A. P., 1985, Kinetics of the enzymatic hydrolysis of cellulose: 1. A mathematical model for a batch reactor process, Enz. Microb. Technol. 7: 346-352), an attrition reactor (Ryu, S. K., and Lee, J. M., 1983, Bioconversion of waste cellulose by using an attrition bioreactor, Biotechnol. Bioeng. 25: 53-65), or a reactor with intensive stirring induced by an electromagnetic field (Gusakov, A. V., Sinitsyn, A. P., Davydkin, I. Y., Davydkin, V. Y., Protas, O. V., 1996, Enhancement of enzymatic cellulose hydrolysis using a novel type of bioreactor with intensive stirring induced by electromagnetic field, Appl. Biochem. Biotechnol. 56: 141-153). Additional reactor types include: fluidized bed, upflow blanket, immobilized, and extruder type reactors for hydrolysis and/or fermentation.

[0223] Pretreatment. In practicing the methods of the present invention, any pretreatment process known in the art can be used to disrupt plant cell wall components of cellulosic and/or xylan-containing material (Chandra et al., 2007, Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics? Adv. Biochem. Engin./Biotechnol. 108: 67-93; Galbe and Zacchi, 2007, Pretreatment of lignocellulosic materials for efficient bioethanol production, Adv. Biochem. Engin. / Biotechnol. 108: 41-65; Hendriks and Zeeman, 2009, Pretreatments to enhance the digestibility of lignocellulosic biomass, Bioresource Technol. 100: 10-18; Mosier et al., 2005, Features of promising technologies for pretreatment of lignocellulosic biomass, Bioresource Technol. 96: 673-686; Taherzadeh and Karimi, 2008, Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review, Int. J. of Mol. Sci. 9: 1621-1651; Yang and Wyman, 2008, Pretreatment: the key to unlocking low-cost cellulosic ethanol,

Biofuels Bioproducts and Biorefining-Biofpr. 2: 26-40).

[0224] Cellulosic or xylan-containing material can also be subjected to particle size reduction, pre-soaking, wetting, washing, or conditioning prior to pretreatment using methods known in the art.

[0225] Conventional pretreatments include, but are not limited to, steam pretreatment (with or without explosion), dilute acid pretreatment, hot water pretreatment, alkaline pretreatment, lime pretreatment, wet oxidation, wet explosion, ammonia fiber explosion, organosolv pretreatment, and biological pretreatment. Additional pretreatments include ammonia percolation, ultrasound, electroporation, microwave, supercritical CO₂, supercritical H₂O, ozone, and gamma irradiation pretreatments.

[0226] Cellulosic or xylan-containing material can be pretreated before hydrolysis and/or fermentation. Pretreatment is preferably performed prior to the hydrolysis. Alternatively, the pretreatment can be carried out simultaneously with enzyme hydrolysis to release fermentable sugars, such as glucose, xylose, and/or cellobiose. In most cases the pretreatment step itself results in some conversion of biomass to fermentable sugars (even in absence of enzymes).

[0227] Steam Pretreatment. In steam pretreatment, cellulosic or xylan-containing material is heated to disrupt the plant cell wall components, including lignin, hemicellulose, and cellulose to make the cellulose and other fractions, e.g., hemicellulose, accessible to enzymes. Cellulosic or xylan-containing material is passed to or through a reaction vessel where steam is injected to increase the temperature to the required temperature and pressure and is retained therein for the desired reaction time. Steam pretreatment is preferably done at 140-230°C, more preferably 160-200°C, and most preferably 170-190°C, where the optimal temperature range depends on any addition of a chemical catalyst. Residence time for the steam pretreatment is preferably 1-15 minutes, more preferably 3-12 minutes, and most preferably 4-10 minutes, where the optimal residence time depends on temperature range and any addition of a chemical catalyst. Steam pretreatment allows for relatively high solids loadings, so that cellulosic or xylan-containing material is generally only moist during the pretreatment. The steam pretreatment is often combined with an explosive discharge of the material after the pretreatment, which is known as steam explosion, that is, rapid flashing to atmospheric pressure and turbulent flow of the material to increase the accessible surface area by fragmentation (Duff and Murray, 1996, *Bioresource Technology* 85: 1-33; Galbe and Zacchi, 2002, *Appl. Microbiol. Biotechnol.* 59: 618-628; U.S. Patent Application No. 20020164730). During steam pretreatment, hemicellulose acetyl groups are cleaved and the resulting acid autocatalyzes partial hydrolysis of the hemicellulose to monosaccharides and oligosaccharides. Lignin is removed to only a limited extent.

[0228] A catalyst such as H₂SO₄ or SO₂ (typically 0.3 to 3% w/w) is often added prior to steam pretreatment, which decreases the time and temperature, increases the recovery, and improves enzymatic hydrolysis (Ballesteros et al., 2006, *Appl. Biochem. Biotechnol.* 129-132: 496-508; Varga et al., 2004, *Appl. Biochem. Biotechnol.* 113-116: 509-523; Sassner et al., 2006, *Enzyme Microb. Technol.* 39: 756-762).

[0229] Chemical Pretreatment: The term "chemical treatment" refers to any chemical pretreatment that promotes the separation and/or release of cellulose, hemicellulose, and/or lignin. Examples of suitable chemical pretreatment processes include, for example, dilute acid pretreatment, lime pretreatment, wet oxidation, ammonia fiber/freeze explosion (AFEX), ammonia percolation (APR), and organosolv pretreatments.

[0230] In dilute acid pretreatment, cellulosic or xylan-containing material is mixed with dilute acid, typically H₂SO₄, and water to form a slurry, heated by steam to the desired temperature, and after a residence time flashed to atmospheric pressure. The dilute acid pretreatment can be performed with a number of reactor designs, e.g., plug-flow reactors, counter-current reactors, or continuous counter-current shrinking bed reactors (Duff and Murray, 1996, *supra*; Schell et al., 2004, *Bioresource Technol.* 91: 179-188; Lee et al., 1999, *Adv. Biochem. Eng. Biotechnol.* 65: 93-115).

[0231] Several methods of pretreatment under alkaline conditions can also be used. These alkaline pretreatments include, but are not limited to, lime pretreatment, wet oxidation, ammonia percolation (APR), and ammonia fiber/freeze explosion (AFEX).

[0232] Lime pretreatment is performed with calcium carbonate, sodium hydroxide, or ammonia at low temperatures of 85-150°C and residence times from 1 hour to several days (Wyman et al., 2005, *Bioresource Technol.* 96: 1959-1966; Mosier et al., 2005, *Bioresource Technol.* 96: 673-686). WO 2006/110891, WO 2006/11899, WO 2006/11900, and WO 2006/110901 disclose pretreatment methods using ammonia.

[0233] Wet oxidation is a thermal pretreatment performed typically at 180-200°C for 5-15 minutes with addition of an oxidative agent such as hydrogen peroxide or over-pressure of oxygen (Schmidt and Thomsen, 1998, *Bioresource Technol.* 64: 139-151;

Palonen et al., 2004, Appl. Biochem. Biotechnol. 117: 1-17; Varga et al., 2004, Biotechnol. Bioeng. 88: 567-574; Martin et al., 2006, J. Chem. Technol. Biotechnol. 81: 1669-1677). The pretreatment is performed at preferably 1-40% dry matter, more preferably 2-30% dry matter, and most preferably 5-20% dry matter, and often the initial pH is increased by the addition of alkali such as sodium carbonate.

[0234] A modification of the wet oxidation pretreatment method, known as wet explosion (combination of wet oxidation and steam explosion), can handle dry matter up to 30%. In wet explosion, the oxidizing agent is introduced during pretreatment after a certain residence time. The pretreatment is then ended by flashing to atmospheric pressure (WO 2006/032282).

[0235] Ammonia fiber explosion (AFEX) involves treating cellulosic or xylan-containing material with liquid or gaseous ammonia at moderate temperatures such as 90-100°C and high pressure such as 17-20 bar for 5-10 minutes, where the dry matter content can be as high as 60% (Gollapalli et al., 2002, Appl. Biochem. Biotechnol. 98: 23-35; Chundawat et al., 2007, Biotechnol. Bioeng. 96: 219-231; Alizadeh et al., 2005, Appl. Biochem. Biotechnol. 121: 1133-1141; Teymouri et al., 2005, Bioresource Technol. 96: 2014-2018). AFEX pretreatment results in the depolymerization of cellulose and partial hydrolysis of hemicellulose. Lignin-carbohydrate complexes are cleaved.

[0236] Organosolv pretreatment delignifies cellulosic or xylan-containing material by extraction using aqueous ethanol (40-60% ethanol) at 160-200°C for 30-60 minutes (Pan et al., 2005, Biotechnol. Bioeng. 90: 473-481; Pan et al., 2006, Biotechnol. Bioeng. 94: 851-861; Kurabi et al., 2005, Appl. Biochem. Biotechnol. 121: 219-230). Sulphuric acid is usually added as a catalyst. In organosolv pretreatment, the majority of hemicellulose is removed.

[0237] Other examples of suitable pretreatment methods are described by Schell et al., 2003, Appl. Biochem. and Biotechnol. Vol. 105-108, p. 69-85, and Mosier et al., 2005, Bioresource Technology 96: 673-686, and U.S. Published Application 2002/0164730.

[0238] In one aspect, the chemical pretreatment is preferably carried out as an acid treatment, and more preferably as a continuous dilute and/or mild acid treatment. The acid is typically sulfuric acid, but other acids can also be used, such as acetic acid, citric acid, nitric acid, phosphoric acid, tartaric acid, succinic acid, hydrogen chloride, or mixtures thereof. Mild acid treatment is conducted in the pH range of preferably 1-5, more preferably 1-4, and most preferably 1-3. In one aspect, the acid concentration is in the range from preferably 0.01 to 20 wt % acid, more preferably 0.05 to 10 wt % acid, even more preferably 0.1 to 5 wt % acid, and most preferably 0.2 to 2.0 wt % acid. The acid is contacted with cellulosic or xylan-containing material and held at a temperature in the range of preferably 160-220°C, and more preferably 165-195°C, for periods ranging from seconds to minutes to, e.g., 1 second to 60 minutes.

[0239] In another aspect, pretreatment is carried out as an ammonia fiber explosion step (AFEX pretreatment step).

[0240] In another aspect, pretreatment takes place in an aqueous slurry. In preferred aspects, cellulosic or xylan-containing material is present during pretreatment in amounts preferably between 10-80 wt%, more preferably between 20-70 wt%, and most preferably between 30-60 wt%, such as around 50 wt%. The pretreated cellulosic or xylan-containing material can be unwashed or washed using any method known in the art, e.g., washed with water.

[0241] Mechanical Pretreatment: The term "mechanical pretreatment" refers to various types of grinding or milling (e.g., dry milling, wet milling, or vibratory ball milling).

[0242] Physical Pretreatment: The term "physical pretreatment" refers to any pretreatment that promotes the separation and/or release of cellulose, hemicellulose, and/or lignin from cellulosic or xylan-containing material. For example, physical pretreatment can involve irradiation (e.g., microwave irradiation), steaming/steam explosion, hydrothermolysis, and combinations thereof.

[0243] Physical pretreatment can involve high pressure and/or high temperature (steam explosion). In one aspect, high pressure means pressure in the range of preferably about 300 to about 600 psi, more preferably about 350 to about 550 psi, and most preferably about 400 to about 500 psi, such as around 450 psi. In another aspect, high temperature means temperatures in the range of about 100 to about 300°C, preferably about 140 to about 235°C. In a preferred aspect, mechanical pretreatment is performed in a batch-process, steam gun hydrolyzer system that uses high pressure and high temperature as defined above, e.g., a Sunds Hydrolyzer available from Sunds Defibrator AB, Sweden.

[0244] Combined Physical and Chemical Pretreatment: Cellulosic or xylan-containing material can be pretreated both physically and chemically. For instance, the pretreatment step can involve dilute or mild acid treatment and high temperature and/or pressure treatment. The physical and chemical pretreatments can be carried out sequentially or simultaneously, as desired. A

mechanical pretreatment can also be included.

[0245] Accordingly, in a preferred aspect, cellulosic or xylan-containing material is subjected to mechanical, chemical, or physical pretreatment, or any combination thereof, to promote the separation and/or release of cellulose, hemicellulose, and/or lignin.

[0246] **Biological Pretreatment:** The term "biological pretreatment" refers to any biological pretreatment that promotes the separation and/or release of cellulose, hemicellulose, and/or lignin from cellulosic or xylan-containing material. Biological pretreatment techniques can involve applying lignin-solubilizing microorganisms (see, for example, Hsu, T.-A., 1996, Pretreatment of biomass, in *Handbook on Bioethanol: Production and Utilization*, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212; Ghosh and Singh, 1993, Physicochemical and biological treatments for enzymatic/microbial conversion of cellulosic biomass, *Adv. Appl. Microbiol.* 39: 295-333; McMillan, J. D., 1994, Pretreating lignocellulosic biomass: a review, in *Enzymatic Conversion of Biomass for Fuels Production*, Himmel, M. E., Baker, J. O., and Overend, R. P., eds., ACS Symposium Series 566, American Chemical Society, Washington, DC, chapter 15; Gong, C. S., Cao, N. J., Du, J., and Tsao, G. T., 1999, Ethanol production from renewable resources, in *Advances in Biochemical Engineering/Biotechnology*, Scheper, T., ed., Springer-Verlag Berlin Heidelberg, Germany, 65: 207-241; Olsson and Hahn-Hagerdal, 1996, Fermentation of lignocellulosic hydrolysates for ethanol production, *Enz. Microb. Tech.* 18: 312-331; and Vallander and Eriksson, 1990, Production of ethanol from lignocellulosic materials: State of the art, *Adv. Biochem. Eng./Biotechnol.* 42: 63-95).

[0247] **Saccharification.** In the hydrolysis step, also known as saccharification, the pretreated cellulosic or xylan-containing material is hydrolyzed to break down cellulose and hemicellulose to fermentable sugars, such as glucose, cellobiose, xylose, xylulose, arabinose, mannose, galactose, and/or soluble oligosaccharides. The hydrolysis is performed enzymatically by an enzyme composition in the presence of a polypeptide having alpha-glucuronidase activity of the present invention. The enzymes of the compositions can also be added sequentially.

[0248] Enzymatic hydrolysis is preferably carried out in a suitable aqueous environment under conditions that can be readily determined by one skilled in the art. In a preferred aspect, hydrolysis is performed under conditions suitable for the activity of the enzyme(s), *i.e.*, optimal for the enzyme(s). The hydrolysis can be carried out as a fed batch or continuous process where the pretreated cellulosic or xylan-containing material (substrate) is fed gradually to, for example, an enzyme containing hydrolysis solution.

[0249] The saccharification is generally performed in stirred-tank reactors or fermentors under controlled pH, temperature, and mixing conditions. Suitable process time, temperature and pH conditions can readily be determined by one skilled in the art. For example, the saccharification can last up to 200 hours, but is typically performed for preferably about 12 to about 96 hours, more preferably about 16 to about 72 hours, and most preferably about 24 to about 48 hours. The temperature is in the range of preferably about 25°C to about 70°C, more preferably about 30°C to about 65°C, and more preferably about 40°C to 60°C, in particular about 50°C. The pH is in the range of preferably about 3 to about 8, more preferably about 3.5 to about 7, and most preferably about 4 to about 6, in particular about pH 5. The dry solids content is in the range of preferably about 5 to about 50 wt %, more preferably about 10 to about 40 wt %, and most preferably about 20 to about 30 wt %.

[0250] The enzyme composition preferably comprises enzymes having cellulolytic activity and/or xylan degrading activity. In one aspect, the enzyme composition comprises one or more xylan degrading enzymes. In another aspect, the enzyme composition comprises one or more cellulolytic enzymes. In another aspect, the enzyme composition comprises one or more xylan degrading enzymes and one or more cellulolytic enzymes.

[0251] The one or more xylan degrading enzymes are preferably selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase. The one or more cellulolytic enzymes are preferably selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

[0252] In another preferred aspect, the enzyme composition further or even further comprises a polypeptide having cellulolytic enhancing activity (see, for example, WO 2005/074647, WO 2005/074656, and WO 2007/089290). In another aspect, the enzyme composition may further or even further comprise one or more additional enzyme activities to improve the degradation of the cellulose-containing material. Preferred additional enzymes are hemicellulases (*e.g.*, alpha-D-glucuronidases, alpha-L-arabinofuranosidases, endo-mannanases, beta-mannosidases, alpha-galactosidases, endo-alpha-L-arabinanases, beta-galactosidases), carbohydrate-esterases (*e.g.*, acetylxylan esterases, acetyl-mannan esterases, ferulic acid esterases, coumaric acid esterases, glucuronoyl esterases), pectinases, proteases, ligninolytic enzymes (*e.g.*, laccases, manganese peroxidases, lignin peroxidases, H₂O₂-producing enzymes, oxidoreductases), expansins, swollenins, or mixtures thereof. In the methods of the present invention, the additional enzyme(s) can be added prior to or during fermentation, *e.g.*, during saccharification or during or

after propagation of the fermenting microorganism(s).

[0253] One or more components of the enzyme composition may be wild-type proteins, recombinant proteins, or a combination of wild-type proteins and recombinant proteins. For example, one or more components may be native proteins of a cell, which is used as a host cell to express recombinantly one or more (several) other components of the enzyme composition. One or more components of the enzyme composition may be produced as monocomponents, which are then combined to form the enzyme composition. The enzyme composition may be a combination of multicomponent and monocomponent protein preparations.

[0254] The enzymes used in the methods of the present invention may be in any form suitable for use in the processes described herein, such as, for example, a crude fermentation broth with or without cells removed, a semi-purified or purified enzyme preparations, or a host cell as a source of the enzymes. The enzyme composition may be a dry powder or granulate, a non-dusting granulate, a liquid, a stabilized liquid, or a stabilized protected enzyme. Liquid enzyme preparations may, for instance, be stabilized by adding stabilizers such as a sugar, a sugar alcohol or another polyol, and/or lactic acid or another organic acid according to established processes.

[0255] The optimum amounts of the enzymes and polypeptides having alpha-glucuronidase activity depend on several factors including, but not limited to, the mixture of component cellulolytic enzymes, the cellulosic or xylan-containing material, the concentration of the cellulosic or xylan-containing material, the pretreatment(s) of the cellulosic or xylan-containing material, temperature, time, pH, and inclusion of fermenting organism (e.g., yeast for Simultaneous Saccharification and Fermentation).

[0256] In a preferred aspect, an effective amount of cellulolytic enzyme(s) and/or xylan-degrading enzyme(s) to cellulosic or xylan-containing material is about 0.5 to about 50 mg, preferably at about 0.5 to about 40 mg, more preferably at about 0.5 to about 25 mg, more preferably at about 0.75 to about 20 mg, more preferably at about 0.75 to about 15 mg, even more preferably at about 0.5 to about 10 mg, and most preferably at about 2.5 to about 10 mg per g of cellulosic or xylan-containing material.

[0257] In another preferred aspect, an effective amount of polypeptide(s) having alpha-glucuronidase activity to cellulosic or xylan-containing material is about 0.01 to about 50.0 mg, preferably about 0.01 to about 40 mg, more preferably about 0.01 to about 30 mg, more preferably about 0.01 to about 20 mg, more preferably about 0.01 to about 10 mg, more preferably about 0.01 to about 5 mg, more preferably at about 0.025 to about 1.5 mg, more preferably at about 0.05 to about 1.25 mg, more preferably at about 0.075 to about 1.25 mg, more preferably at about 0.1 to about 1.25 mg, even more preferably at about 0.15 to about 1.25 mg, and most preferably at about 0.25 to about 1.0 mg per g of cellulosic or xylan-containing material.

[0258] In another preferred aspect, an effective amount of polypeptide(s) having alpha-glucuronidase activity to cellulolytic enzyme(s) and/or xylan-degrading enzyme(s) is about 0.005 to about 1.0 g, preferably at about 0.01 to about 1.0 g, more preferably at about 0.15 to about 0.75 g, more preferably at about 0.15 to about 0.5 g, more preferably at about 0.1 to about 0.5 g, even more preferably at about 0.1 to about 0.5 g, and most preferably at about 0.05 to about 0.2 g per g of cellulolytic enzyme(s).

[0259] The enzymes can be derived or obtained from any suitable origin, including, bacterial, fungal, yeast, plant, or mammalian origin. The term "obtained" means herein that the enzyme may have been isolated from an organism that naturally produces the enzyme as a native enzyme. The term "obtained" also means herein that the enzyme may have been produced recombinantly in a host cell employing methods described herein, wherein the recombinantly produced enzyme is either native or foreign to the host cell or has a modified amino acid sequence, e.g., having one or more amino acids that are deleted, inserted and/or substituted, i.e., a recombinantly produced enzyme that is a mutant and/or a fragment of a native amino acid sequence or an enzyme produced by nucleic acid shuffling processes known in the art. Encompassed within the meaning of a native enzyme are natural variants and within the meaning of a foreign enzyme are variants obtained recombinantly, such as by site-directed mutagenesis or shuffling.

[0260] A polypeptide having cellulolytic enzyme activity or xylan degrading activity may be a bacterial polypeptide. For example, the polypeptide may be a gram positive bacterial polypeptide such as a *Bacillus*, *Streptococcus*, *Streptomyces*, *Staphylococcus*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Clostridium*, *Geobacillus*, or *Oceanobacillus* polypeptide having cellulolytic enzyme activity or xylan degrading activity, or a Gram negative bacterial polypeptide such as an *E. coli*, *Pseudomonas*, *Salmonella*, *Campylobacter*, *Helicobacter*, *Flavobacterium*, *Fusobacterium*, *Ilyobacter*, *Neisseria*, or *Ureaplasma* polypeptide having cellulolytic enzyme activity or xylan degrading activity.

[0261] 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 megaferium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, or *Bacillus thuringiensis* polypeptide having cellulolytic

enzyme activity or xylan degrading activity.

[0262] In another preferred aspect, the polypeptide is a *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, or *Streptococcus equi* subsp. *Zooepidemicus* polypeptide having cellulolytic enzyme activity or xylan degrading activity.

[0263] In another preferred aspect, the polypeptide is a *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, or *Streptomyces lividans* polypeptide having cellulolytic enzyme activity or xylan degrading activity.

[0264] The polypeptide having cellulolytic enzyme activity or xylan degrading activity may also be a fungal polypeptide, and more preferably a yeast polypeptide such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* polypeptide having cellulolytic enzyme activity or xylan degrading activity; or more preferably a filamentous fungal polypeptide such as an *Acremonium*, *Agaricus*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Botryosphaeria*, *Ceriporiopsis*, *Chaetomidium*, *Chrysosporium*, *Claviceps*, *Cochliobolus*, *Coprinopsis*, *Coptotermes*, *Corynascus*, *Cryphonectria*, *Cryptococcus*, *Diplodia*, *Exidia*, *Filibasidium*, *Fusarium*, *Gibberella*, *Holomastigotoides*, *Humicola*, *Irpex*, *Lentinula*, *Leptosphaeria*, *Magnaporthe*, *Melanocarpus*, *Meripilus*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Piromyces*, *Poitrasia*, *Pseudoplectania*, *Pseudotrichonympha*, *Rhizomucor*, *Schizophyllum*, *Scytalidium*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trichoderma*, *Trichophaea*, *Verticillium*, *Volvariella*, or *Xylaria* polypeptide having cellulolytic enzyme activity or xylan degrading activity.

[0265] In a preferred aspect, the polypeptide is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, or *Saccharomyces oviformis* polypeptide having cellulolytic enzyme activity or xylan degrading activity.

[0266] In another preferred aspect, the polypeptide is an *Acremonium cellulolyticus*, *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus fumigatus*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium tropicum*, *Chrysosporium merdarium*, *Chrysosporium inops*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium zonatum*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola grisea*, *Humicola insolens*, *Humicola lanuginosa*, *Irpex lacteus*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium funiculosum*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Thielavia achromatica*, *Thielavia albomyces*, *Thielavia albopilosa*, *Thielavia australeinsis*, *Thielavia fimeti*, *Thielavia microspora*, *Thielavia ovispora*, *Thielavia peruviana*, *Thielavia spededonium*, *Thielavia setosa*, *Thielavia subthermophila*, *Thielavia terrestris*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, *Trichoderma viride*, or *Trichophaea saccata* polypeptide having cellulolytic enzyme activity or xylan degrading activity.

[0267] Chemically modified or protein engineered mutants of polypeptides having cellulolytic enzyme activity or xylan degrading activity may also be used.

[0268] One or more components of the enzyme composition may be a recombinant component, i.e., produced by cloning of a DNA sequence encoding the single component and subsequent cell transformed with the DNA sequence and expressed in a host (see, for example, WO 91/17243 and WO 91/17244). The host is preferably a heterologous host (enzyme is foreign to host), but the host may under certain conditions also be a homologous host (enzyme is native to host). Monocomponent cellulolytic proteins may also be prepared by purifying such a protein from a fermentation broth.

[0269] Examples of commercial cellulolytic protein preparations suitable for use in the present invention include, for example, CELLIC™ Ctec (Novozymes A/S), CELLUCLAST™ (Novozymes A/S), and NOVOZYM™ 188 (Novozymes A/S). Other commercially available preparations comprising cellulase that may be used include CELLUZYME™, CEREFLO™ and ULTRAFLO™ (Novozymes A/S), LAMINEX™ and SPEZYME™ CP (Genencor Int.), ROHAMENT™ 7069 W (Röhm GmbH), and FIBREZYME® LDI, FIBREZYME® LBR, or VISCOSTAR® 150L (Dyadic International, Inc.). The cellulase enzymes are added in amounts effective from about 0.001 to about 5.0 wt % of solids, more preferably from about 0.025 to about 4.0 wt % of solids, and most preferably from about 0.005 to about 2.0 wt % of solids.

[0270] Examples of bacterial endoglucanases that can be used in the methods of the present invention, include, but are not limited to, an *Acidothermus cellulolyticus* endoglucanase (WO 91/05039; WO 93/15186; U.S. Patent No. 5,275,944; WO

96/02551; U.S. Patent No. 5,536,655, WO 00/70031, WO 05/093050); *Thermobifida fusca* endoglucanase III (WO 05/093050); and *Thermobifida fusca* endoglucanase V (WO 05/093050).

[0271] Examples of fungal endoglucanases that can be used in the methods of the present invention, include, but are not limited to, a *Trichoderma reesei* endoglucanase I (Penttilä et al., 1986, Gene 45: 253-263; GENBANK™ accession no. M15665); *Trichoderma reesei* endoglucanase II (Saloheimo, et al., 1988, Gene 63:11-22; GENBANK™ accession no. M19373); *Trichoderma reesei* endoglucanase III (Okada et al., 1988, Appl. Environ. Microbiol. 64: 555-563; GENBANK™ accession no. AB003694); *Trichoderma reesei* endoglucanase IV (Saloheimo et al., 1997, Eur. J. Biochem. 249: 584-591; GENBANK™ accession no. Y11113); and *Trichoderma reesei* endoglucanase V (Saloheimo et al., 1994, Molecular Microbiology 13: 219-228; GENBANK™ accession no. Z33381); *Aspergillus aculeatus* endoglucanase (Ooi et al., 1990, Nucleic Acids Research 18: 5884); *Aspergillus kawachii* endoglucanase (Sakamoto et al., 1995, Current Genetics 27: 435-439); *Erwinia carotovora* endoglucanase (Saarilahti et al., 1990, Gene 90: 9-14); *Fusarium oxysporum* endoglucanase (GENBANK™ accession no. L29381); *Humicola grisea* var. *thermoidea* endoglucanase (GENBANK™ accession no. AB003107); *Melanocarpus albomyces* endoglucanase (GENBANK™ accession no. MAL515703); *Neurospora crassa* endoglucanase (GENBANK™ accession no. XM_324477); *Humicola insolens* endoglucanase V; *Myceliophthora thermophila* CBS 117.65 endoglucanase; basidiomycete CBS 495.95 endoglucanase; basidiomycete CBS 494.95 endoglucanase; *Thielavia terrestris* NRRL 8126 CEL6B endoglucanase; *Thielavia terrestris* NRRL 8126 CEL6C endoglucanase; *Thielavia terrestris* NRRL 8126 CEL7C endoglucanase; *Thielavia terrestris* NRRL 8126 CEL7E endoglucanase; *Thielavia terrestris* NRRL 8126 CEL7F endoglucanase; *Cladorrhinum foecundissimum* ATCC 62373 CEL7A endoglucanase; and *Trichoderma reesei* strain No. VTT-D-80133 endoglucanase (GENBANK™ accession no. M15665).

[0272] Examples of cellobiohydrolases useful in the methods of the present invention include, but are not limited to, *Trichoderma reesei* cellobiohydrolase I; *Trichoderma reesei* cellobiohydrolase II; *Humicola insolens* cellobiohydrolase I, *Myceliophthora thermophila* cellobiohydrolase II, *Thielavia terrestris* cellobiohydrolase II (CEL6A), *Chaetomium thermophilum* cellobiohydrolase I, and *Chaetomium thermophilum* cellobiohydrolase II.

[0273] Examples of beta-glucosidases useful in the methods of the present invention include, but are not limited to, *Aspergillus oryzae* beta-glucosidase; *Aspergillus fumigatus* beta-glucosidase; *Penicillium brasilianum* IBT 20888 beta-glucosidase; *Aspergillus niger* beta-glucosidase; and *Aspergillus aculeatus* beta-glucosidase.

[0274] The *Aspergillus oryzae* polypeptide having beta-glucosidase activity can be obtained according to WO 2002/095014. The *Aspergillus fumigatus* polypeptide having beta-glucosidase activity can be obtained according to WO 2005/047499. The *Penicillium brasilianum* polypeptide having beta-glucosidase activity can be obtained according to WO 2007/019442. The *Aspergillus niger* polypeptide having beta-glucosidase activity can be obtained according to Dan et al., 2000, J. Biol. Chem. 275: 4973-4980. The *Aspergillus aculeatus* polypeptide having beta-glucosidase activity can be obtained according to Kawaguchi et al., 1996, Gene 173: 287-288.

[0275] The beta-glucosidase may be a fusion protein. In one aspect, the beta-glucosidase is the *Aspergillus oryzae* beta-glucosidase variant BG fusion protein or the *Aspergillus oryzae* beta-glucosidase fusion protein obtained according to WO 2008/057637.

[0276] Other endoglucanases, cellobiohydrolases, and beta-glucosidases are disclosed in numerous Glycosyl Hydrolase families using the classification according to Henrissat B., 1991, A classification of glycosyl hydrolases based on amino-acid sequence similarities, Biochem. J. 280: 309-316, and Henrissat B., and Bairoch A., 1996, Updating the sequence-based classification of glycosyl hydrolases, Biochem. J. 316: 695-696.

[0277] Other cellulolytic enzymes that may be used in the present invention are described in EP 495,257, EP 531,315, EP 531,372, WO 89/09259, WO 94/07998, WO 95/24471, WO 96/11262, WO 96/29397, WO 96/034108, WO 97/14804, WO 98/08940, WO 98/012307, WO 98/13465, WO 98/015619, WO 98/015633, WO 98/028411, WO 99/06574, WO 99/10481, WO 99/025846, WO 99/025847, WO 99/031255, WO 2000/009707, WO 2002/050245, WO 2002/0076792, WO 2002/101078, WO. 2003/027306, WO 2003/052054, WO 2003/052055, WO 2003/052056, WO 2003/052057, WO 2003/052118, WO 2004/016760, WO 2004/043980, WO 2004/048592, WO 2005/001065, WO 2005/028636, WO 2005/093050, WO 2005/093073, WO 2006/074005, WO 2006/117432, WO 2007/071818, WO 2007/071820, WO 2008/008070, WO 2008/008793, U.S. Patent No. 4,435,307, U.S. Patent No. 5,457,046, U.S. Patent No. 5,648,263, U.S. Patent No. 5,686,593, U.S. Patent No. 5,691,178, U.S. Patent No. 5,763,254, and U.S. Patent No. 5,776,757.

[0278] In the methods of the present invention, any polypeptide having cellulolytic enhancing activity can be used.

[0279] In a first aspect, the polypeptide having cellulolytic enhancing activity comprises the following motifs:

[ILMV]-P-X(4,5)-G-X-Y-[ILMV]-X-R-X-[EQ]-X(4)-[HNQ] and [FW]-[TF]-K-[AIV],
wherein X is any amino acid, X(4,5) is any amino acid at 4 or 5 contiguous positions, and X(4) is any amino acid at 4 contiguous positions.

[0280] The polypeptide comprising the above-noted motifs may further comprise:

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

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

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

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

[0281] In a preferred aspect, the polypeptide having cellulolytic enhancing activity further comprises H-X(1,2)-G-P-X(3)-[YW]-[AILMV]. In another preferred aspect, the isolated polypeptide having cellulolytic enhancing activity further comprises [EQ]-X-Y-X(2)-C-X-[EHQN]-[FILV]-X-[ILV]. In another preferred aspect, the polypeptide having cellulolytic enhancing activity further comprises H-X(1,2)-G-P-X(3)-[YW]-[AILMV] and [EQ]-X-Y-X(2)-C-X-[EHQN]-[FILV]-X-[ILV].

[0282] In a second aspect, the polypeptide having cellulolytic enhancing activity comprises the following motif:

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

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

[0283] Examples of polypeptides having cellulolytic enhancing activity useful in the methods of the present invention include, but are not limited to, polypeptides having cellulolytic enhancing activity from *Thielavia terrestris* (WO 2005/074647); polypeptides having cellulolytic enhancing activity from *Thermoascus aurantiacus* (WO 2005/074656); and polypeptides having cellulolytic enhancing activity from *Trichoderma reesei* (WO 2007/089290).

[0284] Examples of commercial xylan degrading enzyme preparations suitable for use in the present invention include, for example, SHEARZYME™ (Novozymes A/S), CELLIC™ Htec (Novozymes A/S), VISCOZYME® (Novozymes A/S), ULTRAFLO® (Novozymes A/S), PULPZYME® HC (Novozymes A/S), MULTIFECT® Xylanase (Genencor), ECOPULP® TX-200A (AB Enzymes), HSP 6000 Xylanase (DSM), DEPOL™ 333P (Biocatalysts Limit, Wales, UK), DEPOL™ 740L (Biocatalysts Limit, Wales, UK), and DEPOL™ 762P (Biocatalysts Limit, Wales, UK).

[0285] Examples of xylanases useful in the methods of the present invention include, but are not limited to, *Aspergillus aculeatus* xylanase (GeneSeqP:AAR63790; WO 94/21785), *Aspergillus fumigatus* xylanases (WO 2006/078256), and *Thielavia terrestris* NRRL 8126 xylanases (WO 2009/079210).

[0286] Examples of beta-xylosidases useful in the methods of the present invention include, but are not limited to, *Trichoderma reesei* beta-xylosidase (UniProtKB/TrEMBL accession number Q92458), *Talaromyces emersonii* (SwissProt accession number Q8X212), and *Neurospora crassa* (SwissProt accession number Q7SOW4).

[0287] Examples of acetylxylan esterases useful in the methods of the present invention include, but are not limited to, *Hypocrea jecorina* acetylxylan esterase (WO 2005/001036), *Neurospora crassa* acetylxylan esterase (UniProt accession number q7s259), *Thielavia terrestris* NRRL 8126 acetylxylan esterase (WO 2009/042846), *Chaetomium globosum* acetylxylan esterase (UniProt accession number Q2GWX4), *Chaetomium gracile* acetylxylan esterase (GeneSeqP accession number AAB82124), *Phaeosphaeria nodorum* acetylxylan esterase (UniProt accession number Q0UHU1), and *Humicola insolens* DSM 1800 acetylxylan esterase (WO 2009/073709).

[0288] Examples of ferulic acid esterases useful in the methods of the present invention include, but are not limited to, *Humicola*

insolens DSM 1800 feruloyl esterase (WO 2009/076122), *Neurospora crassa* feruloyl esterase (UniProt accession number Q9HGR3), and *Neosartorya fischeri* feruloyl esterase (UniProt Accession number A1D9T4).

[0289] Examples of arabinofuranosidases useful in the methods of the present invention include, but are not limited to, *Humicola insolens* DSM 1800 arabinofuranosidase (WO 2009/073383) and *Aspergillus niger* arabinofuranosidase (GeneSeqP accession number AAR94170).

[0290] Examples of alpha-glucuronidases include, but are not limited to, *Aspergillus clavatus* alpha-glucuronidase (UniProt accession number alcc12), *Trichoderma reesei* alpha-glucuronidase (Uniprot accession number Q99024), *Talaromyces emersonii* alpha-glucuronidase (UniProt accession number Q8X211), *Aspergillus niger* alpha-glucuronidase (Uniprot accession number Q96WX9), *Aspergillus terreus* alpha-glucuronidase (SwissProt accession number Q0CJP9), and *Aspergillus fumigatus* alpha-glucuronidase (SwissProt accession number Q4WW45).

[0291] The enzymes and proteins used in the methods of the present invention may be produced by fermentation of the above-noted microbial strains on a nutrient medium containing suitable carbon and nitrogen sources and inorganic salts, using procedures known in the art (see, e.g., Bennett, J.W. and LaSure, L. (eds.), *More Gene Manipulations in Fungi*, Academic Press, CA, 1991). Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). Temperature ranges and other conditions suitable for growth and enzyme production are known in the art (see, e.g., Bailey, J.E., and Ollis, D.F., *Biochemical Engineering Fundamentals*, McGraw-Hill Book Company, NY, 1986).

[0292] The fermentation can be any method of cultivation of a cell resulting in the expression or isolation of an enzyme. Fermentation may, therefore, be understood as comprising shake flask cultivation, or small- or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the enzyme to be expressed or isolated. The resulting enzymes produced by the methods described above may be recovered from the fermentation medium and purified by conventional procedures.

[0293] Fermentation. The fermentable sugars obtained from the pretreated and hydrolyzed cellulosic or xylan-containing material can be fermented by one or more fermenting microorganisms capable of fermenting the sugars directly or indirectly into a desired fermentation product. "Fermentation" or "fermentation process" refers to any fermentation process or any process comprising a fermentation step. Fermentation processes also include fermentation processes used in the consumable alcohol industry (e.g., beer and wine), dairy industry (e.g., fermented dairy products), leather industry, and tobacco industry. The fermentation conditions depend on the desired fermentation product and fermenting organism and can easily be determined by one skilled in the art.

[0294] In the fermentation step, sugars, released from cellulosic or xylan-containing material as a result of the pretreatment and enzymatic hydrolysis steps, are fermented to a product, e.g., ethanol, by a fermenting organism, such as yeast. Hydrolysis (saccharification) and fermentation can be separate or simultaneous, as described herein.

[0295] Any suitable hydrolyzed cellulosic or xylan-containing material can be used in the fermentation step in practicing the present invention. The material is generally selected based on the desired fermentation product, i.e., the substance to be obtained from the fermentation, and the process employed, as is well known in the art.

[0296] The term "fermentation medium" is understood herein to refer to a medium before the fermenting microorganism(s) is(are) added, such as, a medium resulting from a saccharification process, as well as a medium used in a simultaneous saccharification and fermentation process (SSF).

[0297] "Fermenting microorganism" refers to any microorganism, including bacterial and fungal organisms, suitable for use in a desired fermentation process to produce a fermentation product. The fermenting organism can be C₆ and/or C₅ fermenting organisms, or a combination thereof. Both C₆ and C₅ fermenting organisms are well known in the art. Suitable fermenting microorganisms are able to ferment, i.e., convert, sugars, such as glucose, xylose, xylulose, arabinose, maltose, mannose, galactose, or oligosaccharides, directly or indirectly into the desired fermentation product.

[0298] Examples of bacterial and fungal fermenting organisms producing ethanol are described by Lin et al., 2006, *Appl. Microbiol. Biotechnol.* 69: 627-642.

[0299] Examples of fermenting microorganisms that can ferment C₆ sugars include bacterial and fungal organisms, such as

yeast. Preferred yeast includes strains of the *Saccharomyces* spp., preferably *Saccharomyces cerevisiae*.

[0300] Examples of fermenting organisms that can ferment C5 sugars include bacterial and fungal organisms, such as yeast. Preferred C5 fermenting yeast include strains of *Pichia*, preferably *Pichia stipitis*, such as *Pichia stipitis* CBS 5773; strains of *Candida*, preferably *Candida boidinii*, *Candida brassicae*, *Candida sheatae*, *Candida diddensii*, *Candida pseudotropicalis*, or *Candida utilis*.

[0301] Other fermenting organisms include strains of *Zymomonas*, such as *Zymomonas mobilis*; *Hansenula*, such as *Hansenula anomala*; *Kluyveromyces*, such as *K. fragilis*; *Schizosaccharomyces*, such as *S. pombe*; and *E. coli*, especially *E. coli* strains that have been genetically modified to improve the yield of ethanol.

[0302] In a preferred aspect, the yeast is a *Saccharomyces* spp. In a more preferred aspect, the yeast is *Saccharomyces cerevisiae*. In another more preferred aspect, the yeast is *Saccharomyces distaticus*. In another more preferred aspect, the yeast is *Saccharomyces uvarum*. In another preferred aspect, the yeast is a *Kluyveromyces*. In another more preferred aspect, the yeast is *Kluyveromyces marxianus*. In another more preferred aspect, the yeast is *Kluyveromyces fragilis*. In another preferred aspect, the yeast is a *Candida*. In another more preferred aspect, the yeast is *Candida boidinii*. In another more preferred aspect, the yeast is *Candida brassicae*. In another more preferred aspect, the yeast is *Candida diddensii*. In another more preferred aspect, the yeast is *Candida pseudotropicalis*. In another more preferred aspect, the yeast is *Candida utilis*. In another preferred aspect, the yeast is a *Clavispora*. In another more preferred aspect, the yeast is *Clavispora lusitaniae*. In another more preferred aspect, the yeast is *Clavispora opuntiae*. In another preferred aspect, the yeast is a *Pachysolen*. In another more preferred aspect, the yeast is *Pachysolen tannophilus*. In another preferred aspect, the yeast is a *Pichia*. In another more preferred aspect, the yeast is a *Pichia stipitis*. In another preferred aspect, the yeast is a *Bretanomyces*. In another more preferred aspect, the yeast is *Bretanomyces clausenii* (Philippidis, G. P., 1996, Cellulose bioconversion technology, in Handbook on Bioethanol: Production and Utilization, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212).

[0303] Bacteria that can efficiently ferment hexose and pentose to ethanol include, for example, *Zymomonas mobilis* and *Clostridium thermocellum* (Philippidis, 1996, *supra*).

[0304] In a preferred aspect, the bacterium is a *Zymomonas*. In a more preferred aspect, the bacterium is *Zymomonas mobilis*. In another preferred aspect, the bacterium is a *Clostridium*. In another more preferred aspect, the bacterium is *Clostridium thermocellum*.

[0305] Commercially available yeast suitable for ethanol production includes, e.g., ETHANOL RED™ yeast (available from Fermentis/Lesaffre, USA), FALI™ (available from Fleischmann's Yeast, USA), SUPERSTART™ and THERMOSACC™ fresh yeast (available from Ethanol Technology, WI, USA), BIOFERM™ AFT and XR (available from NABC - North American Bioproducts Corporation, GA, USA), GERT STRAND™ (available from Gert Strand AB, Sweden), and FERMIO™ (available from DSM Specialties).

[0306] In a preferred aspect, the fermenting microorganism has been genetically modified to provide the ability to ferment pentose sugars, such as xylose utilizing, arabinose utilizing, and xylose and arabinose co-utilizing microorganisms.

[0307] The cloning of heterologous genes into various fermenting microorganisms has led to the construction of organisms capable of converting hexoses and pentoses to ethanol (cofermentation) (Chen and Ho, 1993, Cloning and improving the expression of *Pichia stipitis* xylose reductase gene in *Saccharomyces cerevisiae*, Appl. Biochem. Biotechnol. 39-40: 135-147; Ho et al., 1998, Genetically engineered *Saccharomyces* yeast capable of effectively cofermenting glucose and xylose, Appl. Environ. Microbiol. 64: 1852-1859; Kotter and Ciriacy, 1993, Xylose fermentation by *Saccharomyces cerevisiae*, Appl. Microbiol. Biotechnol. 38: 776-783; Walfridsson et al., 1995, Xylose-metabolizing *Saccharomyces cerevisiae* strains overexpressing the TKL1 and TAL1 genes encoding the pentose phosphate pathway enzymes transketolase and transaldolase, Appl. Environ. Microbiol. 61: 4184-4190; Kuyper et al., 2004, Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle, FEMS Yeast Research 4: 655-664; Beall et al., 1991, Parametric studies of ethanol production from xylose and other sugars by recombinant *Escherichia coli*, Biotech. Bioeng. 38: 296-303; Ingram et al., 1998, Metabolic engineering of bacteria for ethanol production, Biotechnol. Bioeng. 58: 204-214; Zhang et al., 1995, Metabolic engineering of a pentose metabolism pathway in ethanologenic *Zymomonas mobilis*, Science 267: 240-243; Deanda et al., 1996, Development of an arabinose-fermenting *Zymomonas mobilis* strain by metabolic pathway engineering, Appl. Environ. Microbiol. 62: 4465-4470; WO 2003/062430, xylose isomerase).

[0308] In a preferred aspect, the genetically modified fermenting microorganism is *Saccharomyces cerevisiae*. In another preferred aspect, the genetically modified fermenting microorganism is *Zymomonas mobilis*. In another preferred aspect, the

genetically modified fermenting microorganism is *Escherichia coli*. In another preferred aspect, the genetically modified fermenting microorganism is *Klebsiella oxytoca*. In another preferred aspect, the genetically modified fermenting microorganism is *Kluyveromyces* sp.

[0309] The fermenting microorganism is typically added to the degraded lignocellulose or hydrolysate and the fermentation is performed for about 8 to about 96 hours, such as about 24 to about 60 hours. The temperature is typically between about 26°C to about 60°C, in particular about 32°C or 50°C, and at about pH 3 to about pH 8, such as around pH 4-5, 6, or 7.

[0310] In a preferred aspect, the yeast and/or another microorganism is applied to the degraded cellulosic or xylan-containing material and the fermentation is performed for about 12 to about 96 hours, such as typically 24-60 hours. In a preferred aspect, the temperature is preferably between about 20°C to about 60°C, more preferably about 25°C to about 50°C, and most preferably about 32°C to about 50°C, in particular about 32°C or 50°C, and the pH is generally from about pH 3 to about pH 7, preferably around pH 4-7. However, some fermenting organisms, e.g., bacteria, have higher fermentation temperature optima. Yeast or another microorganism is preferably applied in amounts of approximately 10^5 to 10^{12} , preferably from approximately 10^7 to 10^{10} , especially approximately 2×10^8 viable cell count per ml of fermentation broth. Further guidance in respect of using yeast for fermentation can be found in, e.g., "The Alcohol Textbook" (Editors K. Jacques, T.P. Lyons and D.R. Kelsall, Nottingham University Press, United Kingdom 1999).

[0311] For ethanol production, following the fermentation the fermented slurry is distilled to extract the ethanol. The ethanol obtained according to the methods of the invention can be used as, e.g., fuel ethanol, drinking ethanol, *i.e.*, potable neutral spirits, or industrial ethanol.

[0312] A fermentation stimulator can be used in combination with any of the processes described herein to further improve the fermentation process, and in particular, the performance of the fermenting microorganism, such as, rate enhancement and ethanol yield. A "fermentation stimulator" refers to stimulators for growth of the fermenting microorganisms, in particular, yeast. Preferred fermentation stimulators for growth include vitamins and minerals. Examples of vitamins include multivitamins, biotin, pantothenate, nicotinic acid, meso-inositol, thiamine, pyridoxine, para-aminobenzoic acid, folic acid, riboflavin, and Vitamins A, B, C, D, and E. See, for example, Alfenore et al., Improving ethanol production and viability of *Saccharomyces cerevisiae* by a vitamin feeding strategy during fed-batch process, Springer-Verlag (2002), which is hereby incorporated by reference. Examples of minerals include minerals and mineral salts that can supply nutrients comprising P, K, Mg, S, Ca, Fe, Zn, Mn, and Cu.

[0313] Fermentation products: A fermentation product can be any substance derived from the fermentation. The fermentation product can be, without limitation, an alcohol (e.g., arabinitol, butanol, ethanol, glycerol, methanol, 1,3-propanediol, sorbitol, and xylitol); an organic acid (e.g., acetic acid, acetonic acid, adipic acid, ascorbic acid, citric acid, 2,5-diketo-D-gluconic acid, formic acid, fumaric acid, glucaric acid, gluconic acid, glucuronic acid, glutaric acid, 3-hydroxypropionic acid, itaconic acid, lactic acid, malic acid, malonic acid, oxalic acid, propionic acid, succinic acid, and xylonic acid); a ketone (e.g., acetone); an amino acid (e.g., aspartic acid, glutamic acid, glycine, lysine, serine, and threonine); and a gas (e.g., methane, hydrogen (H₂), carbon dioxide (CO₂), and carbon monoxide (CO)). The fermentation product can also be protein as a high value product.

[0314] In a preferred aspect, the fermentation product is an alcohol. It will be understood that the term "alcohol" encompasses a substance that contains one or more hydroxyl moieties. In a more preferred aspect, the alcohol is arabinitol. In another more preferred aspect, the alcohol is butanol. In another more preferred aspect, the alcohol is ethanol. In another more preferred aspect, the alcohol is glycerol. In another more preferred aspect, the alcohol is methanol. In another more preferred aspect, the alcohol is 1,3-propanediol. In another more preferred aspect, the alcohol is sorbitol. In another more preferred aspect, the alcohol is xylitol. See, for example, Gong, C. S., Cao, N. J., Du, J., and Tsao, G. T., 1999, Ethanol production from renewable resources, in *Advances in Biochemical Engineering/Biotechnology*, Scheper, T., ed., Springer-Verlag Berlin Heidelberg, Germany, 65: 207-241; Silveira, M. M., and Jonas, R., 2002, The biotechnological production of sorbitol, *Appl. Microbiol. Biotechnol.* 59: 400-408; Nigam, P., and Singh, D., 1995, Processes for fermentative production of xylitol - a sugar substitute, *Process Biochemistry* 30 (2): 117-124; Ezeji, T. C., Qureshi, N. and Blaschek, H. P., 2003, Production of acetone, butanol and ethanol by *Clostridium beijerinckii* BA101 and in situ recovery by gas stripping, *World Journal of Microbiology and Biotechnology* 19 (6): 595-603.

[0315] In another preferred aspect, the fermentation product is an organic acid. In another more preferred aspect, the organic acid is acetic acid. In another more preferred aspect, the organic acid is acetonic acid. In another more preferred aspect, the organic acid is adipic acid. In another more preferred aspect, the organic acid is ascorbic acid. In another more preferred aspect, the organic acid is citric acid. In another more preferred aspect, the organic acid is 2,5-diketo-D-gluconic acid. In another more preferred aspect, the organic acid is formic acid. In another more preferred aspect, the organic acid is fumaric acid. In another more preferred aspect, the organic acid is glucaric acid. In another more preferred aspect, the organic acid is gluconic acid. In

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

[0316] In another preferred aspect, the fermentation product is a ketone. It will be understood that the term "ketone" encompasses a substance that contains one or more ketone moieties. In another more preferred aspect, the ketone is acetone. See, for example, Qureshi and Blaschek, 2003, *supra*.

[0317] In another preferred aspect, the fermentation product is an amino acid. In another more preferred aspect, the organic acid is aspartic acid. In another more preferred aspect, the amino acid is glutamic acid. In another more preferred aspect, the amino acid is glycine. In another more preferred aspect, the amino acid is lysine. In another more preferred aspect, the amino acid is serine. In another more preferred aspect, the amino acid is threonine. See, for example, Richard, A., and Margaritis, A., 2004, Empirical modeling of batch fermentation kinetics for poly(glutamic acid) production and other microbial biopolymers, Biotechnology and Bioengineering 87 (4): 501-515.

[0318] In another preferred aspect, the fermentation product is a gas. In another more preferred aspect, the gas is methane. In another more preferred aspect, the gas is H₂. In another more preferred aspect, the gas is CO₂. In another more preferred aspect, the gas is CO. See, for example, Kataoka, N., A. Miya, and K. Kiriya, 1997, Studies on hydrogen production by continuous culture system of hydrogen-producing anaerobic bacteria, Water Science and Technology 36 (6-7): 41-47; and Gunaseelan V.N. in Biomass and Bioenergy, Vol. 13 (1-2), pp. 83-114, 1997, Anaerobic digestion of biomass for methane production: A review.

[0319] Recovery. The fermentation product(s) can be optionally recovered from the fermentation medium using any method known in the art including, but not limited to, chromatography, electrophoretic procedures, differential solubility, distillation, or extraction. For example, alcohol is separated from the fermented cellulosic or xylan-containing material and purified by conventional methods of distillation. Ethanol with a purity of up to about 96 vol.% can be obtained, which can be used as, for example, fuel ethanol, drinking ethanol, *i.e.*, potable neutral spirits, or industrial ethanol.

Other Uses

[0320] The polypeptides of the present invention may also be used with limited activity of other xylanolytic enzymes to degrade xylans for production of oligosaccharides. The oligosaccharides may be used as bulking agents, like arabinoxylan oligosaccharides released from cereal cell wall material, or of more or less purified arabinoxylans from cereals.

[0321] The polypeptides of the present invention may also be used in combination with other xylanolytic enzymes to degrade xylans to xylose and other monosaccharides (U.S. Patent No. 5,658,765). The released xylose may be converted to other compounds.

[0322] The polypeptides of the present invention may be used together with other enzymes like glucanases to improve the extraction of oil from oil-rich plant material, like corn-oil from corn-embryos.

[0323] The polypeptides of the present invention may also be used in baking to improve the development, elasticity, and/or stability of dough and/or the volume, crumb structure, and/or anti-staling properties of the baked product (see U.S. Patent No. 5,693,518). The polypeptides may also be used for the preparation of dough or baked products prepared from any type of flour or meal (e.g., based on wheat, rye, barley, oat, or maize). The baked products produced with a polypeptide of the present invention include bread, rolls, baguettes and the like. For baking purposes a polypeptide of the present invention may be used as the only or major enzymatic activity, or may be used in combination with other enzymes such as a xylanase, a lipase, an amylase, an oxidase (e.g., glucose oxidase, peroxidase), a laccase and/or a protease.

[0324] The polypeptides of the present invention may also be used for modification of animal feed and may exert their effect either *in vitro* (by modifying components of the feed) or *in vivo* to improve feed digestibility and increase the efficiency of its

utilization (U.S. Patent No. 6,245,546). The polypeptides may be added to animal feed compositions containing high amounts of arabinoxylans and glucuronoxylans, e.g., feed containing cereals such as barley, wheat, rye, oats, or maize. When added to feed the polypeptide will improve the *in vivo* break-down of plant cell wall material partly due to a reduction of intestinal viscosity (Bedford et al., 1993, Proceedings of the 1 st Symposium on Enzymes in Animal Nutrition, pp. 73-77), whereby improved utilization of the plant nutrients by the animal is achieved. Thereby, the growth rate and/or feed conversion ratio (*i.e.*, the weight of ingested feed relative to weight gain) of the animal is improved.

[0325] The polypeptides of the present invention may also be used in the paper and pulp industry, *inter alia*, in bleaching processes to enhance the brightness of bleached pulps whereby the amount of chlorine used in the bleaching stages is reduced, and to increase the freeness of pulps in the recycled paper process (Eriksson, 1990, Wood Science and Technology 24: 79-101; Paice et al., 1988, Biotechnol. and Bioeng. 32: 235-239, and Pommier et al., 1989, Tappi Journal 187-191). The treatment of lignocellulosic pulp may be performed, for example, as described in U.S. Patent No. 5,658,765, WO 93/08275, WO 91/02839, and WO 92/03608.

[0326] The polypeptides of the present invention may also be used in beer brewing, in particular to improve the filterability of wort containing, for example, barley and/or sorghum malt (WO 2002/24926). The polypeptides may be used in the same manner as pentosanases conventionally used for brewing, *e.g.*, as described by Viëtor et al., 1993, J. Inst. Brew. 99: 243-248; and EP 227159. Furthermore, the polypeptides may be used for treatment of brewers spent grain, *i.e.*, residuals from beer wort production containing barley or malted barley or other cereals, so as to improve the utilization of the residuals for, *e.g.*, animal feed.

[0327] The polypeptides of the present invention may be used for separation of components of plant cell materials, in particular of cereal components such as wheat components. Of particular interest is the separation of wheat into gluten and starch, *i.e.*, components of considerable commercial interest. The separation process may be performed by use of methods known in the art, such as the so-called batter process (or wet milling process) performed as a hydroclone or a decanter process. In the batter process, the starting material is a dilute pumpable dispersion of the plant material such as wheat to be subjected to separation. In a wheat separation process the dispersion is made normally from wheat flour and water.

[0328] The polypeptides of the invention may also be used in the preparation of fruit or vegetable juice in order to increase yield.

[0329] The polypeptides of the present invention may also be used as a component of an enzymatic scouring system for textiles.

[0330] The polypeptides of the present invention may also be used in laundry detergent applications in combination with other enzyme functionalities.

Signal Peptide

[0331] The present invention also relates to an isolated polynucleotide encoding a signal peptide comprising or consisting of amino acids 1 to 15 of SEQ ID NO: 2.

[0332] In a preferred aspect, the isolated polynucleotide encoding a signal peptide comprises or consists of nucleotides 1 to 45 of SEQ ID NO: 1.

[0333] The present invention also relates to nucleic acid constructs comprising a gene encoding a protein, wherein the gene is operably linked to the isolated polynucleotide encoding the signal peptide, wherein the gene is foreign to the polynucleotide encoding the signal peptide.

[0334] The present invention also relates to recombinant expression vectors and recombinant host cells comprising such nucleic acid constructs.

[0335] The present invention also relates to methods of producing a protein, comprising:

1. (a) cultivating a recombinant host cell comprising a gene encoding a protein operably linked to the such a polynucleotide encoding a signal peptide, wherein the gene is foreign to the polynucleotide under conditions conducive for production of the protein; and (b) recovering the protein; and (b) recovering the protein.

[0336] 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 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 proteins and hybrid proteins.

[0337] 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, 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 xylanase.

[0338] The gene may be obtained from any prokaryotic, eukaryotic, or other source.

[0339] The present invention is further described by the following examples that should not be construed as limiting the scope of the invention.

Examples

Materials

[0340] Chemicals used as buffers and substrates were commercial products of at least reagent grade.

Strains

[0341] *Humicola insolens* DSM 1800 was used as the source of a Family 67 glycosidase gene encoding a polypeptide having alpha-glucuronidase activity.

Media

[0342] PDA plates were composed per liter of 39 g of potato dextrose agar.

[0343] YP medium was composed per liter of 10 g of yeast extract and 20 g of Bacto peptone.

Alpha-glucuronidase activity assay

[0344] The assay solution for determining alpha-glucuronidase activity is composed of 0.16 ml of substrate (2 mg of aldotriouronic acid-aldobiuronic acid [80:20] in 0.05 M sodium acetate pH 5.0 buffer) and 0.04 ml of enzyme solution (total volume, 0.2 ml). The assay is initiated by addition of the enzyme sample. After 30 minutes of incubation at 40°C, the reaction is stopped by boiling the sample for 4 minutes. Precipitate is removed by centrifugation (10,000 x g), after which the supernatant is transferred to a new tube. To the tube is added 0.6 ml of copper reagent prepared as described by Milner and Avigad, 1967, Carbohydrate Research 4: 359-351, and then the sample is boiled for 10 minutes and cooled on ice. Subsequently, 0.4 ml of arsenomolybdate reagent prepared as described by Nelson, 1944, J. Biol. Chem. 153: 375-380, is added. The sample is mixed gently, 0.8 ml of H₂O is added, and the absorbance at 600 nm is measured against water. A control is prepared by boiling a complete assay mixture at time zero, before incubation at 40°C. A substrate control is made by adding water instead of enzyme solution. A standard curve is prepared by using D-glucuronic acid.

Example 1: *Humicola insolens* DSM 1800 genomic DNA extraction

[0345] *Humicola insolens* DSM 1800 was grown on PDA plates at 45°C to confluence. Three 4 mm² squares were cut from the PDA plates and inoculated into 25 ml of YP medium containing 2% glucose in a baffled 125 ml shake flask at 41°C and 200 rpm for 2 days. Mycelia were harvested by filtration using MIRACLOTH® (Calbiochem, La Jolla, CA, USA), washed twice in deionized water, and frozen under liquid nitrogen. Frozen mycelia were ground, by mortar and pestle, to a fine powder, and total DNA was isolated using a DNEASY® Plant Maxi Kit (QIAGEN Inc., Valencia, CA, USA).

Example 2: Isolation of a partial fragment of a Family 67 glycosidase alpha-glucuronidase gene from *Humicola insolens* DSM 1800

[0346] Using the Consensus-Degenerate Hybrid Oligonucleotide Primer Program (CODEHOP; Rose et al., 1998, Nucleic Acids Research 26: 1628-1635), degenerate primers were designed to regions of homology with known alpha-glucuronidases. Degenerate primers employed to generate a fragment of the *Humicola insolens* GH67A alpha-glucuronidase gene are shown below.

Primer HinsAglucsense3:

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

Protein translation for degenerate primer HinsAglucsense3:

KYGPIDFQV

Primer HinsAglucAnti2:

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

Protein translation for degenerate primer HinsAglucAnti2:

TPDXLMLWF

[0347] To obtain the initial DNA fragment of the *Humicola insolens* GH67A alpha-glucuronidases gene, gradient PCR was performed at 6 different annealing temperatures ranging from 50°C to 70.5°C. Amplification reactions (25 µl) were composed of 80 ng of *Humicola insolens* DSM 1800 genomic DNA as template, 0.4 mM each of dATP, dTTP, dGTP, and dCTP, 50 pmol each of primer HinsAglucsense3 and primer HinsAglucAnti2, 1X ADVANTAGE® GC-Melt LA Buffer (Clontech Laboratories, Inc., Mountain View, CA, USA), and 1.25 units of ADVANTAGE® GC Genomic Polymerase Mix (Clontech Laboratories, Inc., Mountain View, CA, USA). The amplifications were performed using an EPPENDORF® MASTERCYCLER® 5333 (Eppendorf Scientific, Inc., Westbury, NY, USA) programmed for pre-denaturing at 95°C for 1 minute; 30 cycles each at a denaturing temperature of 95°C for 30 seconds; annealing temperature of 60°C +/- 10°C for 30 seconds (6 gradient options) and elongation at 72°C for 1 minute; and final elongation at 72°C for 6 minutes.

[0348] The reaction products were isolated by 1.0% agarose gel electrophoresis in TBE buffer (10.8 g of Tris base, 5.5 g of boric acid, and 4 ml of 0.5 M EDTA pH 8.0 per liter). A PCR product band of approximately 700 bp from an annealing temperature of 51.4°C was excised from the gel, purified using a QIAQUICK® Gel Extraction Kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's instructions, and sequenced. DNA sequencing of the PCR fragment was performed with a Perkin-Elmer Applied Biosystems Model 377 XL Automated DNA Sequencer (Perkin-Elmer/Applied Biosystems, Inc., Foster City, CA, USA) using dye-terminator chemistry (Giesecke et al., 1992, Journal of Virology Methods 38: 47-60) and primer walking strategy. A partial sequence was obtained, which contained homology to other known alpha-glucuronidases.

Example 3: Isolation of a full-length *Humicola insolens* GH67A alpha-glucuronidase gene

[0349] A full-length Family 67 alpha-glucuronidase gene was isolated from *Humicola insolens* DSM 1800 using a GENOMEWALKER™ Universal Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) according to the manufacturer's instructions. Briefly, total genomic DNA from *Humicola insolens* DSM 1800 was digested separately with four different restriction enzymes (*Dra* I, *Eco* RV, *Pvu* II, and *Stu* I) that leave blunt ends. Each batch of digested genomic DNA was then ligated separately

to the GENOMEWALKER™ Adaptor (Clontech Laboratories, Inc., Mountain View, CA, USA) to create four libraries. These four libraries were then employed as templates in PCR reactions using six gene-specific primers shown below, two for a primary and secondary PCR amplifying a portion upstream of the fragment approaching the 5' end of the gene (walk 1 N-terminus), two for a primary and secondary PCR amplifying upstream through the 5' end encoding the N-terminus of the alpha-glucuronidase gene (walk 2 N-terminus), and two for primary and secondary PCR amplifying downstream of the fragment through the 3' end encoding the C-terminus of the alpha-glucuronidase gene. The following primers were designed based on the partial Family 67 alpha-glucuronidase gene sequence from *Humicola insolens* described in Example 2.

N-terminus:

[0350]

Walk 1:

Primer HinsAGGSP15'R (primary):

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

Primer HinsAGGSP25'R (secondary):

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

Walk 2:

Primer HinsAGGWset2ntermRGSP1 (primary):

5'-ATAGCCACGCTCGACGCTGCCGTGAAT-3' (SEQ ID NO: 7)

Primer HinsAGGW2ntermGSP2R (secondary):

5'-ACTGGTTCACCCAGCGGATGGGAGCAT-3' (SEQ ID NO: 8)

C-terminus:

[0351]

Primer HinsAGGSP13'F (primary):

5'-TCCAGACGCTGACCGACATCCTGTTGG-3' (SEQ ID NO: 9)

Primer HinsAGGSP23'F (secondary):

5'-CCCGAGGTCGCCGAGCTGTATGAACAC-3' (SEQ ID NO: 10)

[0352] The primary amplifications were composed of 1 µl (approximately 6 ng) of each library as template, 0.4 mM each of dATP, dTTP, dGTP, and dCTP, 10 pmol of Adaptor Primer 1 (Clontech Laboratories, Inc., Mountain View, CA, USA), 50 pmol of primer HinsAGGSP15'R or HinsAGGWset2ntermRGSP1 or HinsAGGSP13'F, 1X ADVANTAGE® GC-Melt LA Buffer, and 1.25 units of ADVANTAGE® GC Genomic Polymerase Mix in a final volume of 25 µl. The amplifications were performed using an EPPENDORF® MASTERCYCLER® 5333 programmed for pre-denaturing at 95°C for 1 minute; 7 cycles each at a denaturing temperature of 95°C for 25 seconds; annealing and elongation at 72°C for 5 minutes; 32 cycles each at a denaturing temperature of 95°C for 25 seconds; annealing and elongation at 67°C for 5 minutes; and final elongation at 67°C for 7 minutes.

[0353] The secondary amplifications were composed of 1 µl of each primary PCR product as template, 0.4 mM each of dATP, dTTP, dGTP, and dCTP, 10 pmol of Adaptor Primer 2 (Clontech Laboratories, Inc., Mountain View, CA, USA), 50 pmol of primer HinsAGGSP25'R or HinsAGGW2ntermGSP2R or HinsAGGSP23'F, 1X ADVANTAGE® GC-Melt LA Buffer, and 1.25 units of ADVANTAGE® GC Genomic Polymerase Mix in a final volume of 25 µl. The amplifications were performed using an

EPPENDORF® MASTERCYCLER® 5333 programmed for pre-denaturing at 95°C for 1 minute; 5 cycles each at a denaturing temperature of 95°C for 25 seconds; annealing and elongation at 72°C for 5 minutes; 20 cycles each at a denaturing temperature of 95°C for 25 seconds; annealing and elongation at 67°C for 5 minutes; and final elongation at 67°C for 7 minutes.

[0354] The reaction products were isolated by 1.0% agarose gel electrophoresis in TBE buffer. From the 5' end N-terminus walk 1 PCR amplification, 3 product bands were analyzed: a 500-800 bp smear product band from the *Eco* RV library, an 800 bp product band from the *Pvu* II library, and a 500 bp product band from the *Stu* I library. The 3 product bands were excised from the gel, purified using a QIAQUICK® Gel Extraction Kit, cloned into pCR®2.1-TOPO® vector (Invitrogen, Carlsbad, CA, USA) using a TOPO® TA Cloning Kit (Invitrogen, Carlsbad, CA, USA), and sequenced as described in Example 2. From the 5' end N-terminus walk 2 PCR amplification, 3 product bands were analyzed: a 750 bp product band from the *Eco* RV library, a 400 bp doublet product band from the *Pvu* II library, and a 2.2 kb product band from the *Stu* I library. The 3 product bands were excised from the gel, purified using a QIAQUICK® Gel Extraction Kit, cloned into pCR®2.1-TOPO® vector using a TOPO® TA Cloning Kit, and sequenced as above. From the 3' end C-terminus PCR amplification, 2 product bands were analyzed: a 400 bp product band from the *Pvu* II library, and a 2.2 kb product band from the *Stu* I library. The 2 product bands were excised from the gel, purified using a QIAQUICK® Gel Extraction Kit, cloned into pCR®2.1-TOPO® vector using a TOPO® TA Cloning Kit, and sequenced as above.

Example 4: Construction of pHinsGH67A

[0355] DNA sequencing was performed with a Perkin-Elmer Applied Biosystems Model 377 XL Automated DNA Sequencer using dye-terminator chemistry (Giesecke *et al.*, 1992, *supra*) and primer walking strategy.

[0356] Nucleotide sequence data were scrutinized for quality and all sequences were compared to each other with assistance of PHRED/PHRAP software (University of Washington, Seattle, WA, USA). The PCR fragment sequence results were compared and aligned with the partial Family 67 alpha-glucuronidase gene sequence from *Humicola insolens* described in Example 2.

[0357] A gene model for the *Humicola insolens* alpha-glucuronidase sequence was constructed based on similarity of the encoded protein to known homologs of alpha-glucuronidases. Two synthetic oligonucleotide primers shown below were designed to PCR amplify the *Humicola insolens* DSM 1800 alpha-glucuronidase gene from the genomic DNA prepared in Example 1.

HinsAGBDInfNCO1:

5'-ACACAACCTGGCC**ATGAAGAGCCTCCTGCTGCTCGCGGCCATT**-3' (SEQ ID NO: 11)

HinsAGBDInfPac1:

5'-CAGTCACCTCTAGTTAT**CAATCCACAATCCCCTTTCCATGCG**-3' (SEQ ID NO: 12)

[0358] Bold letters represent coding sequence. The remaining sequence is homologous to the insertion sites of pBM120a (WO 2006/078256) for eventual cloning into the plasmid for expression of the *Humicola insolens* DSM 1800 alpha-glucuronidase gene in *Aspergillus niger* MBin120.

[0359] Fifty picomoles of each of the primers above were used in a PCR reaction composed of 80 ng of *Humicola insolens* genomic DNA, 1X Expand High Fidelity^{plus} Reaction Buffer® (Roche Diagnostics, Mannheim, Germany), 0.4 mM each of dATP, dTTP, dGTP, and dCTP, and 5 units of Expand High Fidelity^{plus} Enzyme Blend® in a final volume of 50 µl. The amplification was performed using an EPPENDORF® MASTERCYCLER® 5333 programmed for 1 cycle at 95°C for 1 minute; 30 cycles each at 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 2 minutes 30 seconds; and a final elongation at 72°C for 7 minutes. The heat block then went to a 4°C soak cycle.

[0360] The reaction products were isolated by 1.0% agarose gel electrophoresis in TBE buffer where an approximately 2.7 kb product band was excised from the gel, and purified using a QIAQUICK® Gel Extraction Kit.

[0361] The purified 2.7 kb gel fragment was cloned into pCR®2.1-TOPO® vector using a TOPO® TA CLONING Kit, to generate pHinsGH67A (Figure 2). The *Humicola insolens* GH67A insert in pHinsGH67A was confirmed by DNA sequencing. *E. coli* pHinsGH67A was deposited with the Agricultural Research Service Patent Culture Collection, Northern Regional Research

Center, 1815 University Street, Peoria, IL, USA, on July 24, 2008 as *E. coli* NRRL B-50155.

Example 5: Characterization of the *Humicola insolens* genomic sequence encoding a GH67A alpha-glucuronidase gene

[0362] The nucleotide sequence (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) of the *Humicola insolens* genomic sequence encoding a GH67A alpha-glucuronidase gene are shown in Figures 1A and 1B. The genomic fragment encodes a polypeptide of 861 amino acids. The % G+C content of the full-length coding sequence and the mature coding sequence are both 64.8%. Using the SignalP software program (Nielsen et al., 1997, Protein Engineering 10: 1-6), a signal peptide of 15 residues was predicted. The predicted mature protein contains 846 amino acids with a molecular mass of 95.6 kDa.

[0363] A comparative pairwise global alignment of amino acid sequences was determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) 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 mature polypeptide of the *Humicola insolens* Family 67 alpha-glucuronidase gene shared 63% identity to the deduced amino acid sequence of an *Aspergillus clavatus* alpha-glucuronidase (UniProt accession number alcc12).

Deposit of Biological Material

[0364] 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, Illinois, 61604, USA, and given the following accession number:

Deposit	Accession Number	Date of Deposit
<i>E. coli</i> pHinsGH67A	NRRL B-50155	July 24, 2008

[0365] 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 constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

[0366] Also disclosed is:

- [1] An isolated polypeptide having alpha-glucuronidase activity, selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 65% identity to the mature polypeptide of SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least medium stringency conditions with the mature polypeptide coding sequence of SEQ ID NO: 1 or its full-length complementary strand; (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 65% 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.
- [2] The polypeptide of paragraph 1, comprising an amino acid sequence having at least 65% identity to the mature polypeptide of SEQ ID NO: 2.
- [3] The polypeptide of paragraph 2, comprising an amino acid sequence having at least 70% identity to the mature polypeptide of SEQ ID NO: 2.
- [4] The polypeptide of paragraph 3, comprising an amino acid sequence having at least 75% identity to the mature polypeptide of SEQ ID NO: 2.
- [5] The polypeptide of paragraph 4, comprising an amino acid sequence having at least 80% identity to the mature polypeptide of SEQ ID NO: 2.
- [6] The polypeptide of paragraph 5, comprising an amino acid sequence having at least 85% identity to the mature polypeptide of SEQ ID NO: 2.
- [7] The polypeptide of paragraph 6, comprising an amino acid sequence having at least 90% identity to the mature polypeptide of SEQ ID NO: 2.
- [8] The polypeptide of paragraph 7, comprising an amino acid sequence having at least 95% identity to the mature polypeptide of SEQ ID NO: 2.

9. [9] The polypeptide of paragraph 8, comprising an amino acid sequence having at least 97% identity to the mature polypeptide of SEQ ID NO: 2.
10. [10] The polypeptide of paragraph 1, comprising or consisting of the amino acid sequence of SEQ ID NO: 2; or a fragment thereof having alpha-glucuronidase activity.
11. [11] The polypeptide of paragraph 10, comprising or consisting of the amino acid sequence of SEQ ID NO: 2.
12. [12] The polypeptide of paragraph 10, comprising or consisting of the mature polypeptide of SEQ ID NO: 2.
13. [13] The polypeptide of paragraph 1, which is encoded by a polynucleotide that hybridizes under at least medium stringency conditions with the mature polypeptide coding sequence of SEQ ID NO: 1 or its full-length complementary strand.
14. [14] The polypeptide of paragraph 13, which is encoded by a polynucleotide that hybridizes under at least medium-high stringency conditions with the mature polypeptide coding sequence of SEQ ID NO: 1 or its full-length complementary strand.
15. [15] The polypeptide of paragraph 14, which is encoded by a polynucleotide that hybridizes under at least high stringency conditions with the mature polypeptide coding sequence of SEQ ID NO: 1 or its full-length complementary strand.
16. [16] The polypeptide of paragraph 15, which is encoded by a polynucleotide that hybridizes under at least very high stringency conditions with the mature polypeptide coding sequence of SEQ ID NO: 1 or its full-length complementary strand.
17. [17] The polypeptide of paragraph 1, which is encoded by a polynucleotide comprising a nucleotide sequence having at least 65% identity to the mature polypeptide coding sequence of SEQ ID NO: 1.
18. [18] The polypeptide of paragraph 17, which is encoded by a polynucleotide comprising a nucleotide sequence having at least 70% identity to the mature polypeptide coding sequence of SEQ ID NO: 1.
19. [19] The polypeptide of paragraph 18, which is encoded by a polynucleotide comprising a nucleotide sequence having at least 75% identity to the mature polypeptide coding sequence of SEQ ID NO: 1.
20. [20] The polypeptide of paragraph 19, which is encoded by a polynucleotide comprising a nucleotide sequence having at least 80% identity to the mature polypeptide coding sequence of SEQ ID NO: 1.
21. [21] The polypeptide of paragraph 20, which is encoded by a polynucleotide comprising a nucleotide sequence having at least 85% identity to the mature polypeptide coding sequence of SEQ ID NO: 1.
22. [22] The polypeptide of paragraph 21, which is encoded by a polynucleotide comprising a nucleotide sequence having at least 90% identity to the mature polypeptide coding sequence of SEQ ID NO: 1.
23. [23] The polypeptide of paragraph 22, which is encoded by a polynucleotide comprising a nucleotide sequence having at least 95% identity to the mature polypeptide coding sequence of SEQ ID NO: 1.
24. [24] The polypeptide of paragraph 23, which is encoded by a polynucleotide comprising a nucleotide sequence having at least 97% identity to the mature polypeptide coding sequence of SEQ ID NO: 1.
25. [25] The polypeptide of paragraph 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 alpha-glucuronidase activity.
26. [26] The polypeptide of paragraph 25, which is encoded by a polynucleotide comprising or consisting of the nucleotide sequence of SEQ ID NO: 1.
27. [27] The polypeptide of paragraph 25, which is encoded by a polynucleotide comprising or consisting of the mature polypeptide coding sequence of SEQ ID NO: 1.
28. [28] The polypeptide of paragraph 1, wherein the polypeptide is a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the mature polypeptide of SEQ ID NO: 2.
29. [29] The polypeptide of paragraph 1, which is encoded by the polynucleotide contained in plasmid pHinsGH67A which is contained in *E. coli* NRRL B-50155.
30. [30] The polypeptide of any of paragraphs 1-29, wherein the mature polypeptide is amino acids 16 to 861 of SEQ ID NO: 2.
31. [31] The polypeptide of any of paragraphs 1-30, wherein the mature polypeptide coding sequence is nucleotides 46 to 2583 of SEQ ID NO: 1.
32. [32] An isolated polynucleotide comprising a nucleotide sequence that encodes the polypeptide of any of paragraphs 1-31.
33. [33] The isolated polynucleotide of paragraph 32, comprising 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.
34. [34] A nucleic acid construct comprising the polynucleotide of paragraph 32 or 33 operably linked to one or more (several) control sequences that direct the production of the polypeptide in an expression host.
35. [35] A recombinant expression vector comprising the nucleic acid construct of paragraph 34.
36. [36] A recombinant host cell comprising the polynucleotide of paragraph 32 or 33 operably linked to one or more (several) control sequences that direct the production of a polypeptide having alpha-glucuronidase activity.
37. [37] A method of producing the polypeptide of any of paragraphs 1-31, 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.

38. [38] A method of producing the polypeptide of any of paragraphs 1-31, 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.
39. [39] A method of producing a mutant of a parent cell, comprising disrupting or deleting a polynucleotide encoding the polypeptide, or a portion thereof, of any of paragraphs 1-31, which results in the mutant producing less of the polypeptide than the parent cell.
40. [40] A mutant cell produced by the method of paragraph 39.
41. [41] The mutant cell of paragraph 40, further comprising a gene encoding a native or heterologous protein.
42. [42] A method of producing a protein, comprising: (a) cultivating the mutant cell of paragraph 41 under conditions conducive for production of the protein; and (b) recovering the protein.
43. [43] The isolated polynucleotide of paragraph 32 or 33, obtained by (a) hybridizing a population of DNA under at least medium stringency conditions with the mature polypeptide coding sequence of SEQ ID NO: 1 or its full-length complementary strand; and (b) isolating the hybridizing polynucleotide, which encodes a polypeptide having alpha-glucuronidase activity.
44. [44] The isolated polynucleotide of paragraph 43, obtained by (a) hybridizing a population of DNA under at least medium-high stringency conditions with the mature polypeptide coding sequence of SEQ ID NO: 1 or its full-length complementary strand; and (b) isolating the hybridizing polynucleotide, which encodes a polypeptide having alpha-glucuronidase activity.
45. [45] The isolated polynucleotide of paragraph 44, obtained by (a) hybridizing a population of DNA under at least high stringency conditions with the mature polypeptide coding sequence of SEQ ID NO: 1 or its full-length complementary strand; and (b) isolating the hybridizing polynucleotide, which encodes a polypeptide having alpha-glucuronidase activity.
46. [46] The isolated polynucleotide of paragraph 45, obtained by (a) hybridizing a population of DNA under at least very high stringency conditions with the mature polypeptide coding sequence of SEQ ID NO: 1 or its full-length complementary strand; and (b) isolating the hybridizing polynucleotide, which encodes a polypeptide having alpha-glucuronidase activity.
47. [47] The isolated polynucleotide of any of paragraphs 43-46, wherein the mature polypeptide coding sequence is nucleotides 46 to 2583 of SEQ ID NO: 1.
48. [48] A method of producing a polynucleotide comprising a mutant nucleotide sequence encoding a polypeptide having alpha-glucuronidase activity, comprising: (a) introducing at least one mutation into the mature polypeptide coding sequence of SEQ ID NO: 1, wherein the mutant nucleotide sequence encodes a polypeptide comprising or consisting of the mature polypeptide of SEQ ID NO: 2; and (b) recovering the polynucleotide comprising the mutant nucleotide sequence.
49. [49] A mutant polynucleotide produced by the method of paragraph 48.
50. [50] A method of producing a polypeptide, comprising: (a) cultivating a cell comprising the mutant polynucleotide of paragraph 49 encoding the polypeptide under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.
51. [51] A method of producing the polypeptide of any of paragraphs 1-31, 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.
52. [52] A transgenic plant, plant part or plant cell transformed with a polynucleotide encoding the polypeptide of any of paragraphs 1-31.
53. [53] A double-stranded inhibitory RNA (dsRNA) molecule comprising a subsequence of the polynucleotide of paragraph 32 or 33, wherein optionally the dsRNA is a siRNA or a miRNA molecule.
54. [54] The double-stranded inhibitory RNA (dsRNA) molecule of paragraph 53, which is about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more duplex nucleotides in length.
55. [55] A method of inhibiting the expression of a polypeptide having alpha-glucuronidase 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 the polynucleotide of paragraph 33 or 34.
56. [56] The method of paragraph 55, wherein the dsRNA is about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more duplex nucleotides in length.
57. [57] An isolated polynucleotide encoding a signal peptide comprising or consisting of amino acids 1 to 15 of SEQ ID NO: 2.
58. [58] A nucleic acid construct comprising a gene encoding a protein operably linked to the polynucleotide of paragraph 57, wherein the gene is foreign to the polynucleotide.
59. [59] A recombinant expression vector comprising the nucleic acid construct of paragraph 58.
60. [60] A recombinant host cell comprising the nucleic acid construct of paragraph 58.
61. [61] A method of producing a protein, comprising: (a) cultivating a recombinant host cell comprising a gene encoding a protein operably linked to the polynucleotide of paragraph 57, wherein the gene is foreign to the polynucleotide under conditions conducive for production of the protein; and (b) recovering the protein.
62. [62] A composition comprising the polypeptide of any of paragraphs 1-31.
63. [63] A method for degrading or converting a cellulosic or xylan-containing material, comprising: treating the cellulosic or xylan-containing material with an enzyme composition in the presence of the polypeptide having alpha-glucuronidase

activity of any of paragraphs 1-31.

64. [64] The method of paragraph 63, wherein the cellulosic or xylan-containing material is pretreated.
65. [65] The method of paragraph 63 or 64, wherein the enzyme composition comprises one or more cellulolytic enzymes, one or more xylan degrading enzymes, or a combination of one or more xylan degrading enzymes and one or more cellulolytic enzymes.
66. [66] The method of paragraph 65, wherein the one or more cellulolytic enzymes are selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.
67. [67] The method of paragraph 65 or 66, wherein the enzyme composition further comprises a GH61 polypeptide having cellulolytic enhancing activity.
68. [68] The method of paragraph 65, wherein the one or more xylan degrading enzymes are selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase.
69. [69] The method of any of paragraphs 63-68, wherein the enzyme composition further comprises one or more enzymes selected from the group consisting of a hemicellulase, an esterase, a protease, a laccase, and a peroxidase.
70. [70] The method of any of paragraphs 63-69, further comprising recovering the degraded cellulosic or xylan-containing material.
71. [71] The method of paragraph 70, wherein the degraded cellulosic or xylan-containing material is a sugar.
72. [72] The method of paragraph 71, wherein the sugar is selected from the group consisting of glucose, xylose, mannose, galactose, and arabinose.
73. [73] A method for producing a fermentation product, comprising: (a) saccharifying a cellulosic or xylan-containing material with an enzyme composition in the presence of the polypeptide having alpha-glucuronidase activity of any of paragraphs 1-31; (b) fermenting the saccharified cellulosic or xylan-containing material with one or more fermenting microorganisms to produce the fermentation product; and (c) recovering the fermentation product from the fermentation.
74. [74] The method of paragraph 73, wherein the cellulosic or xylan-containing material is pretreated.
75. [75] The method of paragraph 73 or 74, wherein the enzyme composition comprises one or more cellulolytic enzymes, one or more xylan degrading enzymes, or a combination of one or more xylan degrading enzymes and one or more cellulolytic enzymes.
76. [76] The method of paragraph 75, wherein the one or more cellulolytic enzymes are selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.
77. [77] The method of paragraph 75 or 76, wherein the enzyme composition further comprises a GH61 polypeptide having cellulolytic enhancing activity.
78. [78] The method of paragraph 75, wherein the one or more xylan degrading enzymes are selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase.
79. [79] The method of any of paragraphs 73-78, wherein the enzyme composition further comprises one or more enzymes selected from the group consisting of a hemicellulase, an esterase, a protease, a laccase, and a peroxidase.
80. [80] The method of any of paragraphs 73-79, wherein steps (a) and (b) are performed simultaneously in a simultaneous saccharification and fermentation.
81. [81] The method of any of paragraphs 73-80, wherein the fermentation product is an alcohol, an organic acid, a ketone, an amino acid, or a gas.
82. [82] A method of fermenting a cellulosic or xylan-containing material, comprising: fermenting the cellulosic or xylan-containing material with one or more fermenting microorganisms, wherein the cellulosic or xylan-containing material is saccharified with an enzyme composition in the presence of the polypeptide having alpha-glucuronidase activity of any of paragraphs 1-31.
83. [83] The method of paragraph 82, wherein the fermenting of the cellulosic or xylan-containing material produces a fermentation product.
84. [84] The method of paragraph 83, further comprising recovering the fermentation product from the fermentation.
85. [85] The method of any of paragraphs 82-84, wherein the cellulosic or xylan-containing material is pretreated.
86. [86] The method of any of paragraphs 82-85, wherein the enzyme composition comprises one or more cellulolytic enzymes, one or more xylan degrading enzymes, or a combination of one or more xylan degrading enzymes and one or more cellulolytic enzymes.
87. [87] The method of paragraph 86, wherein the one or more cellulolytic enzymes are selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.
88. [88] The method of paragraph 86 or 87, wherein the enzyme composition further comprises a GH61 polypeptide having cellulolytic enhancing activity.
89. [89] The method of paragraph 86, wherein the one or more xylan degrading enzymes are selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a

glucuronidase.

90. [90] The method of any of paragraphs 82-89, wherein the enzyme composition further comprises one or more enzymes selected from the group consisting of a hemicellulase, an esterase, a protease, a laccase, and a peroxidase.
91. [91] The method of any of paragraphs 82-90, wherein the fermentation product is an alcohol, an organic acid, a ketone, an amino acid, or a gas.

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Patentkrav

1. Isoleret polypeptid med alfa-glucuronidaseaktivitet, som er valgt fra gruppen bestående af:
 - (a) et polypeptid, som omfatter en aminosyresekvens, der udviser mindst 90% identitet med det modne polypeptid ifølge SEQ ID NO: 2;
 - (b) et polypeptid, som kodes for af et polynukleotid, der under betingelser med mindst høj stringens hybridiserer med det modne polypeptids kodningssekvens ifølge SEQ ID NO: 1 eller den dertil komplementære streng i fuld længde;
 - (c) et polypeptid, som kodes for af et polynukleotid, der omfatter en nukleotidsekvens, som udviser mindst 90% identitet med det modne polypeptids kodningssekvens ifølge SEQ ID NO: 1; og
 - (d) en variant, som omfatter en substitution, deletion og/eller insertion af en eller flere op til et samlet antal på 1 til 10 aminosyrer i det modne polypeptid ifølge SEQ ID NO: 2.
2. Polypeptid ifølge krav 1, som omfatter eller består af aminosyresekvensen ifølge SEQ ID NO: 2; eller et fragment deraf med alfa-glucuronidaseaktivitet.
3. Polypeptid ifølge krav 1, hvilket polypeptid kodes for af polynukleotidet indeholdt i plasmid pHinsGH67A, hvilket plasmid er indeholdt i *E. coli*, som er deponeret i overensstemmelse med Budapesttraktaten under accessionsnummer NRRL B-50155.
4. Isoleret polynukleotid, som omfatter en nukleotidsekvens, der koder for polypeptidet ifølge et hvilket som helst af kravene 1-3.
5. Rekombinant værtscelle, som omfatter polynukleotidet ifølge krav 4, hvilket polynukleotid er operativt forbundet til en eller flere kontrolsekvenser, der styrer fremstillingen af et polypeptid med alfa-glucuronidaseaktivitet.
6. Fremgangsmåde til fremstilling af polypeptidet ifølge et hvilket som helst af kravene 1-3, hvilken fremgangsmåde omfatter: (a) dyrkning af en celle, som i sin vildtypeform fremstiller polypeptidet, under betingelser, der er befordrende for fremstilling af polypeptidet; og (b) indvinding af polypeptidet.
7. Fremgangsmåde til fremstilling af polypeptidet ifølge et hvilket som helst af kravene 1-3, hvilken fremgangsmåde omfatter: (a) dyrkning af en værtscelle, som omfatter en

nukleinsyrekonstruktion, hvilken nukleinsyrekonstruktion omfatter en nukleotidsekvens, der koder for polypeptidet, under betingelser, som er befordrende for fremstilling af polypeptidet; og (b) indvinding af polypeptidet.

- 5 8. Fremgangsmåde til fremstilling af en mutant af en stamcelle, hvilken fremgangsmåde omfatter introduktion af forstyrrelse i eller deletion af et polynukleotid, der koder for polypeptidet, eller en del deraf, ifølge et hvilket som helst af kravene 1-3, hvilket resulterer i, at mutanten fremstiller mindre af polypeptidet end stamcellen.
- 10 9. Fremgangsmåde til fremstilling af polypeptidet ifølge et hvilket som helst af kravene 1-3, hvilken fremgangsmåde omfatter: (a) dyrkning af en transgen plante eller en plantecelle, som omfatter et polynukleotid, hvilket polynukleotid koder for polypeptidet, under betingelser, der er befordrende for fremstilling af polypeptidet; og (b) indvinding af polypeptidet.
- 15 10. Transgen plante, plantedel eller plantecelle transformeret med et polynukleotid, hvilket polynukleotid koder for polypeptidet ifølge et hvilket som helst af kravene 1-3.
11. Dobbeltstrenget hæmmende RNA (dsRNA)-molekyle, som omfatter en undersekvens af polynukleotidet ifølge krav 4, hvor dsRNA'et eventuelt er et siRNA- eller miRNA-molekyle.
- 20 12. Fremgangsmåde til hæmning af ekspressionen af et polypeptid med alfa-glucuronidaseaktivitet i en celle, hvilken fremgangsmåde omfatter administration til cellen eller ekspression i cellen af et dobbeltstrenget RNA (dsRNA)-molekyle, hvor dsRNA'et omfatter en undersekvens af polynukleotidet ifølge krav 4.
- 25 13. Isoleret polynukleotid, som koder for et signalpeptid, der omfatter eller består af aminosyrerne 1 til 15 ifølge SEQ ID NO: 2.
- 30 14. Fremgangsmåde til fremstilling af et protein, hvilken fremgangsmåde omfatter: (a) dyrkning af en rekombinant værtscelle, som omfatter et gen, der koder for et protein, som er operativt forbundet til polynukleotidet ifølge krav 13, hvor genet er fremmed for polynukleotidet, under betingelser, der er befordrende for fremstilling af proteinet; og (b) indvinding af proteinet.
- 35 15. Fremgangsmåde til nedbrydning eller omdannelse af et celluloseholdigt eller xylanholdigt materiale, hvilken fremgangsmåde omfatter: behandling af det celluloseholdige eller xylanholdige

materiale med en enzymsammensætning i nærværelse af polypeptidet med alfa-glucuronidaseaktivitet ifølge et hvilket som helst af kravene 1-3.

16. Fremgangsmåde ifølge krav 15, hvilken fremgangsmåde endvidere omfatter indvinding af
5 det nedbrudte celluloseholdige eller xylanholdige materiale.

17. Fremgangsmåde til fremstilling af et fermenteringsprodukt, hvilken fremgangsmåde omfatter:

(a) forsukring af et celluloseholdigt eller xylanholdigt materiale med en
10 enzymsammensætning i nærværelse af polypeptidet med alfa-glucuronidaseaktivitet ifølge et hvilket som helst af kravene 1-3;

(b) fermentering af det forsukrede celluloseholdige eller xylanholdige materiale med en eller flere fermenterende mikroorganismer for at fremstille fermenteringsproduktet; og

(c) indvinding af fermenteringsproduktet fra fermenteringen.

18. Fremgangsmåde til fermentering af et celluloseholdigt eller xylanholdigt materiale, hvilken fremgangsmåde omfatter: fermentering af det celluloseholdige eller xylanholdige materiale med en eller flere fermenterende mikroorganismer, hvor det celluloseholdige eller xylanholdige materiale er
15
20 forsukret med en enzymsammensætning i nærværelse af polypeptidet med alfa-glucuronidaseaktivitet ifølge et hvilket som helst af kravene 1-3.

19. Fremgangsmåde ifølge krav 18, hvor fermenteringen af det celluloseholdige eller xylanholdige materiale frembringer et fermenteringsprodukt.

20. Fremgangsmåde ifølge krav 19, hvilken fremgangsmåde endvidere omfatter indvinding af fermenteringsproduktet fra fermenteringen.

DRAWINGS

M K S L L L L A A I G L A A A E D G L A A W L R
 1 ATGAAGAGCCTCCTGCTGCTCGCGGCCATTGGCCTGGCCGCCCGGAGGACGGCCTCGCTGCGTGGTTGCGG
 Y A R I D N P R T C V A K L P S T I I T L N A T
 73 TACGCTCGCATCGACAACCCAGGACCTGTGTCGCCAACTCCCGTCCACCATCATACCCCTCAACGCCACC
 K G L P V Y T A G E E L G R G I D G I L G K R L
 145 AAGGGTCTTCCCGTCTACACGCGAGGCGAGGCTCGGCAGGGGATCGATGGAATCCTGGGCAAGAGACTC
 K I D E R D E N G K P R G G K K P T K D S N N A
 217 AAGATTGACGAGCGGATGAGAATGGCAAGCCCCGCGCGGAAAGAGCCTACCAAGGACAGCAACAATGCC
 I T V G T V A A Y T K A N P K E A A K L P K L G
 289 ATCACGGTCGGCACAGTGGCGGCCTACACCAAGCCAACCCCAAGGAAGCCGCAAGCTGCCCAAGCTGGGT
 D D G Y Y L S I S R G K A L I L G Q N E R G T L
 361 GACGACGGTTACTATCTCAGCATCTCTCGCGGAAAGGCTTTGATCCTCGGCCAGAACGAGAGGGGCACGCTG
 Y G A F R W L Q L L G Q G N C S D F T L A S T P
 433 TACGGCGCGTTCGGTGGCTGCAGCTGCTCGGCCAGGSCAAGTCTCGGACTTCACTCTGGCGTCGACCCG
 D A P I R W V N Q W D N L Q D G G I H G S V E R
 505 GATGCTCCATCCGCTGGGTGAACAGTGGGACAACCTCCAGGACGGCGGCTTACGCGCAGCTCGAGCGT
 G Y G G S S I F F W D G Y V R P D L T R A R L Y
 577 GGTATGGCGGACGCTCCATCTTCTTGGGACGGCTACGTCGGGCCGACCTCACGCGCGCAGGCTGTAC
 A R L L A S I G L N A V V V N N V N A D P R T L
 649 GCGCGCTGTTGGCGTCCATCGGCCTCAACGCGGTTGGTCAACAAGTCAACGCGGACCCGCGCACGCTG
 T P E L M D G L A R I A D E F R P Y G V Q L A L
 721 ACCCGGAGTTGATGGACGGCTGGCCCGATTGCCGACGAATCCGGCGGTACGGCGTGCAGCTCGCCCTG
 A L N F A S P Q T L G G L D T F D P L E P S V Q
 793 GCGCTCAACTTCGCTTCTCCGACAGCCCTCGCGCGGCTCGACAGTTCCGACCCGCTCGAGCCTTCGCTCCAG
 A W W D N I T N E L Y R R I P D M A G Y L V K A
 865 GCGTGGTGGACACATCACCAACGAGCTCTACCGCGCATCCGGACATGGCGGGATACCTGGTCAAGGCC
 N S E G Q P G P M T Y N R T L A D G A N M F A R
 937 AACTCGGAGGGCAGCGGGCCCCATGACGTACAACCGCACGCTGGCCGACGGCGCAACATGTTTGGCGC
 S L K P H G G I I L F R A F V Y D H R N L N Q T
 1009 TCGCTCAAGCCGACGGCGGCATCATCTGTTCCGCGCTTCGTGTACGACACCGCAACCTCAACGAGACG
 L S W K A D R A R A A V D Y F D G L D D K F D D
 1081 CTCTCGTGAAGGCGACCGCGCGCGCGCTGGACTACTTTGACGGTCTCGACGACAAGTTCGACGAC
 N V V V Q I K Y G P I D F Q V R E P V S P L F A
 1153 AACGTGGTGCAGATCAAGTACGGCCGATCGACTTCCAGGTCCGCGAGCCGCTGTGCGCGCTGTTTGG
 H L V K T A T A I E L Q I T Q E Y L G Q Q A H L
 1225 CACCTCGTCAAGACGGCCACCGCATCGAGCTGCAGATCACCGAGGAGTACCTCGGCCAGCAGGCTCACCTC
 V Y L A P M W K E L L D F D L R V D G K P S R T
 1297 GTCTACCTCGCGCCATGTGGAAGAACTTCTCGACTTTGACCTGCGCGTTCGACGGCAAGCCCTCGCGCAG
 R D I L N G S R F N R K L G G Y A G V V N V G T
 1369 CGCGACATCCTCAACGGCAGCCGCTTCAACCGCAAGCTGGCGGCTATGCCGGCGTCTCAACGTCGGCACC
 N T T W L G S H L A M S N L Y A Y G R L A W N P
 1441 AACACCACCTGGTGGGACGACCTGGCCATGTCCAACCTGTACGCTACGGCCGGCTGGCGTGGAAACCCG
 S E T S E G I L S E W I S L T F S A D R K V H D
 1513 TCCGAACCTCCGAGGGCATCCTGAGCGAGTGGATCTCGCTGACCTTCAAGCGCGGACCGCAAGGTCCACGAC
 V I T R M S M L S W P A Y E N Y T G N L G I Q T
 1585 GTCATCACGCGATGTCGATGCTCTCCTGGCCGCTACGAAAACCTACAGGGCAACCTCGGCATCCAGACG
 L T D I L L G H Y G P N P A S Q D G N P W G Q W
 1657 CTGACCGACATCCTGTGGGCCACTACGGCCCGAACCCGGCTTCGACGAGACGGCAACCCGTGGGGCCAGTGG
 T R A D E D S I G M D R T V W N G T G F A G Q Y
 1729 ACGCGCGCCGACGAGGACAGCATCGGCATGGACCGCACCGTGTGGAACGGCACGGGCTTCGCGGGGCACTAC
 P P E V A E L Y E H P E T T P D N L L L W F H H
 1801 CCGCCGAGGTGCGCGAGCTGTATGAGCACCCAGAACTACCCCGGACAACTGCTCCTGTGGTTCACCAT
 V P Y T Y V L K S G K T V I Q H F Y D A H Y A G

Fig. 1A

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1873 GTGCCCTACACCTACGTCCTCAAGTCGGCAAGACGGTGATCCAGCACTTCTACGACGCGCACTACGCCGGC
    A E T A Q T F P R L W Q T L K G R K G I D K E R
1945 GCCGAGACGGCCAAACCTTCCCGCGGCTGTGGCAAACCTTCAAAGGCCGCAAGGGCATCGACAAGGAGCGC
    Y E H V L F R L V Y Q A G H A I V W R D S I C N
2017 TACGAGCAGCTCCTCTTCCGCTCGTCTACCAGGCCGGCCACGCCATCGTCTGGCGCGACAGCATCTGCAAC
    F Y F N K S G I P D E L G R V G N Y K Y R I E A
2089 TTCTACTTCAACAAATCCGGCATCCCCGACGAGCTGGGCCGCGTGGGCAACTACAAGTACCGCATCGAGGCC
    E A M Q L D G Y V P Y D V S P F E A A S G T K A
2161 GAGGCCATGCAGCTGGACGGCTACGTGCCGTACGACGTGTGCCCTTCGAGGCGGCGTCGGGGACCAAGGCC
    V V T K T N S T V G T V S A V L K D L K T G L Y
2233 GTCGTGACCAAGACCACTCCACCGTCGGCACCGTCAGCGCGGTGCTCAAGGATCTCAAGACGGGCTGTAC
    D V A V N Y Y D Q A I G N S T W D L Y I G R T H
2305 GACGTGGCGGTGAATTACTACGACCAGGCCATCGGGAACTCAACGTGGGATCTGTACATTGGCAGGACACAC
    V G R W K G D L E Y I L G R A P S P Y I D G Q T
2377 GTGGGCCGTTGGAAGGGAGATCTCGAGTATATCCTTGGCCGGGCGCCGTCGCCGTACATTGACGGGCAGACG
    A A R V T F P K V H I K K G S V L K I V G R P D
2449 GCGGCGAGGGTGACGTTCCCAAGGTGCACATCAAGAAGGGCAGCGTGTCAAGATTGTGGGCGGCGCGGAC
    G E E P A P L D Y V S I F P H G K G I V D *
2521 GGGGAGGAGCGGCGCGCTTGGATTATGTGTCCATCTTCCGCATGAAAGGGGATTGTGGATTGA

```

Fig. 1B

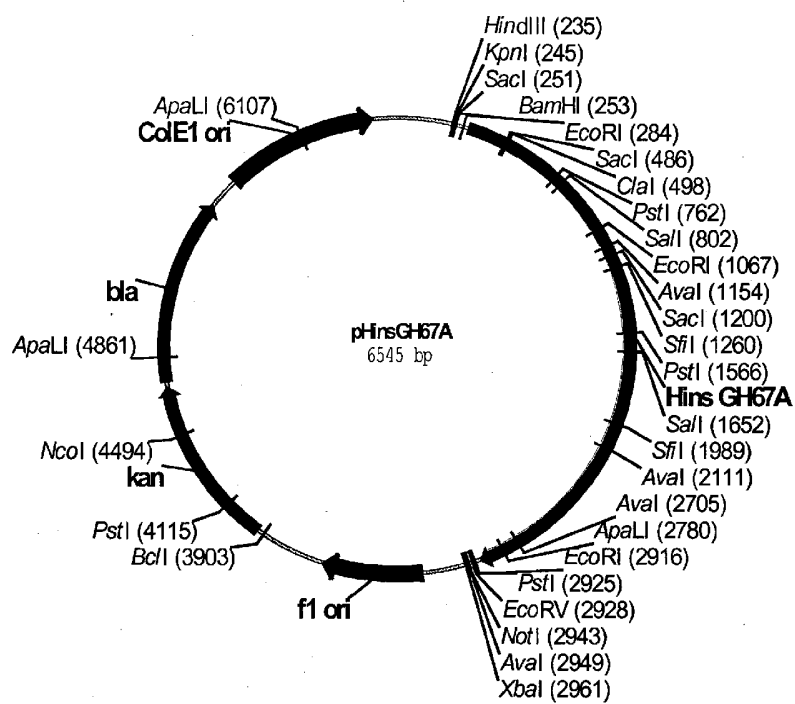


Fig. 2