



- (51) **International Patent Classification:**  
C12N 9/24 (2006.01)
- (21) **International Application Number:**  
PCT/EP2013/058292
- (22) **International Filing Date:**  
22 April 2013 (22.04.2013)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**  
12165165.7 23 April 2012 (23.04.2012) EP
- (71) **Applicant:** NOVOZYMES A/S [DK/DK]; Krogshoejvej 36, DK-2880 Bagsvaerd (DK).
- (72) **Inventors:** BÖRJESSON, Johan; Palmgatan 12 B, S-214 34 Malmö (SE). VIKSOE-NIELSEN, Anders; Lindevej 12, Joerlunde, DK-3550 Slangstrup (DK). SPODSBERG, Nikolaj; Vandkarsevej 62, DK-2880 Bagsvaerd (DK). KROGH, Kristian B. R. M.; Klirevaenget 35, DK-2880 Bagsvaerd (DK).
- (81) **Designated States** (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,

BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

- (84) **Designated States** (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

- without international search report and to be republished upon receipt of that report (Rule 48.2(g))
- with sequence listing part of description (Rule 5.2(a))



WO 2013/160247 A2

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

(57) **Abstract:** The present invention relates to isolated polypeptides having glucuronyl esterase activity, catalytic domains and polynucleotides encoding the polypeptides or catalytic domains. 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 or catalytic domains.

## **POLYPEPTIDES HAVING GLUCURONYL ESTERASE ACTIVITY AND POLYNUCLEOTIDES ENCODING SAME**

### **Reference to a Sequence Listing**

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

### **Background of the Invention**

#### **Field of the Invention**

10           The present invention relates to polypeptides having glucuronyl esterase activity, catalytic domains, binding domains and polynucleotides encoding the polypeptides, catalytic domains or binding domains. 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, catalytic domains and binding domains.

15

#### **Description of the Related Art**

          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).

20           The purpose of the pretreatment is to improve the rate of production as well as the total yield of liberated sugars in the hydrolysis step. In case of chemical pretreatment, like e.g. acid pretreatment or alkali pretreatment, the type of pretreatment will have different effects on lignocelluloses structural components and thus the enzyme composition used for the hydrolysis step may differ dependent on the pretreatment method. The aim of the present method is to improve hydrolysis of pretreated xylan containing material.

25           The present invention provides polypeptides having glucuronyl esterase activity and polynucleotides encoding the polypeptides. The use of polypeptides having glucuronyl esterase activity provides methods for improved hydrolysis of in particular xylan containing material.

30

### **Summary of the Invention**

          The present invention relates to polypeptides having glucuronyl esterase activity, selected from the group consisting of:

(a) a polypeptide having at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide of SEQ ID NO:2; or

at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence  
5 identity to the mature polypeptide of SEQ ID NO:4 or

at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence  
identity to the mature polypeptide of SEQ ID NO:6;

(b) a polypeptide encoded by a polynucleotide that hybridizes under high stringency  
conditions, or very high stringency conditions with (i) the mature polypeptide coding sequence of  
10 SEQ ID NO:1, (ii) the cDNA sequence thereof, or (iii) the full-length complement of (i) or (ii); or

or under very high stringency conditions with (iv) the mature polypeptide coding sequence  
of SEQ ID NO:3, (v) the cDNA sequence thereof, or (vi) the full-length complement of (iv) or (v);

or under very high stringency conditions with (vii) the mature polypeptide coding sequence  
of SEQ ID NO:5, (viii) the cDNA sequence thereof, or (ix) the full-length complement of (vii) or (viii);

15 (c) a polypeptide encoded by a polynucleotide having at least 80%, at least 85%, at  
least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at  
least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature  
polypeptide coding sequence of SEQ ID NO:1 or the cDNA sequence thereof or

having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at  
20 least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature  
polypeptide coding sequence of SEQ ID NO:3 or the cDNA sequence thereof or

having at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at  
least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the  
mature polypeptide coding sequence of SEQ ID NO:5 or the cDNA sequence thereof or

25 (d) a variant of the mature polypeptide of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6  
comprising a substitution, deletion, and/or insertion at one or more positions; and

(e) a fragment of the polypeptide of (a), (b), (c) or (d) that has glucuronyl esterase  
activity.

In a related aspect, the present invention relates to compositions comprising the peptides  
30 having glucuronyl esterase activity as described above.

In yet another related aspect, the present invention relates to a method of producing the  
peptides having glucuronyl esterase activity as described above and or compositions and/or  
recombinant host cells comprising the peptides having glucuronyl esterase activity as described  
above.

Further, the present invention relates to a transgenic plant, plant part or plant cell transformed with a polynucleotide encoding the polypeptide having glucuronyl esterase activity as described above.

Yet, an additional related aspect of the invention relates to a method of producing a mutant  
5 of a parent cell, comprising inactivating a polynucleotide encoding the polypeptide having glucuronyl esterase activity as described above, which results in the mutant producing less of the polypeptide than the parent cell.

The present invention also relates to a double-stranded inhibitory RNA (dsRNA) molecule comprising a subsequence of an isolated polynucleotide encoding the polypeptide having  
10 glucuronyl esterase activity as described above, wherein optionally the dsRNA is an siRNA or an miRNA molecule and methods of inhibiting the expression of a polypeptide having glucuronyl esterase activity in a cell, comprising administering to the cell or expressing in the cell the double-stranded inhibitory RNA (dsRNA) molecule and cells produced by said method.

In a related aspect, present invention relates to an isolated polynucleotide encoding a signal  
15 peptides, recombinant host cells comprising the signal peptide and methods of producing a protein wherein a recombinant host cell comprising the signal peptide is cultivated and the protein is recovered. In particular, the signal peptides of present invention comprise or consist of amino acids 1 to 17 of SEQ ID NO:2 or amino acids 1 to 17 of SEQ ID NO:4 or amino acids 1 to 20 of SEQ ID NO:6.

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

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

The present invention also relates to methods of fermenting a cellulosic material, comprising: fermenting the cellulosic material with one or more fermenting microorganisms,  
30 wherein the cellulosic material is saccharified with an enzyme composition in the presence of a polypeptide having glucuronyl esterase activity.

### Brief Description of the Figures

Figure 1 shows a comparative study of the effect on percentage conversion of pretreated corn fiber after addition four glucuronyl esterases on an enzymatic background of a beta-glucanase and  $\beta$ -xylosidase. Samples: Sample A, *C. unicolor* (SEQ ID NO:2); Sample B, *T. reesei* (SEQ ID NO:4); Sample C, *C. globosum* (SEQ ID NO:6).

5 Figure 2 shows a comparative study of the effect on the release of glucuronic acid (g/kg DM) after addition four glucuronyl esterases on an enzymatic background of beta-glucanase and  $\beta$ -xylosidase. Samples: Sample A, *C. unicolor* (SEQ ID NO:2); Sample B, *T. reesei* (SEQ ID NO:4); Sample C, *C. globosum* (SEQ ID NO:6).

10

### Definitions

**Cellulolytic activity:** The term “cellulolytic activity” means 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.*,  
15 Outlook for cellulase improvement: Screening and selection strategies, 2006, *Biotechnology Advances* 24: 452-481. Total cellulolytic activity is usually measured using insoluble substrates, including Whatman №1 filter paper, microcrystalline cellulose, bacterial cellulose, algal cellulose, cotton, pretreated lignocellulose, *etc.* The most common total cellulolytic activity assay is the filter paper assay using Whatman №1 filter paper as the substrate. The assay was established by the  
20 International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987, Measurement of cellulase activities, *Pure Appl. Chem.* 59: 257-68).

For purposes of the present invention, cellulolytic activity is determined by measuring the increase in hydrolysis of a cellulosic material by cellulolytic enzyme(s) under the following conditions: 1-20 mg of cellulolytic protein/g of cellulose in PCS for 3-7 days at 50-65°C compared to  
25 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 MnSO<sub>4</sub>, 50-65°C, 72 hours, sugar analysis by AMINEX® HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Endoglucanase:** The term “endoglucanase” means an endo-1,4-(1,3;1,4)-beta-D-glucan 4-  
30 glucanohydrolase (E.C. 3.2.1.4), which catalyses endohydrolysis of 1,4-beta-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxymethyl cellulose and hydroxyethyl cellulose), lichenin, beta-1,4 bonds in mixed beta-1,3 glucans such as cereal beta-D-glucans or xyloglucans, and other plant material containing cellulosic components. Endoglucanase activity can be determined by measuring reduction in substrate viscosity or increase in reducing ends determined

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

5           **Cellubiohydrolase:** The term “cellubiohydrolase” means a 1,4-beta-D-glucan cellobiohydrolase (E.C. 3.2.1.91), which catalyzes the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, 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). For purposes of the present invention, cellobiohydrolase activity is determined using a fluorescent disaccharide derivative 4-methylumbelliferyl-β-D-lactoside according to the procedures described by van Tilbeurgh *et al.*, 1982, *FEBS Letters* 149: 152-156 and van Tilbeurgh and Claeysens, 1985, *FEBS Letters* 187: 15 283-288, at pH 5, 40°C.

**Beta-glucosidase:** The term “beta-glucosidase” means a beta-D-glucoside glucohydrolase (E.C. 3.2.1.21), which catalyzes the hydrolysis of terminal non-reducing beta-D-glucose residues with the release of beta-D-glucose. For purposes of the present invention, beta-glucosidase activity is determined according to the basic procedure described by Venturi *et al.*, 2002, *Extracellular beta-D-glucosidase from Chaetomium thermophilum var. coprophilum: production, purification and some biochemical properties*, *J. Basic Microbiol.* 42: 55-66. One unit of beta-glucosidase is defined as 1.0 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 containing 0.01% TWEEN® 20.

**Cellulolytic enhancing activity:** The term “cellulolytic enhancing activity” means a 25 biological activity catalyzed by a GH61 polypeptide that enhances the hydrolysis of a cellulosic material by enzyme having cellulolytic activity. For purposes of the present invention, cellulolytic enhancing activity is determined by measuring the increase in reducing sugars or the increase of the total of cellobiose and glucose from the hydrolysis of a cellulosic material by cellulolytic enzyme under the following conditions: 1-50 mg of total protein/g of cellulose in PCS, wherein total protein is comprised of 50-99.5% w/w cellulolytic protein and 0.5-50% w/w protein of a GH61 polypeptide 30 having cellulolytic enhancing activity for 1-7 day at 50-65°C compared to a control hydrolysis with equal total protein loading without cellulolytic enhancing activity (1-50 mg of cellulolytic protein/g of cellulose in PCS). In a preferred aspect, a mixture of CELLUCLAST® 1.5L (Novozymes A/S, Bagsværd, Denmark) in the presence of 3% of total protein weight *Aspergillus oryzae* beta-

glucosidase (recombinantly produced in *Aspergillus oryzae* according to WO 02/095014) or 3% of total protein weight *Aspergillus fumigatus* beta-glucosidase (recombinantly produced in *Aspergillus oryzae* as described in WO 2002/095014) of cellulase protein loading is used as the source of the cellulolytic activity.

5           The GH61 polypeptides having cellulolytic enhancing activity enhance the hydrolysis of a cellulosic material catalyzed by enzyme having cellulolytic activity by reducing the amount of cellulolytic enzyme required to reach the same degree of hydrolysis preferably at least 1.01-fold, more preferably at least 1.05-fold, more preferably at least 1.10-fold, more preferably at least 1.25-fold, more preferably at least 1.5-fold, more preferably at least 2-fold, more preferably at least 3-  
10 fold, more preferably at least 4-fold, more preferably at least 5-fold, even more preferably at least 10-fold, and most preferably at least 20-fold.

**Family 61 glycoside hydrolase:** The term "Family 61 glycoside hydrolase" or "Family GH61" or "GH61" means a polypeptide falling into the glycoside hydrolase Family 61 according to Henrissat B., 1991, A classification of glycosyl hydrolases based on amino-acid sequence  
15 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.

**Xylan degrading activity:** The terms "xylan degrading activity" or "xylanolytic activity" mean 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  
20 the individual xylanolytic activities (endoxyranases, beta-xylosidases, arabinofuranosidases, alpha-glucuronidases, acetylxylan esterases, feruloyl esterases, and alpha-glucuronoyl esterases). Recent progress in assays of xylanolytic enzymes was summarized in several publications including Biely and Puchard, Recent progress in the assays of xylanolytic enzymes, 2006, *Journal of the Science of Food and Agriculture* 86(11): 1636-1647; Spanikova and Biely, 2006, Glucuronoyl esterase -  
25 Novel carbohydrate esterase produced by *Schizophyllum commune*, *FEBS Letters* 580(19): 4597-4601; Herrmann, Vrsanska, Jurickova, Hirsch, Biely, and Kubicek, 1997, The beta-D-xylosidase of *Trichoderma reesei* is a multifunctional beta-D-xylan xylohydrolase, *Biochemical Journal* 321: 375-381.

Total xylan degrading activity can be measured by determining the reducing sugars formed  
30 from various types of xylan, including, for example, oat spelt, beechwood, and larchwood xylans, or by photometric determination of dyed xylan fragments released from various covalently dyed xylans. The most common total xylanolytic activity assay is based on production of reducing sugars from polymeric 4-O-methyl glucuronoxylan as described in Bailey, Biely, Poutanen, 1992,

Interlaboratory testing of methods for assay of xylanase activity, *Journal of Biotechnology* 23(3): 257-270.

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

10       **Xylanase:** The term "xylanase" means a 1,4-beta-D-xylan-xylohydrolase (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 is defined as 1.0  $\mu$ 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 containing 0.01% TWEEN® 20.

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

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

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



FAE-II. For purposes of the present invention, feruloyl esterase activity is determined using 0.5 mM *p*-nitrophenylferulate as substrate in 50 mM sodium acetate pH 5.0. One unit of feruloyl esterase equals the amount of enzyme capable of releasing 1  $\mu$ mole of *p*-nitrophenolate anion per minute at pH 5, 25°C.

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

The polypeptides of the present invention have at least 60%, e.g. at least 70%, at least 80%, at least 90%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% of the glucuronyl esterase activity of the mature polypeptide of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6.

15        **Alpha-L-arabinofuranosidase:** The term "alpha-L-arabinofuranosidase" means an alpha-L-arabinofuranoside arabinofuranohydrolase (EC 3.2.1.55) that catalyzes the hydrolysis of terminal non-reducing alpha-L-arabinofuranoside residues in alpha-L-arabinosides. The enzyme acts on alpha-L-arabinofuranosides, alpha-L-arabinans containing (1,3)- and/or (1,5)-linkages, arabinoxylans, and arabinogalactans. Alpha-L-arabinofuranosidase is also known as  
20 arabinosidase, alpha-arabinosidase, alpha-L-arabinosidase, alpha-arabinofuranosidase, polysaccharide alpha-L-arabinofuranosidase, alpha-L-arabinofuranoside hydrolase, L-arabinosidase, or alpha-L-arabinanase. For purposes of the present invention, alpha-L-arabinofuranosidase activity is determined using 5 mg of medium viscosity wheat arabinoxylan (Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland) per ml of 100 mM sodium  
25 acetate pH 5 in a total volume of 200  $\mu$ l for 30 minutes at 40°C followed by arabinose analysis by AMINEX® HPX-87H column chromatography (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

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

insoluble crystalline matrix of parallel glucan chains. Hemicelluloses usually hydrogen bond to cellulose, as well as to other hemicelluloses, which help stabilize the cell wall matrix.

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

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

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

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

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

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

**Xylan-containing material:** The term “xylan-containing material” means 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  
5 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.

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

**Allelic variant:** The term “allelic variant” means any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the  
15 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.

**Binding domain:** The term “binding domain” *e.g.*, “cellulose binding domain” means the region of an enzyme that mediates binding of the enzyme to amorphous regions of a cellulose substrate. The cellulose binding domain (CBD) is typically found either at the N-terminal or at the  
20 C-terminal extremity of an glucuronyl esterase.

**Catalytic domain:** The term “catalytic domain” means the region of an enzyme containing the catalytic machinery of the enzyme.

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

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

**Control sequences:** The term “control sequences” means nucleic acid sequences necessary for expression of a polynucleotide encoding a mature polypeptide of the present

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

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

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

**Fragment:** The term "fragment" means a polypeptide or a catalytic or cellulose binding  
15 domain having one or more (*e.g.*, several) amino acids deleted from the amino and/or carboxyl terminus of a mature polypeptide or domain; wherein the fragment has glucuronyl esterase or cellulose binding activity. In one aspect, a fragment contains at least 85%, 90%, and 95% of the number of amino acids of the mature polypeptide of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6.

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

**Isolated or purified:** The term "isolated" or "purified" means a polypeptide or polynucleotide  
25 that is removed from at least one component with which it is naturally associated. For example, a polypeptide may be at least 1% pure, *e.g.*, at least 5% pure, at least 10% pure, at least 20% pure, at least 40% pure, at least 60% pure, at least 80% pure, at least 90% pure, or at least 95% pure, as determined by SDS-PAGE, and a polynucleotide may be at least 1% pure, *e.g.*, at least 5% pure, at least 10% pure, at least 20% pure, at least 40% pure, at least 60% pure, at least 80% pure, at least  
30 90% pure, or at least 95% pure, as determined by agarose electrophoresis.

**Mature polypeptide:** The term "mature polypeptide" means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. In one aspect, the mature polypeptide is amino acids 1 to 474 of SEQ ID NO:2, amino acids 1 to 460 of SEQ ID NO:4 or amino acids 1 to

392 of SEQ ID NO:6 as predicted using SignalP (Nielsen *et al.*, 1997, *Protein Engineering* 10: 1-6) that predicts amino acids 1 to 17 of SEQ ID NO:2, 1 to 17 of SEQ ID NO:4, amino acids 21 to 690 of SEQ ID NO:4 or amino acids 1 to 20 of SEQ ID NO:6; are a signal peptides. It is known in the art that a host cell may produce a mixture of two or more different mature polypeptides (*i.e.*, with a different C-terminal and/or N-terminal amino acid) expressed by the same polynucleotide.

**Mature polypeptide coding sequence:** The term "mature polypeptide coding sequence" means a polynucleotide that encodes a mature polypeptide having glucuronyl esterase activity. In one aspect, the mature polypeptide coding sequence is nucleotides 1 to 2544 of SEQ ID NO:1, nucleotides 1 to 2526 of SEQ ID NO:3, nucleotides 1 to 2508 of SEQ ID NO:5, nucleotides 1 to 2124 of SEQ ID NO:7 or the cDNA sequence thereof; based on the SignalP program (Nielsen *et al.*, 1997, *supra*) that predicts nucleotides 1 to 66 of SEQ ID NO:1, nucleotides 1 to 60 of SEQ ID NO:3, nucleotides 1 to 45 of SEQ ID NO:5, nucleotides 1 to 81 of SEQ ID NO:7, encode a signal peptide.

**Nucleic acid construct:** The term "nucleic acid construct" means a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or is modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature or which is synthetic, which comprises one or more control sequences.

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

**Sequence identity:** The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "sequence identity".

For purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the `-nobrief` option) is used as the percent identity and is calculated as follows:

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

For purposes of the present invention, the sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS

package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 5.0.0 or later. The 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  
 5 as the percent identity and is calculated as follows:

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

**Subsequence:** The term “subsequence” means a polynucleotide having one or more (*e.g.*, several) nucleotides deleted from the 5' and/or 3' end of a mature polypeptide coding sequence; wherein the subsequence encodes a fragment having glucuronyl esterase activity. In one aspect, a  
 10 subsequence contains at least 85%, 90%, and 95% of the number of amino acids of the mature polypeptide of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6.

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

### Detailed Description of the Invention

#### 20 Polypeptides Having glucuronyl esterase Activity

The present invention relates to isolated polypeptides having glucuronyl esterase activity selected from the group consisting of:

- (a) a polypeptide having at least 80% sequence identity to the mature polypeptide of SEQ ID NO:2;
- 25 (b) a polypeptide encoded by a polynucleotide that hybridizes under high, or very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO:1, (ii) the cDNA sequence thereof, or (iii) the full-length complement of (i) or (ii);
- (c) a polypeptide encoded by a polynucleotide having at least 80% sequence identity to the mature polypeptide coding sequence of SEQ ID NO:1 or the cDNA sequence thereof;
- 30 (d) a variant of the mature polypeptide of SEQ ID NO:2 comprising a substitution, deletion, and/or insertion at one or more (*e.g.*, several) positions; and
- (e) a fragment of the polypeptide of (a), (b), (c) or (d) that has glucuronyl esterase activity.

The present invention also relates to isolated polynucleotides encoding the polypeptides of the present invention; nucleic acid constructs; recombinant expression vectors; recombinant host cells comprising the polynucleotides; and methods of producing the polypeptides.

5 The present invention also relates to a polynucleotide encoding a signal peptide comprising or consisting of amino acids 1 to 17 of SEQ ID NO:2, a polynucleotide encoding a propeptide comprising or consisting of amino acids 101 to 474 of SEQ ID NO:2, or a polynucleotide encoding a signal peptide and a propeptide comprising or consisting of amino acids 1 to 474 of SEQ ID NO:2, each of which is operably linked to a gene encoding a protein; nucleic acid constructs, expression vectors, and recombinant host cells comprising the polynucleotides; and methods of producing a  
10 protein.

The present invention relates to isolated polypeptides having glucuronyl esterase activity selected from the group consisting of:

- (a) a polypeptide having at least 80% sequence identity to the mature polypeptide of SEQ ID NO:4;
- 15 (b) a polypeptide encoded by a polynucleotide that hybridizes under high, or very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO:3, (ii) the cDNA sequence thereof, or (iii) the full-length complement of (i) or (ii);
- (c) a polypeptide encoded by a polynucleotide having at least 80% sequence identity to the mature polypeptide coding sequence of SEQ ID NO:4 or the cDNA sequence thereof;
- 20 (d) a variant of the mature polypeptide of SEQ ID NO:4 comprising a substitution, deletion, and/or insertion at one or more (*e.g.*, several) positions; and
- (e) a fragment of the polypeptide of (a), (b), (c) or (d) that has glucuronyl esterase activity.

25 The present invention also relates to isolated polynucleotides encoding the polypeptides of the present invention; nucleic acid constructs; recombinant expression vectors; recombinant host cells comprising the polynucleotides; and methods of producing the polypeptides.

30 The present invention also relates to a polynucleotide encoding a signal peptide comprising or consisting of amino acids 1 to 17 of SEQ ID NO:4, a polynucleotide encoding a propeptide comprising or consisting of amino acids 17 to 460 of SEQ ID NO:4, or a polynucleotide encoding a signal peptide and a propeptide comprising or consisting of amino acids 1 to 460 of SEQ ID NO:4, each of which is operably linked to a gene encoding a protein; nucleic acid constructs, expression vectors, and recombinant host cells comprising the polynucleotides; and methods of producing a protein.

The present invention relates to isolated polypeptides having glucuronyl esterase activity selected from the group consisting of:

- (a) a polypeptide having at least 80% sequence identity to the mature polypeptide of SEQ ID NO:6;
- 5 (b) a polypeptide encoded by a polynucleotide that hybridizes under high, or very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO:6, (ii) the cDNA sequence thereof, or (iii) the full-length complement of (i) or (ii);
- (c) a polypeptide encoded by a polynucleotide having at least 80% sequence identity to the mature polypeptide coding sequence of SEQ ID NO:6 or the cDNA sequence thereof;
- 10 (d) a variant of the mature polypeptide of SEQ ID NO:6 comprising a substitution, deletion, and/or insertion at one or more (*e.g.*, several) positions; and
- (e) a fragment of the polypeptide of (a), (b), (c) or (d) that has glucuronyl esterase activity.

The present invention also relates to isolated polynucleotides encoding the polypeptides of  
15 the present invention; nucleic acid constructs; recombinant expression vectors; recombinant host cells comprising the polynucleotides; and methods of producing the polypeptides.

The present invention also relates to a polynucleotide encoding a signal peptide comprising or consisting of amino acids 1 to 20 of SEQ ID NO:6, a polynucleotide encoding a propeptide comprising or consisting of amino acids 21 to 392 of SEQ ID NO:6, or a polynucleotide encoding a  
20 signal peptide and a propeptide comprising or consisting of amino acids 1 to 392 of SEQ ID NO:6, each of which is operably linked to a gene encoding a protein; nucleic acid constructs, expression vectors, and recombinant host cells comprising the polynucleotides; and methods of producing a protein.

The present invention also relates to methods of inhibiting expression or producing one or  
25 more of the peptides having at least 68% such as *e.g.* 70%, 75%, 80%, 85%, 90%, 95%, 99% or 100% sequence identity to any of the sequences SEQ ID: NO2, SEQ ID NO: 4, or SEQ ID NO: 6.

Furthermore, present invention relates to a method for degrading or converting a cellulosic material, comprising: treating the cellulosic material with an enzyme composition in the presence of the polypeptide having glucuronyl esterase activity having at least 68% such as *e.g.* 70%, 75%,  
30 80%, 85%, 90%, 95%, 99% or 100% sequence identity to any of the sequences SEQ ID: NO2, SEQ ID NO: 4 or SEQ ID NO: 6.

In an embodiment, the present invention relates to isolated polypeptides having a sequence identity to the mature polypeptide of SEQ ID NO:2 of at least 80%, *e.g.* at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at



least 98%, at least 99%, or 100%, which have glucuronyl esterase activity. In one aspect, the polypeptides differ by no more than ten amino acids, *e.g.*, nine amino acids, eight amino acids, seven amino acids, six amino acids, five amino acids, four amino acids, three amino acids, two amino acids, or one amino acid from the mature polypeptide of SEQ ID NO:2.

5 A polypeptide of the present invention preferably comprises or consists of the amino acid sequence of SEQ ID NO:2 or an allelic variant thereof; or is a fragment thereof having glucuronyl esterase activity. In another aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO:2. In another preferred aspect, the polypeptide comprises or consists of amino acids 101 to 474 of SEQ ID NO:2.

10 In an embodiment, the present invention relates to isolated polypeptides having a sequence identity to the mature polypeptide of SEQ ID NO:4 of at least 95%, *e.g.* at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have glucuronyl esterase activity. In one aspect, the polypeptides differ by no more than ten amino acids, *e.g.*, nine amino acids, eight amino acids, seven amino acids, six amino acids, five amino acids, four amino acids, three amino acids, two amino acids, or one amino acid from the mature polypeptide of SEQ ID NO:4.

15 A polypeptide of the present invention preferably comprises or consists of the amino acid sequence of SEQ ID NO:4 or an allelic variant thereof; or is a fragment thereof having glucuronyl esterase activity. In another aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO:4. In another preferred aspect, the polypeptide comprises or consists of amino acids 94 to 460 of SEQ ID NO:4.

20 In an embodiment, the present invention relates to isolated polypeptides having a sequence identity to the mature polypeptide of SEQ ID NO:6 of at least 92%, *e.g.* at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have glucuronyl esterase activity. In one aspect, the polypeptides differ by no more than ten amino acids, *e.g.*, nine amino acids, eight amino acids, seven amino acids, six amino acids, five amino acids, four amino acids, three amino acids, two amino acids, or one amino acid from the mature polypeptide of SEQ ID NO:6.

25 A polypeptide of the present invention preferably comprises or consists of the amino acid sequence of SEQ ID NO:6 or an allelic variant thereof; or is a fragment thereof having glucuronyl esterase activity. In another aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO:6. In another preferred aspect, the polypeptide comprises or consists of amino acids 21 to 392 of SEQ ID NO:6.

30 In another embodiment, the present invention relates to isolated polypeptides having glucuronyl esterase activity that are encoded by a polynucleotide that hybridizes under very low

stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 (ii) the cDNA sequence thereof, or (iii) the full-length complement of (i) or (ii) (Sambrook *et al.*, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York).

The polynucleotide of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 or a subsequence thereof, as well as the polypeptide of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 or a fragment thereof, may be used to design nucleic acid probes to identify and clone DNA encoding polypeptides having glucuronyl esterase activity from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic DNA or cDNA of a cell 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 15, *e.g.*, at least 25, at least 35, or at least 70 nucleotides in length. Preferably, the nucleic acid probe is at least 100 nucleotides in length, *e.g.*, at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500 nucleotides, at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, or at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with  $^{32}\text{P}$ ,  $^3\text{H}$ ,  $^{35}\text{S}$ , biotin, or avidin). Such probes are encompassed by the present invention.

A genomic DNA or cDNA library prepared from such other strains may be screened for DNA that hybridizes with the probes described above and encodes a polypeptide having glucuronyl esterase activity. Genomic or other DNA from such other strains may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA that is homologous with SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 or a subsequence thereof, the carrier material is preferably used in a Southern blot.

For purposes of the present invention, hybridization indicates that the polynucleotide hybridizes to a labeled nucleic acid probe corresponding to (i) SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5; (ii) the mature polypeptide coding sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5; (iii) the cDNA sequence; (iv) the full-length complement thereof; or (v) a subsequence thereof; under very low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions can be detected using, for example, X-ray film.

In another aspect, the nucleic acid probe is a polynucleotide that encodes the polypeptide of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6; the mature polypeptide thereof; or a fragment thereof.

In another aspect, the nucleic acid probe is SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 or the cDNA sequence thereof.

5 For probes of at least 100 nucleotides in length, very low stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 45°C.

10 For probes of at least 100 nucleotides in length, low stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 50°C.

15 For probes of at least 100 nucleotides in length, medium stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 55°C.

20 For probes of at least 100 nucleotides in length, medium-high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and either 35% formamide, following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 60°C.

25 For probes of at least 100 nucleotides in length, high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 65°C.

30 For probes of at least 100 nucleotides in length, very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 70°C.

In another embodiment, the present invention relates to isolated polypeptides having glucuronyl esterase activity encoded by polynucleotides having a sequence identity to the mature

polypeptide coding sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 of at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

5 In another embodiment, the present invention relates to variants of the mature polypeptide of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 comprising a substitution, deletion, and/or insertion at one or more (*e.g.*, several) positions. Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small  
10 amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the groups of basic amino acids (arginine,  
15 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*,  
20 *The Proteins*, Academic Press, New York. Common substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

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

Essential amino acids in a 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  
30 introduced at every residue in the molecule, and the resultant mutant molecules are tested for glucuronyl esterase activity to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron

diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Lett.* 309: 59-64. The identity of essential amino acids can also be inferred from an alignment with a related polypeptide.

5 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  
10 display (e.g., Lowman *et al.*, 1991, *Biochemistry* 30: 10832-10837; U.S. Patent No. 5,223,409; WO 92/06204), and region-directed mutagenesis (Derbyshire *et al.*, 1986, *Gene* 46: 145; Ner *et al.*, 1988, *DNA* 7: 127).

Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells  
15 (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.

In an embodiment, the number of amino acid substitutions, deletions and/or insertions  
20 introduced into the mature polypeptide of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 is not more than 10, e.g., 1, 2, 3, 4, 5, 6, 7, 8 or 9.

The polypeptide may be a hybrid polypeptide in which a region of one polypeptide is fused at the N-terminus or the C-terminus of a region of another polypeptide.

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

A fusion polypeptide can further comprise a cleavage site between the two polypeptides. Upon secretion of the fusion protein, the site is cleaved releasing the two polypeptides. Examples

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

### Sources of Polypeptides Having glucuronyl esterase Activity

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

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

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

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

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

The polypeptide may also be a fungal polypeptide. For example, the polypeptide may be a yeast polypeptide such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* polypeptide; or a filamentous fungal polypeptide such as an *Acremonium*, *Agaricus*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Botryosphaeria*, *Ceriporiopsis*, *Chaetomidium*, *Chrysosporium*, *Claviceps*, *Cochliobolus*, *Coprinopsis*, *Coptotermes*, *Corynascus*, *Cryphonectria*, *Cryptococcus*, *Diplodia*, *Exidia*, *Filibasidium*, *Fusarium*, *Gibberella*,

*Holomastigotoides*, *Humicola*, *Irpex*, *Lentinula*, *Leptosphaeria*, *Magnaporthe*, *Melanocarpus*,  
*Meripilus*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*,  
*Phanerochaete*, *Piromyces*, *Poitrasia*, *Pseudoplectania*, *Pseudotriconympha*, *Rhizomucor*,  
*Schizophyllum*, *Scytalidium*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trichoderma*,  
5 *Trichophaea*, *Verticillium*, *Volvariella*, or *Xylaria* polypeptide.

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

In another aspect, the polypeptide is an *Acremonium cellulolyticus*, *Aspergillus aculeatus*,  
10 *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*,  
*Aspergillus niger*, *Aspergillus oryzae*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*,  
*Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*,  
*Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium*  
15 *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*  
20 *aurantiogriseum*, *Penicillium funiculosum*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*,  
*Thielavia achromatica*, *Thielavia albomyces*, *Thielavia albopilosa*, *Thielavia australeinsis*, *Thielavia fimeti*,  
*Thielavia microspora*, *Thielavia ovispora*, *Thielavia peruviana*, *Thielavia setosa*, *Thielavia spededonium*,  
*Thielavia subthermophila*, *Thielavia terrestris*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*,  
*Trichoderma reesei*,  
25 or *Trichoderma viride* polypeptide.

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.

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

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

## 10 Domains

The present invention also relates to catalytic domains.

In an embodiment, the catalytic domain has a sequence identity to amino acids 101 to 474 of SEQ ID NO:2 of at least 80%, *e.g.* at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. In an aspect, the catalytic domain comprises an amino acid sequence that differs by ten amino acids, *e.g.*, nine amino acids, eight amino acids, seven amino acids, six amino acids, five amino acids, four amino acids, three amino acids, two amino acids, or one amino acid from amino acids 101 to 474 of SEQ ID NO:2.

The catalytic domain preferably comprises or consists of amino acids 101 to 474 of SEQ ID NO:2 or an allelic variant thereof; or is a fragment thereof having glucuronyl esterase activity.

In another embodiment, the catalytic domain is encoded by a polynucleotide that hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions (as defined above) with (i) the nucleotides 33 to 1457 OF SEQ ID NO:1, (ii) the cDNA sequence thereof, or (iii) the full-length complement of (i) or (ii) (Sambrook *et al.*, 1989, *supra*).

In another embodiment, the catalytic domain is encoded by a polynucleotide having a sequence identity to nucleotides 33 to 1457 OF SEQ ID NO:1 or the cDNA sequence thereof at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

In another aspect, the polynucleotide encoding the catalytic domain comprises or consists of nucleotides 33 to 1457 OF SEQ ID NO:1.

In another embodiment, the catalytic domain is a variant of amino acids 101 to 474 of SEQ ID NO:2 comprising a substitution, deletion, and/or insertion at one or more (*e.g.*, several) positions. In an embodiment, the number of amino acid substitutions, deletions and/or insertions



introduced into the sequence of amino acids 101 to 474 of SEQ ID NO:2 is 10, e.g., 1, 2, 3, 4, 5, 6, 8, or 9.

In an embodiment, the catalytic domain has a sequence identity to amino acids 94 to 460 of SEQ ID NO:4 of at least 95%, e.g. at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

5 In an aspect, the catalytic domain comprises an amino acid sequence that differs by ten amino acids, e.g., nine amino acids, eight amino acids, seven amino acids, six amino acids, five amino acids, four amino acids, three amino acids, two amino acids, or one amino acid from amino acids 94 to 460 of SEQ ID NO:4.

10 The catalytic domain preferably comprises or consists of amino acids 94 to 460 of SEQ ID NO:4 or an allelic variant thereof; or is a fragment thereof having glucuronyl esterase activity.

In another embodiment, the catalytic domain is encoded by a polynucleotide that hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions (as defined above) with (i) the nucleotides 81 to 1463 of SEQ ID NO:3, (ii) the cDNA sequence thereof, or (iii) the full-length complement of (i) or (ii) (Sambrook *et al.*, 1989, *supra*).

15 In another embodiment, the catalytic domain is encoded by a polynucleotide having a sequence identity to nucleotides 81 to 1463 of SEQ ID NO:3 or the cDNA sequence thereof at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

20 In another aspect, the polynucleotide encoding the catalytic domain comprises or consists of nucleotides 81 to 1463 of SEQ ID NO:3.

In another embodiment, the catalytic domain is a variant of amino acids 94 to 460 of SEQ ID NO:4 comprising a substitution, deletion, and/or insertion at one or more (e.g., several) positions. In an embodiment, the number of amino acid substitutions, deletions and/or insertions introduced into the sequence of amino acids 94 to 460 of SEQ ID NO:4 is 10, e.g., 1, 2, 3, 4, 5, 6, 8, or 9.

25 In an embodiment, the catalytic domain has a sequence identity to amino acids 26 to 392 of SEQ ID NO:6 of at least 90%, e.g. at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. In an aspect, the catalytic domain comprises an amino acid sequence that differs by ten amino acids, e.g., nine amino acids, eight amino acids, seven amino acids, six amino acids, five amino acids, four amino acids, three amino acids, two amino acids, or one amino acid from amino acids 26 to 392 of SEQ ID NO:6.

30 The catalytic domain preferably comprises or consists of amino acids 26 to 392 of SEQ ID NO:6 or an allelic variant thereof; or is a fragment thereof having glucuronyl esterase activity.

In another embodiment, the catalytic domain is encoded by a polynucleotide that hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions (as defined above) with (i) the nucleotides 235 to 1491 of SEQ ID NO:5, (ii) the cDNA sequence thereof, or (iii) the full-length complement of (i) or (ii) (Sambrook *et al.*, 1989, *supra*).

In another embodiment, the catalytic domain is encoded by a polynucleotide having a sequence identity to nucleotides 235 to 1491 of SEQ ID NO:5 or the cDNA sequence thereof at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

In another aspect, the polynucleotide encoding the catalytic domain comprises or consists of nucleotides 235 to 1491 of SEQ ID NO:5.

In another embodiment, the catalytic domain is a variant of amino acids 25 to 392 of SEQ ID NO:6 comprising a substitution, deletion, and/or insertion at one or more (*e.g.*, several) positions. In an embodiment, the number of amino acid substitutions, deletions and/or insertions introduced into the sequence of amino acids 26 to 392 of SEQ ID NO:6 is 10, *e.g.*, 1, 2, 3, 4, 5, 6, 8, or 9.

The present invention also relates to isolated polypeptides comprising a catalytic domain selected from the group consisting of: glucuronyl esterases (EC 2.4.1.17)

- (a) a catalytic domain having at least 80% sequence identity to amino acids 101 to 474 of SEQ ID NO:2;
- (b) a catalytic domain encoded by a polynucleotide that hybridizes under high, or very high stringency conditions with (i) nucleotides 33 to 1457 OF SEQ ID NO:1, (ii) the cDNA sequence thereof, or (iii) the full-length complement of (i) or (ii);
- (c) a catalytic domain encoded by a polynucleotide having at least 80% sequence identity to nucleotides 33 to 1457 OF SEQ ID NO:1 or the cDNA sequence thereof;
- (e) a variant of amino acids 101 to 474 of SEQ ID NO:2 comprising a substitution, deletion, and/or insertion at one or more (*e.g.*, several) positions; and
- (f) a fragment of the catalytic domain of (a), (b), (c), (d) or (e) that has glucuronyl esterase activity.

The present invention also relates to isolated polypeptides comprising a catalytic domain selected from the group consisting of: glucuronyl esterases (EC 2.4.1.17)

- (a) a catalytic domain having at least 80% sequence identity to amino acids 94 to 460 of SEQ ID NO:4;

(b) a catalytic domain encoded by a polynucleotide that hybridizes under high, or very high stringency conditions with (i) nucleotides 81 to 1463 of SEQ ID NO:3, (ii) the cDNA sequence thereof; or (iii) the full-length complement of (i) or (ii);

(c) a catalytic domain encoded by a polynucleotide having at least 80% sequence identity to nucleotides 81 to 1463 of SEQ ID NO:3 or the cDNA sequence thereof;

(e) a variant of amino acids 94 to 460 of SEQ ID NO:4 comprising a substitution, deletion, and/or insertion at one or more (e.g., several) positions; and

(f) a fragment of the catalytic domain of (a), (b), (c), (d) or (e) that has glucuronyl esterase activity.

10 The present invention also relates to isolated polypeptides comprising a catalytic domain selected from the group consisting of: glucuronyl esterases (EC 2.4.1.17)

(a) a catalytic domain having at least 80% sequence identity to amino acids 48 to 392 of SEQ ID NO:6;

(b) a catalytic domain encoded by a polynucleotide that hybridizes under high, or very high stringency conditions with (i) nucleotides 235 to 1491 of SEQ ID NO:5, (ii) the cDNA sequence thereof, or (iii) the full-length complement of (i) or (ii);

(c) a catalytic domain encoded by a polynucleotide having at least 80% sequence identity to nucleotides 235 to 1491 of SEQ ID NO:5 or the cDNA sequence thereof;

(d) a variant of the mature polypeptide of SEQ ID NO:6 comprising a substitution, deletion, and/or insertion at one or more (e.g., several) positions; and

(e) a variant of amino acids 21 to 392 of SEQ ID NO:6 comprising a substitution, deletion, and/or insertion at one or more (e.g., several) positions; and

(f) a fragment of the catalytic domain of (a), (b), (c), (d) or (e) that has glucuronyl esterase activity.

25

**The present invention also relates to cellulose binding domains.**

In another embodiment, the cellulose binding domain is encoded by a polynucleotide that hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions (as defined above) with (i) the nucleotides of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 (ii) the cDNA sequence thereof, or (iii) the full-length complement of (i) or (ii) (Sambrook *et al.*, 1989, *supra*).

30 In another embodiment, the cellulose binding domain is a variant of SEQ ID NO:2 comprising a substitution, deletion, and/or insertion at one or more (e.g., several) positions. In an

aspect, the number of amino acid substitutions, deletions and/or insertions introduced into SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 is 10, *e.g.*, 1, 2, 3, 4, 5, 6, 8, or 9.

A catalytic domain operably linked to the cellulose binding domain may be from a hydrolase, isomerase, ligase, lyase, oxidoreductase, or transferase, *e.g.*, an aminopeptidase, amylase, 5 carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, xylanase, or beta-xylosidase. The polynucleotide encoding the catalytic domain may be obtained from any 10 prokaryotic, eukaryotic, or other source.

### Polynucleotides

The present invention also relates to isolated polynucleotides encoding a polypeptide, a 15 catalytic domain, or cellulose binding domain of the present invention, as described above.

The techniques used to isolate or clone a polynucleotide encoding a polypeptide are known in the art and include isolation from genomic DNA or cDNA, or a combination thereof. The cloning of the polynucleotides from 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 20 DNA fragments with shared structural features. See, *e.g.*, Innis *et al.*, 1990, *PCR: A Guide to Methods and Application*, Academic Press, New York. Other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligation activated transcription (LAT) and polynucleotide-based amplification (NASBA) may be used. The polynucleotides may be cloned from any relevant microorganism and thus, for example, may be an allelic or species variant of the polypeptide 25 encoding region of the polynucleotide.

Modification of a polynucleotide encoding a polypeptide of the present invention may be necessary for synthesizing 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 30 source, *e.g.*, variants that differ in specific activity, thermostability, pH optimum, or the like. The variants may be constructed on the basis of the polynucleotide presented as the mature polypeptide coding sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 or the cDNA sequence thereof, *e.g.*, a subsequence thereof, and/or by introduction of nucleotide substitutions that do not result in a change in the amino acid sequence of the polypeptide, but which correspond to the

codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions that may give rise to a different amino acid sequence. For a general description of nucleotide substitution, see, e.g., Ford *et al.*, 1991, *Protein Expression and Purification 2*: 95-107.

5

### Nucleic Acid Constructs

The present invention also relates to nucleic acid constructs comprising a polynucleotide of the present invention operably linked to one or more control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

10 A polynucleotide may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotides utilizing recombinant DNA methods are well known in the art.

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

20 Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a bacterial host cell are the promoters obtained from the *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus subtilis* *xylA* and *xylB* genes, *E. coli lac* operon, *E. coli trc* promoter (Egon *et al.*, 1988, *Gene* 69: 301-315), *Streptomyces coelicolor* agarase gene (*dagA*), and prokaryotic beta-lactamase gene (Villa-Kamaroff *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75: 3727-3731), as well as the *tac* promoter (DeBoer *et al.*, 1983, *Proc. Natl. Acad. Sci. USA* 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in Gilbert *et al.*, 1980, *Scientific American*, 242: 74-94; and in Sambrook *et al.*, 1989, *supra*.

30 Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid

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

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

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

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

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*.

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

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

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

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

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

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

The control sequence may also be a signal peptide coding region that encodes a signal peptide linked to the N-terminus of a polypeptide and directs the polypeptide into the cell's secretory pathway. The 5'-end of the coding sequence of the polynucleotide may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of  
25 the coding sequence that encodes the polypeptide. Alternatively, the 5'-end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. A foreign signal peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding sequence. Alternatively, a foreign signal peptide coding sequence may simply replace the natural signal peptide coding sequence in order to enhance secretion of the  
30 polypeptide. However, any signal peptide coding sequence that directs the expressed polypeptide into the secretory pathway of a host cell of choice may be used.

Effective signal peptide coding sequences for bacterial host cells are the signal peptide coding sequences obtained from the genes for *Bacillus* NCIB 11837 maltogenic amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* alpha-

amylase, *Bacillus stearothermophilus* neutral proteases (*nprT*, *nprS*, *nprM*), and *Bacillus subtilis* *prsA*. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57: 109-137.

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

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

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

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

It may also be desirable to add regulatory sequences that regulate expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those that cause expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems include the *lac*, *tac*, and *trp* operator systems. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the *Aspergillus niger* glucoamylase promoter, *Aspergillus oryzae* TAKA alpha-amylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used. Other examples of regulatory sequences are those that allow for gene amplification. In eukaryotic systems, these regulatory sequences include the dihydrofolate reductase gene that is amplified in the presence of methotrexate, and the metallothionein genes that are amplified with heavy metals. In these cases, the polynucleotide encoding the polypeptide would be operably linked with the regulatory sequence.



## Expression Vectors

The 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 nucleotide and control sequences may be joined together to produce a recombinant expression vector that may include one or more convenient restriction sites to allow for insertion or substitution of the polynucleotide encoding the polypeptide at such sites. Alternatively, the polynucleotide may be expressed by inserting the polynucleotide or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

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

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

The vector preferably contains one or more selectable markers that permit easy selection of transformed, transfected, transduced, or the like cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

Examples of bacterial selectable markers are the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers that confer antibiotic resistance such as ampicillin, chloramphenicol, kanamycin, or tetracycline resistance. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for use in a filamentous fungal host cell include, but are not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hph* (hygromycin phosphotransferase), *niaD* (nitrate

reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in an *Aspergillus* cell are *Aspergillus nidulans* or *Aspergillus oryzae amdS* and *pyrG* genes and a *Streptomyces hygrosopicus bar* gene.

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

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

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

Examples of bacterial origins of replication are the origins of replication of plasmids  
25 pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*, and pUB110, pE194, pTA1060, and pAM $\beta$ 1 permitting replication in *Bacillus*.

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

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

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

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

### Host Cells

The present invention also relates to recombinant host cells, comprising a polynucleotide of the present invention operably linked to one or more control sequences that direct the production of  
15 a polypeptide of the present invention. A construct or vector comprising a polynucleotide 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  
20 encoding the polypeptide and its source.

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

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

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

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

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

The introduction of DNA into a *Bacillus* cell may be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Mol. Gen. Genet.* 168: 111-115), using competent cells (see, e.g., Young and Spizizen, 1961, *J. Bacteriol.* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *J. Mol. Biol.* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thorne, 1987, *J. Bacteriol.* 169: 5271-5278). The introduction of DNA into an *E. coli* cell may 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 be effected by protoplast transformation and electroporation (see, e.g., Gong *et al.*, 2004, *Folia Microbiol. (Praha)* 49: 399-405), conjugation (see, e.g., Mazodier *et al.*, 1989, *J. Bacteriol.* 171: 3583-3585), or 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 be effected by electroporation (see, e.g., Choi *et al.*, 2006, *J. Microbiol. Methods* 64: 391-397) or conjugation (see, e.g., Pinedo and Smets, 2005, *Appl. Environ. Microbiol.* 71: 51-57). The introduction of DNA into a *Streptococcus* cell may be effected by natural competence (see, e.g., Perry and Kuramitsu, 1981, *Infect. Immun.* 32: 1295-1297), protoplast transformation (see, e.g., Catt and Jollick, 1991, *Microbios* 68: 189-207), electroporation (see, e.g., Buckley *et al.*, 1999, *Appl. Environ. Microbiol.* 65: 3800-3804) or conjugation (see, e.g., Clewell, 1981, *Microbiol. Rev.* 45: 409-436). However, any method known in the art for introducing DNA into a host cell can be used.

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

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

The fungal host cell may be a yeast cell. "Yeast" as used herein includes 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).

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

The fungal host cell may be a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*,  
10 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.

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

For example, the filamentous fungal host cell may be an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*,  
20 *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermisporea*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*,  
25 *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 insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes*

*versicolor*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described in EP 238023, Yelton *et al.*, 1984, *Proc. Natl. Acad. Sci. USA* 81: 1470-1474, and Christensen *et al.*, 1988, *Bio/Technology* 6: 1419-1422. 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, *J. Bacteriol.* 153: 163; and Hinnen *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75: 1920.

### Methods of Production

The present invention also relates to methods of producing a polypeptide of the present invention, comprising: (a) cultivating a cell, which in its wild-type form produces the polypeptide, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide. In a preferred aspect, the cell is an *Cerreana* (for SEQ ID NO:2), *Trichoderma* (for SEQ ID NO:4) or *Chaetomium* (for SEQ ID NO:6) cell. In a more preferred aspect, the cell is an *Cerreana unicolor* (for SEQ ID NO:2), *Trichoderma reesei* (for SEQ ID NO:4) or *Chaetomium globosum* (for SEQ ID NO:6).

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

The host 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, it can be recovered from cell lysates.

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

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

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

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

## Plants

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

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

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

Examples of plant parts are stem, callus, leaves, root, fruits, seeds, and tubers as well as the individual tissues comprising these parts, e.g., epidermis, mesophyll, parenchyme, vascular tissues, meristems. Specific plant cell compartments, such as chloroplasts, apoplasts, mitochondria, vacuoles, peroxisomes and cytoplasm are also considered to be a plant part.

Furthermore, any plant cell, whatever the tissue origin, is considered to be a plant part. Likewise, plant parts such as specific tissues and cells isolated to facilitate the utilization of the invention are also considered plant parts, e.g., embryos, endosperms, aleurone and seed coats.

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

The transgenic plant or plant cell expressing the polypeptide or domain 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 expression constructs encoding the polypeptide or domain into the plant host genome or chloroplast genome and propagating the resulting modified plant or plant cell into a transgenic plant or plant cell.

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

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

For constitutive expression, the 35S-CaMV, the maize ubiquitin 1, or 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 Cell Physiol.* 39: 885-889), a *Vicia faba* promoter from the legumin B4 and the unknown seed protein gene from *Vicia faba* (Conrad *et al.*, 1998, *J. Plant Physiol.* 152: 708-711), a promoter from a seed oil body protein (Chen *et al.*, 1998, *Plant Cell Physiol.* 39: 935-941), the storage protein *napA* promoter from



*Brassica napus*, or any other seed specific promoter known in the art, e.g., as described in WO 91/14772. Furthermore, the promoter may be a leaf specific promoter such as the *rbcS* promoter from rice or tomato (Kyoizuka *et al.*, 1993, *Plant Physiol.* 102: 991-1000), the chlorella virus adenine methyltransferase gene promoter (Mitra and Higgins, 1994, *Plant Mol. Biol.* 26: 85-93), the *aldP* gene promoter from rice (Kagaya *et al.*, 1995, *Mol. Gen. Genet.* 248: 668-674), or a wound inducible promoter such as the potato *pin2* promoter (Xu *et al.*, 1993, *Plant Mol. Biol.* 22: 573-588). Likewise, the promoter may be induced by abiotic treatments such as temperature, drought, or alterations in salinity or induced by exogenously applied substances that activate the promoter, e.g., ethanol, oestrogens, plant hormones such as ethylene, abscisic acid, and gibberellic acid, and heavy metals.

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

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

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

Presently, *Agrobacterium tumefaciens*-mediated gene transfer is the method of choice for generating transgenic dicots (for a review, see Hooykas and Schilperoort, 1992, *Plant Mol. Biol.* 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 J.* 2: 275-281; Shimamoto, 1994, *Curr. Opin. Biotechnol.* 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 Mol. Biol.* 21: 415-428. Additional transformation methods for use in accordance with the present disclosure include those described in U.S. Patent Nos. 6,395,966 and 7,151,204 (both of which are herein incorporated by reference in their entirety).

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.

In addition to direct transformation of a particular plant genotype with a construct of the present invention, transgenic plants may be made by crossing a plant having the construct to a second plant lacking the construct. For example, a construct encoding a polypeptide or domain can be introduced into a particular plant variety by crossing, without the need for ever directly transforming a plant of that given variety. Therefore, the present invention encompasses not only a plant directly regenerated from cells which have been transformed in accordance with the present invention, but also the progeny of such plants. As used herein, progeny may refer to the offspring of any generation of a parent plant prepared in accordance with the present invention. Such progeny may include a DNA construct prepared in accordance with the present invention. Crossing results in the introduction of a transgene into a plant line by cross pollinating a starting line with a donor plant line. Non-limiting examples of such steps are described in U.S. Patent No. 7,151,204.

Plants may be generated through a process of backcross conversion. For example, plants include plants referred to as a backcross converted genotype, line, inbred, or hybrid.

Genetic markers may be used to assist in the introgression of one or more transgenes of the invention from one genetic background into another. Marker assisted selection offers advantages relative to conventional breeding in that it can be used to avoid errors caused by phenotypic variations. Further, genetic markers may provide data regarding the relative degree of elite germplasm in the individual progeny of a particular cross. For example, when a plant with a desired trait which otherwise has a non-agronomically desirable genetic background is crossed to an elite parent, genetic markers may be used to select progeny which not only possess the trait of interest, but also have a relatively large proportion of the desired germplasm. In this way, the number of generations required to introgress one or more traits into a particular genetic background is minimized.

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

### Removal or Reduction of Glucuronyl Esterase Activity

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.

The mutant cell may be constructed by reducing or eliminating expression of the polynucleotide using methods well known in the art, for example, insertions, disruptions, replacements, or deletions. In a preferred aspect, the polynucleotide is inactivated. The polynucleotide 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 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 polynucleotide. 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.

Modification or inactivation of the polynucleotide may be performed by subjecting the parent cell to mutagenesis and selecting for mutant cells in which expression of the polynucleotide 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.

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.

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

Modification or inactivation of the polynucleotide may be accomplished by insertion, substitution, or deletion of one or more nucleotides in the gene or a regulatory element required for 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 polynucleotide to be modified, it is preferred that the modification be performed *in vitro* as exemplified below.

An example of a convenient way to eliminate or reduce expression of a polynucleotide 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 polynucleotide 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 polynucleotide. It may be desirable that the defective polynucleotide also encodes a marker that may be used for selection of transformants in which the polynucleotide has been modified or destroyed. In an aspect, the polynucleotide is disrupted with a selectable marker such as those described herein.

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

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

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 the 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).

The dsRNAs of the present invention can be used in gene-silencing. In one aspect, the invention provides methods to selectively degrade RNA using a dsRNAi 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, an organ or an animal. Methods for

making and using dsRNA molecules to selectively degrade RNA are well known in the art; see, for example, U.S. Patent Nos. 6,489,127; 6,506,559; 6,511,824; and 6,515,109.

5 The present invention further relates to a mutant cell of a parent cell that comprises a disruption or deletion of a polynucleotide encoding the polypeptide or a control sequence thereof or a silenced gene encoding the polypeptide, which results in the mutant cell producing less of the polypeptide or no polypeptide compared to the parent cell.

10 The polypeptide-deficient mutant cells are particularly useful as host cells for expression of native and heterologous polypeptides. Therefore, the present invention further relates to methods of producing a native or heterologous polypeptide, comprising: (a) cultivating the mutant cell under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide. The term "heterologous polypeptides" means polypeptides that are not native to the host cell, *e.g.*, a variant of a native protein. The host cell may comprise more than one copy of a polynucleotide encoding the native or heterologous polypeptide.

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

20 The methods of the present invention for producing an essentially glucuronyl esterase-free product is of particular interest in the production of eukaryotic polypeptides, in particular fungal proteins such as enzymes. The glucuronyl esterase-deficient cells may also be used to express heterologous proteins of pharmaceutical interest such as hormones, growth factors, receptors, and the like. 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.

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

### **Methods of Processing Cellulosic Material**

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

The present invention also relates to methods of producing a fermentation product, comprising: (a) saccharifying a cellulosic material with an enzyme composition in the presence of a polypeptide having glucuronyl esterase activity of the present invention; (b) fermenting the

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

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

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

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

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

temperature that the fermentation strain can tolerate. DMC combines all three processes (enzyme production, hydrolysis, and fermentation) in one or more (several) steps where the same organism is used to produce the enzymes for conversion of the cellulosic material to fermentable sugars and to convert the fermentable sugars into a final product (Lynd, L. R., Weimer, P. J., van Zyl, W. H., and Pretorius, I. S., 2002, Microbial cellulose utilization: Fundamentals and biotechnology, *Microbiol. Mol. Biol. Reviews* 66: 506-577). It is understood herein that any method known in the art comprising pretreatment, enzymatic hydrolysis (saccharification), fermentation, or a combination thereof, can be used in the practicing the methods of the present invention.

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

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

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

Conventional pretreatments include, but are not limited to, steam pretreatment (with or without explosion), dilute acid pretreatment, hot water pretreatment, alkaline pretreatment, lime pretreatment, wet oxidation, wet explosion, ammonia fiber explosion, organosolv pretreatment, and biological pretreatment. Additional pretreatments include ammonia percolation, ultrasound, 5 electroporation, microwave, supercritical CO<sub>2</sub>, supercritical H<sub>2</sub>O, ozone, and gamma irradiation pretreatments.

The cellulosic material can be pretreated before hydrolysis and/or fermentation. Pretreatment is preferably performed prior to the hydrolysis. Alternatively, the pretreatment can be carried out 10 simultaneously with enzyme hydrolysis to release fermentable sugars, such as glucose, xylose, and/or cellobiose. In most cases the pretreatment step itself results in some conversion of biomass to fermentable sugars (even in absence of enzymes).

Steam Pretreatment. In steam pretreatment, cellulosic material is heated to disrupt the plant cell wall components, including lignin, hemicellulose, and cellulose to make the cellulose and other fractions, e.g., hemicellulose, accessible to enzymes. Cellulosic material is passed to or through a 15 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 20 minutes, where the optimal residence time depends on temperature range and any addition of a chemical catalyst. Steam pretreatment allows for relatively high solids loadings, so that cellulosic material is generally only moist during the pretreatment. The steam pretreatment is often combined with an explosive discharge of the material after the pretreatment, which is known as steam explosion, that is, rapid flashing to atmospheric pressure and turbulent flow of the material to 25 increase the accessible surface area by fragmentation (Duff and Murray, 1996, *Bioresource Technology* 855: 1-33; Galbe and Zacchi, 2002, *Appl. Microbiol. Biotechnol.* 59: 618-628; U.S. Patent Application No. 20020164730). During steam pretreatment, hemicellulose acetyl groups are cleaved and the resulting acid autocatalyzes partial hydrolysis of the hemicellulose to monosaccharides and oligosaccharides. Lignin is removed to only a limited extent.

30 A catalyst such as H<sub>2</sub>SO<sub>4</sub> or SO<sub>2</sub> (typically 0.3 to 3% w/w) is often added prior to steam pretreatment, which decreases the time and temperature, increases the recovery, and improves enzymatic hydrolysis (Ballesteros *et al.*, 2006, *Appl. Biochem. Biotechnol.* 129-132: 496-508; Varga *et al.*, 2004, *Appl. Biochem. Biotechnol.* 113-116: 509-523; Sassner *et al.*, 2006, *Enzyme Microb. Technol.* 39: 756-762).



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

In dilute acid pretreatment, cellulosic material is mixed with dilute acid, typically H<sub>2</sub>SO<sub>4</sub>, and water to form a slurry, heated by steam to the desired temperature, and after a residence time flashed to atmospheric pressure. The dilute acid pretreatment can be performed with a number of reactor designs, e.g., plug-flow reactors, counter-current reactors, or continuous counter-current shrinking bed reactors (Duff and Murray, 1996, *supra*; Schell *et al.*, 2004, *Bioresource Technol.* 91: 179-188; Lee *et al.*, 1999, *Adv. Biochem. Eng. Biotechnol.* 65: 93-115).

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

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

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

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

Ammonia fiber explosion (AFEX) involves treating cellulosic material with liquid or gaseous ammonia at moderate temperatures such as 90-100°C and high pressure such as 17-20 bar for 5-10 minutes, where the dry matter content can be as high as 60% (Gollapalli *et al.*, 2002, *Appl. Biochem. Biotechnol.* 98: 23-35; Chundawat *et al.*, 2007, *Biotechnol. Bioeng.* 96: 219-231; Alizadeh *et al.*, 2005,

*Appl. Biochem. Biotechnol.* 121: 1133-1141; Teymouri *et al.*, 2005, *Bioresource Technol.* 96: 2014-2018). AFEX pretreatment results in the depolymerization of cellulose and partial hydrolysis of hemicellulose. Lignin-carbohydrate complexes are cleaved.

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

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

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

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

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

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

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

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

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

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

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

Saccharification. In the hydrolysis step, also known as saccharification, the cellulosic material, e.g., pretreated, is hydrolyzed to break down cellulose and alternatively also hemicellulose to fermentable sugars, such as glucose, cellobiose, xylose, xylulose, arabinose, mannose, galactose, and/or soluble oligosaccharides. The hydrolysis is performed enzymatically by an enzyme

composition in the presence of a polypeptide having glucuronyl esterase activity of the present invention. The composition can further comprise one or more (several) hemicellulolytic or xylan degrading enzymes. The enzymes of the compositions can also be added sequentially.

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

10 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  
15 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 %.

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

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

30 In another 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 (several) 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., acetyl-xylan esterases, acetyl-mannan esterases, ferulic acid esterases, coumaric acid esterases, glucuronoyl esterases), pectinases, proteases, ligninolytic enzymes (e.g., laccases, manganese peroxidases, lignin peroxidases, H<sub>2</sub>O<sub>2</sub>-producing enzymes, oxidoreductases), expansins, swollenins, or mixtures thereof. In the methods of the present invention, the additional enzyme(s) can be added prior to or during fermentation, e.g., during saccharification or during or after propagation of the fermenting microorganism(s).

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

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

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

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

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

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

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

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

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

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

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

The polypeptide having cellulolytic enzyme activity or xylan degrading activity may also be a fungal polypeptide, and more preferably a yeast polypeptide such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* polypeptide 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*, *Pseudotriconympha*, *Rhizomucor*, *Schizophyllum*, *Scytalidium*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trichoderma*, *Trichophaea*, *Verticillium*, *Volvariella*, or *Xylaria* polypeptide having cellulolytic enzyme activity or xylan degrading activity.

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

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*,  
5 *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*,  
10 *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.

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

One or more (several) components of the enzyme composition may be a recombinant  
15 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  
20 fermentation broth.

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

Examples of bacterial endoglucanases that can be used in the methods of the present  
invention, include, but are not limited to, an *Acidothermus cellulolyticus* endoglucanase (WO



91/05039; WO 93/15186; U.S. Patent No. 5,275,944; WO 96/02551; U.S. Patent No. 5,536,655, WO 00/70031, WO 05/093050); *Thermobifida fusca* endoglucanase III (WO 05/093050); and *Thermobifida fusca* endoglucanase V (WO 05/093050).

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

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.

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.

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.

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.

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.

Other cellulolytic enzymes that may be used in the present invention are described in EP 495 257, EP 531 315, EP 531 372, WO 1989/09259, WO 1994/07998, WO 1995/24471, WO 1996/11262, WO 1996/29397, WO 1996/034108, WO 1997/14804, WO 1998/08940, WO 1998/012307, WO 1998/13465, WO 1998/015619, WO 1998/015633, WO 1998/028411, WO 1999/06574, WO 1999/10481, WO 1999/025846, WO 1999/025847, WO 1999/031255, WO 2000/009707, WO 2002/050245, WO 2002/0076792, WO 2002/101078, WO 2003/027306, WO 2003/052054, WO 2003/052055, WO 2003/052056, WO 2003/052057, WO 2003/052118, WO 2004/016760, WO 2004/043980, WO 2004/048592, WO 2005/001065, WO 2005/028636, WO 2005/093050, WO 2005/093073, WO 2006/074005, WO 2006/117432, WO 2007/071818, WO 2007/071820, WO 2008/008070, WO 2008/008793, U.S. Patent No. 4,435,307, U.S. Patent No. 5,457,046, U.S. Patent No. 5,648,263, U.S. Patent No. 5,686,593, U.S. Patent No. 5,691,178, U.S. Patent No. 5,763,254, and U.S. Patent No. 5,776,757.

In the methods of the present invention, any GH61 polypeptide having cellulolytic enhancing activity can be used.

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

[ILMV]-P-X(4,5)-G-X-Y-[LMV]-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.

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.

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].

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.

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); polypeptides having cellulolytic enhancing activity from *Trichoderma reesei* (WO 2007/089290); and polypeptides having cellulolytic enhancing activity from *Myceliophthora thermophila* (WO 2009/085935; WO 2009/085859; WO 2009/085864; WO 2009/085868).

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).

Examples of xylanases useful in the methods of the present invention include, but are not limited to, *Aspergillus aculeatus* xylanase (GeneSeqP:AAR63790; WO 94/21785), *Aspergillus*

*fumigatus* xylanases (WO 2006/078256), and *Thielavia terrestris* NRRL 8126 xylanases (WO 2009/079210).

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

Examples of acetylxylan esterases useful in the methods of the present invention include, but are not limited to, *Hypocrea jecorina* acetylxylan esterase (WO 2005/001036), *Neurospora crassa* acetylxylan esterase (UniProt accession number q7s259), *Thielavia terrestris* NRRL 8126 acetylxylan esterase (WO 2009/042846), *Chaetomium globosum* acetylxylan esterase (Uniprot accession number Q2GWX4), *Chaetomium gracile* acetylxylan esterase (GeneSeqP accession number AAB82124), *Phaeosphaeria nodorum* acetylxylan esterase (Uniprot accession number Q0UJH1), and *Humicola insolens* DSM 1800 acetylxylan esterase (WO 2009/073709).

Examples of ferulic acid esterases useful in the methods of the present invention include, but are not limited to, *Humicola insolens* DSM 1800 feruloyl esterase (WO 2009/076122), *Neurospora crassa* feruloyl esterase (UniProt accession number Q9HGR3), and *Neosartorya fischeri* feruloyl esterase (UniProt Accession number A1D9T4).

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).

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

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).

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  
5 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.

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

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

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

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

30 "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 C<sub>6</sub> and/or C<sub>5</sub> fermenting organisms, or a combination thereof. Both C<sub>6</sub> and C<sub>5</sub> fermenting organisms are well known in the art. Suitable fermenting microorganisms are able to ferment, i.e., convert, sugars, such as glucose, xylose, xylulose, arabinose, maltose,

mannose, galactose, or oligosaccharides, directly or indirectly into the desired fermentation product.

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

5           Examples of fermenting microorganisms that can ferment C<sub>6</sub> sugars include bacterial and fungal organisms, such as yeast. Preferred yeast includes strains of the *Saccharomyces* spp., preferably *Saccharomyces cerevisiae*.

10           Examples of fermenting organisms that can ferment C<sub>5</sub> sugars include bacterial and fungal organisms, such as yeast. Preferred C<sub>5</sub> fermenting yeast include strains of *Pichia*, preferably *Pichia stipitis*, such as *Pichia stipitis* CBS 5773; strains of *Candida*, preferably *Candida boidinii*, *Candida brassicae*, *Candida sheatae*, *Candida diddensii*, *Candida pseudotropicalis*, or *Candida utilis*.

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

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

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

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

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

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

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

In a preferred aspect, the genetically modified fermenting microorganism is *Saccharomyces cerevisiae*. In another preferred aspect, the genetically modified fermenting microorganism is *Zymomonas mobilis*. In another preferred aspect, the genetically modified fermenting microorganism is *Escherichia coli*. In another preferred aspect, the genetically modified fermenting

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

It is well known in the art that the organisms described above can also be used to produce other substances, as described herein.

5 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.

10 In a preferred aspect, the yeast and/or another microorganism is applied to the degraded cellulosic material and the fermentation is performed for about 12 to about 96 hours, such as typically 24-60 hours. In a preferred aspect, the temperature is preferably between about 20°C to about 60°C, more preferably about 25°C to about 50°C, and most preferably about 32°C to about 50°C, in particular about 32°C or 50°C, and the pH is generally from about pH 3 to about pH 7, preferably around pH 4-7. However, some fermenting organisms, e.g., bacteria, have higher  
15 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  $2 \times 10^8$  viable cell count per ml of fermentation broth. Further guidance in respect of using yeast for fermentation can be found in, e.g., "The Alcohol Textbook" (Editors K. Jacques, T.P. Lyons and D.R. Kelsall, Nottingham University Press, United Kingdom 1999), which is hereby incorporated by  
20 reference.

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

25 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-  
30 aminobenzoic acid, folic acid, riboflavin, and Vitamins A, B, C, D, and E. See, for example, Alfenore *et al.*, Improving ethanol production and viability of *Saccharomyces cerevisiae* by a vitamin feeding strategy during fed-batch process, Springer-Verlag (2002), which is hereby incorporated by reference. Examples of minerals include minerals and mineral salts that can supply nutrients comprising P, K, Mg, S, Ca, Fe, Zn, Mn, and Cu.



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

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

In another preferred aspect, the fermentation product is an organic acid. In another more preferred aspect, the organic acid is acetic acid. In another more preferred aspect, the organic acid is acetonetic 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 and Lee, 1997, Membrane-mediated extractive fermentation for lactic acid production from cellulosic biomass, *Appl. Biochem. Biotechnol.* 63-65: 435-448.

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

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

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

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

## Signal Peptide

The present invention also relates to an isolated polynucleotide encoding a signal peptide comprising or consisting of amino acids 1 to 17 of SEQ ID NO:2, amino acids 1 to 17 of SEQ ID NO:4 or amino acids 1 to 26 of SEQ ID NO:6. The polynucleotides may further comprise a gene  
5 encoding a protein, which is operably linked to the signal peptide and/or propeptide. The protein is preferably foreign to the signal peptide. In one aspect, the polynucleotide encoding the signal peptide is nucleotides 33 to 83 of SEQ ID NO:1, amino acids 81 to 131 of SEQ ID NO:3, amino acids 235 to 312 of SEQ ID NO:5.

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

The present invention also relates to methods of producing a protein, comprising: (a) cultivating a recombinant host cell comprising such polynucleotide; and (b) recovering the protein.

The protein may be native or heterologous to a host cell. The term "protein" is not meant herein to refer to a specific length of the encoded product and, therefore, encompasses peptides,  
15 oligopeptides, and polypeptides. The term "protein" also encompasses two or more polypeptides combined to form the encoded product. The proteins also include hybrid polypeptides and fused polypeptides.

Preferably, the protein is a hormone or variant thereof, enzyme, receptor or portion thereof, antibody or portion thereof, or reporter. For example, the protein may be a hydrolase, isomerase,  
20 ligase, lyase, oxidoreductase, or transferase, e.g., an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase,  
25 proteolytic enzyme, ribonuclease, transglutaminase, xylanase, or beta-xylosidase.

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

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

## 30 Examples

### Strains

The enzymes included herein are isolated from a diverse range of microorganisms including *Cerrena unicolor* (SEQ ID NO:1+2), *Trichoderma reesei* (SEQ ID NO:3+4) and *Chaetomium globosum* (SEQ ID NO:5+6).

### Media and Solutions

The reaction conditions, media and solutions provided herein are included for inspiration and may be replaced by alternative methods, reaction conditions and media where the skilled person finds it applicable.

### Hydrolysis conditions

Conditions	
Total reaction volume	2 ml
Hydrolysis time	24 or 48 h
Beta-glucanase composition ( <i>H. insolens</i> )	5 mg protein/g DM
Beta-xylosidase ( <i>Trichoderma reesei</i> )	1 mg protein/g DM
$\alpha$ -glucuronidase (if added)	1 mg protein/g DM
Substrate	Pretreated corn fiber (140 °C, 150 min)
Substrate loading	2.5 %
Buffer	50 mM Succinic acid pH 5.0
Instruments	Thermomixer at 50 °C and 1300 rpm

The 0.05 g pretreated corn fiber was transferred to plastic vials. Enzymes and buffer was added and the plastic vials containing a total reaction volume of 2 ml was placed on a thermomixer at 50°C and 1300 rpm for 24 or 48 hours.

### Determination of arabinose and xylose

Arabinose and xylose were determined by carbohydrate hydrolysis using dilute hydrochloric acid. The pretreated corn fiber was transferred to 125 ml conical flasks and diluted to contain approximately 10% dry matter. The corn fiber sample was preheated at 100°C in an oil bath. Hydrolysis was started by adding 5 ml of 2 M hydrochloric acid for 2 hours at 100°C. After incubation the flasks were cooled on ice and neutralized with 4 M sodium hydroxide. Samples were filtered with a MINISART® 0.2 micron syringe filter (Sartorius AG, Goettingen, Germany) and analyzed for arabinose and xylose on a DIONEX BIOLC® System (Dionex Corporation, Sunnyvale, CA, USA).

### Determination of glucose

Glucose concentration was determined with a DIONEX® BIOLC® System according to the following method. Samples (10 µl) were loaded onto a DIONEX BIOLC® System equipped with a DIONEX® CARBOPAC™ PA1 analytical column (4 x 250 mm) (Dionex Corporation, Sunnyvale, CA, USA) combined with a CARBOPAC™ PA1 guard column (4 x 50 mm) (Dionex Corporation, Sunnyvale, CA, USA). The monosaccharides were separated isocratically with 10 mM potassium hydroxide at a flow rate of 1 ml per minute and detected by a pulsed electrochemical detector in the pulsed amperometric detection mode. The potential of the electrode was programmed for +0.1 volt (t=0-0.4 second) to -2.0 volt (t=0.41-0.42 second) to 0.6 volt (t=0.43 second) and finally -0.1 volt (t=0.44-0.50 second), while integrating the resulting signal from t=0.2-0.4 second.

### Determination of glucuronic acid

Glucuronic acid concentration was determined with a DIONEX® ICS3000® System according to the following method. Samples (10 µl) were loaded onto a DIONEX ICS3000® System equipped with a DIONEX® CARBOPAC™ PA1 analytical column (4 x 250 mm) (Dionex Corporation, Sunnyvale, CA, USA) combined with a CARBOPAC™ PA1 guard column (4 x 50 mm) (Dionex Corporation, Sunnyvale, CA, USA). Glucuronic acid was separated isocratically with 101 mM sodium hydroxide and 160 mM sodium acetate at a flow rate of 1 ml per minute and detected by a pulsed electrochemical detector in the pulsed amperometric detection mode. The potential of the electrode was programmed for +0.1 volt (t=0-0.4 second) to -2.0 volt (t=0.41-0.42 second) to 0.6 volt (t=0.43 second) and finally -0.1 volt (t=0.44-0.50 second), while integrating the resulting signal from t=0.2-0.4 second. Pure glucuronic acid dissolved in deionised water was used as a standard. Standards of the following concentration were used: 5, 10, 25, 50, 100, 250 and 500 µg/ml were used to determine the concentration of glucuronic acid in the hydrolysed samples.

### Example 1, Effect of glucuronyl esterase on hydrolysis of pretreated corn fiber

Figure 1 shows the conversion of pretreated corn fiber after hydrolysis for 48 hours with and without addition of glucuronyl esterase.

As apparent from figure 1, addition of glucuronyl esterase to hydrolysis mixtures comprising β-glucanase and β-xylosidase enhances the total hydrolysis.

The effect of glucuronyl esterase on hydrolysis of pretreated corn fiber was evaluated. Corn fiber is a fraction from the wet milling of corn kernels. Corn fiber is the seed coat and residual endosperm left after starch is removed and further processed. Corn fiber was pretreated by autoclaving at 140°C for 150 minutes. The amount of theoretical arabinose, glucose and xylose in

the substrate was determined to be 114, 302, and 204 g per kg dry matter using the following methods.

Arabinose and xylose were determined by carbohydrate hydrolysis using dilute hydrochloric acid. The pretreated corn fiber was transferred to 125 ml conical flasks and diluted to contain approximately 10% dry matter. The corn fiber sample was preheated at 100°C in an oil bath. Hydrolysis was started by adding 5 ml of 2 M hydrochloric acid for 2 hours at 100°C. After incubation the flasks were cooled on ice and neutralized with 4 M sodium hydroxide. Samples were filtered with a MINISART® 0.2 micron syringe filter (Sartorius AG, Goettingen, Germany) and analyzed for arabinose and xylose on a DIONEX BIOLC® System (Dionex Corporation, Sunnyvale, CA, USA).

The hydrolysis of the pretreated corn fiber was conducted with a *H. insolens* beta-glucanase composition and a *Trichoderma reesei* beta-xylosidase. The *Trichoderma reesei* beta-xylosidase was obtained recombinantly by expression in *Aspergillus oryzae* as described in Rasmussen *et al.*, 2006, *Biotechnology and Bioengineering* 94: 869-876 using standard cultivation methods for *Aspergillus oryzae*.

The hydrolysis of the pretreated corn fiber was performed in 2 ml EPPENDORF® tubes (Eppendorf AG, Germany) at a temperature of 50°C and a pH of 5.0 in 50 mM succinic acid. Samples were incubated in a THERMOMIXER® Comfort (Eppendorf AG, Germany) that subjected each sample with constant heating and mixing. The substrate amount used was 2.5 w/w % in a total sample volume of 2 ml. The *Chaetomium globosum* glucuronyl esterase was added at an enzyme loading of 1 mg enzyme per g of dry matter on top of both the *H. insolens* beta-glucanase composition and the *Trichoderma reesei* beta-xylosidase. *H. insolens* beta-glucanase composition was added at a loading of 5 mg enzyme per g of dry matter and the *Trichoderma reesei* beta-xylosidase at a loading of 1 mg enzyme per g of dry matter. Hydrolysis was terminated after 48 hours by heating the samples for 10 minutes at 100°C in a heat block (Techne Inc., Burlington NJ, USA).

Conversion was calculated by determining the amount of sugars released from the substrate as a percentage of what was added from the start using the formula below but not including initial monomeric sugars. T-tests were performed with a two tailed distribution and equal variance of sample data.

$$\text{Conversion (\%)} = (\text{Sugar amount in hydrolysate} / \text{Sugar amount in added substrate}) \times 100$$

Comparing the conversion of pretreated corn fiber when adding the *Chaetomium globosum* glucuronyl esterase at an enzyme loading of 1 mg of enzyme per gram dry matter together with 1 mg enzyme per g of dry matter of *Trichoderma reesei* beta-xylosidase and 5 mg enzyme per g of

dry matter of *H. insolens* beta-glucanase composition to just adding 1 mg enzyme per g of dry matter of beta-xylosidase from *Trichoderma reesei* and 5 mg enzyme per g of dry matter of *H. insolens* beta-glucanase composition demonstrated a significant ( $P \leq 0.0323$ ) increase in conversion from 44.1 to 46.1 (Table 1).

5

**Table 1**

Samples	Mean Conversion	Standard deviation	T-test
<i>H. insolens</i> beta-glucanase composition and <i>Trichoderma reesei</i> beta-xylosidase	44.1	1.0	0.0323
<i>H. insolens</i> beta-glucanase composition, <i>Trichoderma reesei</i> beta-xylosidase, and <i>Chaetomium globosum</i> glucuronyl esterase	46.1	0.3	

**Example 2:**

In a further aspect, the invention relates to the enhanced release of glucuronic acid of pretreated corn fiber after hydrolysis with addition of glucuronyl esterase. As shown in figure 2, addition of glucuronyl esterase stimulates the release of glucuronic acid during hydrolysis of pretreated corn fiber.

Comparing the release of glucuronic acid from pretreated corn fiber when adding the *Chaetomium globosum* glucuronyl esterase at an enzyme loading of 1 mg of enzyme per gram dry matter together with 1 mg enzyme per g of dry matter of *Trichoderma reesei* beta-xylosidase and 5 mg enzyme per g of dry matter of *H. insolens* beta-glucanase composition to just adding 1 mg enzyme per g of dry matter of beta-xylosidase from *Trichoderma reesei* and 5 mg enzyme per g of dry matter of *H. insolens* beta-glucanase composition demonstrated a significant ( $P \leq 0.0449$ ) increase in glucuronic acid release from 4.0 to 5.3 g/kg DM (Table 2).

20

**Table 2**

Samples	Mean release (g/kg DM)	Standard deviation	T-test
---------	------------------------	--------------------	--------

<i>H. insolens</i> beta-glucanase composition and <i>Trichoderma reesei</i> beta-xylosidase	4.0	0.2	0.0449
<i>H. insolens</i> beta-glucanase composition, <i>Trichoderma reesei</i> beta-xylosidase, and <i>Chaetomium globosum</i> glucuronyl esterase	5.3	0,7	

**Aspects**

Further, the present invention relates to the following aspects:

Aspect 1. An isolated polypeptide having glucuronyl esterase activity, selected from the group consisting of:

(a) a polypeptide having at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide of SEQ ID NO:2; or

at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide of SEQ ID NO:4 or

at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide of SEQ ID NO:6;

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

or under very high stringency conditions with (iv) the mature polypeptide coding sequence of SEQ ID NO:3, (v) the cDNA sequence thereof, or (vi) the full-length complement of (iv) or (v);

or under very high stringency conditions with (vii) the mature polypeptide coding sequence of SEQ ID NO:5, (viii) the cDNA sequence thereof, or (ix) the full-length complement of (vii) or (viii);

or under medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with (x) the mature polypeptide coding sequence of SEQ ID NO:7, (xi) the cDNA sequence thereof, or (xii) the full-length complement of (x) or (xi);

(c) a polypeptide encoded by a polynucleotide having at least 80%, at least 85%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide coding sequence of SEQ ID NO:1 or the cDNA sequence thereof or



having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide coding sequence of SEQ ID NO:3 or the cDNA sequence thereof or

5 having at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide coding sequence of SEQ ID NO:5 or the cDNA sequence thereof or

10 having at least 80%, at least 85%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide coding sequence of SEQ ID NO:7 or the cDNA sequence thereof.

(d) a variant of the mature polypeptide of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 comprising a substitution, deletion, and/or insertion at one or more positions; and

15 (e) a fragment of the polypeptide of (a), (b), (c) or (d) that has glucuronyl esterase activity.

Aspect 2. The polypeptide of aspect 1, comprising or consisting one of the sequences SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6.

20 Aspect 3. The polypeptide of any of aspects 1-2, comprising or consisting of the mature polypeptide of one of the sequences SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6.

Aspect 4. The polypeptide of aspect 3, wherein the mature polypeptide is amino acids 101 to 474 of SEQ ID NO:2, 94 to 460 of SEQ ID NO:4 or 21 to 392 of SEQ ID NO:6.

25 Aspect 5. The polypeptide of any of aspects 1-4, which is a fragment of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 wherein the fragment has glucuronyl esterase activity.

Aspect 6. A composition comprising the polypeptide of any of aspects 1-5.

30 Aspect 7. An isolated polynucleotide encoding the polypeptide of any of aspects 1-5.

Aspect 8. A nucleic acid construct or expression vector comprising the polynucleotide of aspect 7 operably linked to one or more control sequences that direct the production of the polypeptide in an expression host.

- Aspect 9. A recombinant host cell comprising the polynucleotide of aspect 7 operably linked to one or more control sequences that direct the production of the polypeptide.
- 5 Aspect 10. A method of producing the polypeptide of any of aspects 1-5, comprising:  
(a) cultivating a cell, which in its wild-type form produces the polypeptide, under conditions conducive for production of the polypeptide; and  
(b) recovering the polypeptide.
- 10 Aspect 11. A method of producing a polypeptide having glucuronyl esterase activity, comprising:  
(a) cultivating the host cell of aspect 9 under conditions conducive for production of the polypeptide; and  
(b) recovering the polypeptide.
- 15 Aspect 12. A transgenic plant, plant part or plant cell transformed with a polynucleotide encoding the polypeptide of any of aspects 1-5.
- Aspect 13. A method of producing a polypeptide having glucuronyl esterase activity, comprising:  
(a) cultivating the transgenic plant or plant cell of aspect 12 under conditions conducive  
20 for production of the polypeptide; and  
(b) recovering the polypeptide.
- Aspect 14. A method of producing a mutant of a parent cell, comprising inactivating a polynucleotide encoding the polypeptide of any of aspects 1-5, which results in the mutant  
25 producing less of the polypeptide than the parent cell.
- Aspect 15. A mutant cell produced by the method of aspect 14.
- Aspect 16. The mutant cell of aspect 15, further comprising a gene encoding a native or  
30 heterologous protein.
- Aspect 17. A method of producing a protein, comprising:  
(a) cultivating the mutant cell of aspect 15 or 16 under conditions conducive for production of the protein; and

(b) recovering the protein.

Aspect 18. A double-stranded inhibitory RNA (dsRNA) molecule comprising a subsequence of the polynucleotide of aspect 7, wherein optionally the dsRNA is an siRNA or an miRNA molecule.

5

Aspect 19. The double-stranded inhibitory RNA (dsRNA) molecule of aspect 18, which is about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more duplex nucleotides in length.

Aspect 20. A method of inhibiting the expression of a polypeptide having glucuronyl esterase activity in a cell, comprising administering to the cell or expressing in the cell the double-stranded inhibitory RNA (dsRNA) molecule of aspect 18 or 19.

10

Aspect 21. The method of aspect 20, wherein the dsRNA is about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more duplex nucleotides in length.

15

Aspect 22. A cell produced by the method of aspect 20 or 21.

Aspect 23. The cell of aspect 22, further comprising a gene encoding a native or heterologous protein.

20

Aspect 24. A method of producing a protein, comprising:

(a) cultivating the cell of aspect 22 or 23 under conditions conducive for production of the protein; and

(b) recovering the protein.

25

Aspect 25. An isolated polynucleotide encoding a signal peptide comprising or consisting of amino acids 1 to 17 of SEQ ID NO:2 or amino acids 1 to 17 of SEQ ID NO:4 or amino acids 1 to 20 of SEQ ID NO:6.

Aspect 26. A nucleic acid construct or expression vector comprising a gene encoding a protein operably linked to the polynucleotide of aspect 25, wherein the gene is foreign to the polynucleotide encoding the signal peptide.

30

Aspect 27. A recombinant host cell comprising a gene encoding a protein operably linked to the polynucleotide of aspect 25, wherein the gene is foreign to the polynucleotide encoding the signal peptide.

- 5 Aspect 28. 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 aspect 24 or 25, wherein the gene is foreign to the polynucleotide encoding the signal peptide, under conditions conducive for production of the protein; and
  - (b) recovering the protein.

10 Aspect 29. A method for degrading or converting a cellulosic material, comprising: treating the cellulosic material with an enzyme composition in the presence of the polypeptide having glucuronyl esterase activity of any of aspects 1-5.

15 Aspect 30. The method of aspect 29, wherein the cellulosic material is pretreated.

Aspect 31. The method of aspect 29 or 30, wherein the enzyme composition comprises one or more enzymes selected from the group consisting of a cellulase, a polypeptide having cellulolytic enhancing activity, a hemicellulase, an esterase, a protease, a laccase, or a peroxidase.

20 Aspect 32. The method of aspect 31, wherein the cellulase is one or more enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

25 Aspect 33. The method of aspect 31, wherein the hemicellulase is one or more enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase.

30 Aspect 34. The method of any of aspects 29-33, further comprising recovering the degraded cellulosic material.

Aspect 35. The method of aspect 34, wherein the degraded cellulosic material is a sugar.

Aspect 36. The method of aspect 35, wherein the sugar is selected from the group consisting of glucose, xylose, mannose, galactose, and arabinose.

Aspect 37. A method for producing a fermentation product, comprising:

(a) saccharifying a cellulosic material with an enzyme composition in the presence of the polypeptide having glucuronyl esterase activity of any of aspects 1-5;

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

(c) recovering the fermentation product from the fermentation.

Aspect 38. The method of aspect 37, wherein the cellulosic material is pretreated.

10

Aspect 39. The method of aspect 37 or 38, wherein the enzyme composition comprises one or more enzymes selected from the group consisting of a cellulase, a polypeptide having cellulolytic enhancing activity, a hemicellulase, an esterase, a protease, a laccase, or a peroxidase.

15 Aspect 40. The method of aspect 39, wherein the cellulase is one or more enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

Aspect 41. The method of aspect 39, wherein the hemicellulase is one or more enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase.

20

Aspect 42. The method of any of aspects 37-41, wherein steps (a) and (b) are performed simultaneously in a simultaneous saccharification and fermentation.

25 Aspect 43. The method of any of aspects 37-42, wherein the fermentation product is an alcohol, an organic acid, a ketone, an amino acid, or a gas.

Aspect 44. A method of fermenting a cellulosic material, comprising: fermenting the cellulosic material with one or more fermenting microorganisms, wherein the cellulosic material is saccharified with an enzyme composition in the presence of a polypeptide having glucuronyl esterase activity of any of aspects 1-5.

30

Aspect 45. The method of aspect 44, wherein the fermenting of the cellulosic material produces a fermentation product.

Aspect 46. The method of aspect 45, further comprising recovering the fermentation product from the fermentation.

5 Aspect 47. The method of any of aspects 44-46, wherein the cellulosic material is pretreated before saccharification.

10 Aspect 48. The method of any of aspects 44-47, wherein the enzyme composition comprises one or more enzymes selected from the group consisting of a cellulase, a polypeptide having cellulolytic enhancing activity, a hemicellulase, an esterase, a protease, a laccase, or a peroxidase.

Aspect 49. The method of aspect 48, wherein the cellulase is one or more enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

15 Aspect 50. The method of aspect 48, wherein the hemicellulase is one or more enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase.

20 Aspect 51. The method of any of aspects 45-50, wherein the fermentation product is an alcohol, an organic acid, a ketone, an amino acid, or a gas.

**Claims**

1. An isolated polypeptide having glucuronyl esterase activity, selected from the group consisting of:
- 5 (a) a polypeptide having at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide of SEQ ID NO:2; or  
at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide of SEQ ID NO:4 or  
10 at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide of SEQ ID NO:6;
- (b) a polypeptide encoded by a polynucleotide that hybridizes under high stringency conditions, or very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO:1, (ii) the cDNA sequence thereof, or (iii) the full-length complement of (i) or (ii); or  
15 or under very high stringency conditions with (iv) the mature polypeptide coding sequence of SEQ ID NO:3, (v) the cDNA sequence thereof, or (vi) the full-length complement of (iv) or (v);  
or under very high stringency conditions with (vii) the mature polypeptide coding sequence of SEQ ID NO:5, (viii) the cDNA sequence thereof, or (ix) the full-length complement of (vii) or (viii);
- (c) a polypeptide encoded by a polynucleotide having at least 80%, at least 85%, at  
20 least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide coding sequence of SEQ ID NO:1 or the cDNA sequence thereof or  
having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature  
25 polypeptide coding sequence of SEQ ID NO:3 or the cDNA sequence thereof or  
having at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide coding sequence of SEQ ID NO:5 or the cDNA sequence thereof or
- (d) a variant of the mature polypeptide of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6  
30 comprising a substitution, deletion, and/or insertion at one or more positions; and
- (e) a fragment of the polypeptide of (a), (b), (c) or (d) that has glucuronyl esterase activity.

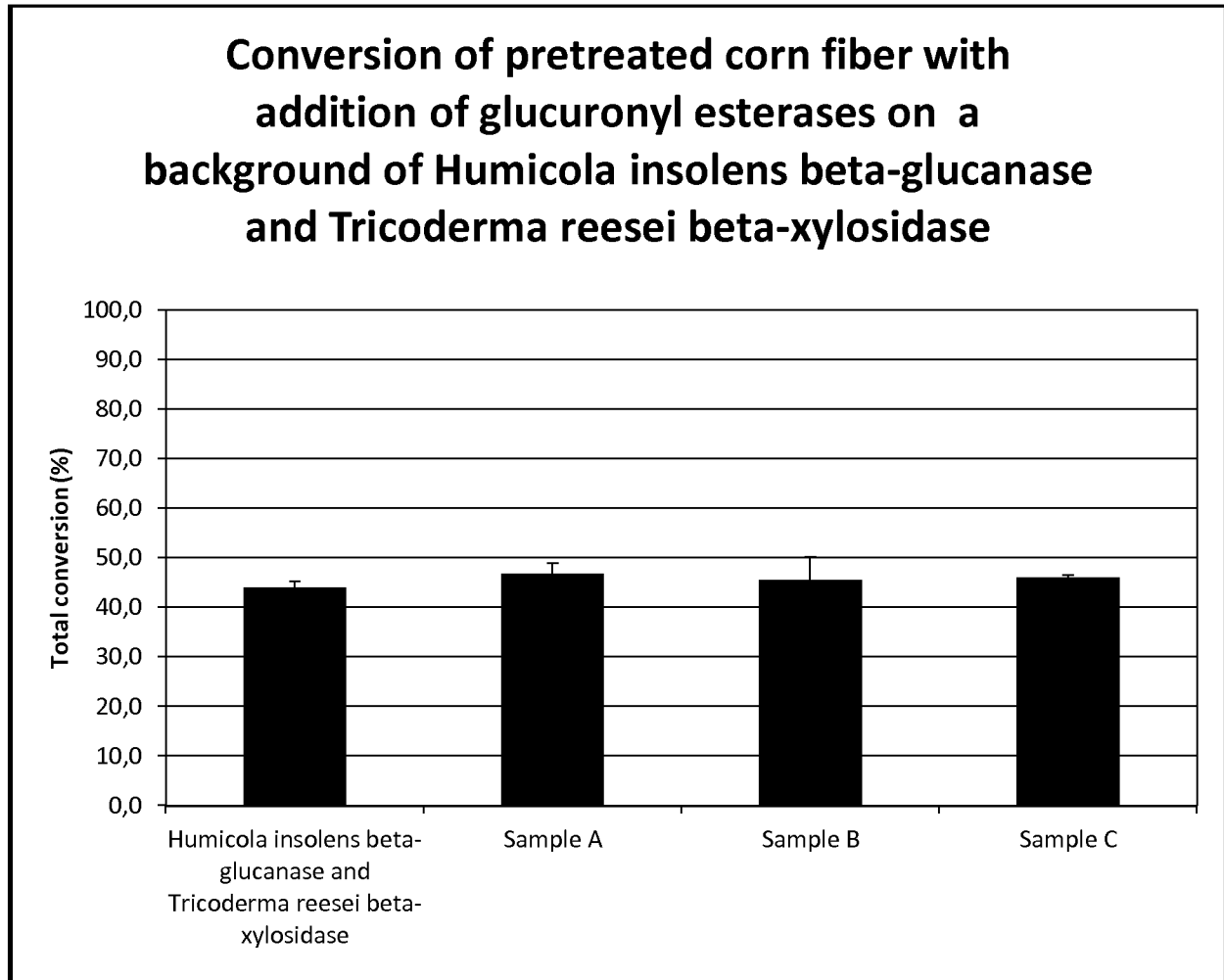
2. The polypeptide of claim 1, comprising or consisting one of the sequences SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6.
3. The polypeptide of any of claims 1-2, comprising or consisting of the mature polypeptide of one of the sequences SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6.
4. The polypeptide of claim 3, wherein the mature polypeptide is amino acids 101 to 474 of SEQ ID NO:2, 94 to 460 of SEQ ID NO:4 or 21 to 392 of SEQ ID NO:6.
5. The polypeptide of any of claims 1-4, which is a fragment of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 wherein the fragment has glucuronyl esterase activity.
6. A composition comprising the polypeptide of any of claims 1-5.
7. An isolated polynucleotide encoding the polypeptide of any of claims 1-5.
8. A nucleic acid construct or expression vector comprising the polynucleotide of claim 7 operably linked to one or more control sequences that direct the production of the polypeptide in an expression host.
9. A recombinant host cell comprising the polynucleotide of claim 7 operably linked to one or more control sequences that direct the production of the polypeptide.
10. A method of producing the polypeptide of any of claims 1-5, 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.
11. A method of producing a polypeptide having glucuronyl esterase activity, comprising:
- (a) cultivating the host cell of claim 9 under conditions conducive for production of the polypeptide; and
  - (b) recovering the polypeptide.



12. A method for degrading or converting a cellulosic material, comprising: treating the cellulosic material with an enzyme composition in the presence of the polypeptide having glucuronyl esterase activity of any of claims 1-5.
- 5 13. The method of claim 12, wherein the cellulosic material is pretreated.
14. The method of claim 12 or 13, wherein the enzyme composition comprises one or more enzymes selected from the group consisting of a cellulase, a polypeptide having cellulolytic enhancing activity, a hemicellulase, an esterase, a protease, a laccase, or a peroxidase.
- 10 15. A method for producing a fermentation product, comprising:
- (a) saccharifying a cellulosic material with an enzyme composition in the presence of the polypeptide having glucuronyl esterase activity of any of claims 1-5;
  - (b) fermenting the saccharified cellulosic material with one or more fermenting
  - 15 microorganisms to produce the fermentation product; and
  - (c) recovering the fermentation product from the fermentation.

1/ 2

Figure 1



2/ 2

Figure 2

