(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 February 2012 (16.02.2012)

PCT

(10) International Publication Number
WO 2012/021410 A1

(51) International Patent Classification:
C12P 7/10 (2006.01) *C12N 9/42* (2006.01)
C12P 19/14 (2006.01) *C12P 19/02* (2006.01)

(21) International Application Number:
PCT/US2011/046795

(22) International Filing Date:
5 August 2011 (05.08.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

61/373,170	12 August 2010 (12.08.2010)	US
61/373,166	12 August 2010 (12.08.2010)	US
61/373,157	12 August 2010 (12.08.2010)	US
61/373,150	12 August 2010 (12.08.2010)	US
61/373,145	12 August 2010 (12.08.2010)	US
61/373,128	12 August 2010 (12.08.2010)	US
61/373,124	12 August 2010 (12.08.2010)	US
61/373,210	12 August 2010 (12.08.2010)	US

(71) Applicants *(for all designated States except US)*: **NOVOZYMES, INC.** [US/US]; 1445 Drew Avenue, Davis, California 95618 (US). **NOVOZYMES NORTH AMERICA, INC.** [US/US]; 77 Perry Chapel Church Road, Franklinton, North Carolina 27525 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **QUINLAN, Jason** [CA/US]; 839 West Lincoln Avenue, Apt 617, Woodland, California 95695 (US). **XU, Feng** [US/US]; 1555 Santa Rosa, Davis, California 95616 (US). **SWEENEY, Matthew** [US/US]; 517 14th Street, Sacramento, California 95814 (US). **HIGGINS, Don** [US/US]; 4060 Ridgeway Drive, Franklinton, North Carolina 27525 (US). **XU, Hui** [US/US]; 3121 Gross Avenue, Wake Forest, North Carolina 27587 (US).

(74) Agent: **STARNES, Robert**; Novozymes, Inc., 1445 Drew Avenue, Davis, CA 95618 (US).

(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, 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, PE, PG, PH, PL, PT, QA, RO, RS, RU, 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.

[Continued on next page]

(54) Title: COMPOSITIONS COMPRISING A POLYPEPTIDE HAVING CELLULOLYTIC ENHANCING ACTIVITY AND A LIQUOR AND USES THEREOF

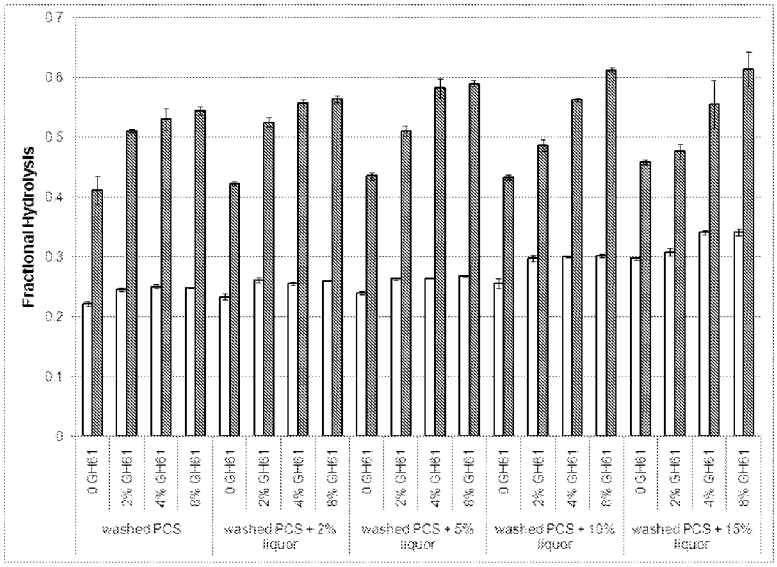

Fig. 2A

(57) Abstract: The present invention relates to compositions comprising: a polypeptide having cellulolytic enhancing activity and a liquor. The present invention also relates to methods of using the compositions.

WO 2012/021410 A1

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, 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).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

COMPOSITIONS COMPRISING A POLYPEPTIDE HAVING CELLULOLYTIC ENHANCING ACTIVITY AND A LIQUOR AND USES THEREOF

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

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

Cross-Reference to Related Applications

This application claims the benefit of U.S. Provisional Application Serial No. 61/373,124, filed August 12, 2010, U.S. Provisional Application Serial No. 61/373,128, filed August 12, 2010, U.S. Provisional Application Serial No. 61/373,145, filed August 12, 2010, U.S. Provisional Application Serial No. 61/373,150, filed August 12, 2010, U.S. Provisional Application Serial No. 61/373,157, filed August 12, 2010, U.S. Provisional Application Serial No. 61/373,166, filed August 12, 2010, U.S. Provisional Application Serial No. 61/373,170, filed August 12, 2010, and U.S. Provisional Application Serial No. 61/373,210, filed August 12, 2010, which applications are incorporated herein by reference.

Reference to a Sequence Listing

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

Background of the Invention

Field of the Invention

The present invention relates to compositions comprising a polypeptide having cellulolytic enhancing activity and a liquor, and to methods of using the compositions.

Description of the Related Art

Cellulose is a polymer of the simple sugar glucose covalently linked by beta-1,4-bonds. Many microorganisms produce enzymes that hydrolyze beta-linked glucans. These enzymes include endoglucanases, cellobiohydrolases, and beta-glucosidases. Endoglucanases digest the cellulose polymer at random locations, opening it to attack by cellobiohydrolases. Cellobiohydrolases sequentially release molecules of cellobiose from the ends of the cellulose polymer. Cellobiose is a water-soluble beta-1,4-linked dimer of glucose.

Beta-glucosidases hydrolyze cellobiose to glucose.

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

WO 2005/074647, WO 2008/148131, WO 2011/035027 disclose isolated GH61 polypeptides having cellulolytic enhancing activity and the polynucleotides thereof from *Thielavia terrestris*. WO 2005/074656 and WO 2010/065830 disclose isolated GH61 polypeptides having cellulolytic enhancing activity and the polynucleotides thereof from *Thermoascus aurantiacus*. WO 2007/089290 discloses an isolated GH61 polypeptide having cellulolytic enhancing activity and the polynucleotide thereof from *Trichoderma reesei*. WO 2009/085935, WO 2009/085859, WO 2009/085864, and WO 2009/085868 disclose isolated GH61 polypeptides having cellulolytic enhancing activity and the polynucleotides thereof from *Myceliophthora thermophila*. WO 2010/138754 discloses isolated GH61 polypeptides having cellulolytic enhancing activity and the polynucleotides thereof from *Aspergillus fumigatus*. WO 2011/005867 discloses isolated GH61 polypeptides having cellulolytic enhancing activity and the polynucleotides thereof from *Penicillium pinophilum*. WO 2011/039319 discloses isolated GH61 polypeptides having cellulolytic enhancing activity and the polynucleotides thereof from *Thermoascus* sp. WO 2011/041397 discloses isolated GH61 polypeptides having cellulolytic enhancing activity and the polynucleotides thereof from *Penicillium* sp. WO 2011/041504 discloses isolated GH61 polypeptides having cellulolytic enhancing activity and the polynucleotides thereof from *Thermoascus crustaceous*. WO 2008/151043 discloses methods of increasing the activity of a GH61 polypeptide having cellulolytic enhancing activity by adding a soluble activating divalent metal cation to a composition comprising the polypeptide.

It would be advantageous in the art to improve the ability of polypeptides having cellulolytic enhancing activity to enhance enzymatic hydrolysis of lignocellulosic feedstocks.

The present invention relates to compositions comprising a polypeptide having cellulolytic enhancing activity and a liquor, and to methods of using the compositions.

Summary of the Invention

The present invention relates to compositions comprising: (a) a polypeptide having cellulolytic enhancing activity; and (b) a liquor, wherein the combination of the polypeptide

having cellulolytic enhancing activity and the liquor enhances hydrolysis of a cellulosic material by a cellulolytic enzyme.

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 cellulolytic enhancing activity and a liquor, wherein the combination of the polypeptide having cellulolytic enhancing activity and the liquor enhances hydrolysis of the cellulosic material by the enzyme composition.

The present invention also relates to methods for producing a fermentation product, comprising:

(a) saccharifying a cellulosic material with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity and a liquor, wherein the combination of the polypeptide having cellulolytic enhancing activity and the liquor enhances hydrolysis of the cellulosic material by the enzyme composition;

(b) fermenting the saccharified cellulosic material with one or more (*e.g.*, 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 (*e.g.*, several) fermenting microorganisms, wherein the cellulosic material is saccharified with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity and a liquor, wherein the combination of the polypeptide having cellulolytic enhancing activity and the liquor enhances hydrolysis of the cellulosic material by the enzyme composition.

Brief Description of the Figures

Figure 1 shows the fractional hydrolysis of washed and unwashed pretreated corn stover by a *Trichoderma reesei* cellulase composition with various concentrations of *Thermoascus aurantiacus* GH61A polypeptide having cellulolytic enhancement activity. Open symbols: 1 day of hydrolysis; closed symbols: 3 days of hydrolysis; squares: milled, water-washed pretreated corn stover; circles: milled, unwashed pretreated corn stover; triangles: hot-water washed, milled pretreated corn stover.

Figures 2A and 2B show the effect of acid-pretreated corn stover liquor on GH61 polypeptide-enhancement of cellulolysis of milled, water-washed pretreated corn stover by a *T. reesei* cellulase composition. Panel A: fractional hydrolysis. White bars: 1 day hydrolysis; gray bars: 3 day hydrolysis. Panel B: open symbols, 1 day hydrolysis; solid symbols: 3 day hydrolysis. Circles: no added liquor; squares: 2% (v/v) liquor; diamonds: 5% (v/v) liquor; triangles: 10% liquor; inverted triangles: 15% liquor. Data were fit linearly or by a modified

saturation-binding model as described.

Figures 3A and 3B show acid-pretreated corn stover liquor dependence of *T. aurantiacus* GH61A polypeptide-enhancement of hydrolysis of pretreated corn stover. Panel A: AVICEL® + various concentrations of the acid-pretreated corn stover liquor as a function of GH61 polypeptide concentrations. Panel B: AVICEL® + synthetic liquor as a function of GH61 polypeptide concentration. Open symbols: 1 day hydrolysis; closed symbols: 3 days hydrolysis. Circles: no liquor; diamonds: 5% (v/v) liquor; triangles: 10% liquor; inverted triangles: 15% liquor; squares: 5% synthetic liquor containing no phenol; right triangles: 15% synthetic liquor containing no phenol. Data were fit linearly or using Equation 2, as described.

Figures 4A, 4B, and 4C show the effect of enzymatically treated or not enzymatically-treated pretreated corn stover liquors on enhancement of cellulolysis of pretreated corn stover by the *Thelavia terrestris* GH61E polypeptide. Panel A: un-treated liquor; Panel B: *T. reesei* cellulase-treated liquor; and Panel C: *T. reesei* cellulase and *Thelavia terrestris* GH61E polypeptide-treated liquor. Circles: no added liquor; squares: 5% (v/v) liquor; diamonds: 10% (v/v) liquor; triangles: 15% (v/v) liquor. Data were fit linearly or using Equation 2, as described.

Figures 5A and 5B show the fractional hydrolysis of microcrystalline cellulose by the *T. reesei* cellulase composition with various *T. aurantiacus* GH61A polypeptide concentrations, comparing impact of addition of dilute-acid and steam pretreatment liquors at 5 days of hydrolysis. Panel A: NREL acid-pretreated corn stover liquor; Panel B: steam explosion-pretreated corn stover liquor. Open symbols: 5% (v/v) liquor; closed symbols: 15% (v/v) liquor. Circles: whole liquor; squares: low molecular weight fraction, triangles: high molecular weight fraction. Data were fit using Equation 2, as described.

Figure 6 shows the effect of retentates and flow-through samples of molecular weight-filtered acid-pretreated corn stover liquor on GH61 polypeptide cellulolytic enhancing activity. White bars: 1 day of saccharification; gray bars: 6 days of saccharification. Concentrations listed refer to the *T. reesei* cellulase composition and the GH61 polypeptide concentration, respectively, in mg per gram cellulose.

Figure 7 shows (A) the fractional hydrolysis of microcrystalline cellulose by individual *T. reesei* cellulase monocomponents and mixtures thereof, and the effects of the *T. aurantiacus* GH61A polypeptide and NREL acid-pretreated corn stover liquor thereon and (B) the *GH61 effect* on the individual cellulases and mixtures of cellulases. White bars: 3 days of hydrolysis; gray bars: 5 days of hydrolysis; black bars: 7 days of hydrolysis.

Figure 8A shows HPLC chromatography of NREL acid-pretreated corn stover liquor. Fractional hydrolysis and absorbance are plotted for the various HPLC fractions. Solid line: fractional hydrolysis; light dashed line: absorbance at 210 nm; heavy dashed line:

absorbance at 280 nm. The average hydrolysis for all samples is indicated by the solid, horizontal line. Figure 8B shows the fractional hydrolysis for the *T. reesei* cellulase composition with increasing GH61A polypeptide concentrations in the presence of 3 kDa MWCO flow-through fractions of NREL acid-pretreated corn stover liquor incubated with microcrystalline cellulose and eluted with successive water and organic solvent washes. Gray bars: 3 days of hydrolysis; black bars: 7 days of hydrolysis.

Figure 9 shows a standard curve of AVICEL® height vs. added mass.

Figure 10 shows the effect of various pooled, HPLC-separated NREL acid-pretreated corn stover liquor fractions and the *T. aurantiacus* GH61A polypeptide on hydrolysis of microcrystalline cellulose by the *T. reesei* cellulase composition. Solid lines: height of AVICEL® incubated with the GH61 polypeptide; dashed lines: $A_{(600nm)}$ of washed, BCA reagent-reacted AVICEL®.

Figure 11 shows (A) a standard curve of reducing sugar equivalents and (B) the reducing sugar equivalents measured in the solid microcrystalline cellulose incubated with the *T. aurantiacus* GH61A polypeptide and the indicated HPLC fractions.

Figure 12 shows the results of a microcrystalline cellulose hydrolysis assay with the *T. reesei* cellulase composition in the presence of the *T. aurantiacus* GH61A polypeptide and chromatographed fractions of acid-pretreated xylan. Solid line: fractional hydrolysis with GH61; dashed line: mean fractional hydrolysis.

Figure 13 shows a LC-MS chromatogram of a representative HPLC fraction of acid-pretreated xylan.

Figure 14 shows liquid chromatography-mass spectrometry chromatograms of GH61 polypeptide-affinity enriched acid-pretreated corn stover. Panel A: diode array detection; Panel B: TOF MS/MS total ion current 17.5; Panel C: TOF MS/MS ES-total ion current 273; Panel D: TOF MS ES-total ion survey.

Figure 15 shows ion chromatograms of microcrystalline cellulose or phosphoric acid-swollen cellulose incubated with the *T. aurantiacus* GH61A polypeptide and NREL acid-pretreated corn stover liquor. Panel A: reaction samples; Panel B: comparison to analytical standards. Panel A: solid lines: AVICEL® incubations; dashed lines: PASC incubations; light gray: AVICEL®; medium gray: AVICEL® + NREL acid-pretreated corn stover liquor; dark gray: AVICEL® + GH61 polypeptide; black: AVICEL® + GH61 polypeptide + liquor. Panel B: black: AVICEL® + GH61 polypeptide + liquor; dark gray solid lines: liquor; dark gray dashed lines: cellopentaose, cellotetraose and cellotriose; light gray: cellopentaonic acid, cellotetraonic acid, gluconic acid, galactonic acid and xylotetraose, peaks as indicated.

Figure 16A shows the fractional hydrolysis of AVICEL® by a *T. reesei* cellulase composition with the indicated *T. aurantiacus* GH61A polypeptide concentration in the presence of alkaline-pretreated corn stover generated using the indicated pretreatment

concentration of sodium hydroxide. White bars: 1 day of hydrolysis; gray bars: 3 days of hydrolysis; black bars: 7 days of hydrolysis. Figure 16B shows the fractional hydrolysis of AVICEL® by the *T. reesei* cellulase composition with the indicated GH61 polypeptide concentration in the presence of water-extracted, acid-pretreated corn stover liquors generated using the indicated extraction temperature. Gray bars: 1 day of hydrolysis; white bars: 3 days of hydrolysis.

Figures 17A and 17B show the fractional hydrolysis of AVICEL® by a *T. reesei* cellulase composition with various concentrations of the *T. aurantiacus* GH61A polypeptide in the presence of acid-pretreated components of biomass or mixtures thereof. White bars: 1 day of hydrolysis; dark gray bars: 5 days of hydrolysis.

Figure 18 shows the fractional hydrolysis of AVICEL® by a *T. reesei* cellulase composition with various concentrations of the *T. aurantiacus* GH61A polypeptide in the presence of post-fermentation liquors. White bars: 1 day of hydrolysis; gray bars: 3 days of hydrolysis; black bars: 7 days of hydrolysis.

Figures 19A and 19B show the fractional hydrolysis of AVICEL® by a *T. reesei* cellulase composition with various concentrations of the *T. aurantiacus* GH61A polypeptide in the presence of various severity acid-pretreatments of corn stover. Gray bars: 3 days of hydrolysis; black bars: 7 days of hydrolysis. Figure 19C shows the fractional hydrolysis of AVICEL® by the *T. reesei* cellulase composition with *T. aurantiacus* GH61A polypeptide and liquors generated by acid pretreatment of cellulose at various severities between 110°C and 190°C at GH61A polypeptide concentrations of 50% ( ), 24% ( ); 8% ( ); 4% ( ); 2% ( ); and 0 ( ) (w/w).

Figure 20A, 20B, and 20C shows the fractional hydrolysis of AVICEL® by *T. reesei* cellulase compositions with increasing GH61 polypeptide concentrations plus added acid-pretreated xylan of various severities. Panel A shows 7 days of hydrolysis data for a broad range of severities. Panel B shows more severe pretreatments, gray bars: 3 days of hydrolysis; black bars: 7 days of hydrolysis. Panel C shows the fractional hydrolysis of AVICEL® by the *T. reesei* cellulase composition with *T. aurantiacus* GH61A polypeptide and liquors generated by acid pretreatment of xylan at various severities between 110°C and 190°C at GH61A polypeptide concentrations of 50% ( ), 24% ( ); 8% ( ); 4% ( ); 2% ( ); and 0 ( ) (w/w).

Figure 21 shows the fractional hydrolysis of AVICEL® by *T. reesei* cellulase compositions with and without 50% (w/w) *T. aurantiacus* GH61A polypeptide concentrations plus added solid-phase extracted NREL acid-pretreated corn stover or acid-pretreated xylan as indicated. Gray bars: 3 days of hydrolysis; black bars: 7 days of hydrolysis.

Figure 22 shows the fractional hydrolysis of AVICEL® by a *T. reesei* cellulase composition with various concentrations of *T. aurantiacus* GH61A in the presence of 10%

(v/v) of the indicated pretreatment liquor or electrodialyzed pretreatment liquor. Gray bars: 3 days of hydrolysis; black bars: 7 days of hydrolysis.

Figure 23 shows the fractional hydrolysis of various biomass substrates by the *T. reesei* cellulase composition with increasing concentrations of the *T. aurantiacus* GH61A polypeptide with and without NREL acid-pretreated corn stover liquor. Panel A: low and medium severity organosolv ethanol pretreated corn stover; Panel B: medium severity glycerol and water pretreated corn stover, and 5% total solids water pretreated corn stover; Panel C: 5% total solids sugarcane bagasse; Panel D: alkaline pretreated corn stover plus no liquor and NREL milled washed pretreated corn stover controls. White bars: 1 day of hydrolysis; gray bars: 3 days of hydrolysis; black bars: 7 days of hydrolysis.

Figure 24 shows the fractional hydrolysis of AVICEL® by the *T. reesei* cellulase composition with either zero or 24% *T. aurantiacus* GH61A polypeptide in the presence of the indicated acid-pretreated monosaccharides. Gray bars: 3 days of hydrolysis; black bars: 7 days of hydrolysis.

Figure 25 shows the fractional hydrolysis of AVICEL® by the *T. reesei* cellulase composition with various concentrations of the indicated GH61 polypeptides with 10% (v/v) NREL PCS liquor. White symbols: 3 days of hydrolysis; black symbols: 7 days of hydrolysis; *Thermoascus aurantiacus* GH61A: circles; *Aspergillus fumigatus* GH61B polypeptide: diamonds; *Penicillium pinophilum* GH61 polypeptide: squares.

Figure 26 shows the glucose produced by hydrolysis of AVICEL® by a *T. reesei* cellulase composition with *T. aurantiacus* GH61A polypeptide in the presence or absence of Kraft lignin. Solid symbols: *T. reesei* cellulase composition + *T. aurantiacus* GH61A polypeptide. Open symbols: *T. reesei* cellulase composition + *T. aurantiacus* GH61A polypeptide supplemented with additional 15% (w/w) *T. aurantiacus* GH61A polypeptide. Circles: no lignin; squares: 0.1% (w/w) Kraft lignin; diamonds: 0.1% (w/w) oxidized Kraft lignin.

Figure 27 shows the concentrations of glucose and xylose from 120 hours of saccharification of washed, milled alkaline pretreated corn stover by the *T. reesei* cellulase composition supplemented with *T. aurantiacus* GH61A polypeptide, replaced with increasing concentrations of *T. aurantiacus* GH61A. Solid squares: xylose, open diamonds: glucose.

Figure 28 shows the conversion of high total solids (15% TS) dilute acid pretreated corn stover of various pretreatment severities as indicated. The pretreated corn stovers were hydrolyzed by either a composition containing a blend of an *Aspergillus aculeatus* GH10 xylanase and a *Trichoderma reesei* cellulase preparation containing *Aspergillus fumigatus* beta-glucosidase and *Thermoascus aurantiacus* GH61A polypeptide or this mixture replaced with 20% additional *T. aurantiacus* GH61A. For each severity pretreatment other than the least severe, replacement of the cellulase-GH61A polypeptide mixture with additional

GH61A polypeptide yielded a greater conversion. Grey bars: 120 hours of saccharification; black bars: 216 hours of saccharification.

Figure 29 shows the conversion of high total solids (15% TS) dilute acid pretreated *Arundo donax* of various pretreatment severities as indicated. The variously pretreated *A. donax* were hydrolyzed by either a composition containing a blend of an *Aspergillus aculeatus* GH10 xylanase (WO 94/021785) and a *Trichoderma reesei* cellulase preparation containing *Aspergillus fumigatus* beta-glucosidase (WO 2005/047499) and *Thermoascus aurantiacus* GH61A polypeptide (WO 2005/074656) or this mixture replaced by 20% additional *T. aurantiacus* GH61A polypeptide. For each severity pretreatment, replacement of the cellulase-GH61A polypeptide mixture with additional GH61A polypeptide yielded a greater conversion. Grey bars: 120 hours of saccharification; black bars: 216 hours of saccharification.

Definitions

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-napthyl 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 (polyoxyethylene sorbitan monolaurate). One unit of acetylxylan esterase is defined as the amount of enzyme capable of releasing 1 µmole of *p*-nitrophenolate anion per minute at pH 5, 25°C.

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

Alpha-L-arabinofuranosidase: The term "alpha-L-arabinofuranosidase" means an alpha-L-arabinofuranoside arabinofuranohydrolase (EC 3.2.1.55) that catalyzes the hydrolysis of terminal non-reducing alpha-L-arabinofuranoside residues in alpha-L-arabinosides. The enzyme acts on alpha-L-arabinofuranosides, alpha-L-arabinans containing (1,3)- and/or (1,5)-linkages, arabinoxylans, and arabinogalactans. Alpha-L-arabinofuranosidase is also known as arabinosidase, alpha-arabinosidase, alpha-L-arabinosidase, alpha-arabinofuranosidase, polysaccharide alpha-L-arabinofuranosidase, alpha-L-arabinofuranoside hydrolase, L-arabinosidase, or alpha-L-arabinanase. For purposes of the present invention, alpha-L-arabinofuranosidase activity is determined using

5 mg of medium viscosity wheat arabinoxylan (Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland) per ml of 100 mM sodium acetate pH 5 in a total volume of 200 µl for 30 minutes at 40°C followed by arabinose analysis by AMINEX® HPX-87H column chromatography (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

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 µmole of glucuronic or 4-O-methylglucuronic acid per minute at pH 5, 40°C.

Beta-glucosidase: The term "beta-glucosidase" means a beta-D-glucoside glucohydrolase (E.C. 3.2.1.21) that 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 using *p*-nitrophenyl-beta-D-glucopyranoside as substrate according to the procedure of 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*-nitrophenolate anion produced per minute at 25°C, pH 4.8 from 1 mM *p*-nitrophenyl-beta-D-glucopyranoside as substrate in 50 mM sodium citrate containing 0.01% TWEEN® 20.

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→4)-xylooligosaccharides, to remove successive D-xylose residues from non-reducing termini. For purposes of the present invention, one unit of beta-xylosidase is defined as 1.0 µmole of *p*-nitrophenolate anion produced per minute at 40°C, pH 5 from 1 mM *p*-nitrophenyl-beta-D-xyloside as substrate in 100 mM sodium citrate containing 0.01% TWEEN® 20.

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

Cellobiohydrolase: The term "cellobiohydrolase" means a 1,4-beta-D-glucan cellobiohydrolase (E.C. 3.2.1.91) that catalyzes the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, cellooligosaccharides, or any beta-1,4-linked glucose containing polymer, releasing cellobiose from the reducing or non-reducing ends of the chain (Teeri, 1997, Crystalline cellulose degradation: New insight into the function of cellobiohydrolases,

Trends in Biotechnology 15: 160-167; Teeri *et al.*, 1998, *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose?, *Biochem. Soc. Trans.* 26: 173-178). For purposes of the present invention, cellobiohydrolase activity is determined according to the procedures described by Lever *et al.*, 1972, *Anal. Biochem.* 47: 273-279; van Tilbeurgh *et al.*, 1982, *FEBS Letters,* 149: 152-156; van Tilbeurgh and Claeyssens, 1985, *FEBS Letters,* 187: 283-288; and Tomme *et al.*, 1988, *Eur. J. Biochem.* 170: 575-581. In the present invention, the Lever *et al.* method can be employed to assess hydrolysis of cellulose in corn stover, while the methods of van Tilbeurgh *et al.* and Tomme *et al.* can be used to determine the cellobiohydrolase activity on a fluorescent disaccharide derivative, 4-methylumbelliferyl-β-D-lactoside.

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

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

Cellulolytic enzyme or cellulase: The term "cellulolytic enzyme" or "cellulase" means one or more (*e.g.*, several) enzymes that hydrolyze a cellulosic material. Such enzymes include endoglucanase(s), cellobiohydrolase(s), beta-glucosidase(s), or combinations thereof. The two basic approaches for measuring cellulolytic activity include:

(1) measuring the total cellulolytic activity, and (2) measuring the individual cellulolytic activities (endoglucanases, cellobiohydrolases, and beta-glucosidases) as reviewed in Zhang *et al.*, Outlook for cellulase improvement: Screening and selection strategies, 2006, *Biotechnology Advances* 24: 452-481. Total cellulolytic activity is usually measured using insoluble substrates, including Whatman №1 filter paper, microcrystalline cellulose, bacterial cellulose, algal cellulose, cotton, pretreated lignocellulose, *etc.* The most common total cellulolytic activity assay is the filter paper assay using Whatman №1 filter paper as the substrate. The assay was established by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987, Measurement of cellulase activities, *Pure Appl. Chem.* 59: 257-68).

For purposes of the present invention, cellulolytic enzyme activity is determined by measuring the increase in hydrolysis of a cellulosic material by cellulolytic enzyme(s) under the following conditions: 1-20 mg of cellulolytic enzyme protein/g of cellulose in PCS for 3-7 days at 50ºC compared to a control hydrolysis without addition of cellulolytic enzyme protein. Typical conditions are 1 ml reactions, washed or unwashed PCS, 5% insoluble solids, 50 mM sodium acetate pH 5, 1 mM $MnSO_4$, 50°C, 72 hours, sugar analysis by AMINEX® HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

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

Cellulose is generally found, for example, in the stems, leaves, hulls, husks, and cobs of plants or leaves, branches, and wood of trees. The cellulosic material can be, but is not limited to, agricultural residue, herbaceous material (including energy crops), municipal solid waste, pulp and paper mill residue, waste paper, and wood (including forestry 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 any biomass material. In another preferred aspect, the cellulosic material is lignocellulose, which comprises cellulose, hemicelluloses, and lignin.

In one aspect, the cellulosic material is agricultural residue. In another aspect, the cellulosic material is herbaceous material (including energy crops). In another aspect, the cellulosic material is municipal solid waste. In another aspect, the cellulosic material is pulp and paper mill residue. In another aspect, the cellulosic material is waste paper. In another aspect, the cellulosic material is wood (including forestry residue).

In another aspect, the cellulosic material is arundo. In another aspect, the cellulosic material is bagasse. In another aspect, the cellulosic material is bamboo. In another aspect, the cellulosic material is corn cob. In another aspect, the cellulosic material is corn fiber. In another aspect, the cellulosic material is corn stover. In another aspect, the cellulosic material is miscanthus. 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 switchgrass. In another aspect, the cellulosic material is wheat straw.

In another aspect, the cellulosic material is aspen. In another aspect, the cellulosic material is eucalyptus. In another aspect, the cellulosic material is fir. In another aspect, the cellulosic material is pine. In another aspect, the cellulosic material is poplar. In another aspect, the cellulosic material is spruce. In another aspect, the cellulosic material is willow.

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

In another aspect, the cellulosic material is an aquatic biomass. As used herein the term "aquatic biomass" means biomass produced in an aquatic environment by a photosynthesis process. The aquatic biomass can be algae, emergent plants, floating-leaf plants, or submerged plants.

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.

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

codon such as TAA, TAG, and TGA. The coding sequence may be a DNA, cDNA, synthetic, or recombinant polynucleotide.

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

Endoglucanase: The term "endoglucanase" means an endo-1,4-(1,3;1,4)-beta-D-glucan 4-glucanohydrolase (E.C. 3.2.1.4) that catalyzes 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, 40ºC.

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

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

Family 61 glycoside hydrolase: The term "Family 61 glycoside hydrolase" or "Family GH61" or "GH61" means a polypeptide falling into the glycoside hydrolase Family 61 according to Henrissat B., 1991, A classification of glycosyl hydrolases based on amino-acid sequence similarities, *Biochem. J.* 280: 309-316, and Henrissat B., and Bairoch A., 1996, Updating the sequence-based classification of glycosyl hydrolases, *Biochem. J.* 316: 695-696. The enzymes in this family were originally classified as a glycoside hydrolase family based on measurement of very weak endo-1,4-beta-D-glucanase activity in one family member. The structure and mode of action of these enzymes are non-canonical and they cannot be considered as bona fide glycosidases. However, they are kept in the CAZy

classification on the basis of their capacity to enhance the breakdown of lignocellulose when used in conjunction with a cellulase or a mixture of cellulases.

Feruloyl esterase: The term "feruloyl esterase" means a 4-hydroxy-3-methoxycinnamoyl-sugar hydrolase (EC 3.1.1.73) that catalyzes the hydrolysis of 4-hydroxy-3-methoxycinnamoyl (feruloyl) groups from 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 µmole of *p*-nitrophenolate anion per minute at pH 5, 25°C.

Hemicellulolytic enzyme or hemicellulase: The term "hemicellulolytic enzyme" or "hemicellulase" means one or more (*e.g.*, several) enzymes that hydrolyze a hemicellulosic material. See, for example, Shallom, D. and Shoham, Y. Microbial hemicellulases. *Current Opinion In Microbiology*, 2003, 6(3): 219-228). Hemicellulases are key components in the degradation of plant biomass. Examples of hemicellulases include, but are not limited to, an acetylmannan esterase, an acetyxylan esterase, an arabinanase, an arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase. The substrates of these enzymes, the hemicelluloses, are a heterogeneous group of branched and linear polysaccharides that are bound via hydrogen bonds to the cellulose microfibrils in the plant cell wall, crosslinking them into a robust network. Hemicelluloses are also covalently attached to lignin, forming together with cellulose a highly complex structure. The variable structure and organization of hemicelluloses require the concerted action of many enzymes for its complete degradation. The catalytic modules of hemicellulases are either glycoside hydrolases (GHs) that hydrolyze glycosidic bonds, or carbohydrate esterases (CEs), which hydrolyze ester linkages of acetate or ferulic acid side groups. These catalytic modules, based on homology of their primary sequence, can be assigned into GH and CE families marked by numbers. Some families, with an overall similar fold, can be further grouped into clans, marked alphabetically (*e.g.*, GH-A). A most informative and updated classification of these and other carbohydrate active enzymes is available in the Carbohydrate-Active Enzymes (CAZy) database. Hemicellulolytic enzyme activities can be measured according to Ghose and Bisaria, 1987, *Pure & Appl. Chem.* 59: 1739-1752.

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

mutations that occur during replication.

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

Liquor: The term “liquor” means the solution phase, either aqueous, organic, or a combination thereof, arising from treatment of a lignocellulose and/or hemicellulose and/or lignacious material or feedstock, or monosaccharides thereof, *e.g.,* xylose, arabinose, mannose, *etc.*, under conditions as described herein, and the soluble contents thereof.

Mature polypeptide: The term “mature polypeptide” means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. It is known in the art that a host cell may produce a mixture of two of more different mature polypeptides (*i.e.*, with a different C-terminal and/or N-terminal amino acid) expressed by the same polynucleotide. The mature polypeptide can be predicted using the SignalP program (Nielsen *et al.*, 1997, *Protein Engineering* 10: 1-6).

Mature polypeptide coding sequence: The term “mature polypeptide coding sequence” is defined herein as a nucleotide sequence that encodes a mature polypeptide having biological activity. The mature polypeptide coding sequence can be predicted using the SignalP program (Nielsen *et al.*, 1997, *supra*).

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

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.

Polypeptide fragment: The term “fragment” means a polypeptide having one or more (*e.g.*, several) amino acids deleted from the amino and/or carboxyl terminus of a mature polypeptide; wherein the fragment has biological activity.

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, alkaline pretreatment, or neutral pretreatment..

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

For purposes of the present invention, the degree of sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 3.0.0, 5.0.0, or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

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

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

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

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 biological activity.

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

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 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.

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

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

Total xylan degrading activity can be measured by determining the reducing sugars formed from various types of xylan, including, for example, oat spelt, beechwood, and larchwood xylans, or by photometric determination of dyed xylan fragments released from various covalently dyed xylans. The most common total xylanolytic activity assay is based on production of reducing sugars from polymeric 4-O-methyl glucuronoxylan as described in Bailey, Biely, Poutanen, 1992, Interlaboratory testing of methods for assay of xylanase activity, *Journal of Biotechnology* 23(3): 257-270. Xylanase activity can also be determined with 0.2% AZCL-arabinoxylan as substrate in 0.01% TRITON® X-100 (4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol) and 200 mM sodium phosphate buffer pH 6 at 37°C. One unit of xylanase activity is defined as 1.0 μmole of azurine produced per minute at 37°C, pH 6 from 0.2% AZCL-arabinoxylan as substrate in 200 mM sodium phosphate pH 6 buffer.

For purposes of the present invention, xylan degrading activity is determined by measuring the increase in hydrolysis of birchwood xylan (Sigma Chemical Co., Inc., St.

Louis, MO, USA) by xylan-degrading enzyme(s) under the following typical conditions: 1 ml reactions, 5 mg/ml substrate (total solids), 5 mg of xylanolytic protein/g of substrate, 50 mM sodium acetate pH 5, 50°C, 24 hours, sugar analysis using *p*-hydroxybenzoic acid hydrazide (PHBAH) assay as described by Lever, 1972, A new reaction for colorimetric determination of carbohydrates, *Anal. Biochem* 47: 273-279.

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

Detailed Description of the Invention

The present invention relates to compositions comprising: (a) a polypeptide having cellulolytic enhancing activity; and (b) a liquor, wherein the combination of the polypeptide having cellulolytic enhancing activity and the liquor enhances hydrolysis of a cellulosic material by a cellulolytic enzyme. In one aspect, the compositions further comprise (c) one or more (*e.g.*, several) enzymes selected from the group consisting of a cellulase, a hemicellulase, an esterase, an expansin, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.

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 cellulolytic enhancing activity and a liquor, wherein the combination of the polypeptide having cellulolytic enhancing activity and the liquor enhances hydrolysis of the cellulosic material by the enzyme composition. In one aspect, the method above further comprises recovering the degraded or converted cellulosic material. Soluble products of degradation or conversion of the cellulosic material can be separated from the insoluble cellulosic material using technology well known in the art such as, for example, centrifugation, filtration, and gravity settling.

The present invention also relates to methods for producing a fermentation product, comprising:

(a) saccharifying a cellulosic material with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity and a liquor, wherein the combination of the polypeptide having cellulolytic enhancing activity and the liquor enhances hydrolysis of the cellulosic material by the enzyme composition;

(b) fermenting the saccharified cellulosic material with one or more (*e.g.*, 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 (*e.g.*, several) fermenting microorganisms, wherein the cellulosic material is saccharified with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity and a liquor, wherein the combination of the polypeptide having cellulolytic enhancing activity and the liquor enhances hydrolysis of the cellulosic material by the enzyme composition. In one aspect, the fermenting of the cellulosic material produces a fermentation product. In another aspect, the method further comprises recovering the fermentation product from the fermentation.

Liquors

The term "liquor" means the solution phase, either aqueous, organic, ionic liquid, or combinations thereof, arising from treatment of a lignocellulose and/or hemicellulose and/or lignacious material or feedstock in a slurry, or monosaccharides thereof, *e.g.,* xylose, arabinose, mannose, *etc.*, under conditions as described herein, and the soluble contents thereof. A liquor for cellulolytic enhancement of a GH61 polypeptide can be produced by treating a lignocellulosic, hemicellulosic, or lignacious material (or feedstock) by applying heat and/or pressure, optionally in the presence of a catalyst, *e.g.*, acid, optionally in the presence of an organic solvent, and optionally in combination with physical disruption of the material, and then separating the solution from the residual solids. Alternatively, the lignocellulosic, hemicellulosic, or lignacious material can be slurried and incubated in aqueous, organic, or ionic liquids or combinations thereof as either solutions or suspensions without addition of heat or pressure. Such conditions determine the degree of cellulolytic enhancement obtainable through the combination of liquor and a GH61 polypeptide during hydrolysis of a cellulosic substrate by a cellulase preparation. The liquor can be separated from the treated material using methods standard in the art, such as filtration, sedimentation, or centifugation.

In one aspect, the material for producing the liquor is herbaceous material. In another aspect, the material is agricultural residue. In another aspect, the material is forestry residue. In another aspect, the material is municipal solid waste. In another aspect, the material is waste paper. In another aspect, the material is pulp and paper mill residue. In another aspect, the material is pulping liquor. In another aspect, the material is mixed wood waste.

In another aspect, the material for producing the liquor is corn stover. In another aspect, the material is corn fiber. In another aspect, the material is corn cob. In another aspect, the material is orange peel. In another aspect, the material is rice straw. In another

aspect, the material is wheat straw. In another aspect, the material is switch grass. In another aspect, the material is miscanthus. In another aspect, the material is sugar cane bagasse. In another aspect, the material is energy cane. In another aspect, the material is sorghum. In another aspect, the material is algae biomass.

In another aspect, the material for producing the liquor is softwood. In another aspect, the material is hardwood. In another aspect, the material is poplar. In another aspect, the material is pine. In another aspect, the material is spruce. In another aspect, the material is fir. In another aspect, the material is willow. In another aspect, the material is eucalyptus.

In another aspect, the material for producing the liquor is a hemicellulose. In another aspect, the material is a hemicellulose-rich lignocellulose. In another aspect, the material is a xylan. In another aspect, the material is beechwood xylan. In another aspect, the material is birch xylan. In another aspect, the material is spruce xylan. In another aspect, the material is arabinoxylan. In another aspect, the material is mannan. In another aspect, the material is glucomannan. In another aspect, the material contains beta-(1,4)-linked xylan. In another aspect, the material contains beta-(1,4)-linked mannan. In another aspect, the material contains branched beta-(1,4)-linked xylan, such as arabinoxylan and arabino-(glucoryono-)xylan. In another aspect, the material contains branched beta-(1,4)-linked mannan, such as galactoglucomannan.

In another aspect, the material for producing the liquor is a C5 monosaccharide (pentose). In another aspect, the material is arabinose. In another aspect, the material is xylose. In another aspect, the material is xylulose. In another aspect, the material is ribose. In another aspect, the material is ribulose. In another aspect, the material is acetyl-xylose. In another aspect, the material is ferulyl-xylose. In another aspect, the material is glucurono-xylose or methyl-glucurono-xylose.

In another aspect, the material for producing the liquor is a C6 monosaccharide (hexose). In another aspect, the material is glucose. In another aspect, the material is mannose. In another aspect, the material is fructose. In another aspect, the material is gulose. In another aspect, the material is allose. In another aspect, the material is altrose. In another aspect, the material is idose. In another aspect, the material is talose. In another aspect, the material is galactose. In another aspect, the material is gluconic acid. In another aspect, the material is glucuronic acid. In another aspect, the material is galactonic acid. In another aspect, the material is galacturonic acid. In another aspect, the material is psicose. In another aspect, the material is fructose. In another aspect, the material is sorbose. In another aspect, the material is tagatose.

In another aspect, the material for producing the liquor is a C4 monosaccharide. In another aspect, the material is erythrose. In another aspect, the material is threose. In

another aspect, the material is erythrulose.

In another aspect, the material for producing the liquor is a C3 monosaccharide. In another aspect, the material is glyceraldehyde. In another aspect, the material is dihydroxyacetone.

In another aspect, the material for producing the liquor is lignin. In another aspect, the material is Kraft (Indulin) lignin. In another aspect, the material is *p*-hydroxyphenyl (H) rich lignin. In another aspect, the material is guaiacyl (G) rich lignin. In another aspect, the material is syringal (S) rich lignin. In another aspect, the material is lignosulfonate. In another aspect the material is black liquor or components thereof. In another aspect, the material is tall oil or components thereof.

In another aspect, the material for producing the liquor is a post-pretreatment residue of biomass (the solid waste after pretreatment. In another aspect, the material for producing the liquor is a post-saccharification residue of biomass (the solid waste after saccharification). In another aspect, the material for producing the liquor is a post-fermentation residue of biomass (the solid waste after fermentation). In another aspect, the material for producing the liquor is a post-distillation residue of biomass (the solid waste after distillation).

In a non-limiting aspect, the material is treated using acid in the range of about 0.5 to about 5% (w/v), *e.g.*, about 0.5 to about 4.5% (w/v), about 0.75 to about 4% (w/v), about 1.0 to about 3.5% (w/v), about 1.25 to about 3.0% (w/v), or about 1.5 to about 2.5% (w/v); a pH of about 0 to about 3, *e.g.,* about 0.5 to about 3, about 0.5 to about 2.5, about 1 to about 2, or about 1 to about 1.5; a time period of about 1 to about 15 minutes, *e.g.*, about 1 to about 12 minutes, about 2 to about 10 minutes, about 3 to about 9 minutes, about 4 to about 9 minutes, about 5 to about 9 minutes, or about 6 to about 8 minutes; at a temperature at about 130ºC to about 250ºC, *e.g.*, about 140ºC to about 220ºC, about 150ºC to about 200ºC, about 160ºC to about 190ºC, about 160ºC to about 185ºC, about 165ºC to about 180ºC, about 165ºC to about 175ºC, or about 165ºC to about 170ºC; and a pressure of about 100 to about 600 psi, *e.g.*, about 50 to about 1700 psi, about 100 to about 1500 psi, about 100 to about 1200 psi, about 100 to about 1000 psi, about 100 to about 500 psi, about 100 to about 400 psi, about 100 to about 300 psi, about 100 to about 200 psi, about 100 to about 150 psi, or about 100 to about 120 psi. In another aspect, the acid is sulfuric acid. In another aspect, the acid is hydrochloric acid. In another aspect, the acid is nitric acid. In another aspect, the acid is phosphoric acid. In another aspect, the acid is acetic acid. In another aspect, the acid is citric acid. In another aspect, the acid is succinic acid. In another aspect, the acid is tartaric acid. In another aspect, the acid is mixtures of any of the above acids. It is understood herein that the conditions described above may need to be optimized depending on the material being treated and the reactor used to produce the liquor. Such optimization is well within the

skill in the art. Conditions used to pretreat a cellulosic material as described herein may also be used to generate liquor from a particular feedstock.

In a preferred aspect, the material is treated using 1.4% (w/v) sulfuric acid for 8 minutes at 165ºC and 107 psi.

Treatment of a material to produce such liquor may also generate other compounds that are inhibitory to cellulases and/or hemicellulases, *e.g.*, organic acids and lignin-derived compounds. Conditions can be selected that balance cellulolytic enhancing activity of a GH61 polypeptide with production of inhibitor compounds of cellulases and/or hemicellulases. Such conditions can vary depending on the material used for producing the liquor. However, the liquor can be subjected to a molecular weight filter(s) or dialysis, *e.g.* electrodialysis, using a membrane with nominal molecular weight cut-off of in the range of about 0.1 kDa to about 10 kDa, *e.g.*, about 0.5 kDa to about 7 kDa, about 0.5 kDa to about 5 kDa, and about 1 kDa to about 3 kDa, to reduce the amount of the inhibitory compounds. Any method known in the art can be used to reduce the amount of the inhibitory compounds. In one aspect, the liquor is further processed to remove inhibitors of a cellulase, a hemicellulase, or a combination thereof.

In other aspects of the present invention, the liquor can be generated *in situ* by pretreating a cellulosic material that will be saccharified by a cellulase preparation. However, in such instances the amount of effective liquor generated *in situ* may be insufficient with regard to the GH61 polypeptide having cellulolytic enhancing activity, cellulolytic enzyme(s), and cellulose. For example, pretreatment of a cellulosic material under mild conditions, *e.g.*, auto-catalyzed steam explosion, alkaline pretreatment, auto-hydrolysis, jet cooking, hot water-pretreatment, organosolv using ethanol, glycerol, *etc.*, dilute acid pretreatment, and the like ("low severity conditions") or a pretreatment that includes a wash or rinse step or unpretreated cellulosic material compared to harsh conditions ("high severity conditions") to produce *in situ* a liquor may be inadequate for optimizing the cellulolytic enhancing activity of a GH61 polypeptide. Conditions employed to produce NREL preatreated corn stover, *i.e.*, 1.4 wt % sulfuric acid for 8 minutes at 165ºC and 107 psi (Example 1) would be considered high severity conditions. In such circumstances a liquor obtained using treatment conditions different from the pretreatment conditions of the cellulosic material can be added to the saccharification reaction. In other aspects of the invention, a low-severity extraction treatment that extracts liquor can be used instead of conventional treatment techniques listed herein, and this liquor can then be added to the saccharification reaction.

In one aspect, the liquor is obtained from a material that is the same as the cellulosic material to be subjected to saccharification by a cellulase composition. In another aspect, the liquor is obtained from a material that is different than the cellulosic material to be subjected to saccharification by a cellulase composition.

In another aspect, the liquor is obtained from a material that is the same as the cellulosic material to be subjected to saccharification by a cellulase composition, but the treatment conditions used to produce the liquor are different from the pretreatment conditions of the cellulosic material. In another aspect, the liquor is obtained from a material that is the same as the cellulosic material to be subjected to saccharification by a cellulase composition, and the treatment conditions used to produce the liquor are the same as the pretreatment conditions of the cellulosic material. In another aspect, the liquor is obtained from a material that is the same as the cellulosic material to be subjected to saccharification by a cellulase composition, and the treatment conditions used to produce the liquor are the same as the pretreatment conditions of the cellulosic material, and the liquor is further processed, *e.g.*, concentrated, filtered to remove cellulase inhibitors, filtered and concentrated, *etc*.

In another aspect, the liquor is obtained from a material that is different than the cellulosic material to be subjected to saccharification by a cellulase composition, and the treatment conditions used to produce the liquor are different from the pretreatment conditions of the cellulosic material. In another aspect, the liquor is obtained from a material that is different than the cellulosic material to be subjected to saccharification by a cellulase composition, and the treatment conditions used to produce the liquor are the same as the pretreatment conditions of the cellulosic material. In another aspect, the liquor is obtained from a material that is different than the cellulosic material to be subjected to saccharification by a cellulase composition, and the treatment conditions used to produce the liquor are the same as the pretreatment conditions of the cellulosic material, and the liquor is further processed, *e.g.,* concentrated, filtered to remove cellulase inhibitors, filtered and concentrated, *etc*. Further processing to remove cellulose inhibitors can be accomplished using any method known in the art.

In another aspect, liquors generated *in situ* may be washed or diluted and replaced with liquors generated *ex situ* to a greater or lesser extent, so the liquor composition/content is optimized for the cellulolytic enhancing effect of a GH61 polypeptide. In another aspect, the solids content of subsequent saccharifications is altered to optimize the liquor content for the cellulolytic enhancing effect of a GH61 polypeptide.

The effective amount of the liquor can depend on one or more (*e.g.*, 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, non-cellulosic components (*e.g.*, native or degraded lignin or hemicellulose), non-cellulase components, temperature, reaction time, and the liquor (*e.g.*, filtered to remove cellulase and/or hemicellulase inhibitors).

The liquor is preferably present in an amount that is not limiting with regard to the

GH61 polypeptide having cellulolytic enhancing activity, cellulolytic enzyme(s), and cellulose. In one aspect, the liquor is present in an amount that is not limiting with regard to the GH61 polypeptide having cellulolytic enhancing activity. In another aspect, the liquor is present in an amount that is not limiting with regard to the cellulolytic enzyme(s). In another aspect, the liquor is present in an amount that is not limiting with regard to the cellulose. In another aspect, the liquor is present in an amount that is not limiting with regard to the GH61 polypeptide having cellulolytic enhancing activity and the cellulolytic enzyme(s). In another aspect, the liquor is present in an amount that is not limiting with regard to the GH61 polypeptide having cellulolytic enhancing activity and the cellulose. In another aspect, the liquor is present in an amount that is not limiting with regard to the cellulolytic enzyme(s) and the cellulose. In another aspect, the liquor is present in an amount that is not limiting with regard to the GH61 polypeptide having cellulolytic enhancing activity, the cellulolytic enzyme(s), and the cellulose.

The liquor is preferably present in an amount that optimizes the cellulolytic enhancing activity of a GH61 polypeptide during saccharification with a cellulase composition. In one aspect, the liquor optimizes the cellulolytic enhancing activity of a GH61 polypeptide with a *GH61 effect* as defined by Equation 3 (the ratio of fractional hydrolysis in the presence to the absence of the GH61 polypeptide) of preferably at least 1.05, more preferably at least 1.10, more preferably at least 1.15, more prefereably at least 1.2, more preferably at least 1.25, more preferably at least 1.3, more preferably at least 1.35, more preferably at least 1.4, more preferably at least 1.45, more preferably at least 1.5, more preferably at least 1.55, more preferably at least 1.6, more preferably at least 1.65, more preferably at least 1.7, more preferably at least 1.75, more preferably at least 1.8, more preferably at least 1.85, more preferably at least 1.9, most preferably at least 1.95, and even most preferably at least 2. An increase in the GH61 effect is obtained when liquor is added, relative to when liquor is not added, during hydrolysis. In another aspect, the amount of GH61 polypeptide is optimized for a given concentration of liquor. Such optimization is accomplished by varying the concentration of each component to determine the optimal ratio of the components during saccharification.

In another aspect, an effective amount of the liquor to cellulose is about 10^{-6} to about 10 g per g of cellulose, *e.g.*, about 10^{-6} to about 7.5 g, about 10^{-6} to about 5, about 10^{-6} to about 2.5 g, about 10^{-6} to about 1 g, about 10^{-5} to about 1 g, about 10^{-5} to about 10^{-1} g, about 10^{-4} to about 10^{-1} g, about 10^{-3} to about 10^{-1} g, and about 10^{-3} to about 10^{-2} g per g of cellulose.

In another aspect, the amount of liquor present that minimizes inhibition of a cellulase composition and in combination with a GH61 polypeptide enhances hydrolysis by an enzyme composition is about 1 to about 20% (v/v), *e.g.*, about 1 to about 15%, about 1 to about 10%,

about 2 to about 7%, about 2 to about 5%, and about 3 to about 5%.

In the methods of the present invention, the term "liquor" encompasses one or more (*e.g.*, several) liquors from different materials based on the same or different conditions of treatment to produce the liquors.

In the methods of the present invention, the liquor is preferably present when a GH61 polypeptide is present, for example, is added with the GH61 polypeptide. The liquor can also be added at different stages of a saccharification. The liquor can also be redosed at different stages of saccharification, *e.g.,* daily, to maintain the presence of an effective concentration of the liquor. The liquor can also be removed or washed to various degrees, or may be diluted at different stages of saccharification. Liquors generated *in situ* may be washed and replaced with liquors generated *ex situ* to a greater or lesser extent, at various times during saccharification

In another aspect of the present invention, the liquor may be recycled from a completed saccharification or completed saccharification and fermentation to a new saccharification. The liquor can be recovered using standard methods in the art, *e.g.*, filtration/centrifugation, sedimentation, and/or flocculation of solids materials pre- or post-distillation, to remove residual solids, cellular debris, *etc.* and then recirculated to the new saccharification.

Polypeptides Having Cellulolytic Enhancing Activity and Polynucleotides Thereof

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-[ILMV]-X-R-X-[EQ]-X(4)-[HNQ] (SEQ ID NO: 127 or SEQ ID NO: 128) 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 isolated polypeptide comprising the above-noted motifs may further comprise:

H-X(1,2)-G-P-X(3)-[YW]-[AILMV] (SEQ ID NO: 129 or SEQ ID NO: 130),

[EQ]-X-Y-X(2)-C-X-[EHQN]-[FILV]-X-[ILV] (SEQ ID NO: 131), or

H-X(1,2)-G-P-X(3)-[YW]-[AILMV] (SEQ ID NO: 132 or SEQ ID NO: 133) and [EQ]-X-Y-X(2)-C-X-[EHQN]-[FILV]-X-[ILV] (SEQ ID NO: 134),

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 embodiment, the isolated GH61 polypeptide having cellulolytic enhancing activity further comprises H-X(1,2)-G-P-X(3)-[YW]-[AILMV] (SEQ ID NO: 135 or SEQ ID NO: 136). In another preferred embodiment, the isolated GH61 polypeptide having cellulolytic enhancing activity further comprises [EQ]-X-Y-X(2)-C-X-[EHQN]-[FILV]-X-[ILV] (SEQ ID NO: 137). In another preferred embodiment, the isolated GH61 polypeptide having cellulolytic enhancing activity further comprises H-X(1,2)-G-P-X(3)-[YW]-[AILMV] (SEQ ID NO: 138 or SEQ ID NO: 139) and [EQ]-X-Y-X(2)-C-X-[EHQN]-[FILV]-X-[ILV] (SEQ ID NO: 140).

In a second aspect, isolated polypeptides having cellulolytic enhancing activity, comprise the following motif:

[ILMV]-P-X(4,5)-G-X-Y-[ILMV]-X-R-X-[EQ]-X(3)-A-[HNQ] (SEQ ID NO: 141 or SEQ ID NO: 142),

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.

In a third aspect, the polypeptide having cellulolytic enhancing activity comprises an amino acid sequence that has a degree of identity to the mature polypeptide of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 144, SEQ ID NO: 146, SEQ ID NO: 148, SEQ ID NO: 150, SEQ ID NO: 152, SEQ ID NO: 154, SEQ ID NO: 156, SEQ ID NO: 158, SEQ ID NO: 160, SEQ ID NO: 162, SEQ ID NO: 164, or SEQ ID NO: 166 of preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 91%, at least 92%, at least 93%, at least 94%, or at least 95%, or at least 100% and even most preferably at least 96%, at least 97%, at least 98%, at least 99%, or at least 100%.

In a preferred aspect, the mature polypeptide is amino acids 20 to 326 of SEQ ID NO: 2, amino acids 18 to 239 of SEQ ID NO: 4, amino acids 20 to 258 of SEQ ID NO: 6, amino acids 19 to 226 of SEQ ID NO: 8, amino acids 20 to 304 of SEQ ID NO: 10, amino acids 23 to 250 of SEQ ID NO: 12, amino acids 22 to 249 of SEQ ID NO: 14, amino acids 20 to 249 of SEQ ID NO: 16, amino acids 18 to 232 of SEQ ID NO: 18, amino acids 16 to 235 of SEQ ID NO: 20, amino acids 19 to 323 of SEQ ID NO: 22, amino acids 16 to 310 of SEQ ID NO: 24, amino acids 20 to 246 of SEQ ID NO: 26, amino acids 22 to 354 of SEQ ID NO: 28,

amino acids 22 to 250 of SEQ ID NO: 30, or amino acids 22 to 322 of SEQ ID NO: 32, amino acids 24 to 444 of SEQ ID NO: 34, amino acids 26 to 253 of SEQ ID NO: 36, amino acids 20 to 223 of SEQ ID NO: 38, amino acids 18 to 246 of SEQ ID NO: 40, amino acids 20 to 334 of SEQ ID NO: 42, amino acids 18 to 227 of SEQ ID NO: 44, amino acids 22 to 368 of SEQ ID NO: 46, amino acids 25 to 330 of SEQ ID NO: 48, amino acids 17 to 236 of SEQ ID NO: 50, amino acids 17 to 250 of SEQ ID NO: 52, amino acids 23 to 478 of SEQ ID NO: 54, amino acids 17 to 230 of SEQ ID NO: 56, amino acids 20 to 257 of SEQ ID NO: 58, amino acids 23 to 251 of SEQ ID NO: 60, amino acids 19 to 349 of SEQ ID NO: 62, amino acids 24 to 436 of SEQ ID NO: 64, amino acids 21 to 344 of SEQ ID NO: 144, amino acids 21 to 389 of SEQ ID NO: 146, amino acids 22 to 406 of SEQ ID NO: 148, amino acids 20 to 427 of SEQ ID NO: 150, amino acids 18 to 267 of SEQ ID NO: 152, amino acids 21 to 273 of SEQ ID NO: 154, amino acids 21 to 322 of SEQ ID NO: 156, amino acids 18 to 234 of SEQ ID NO: 158, amino acids 24 to 233 of SEQ ID NO: 160, amino acids 17 to 237 of SEQ ID NO: 162, amino acids 20 to 484 of SEQ ID NO: 164, or amino acids 22 to 320 of SEQ ID NO: 166.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 2 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 2. In another preferred 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 20 to 326 of SEQ ID NO: 2, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 326 of SEQ ID NO: 2.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 4 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 4. In another preferred 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 18 to 239 of SEQ ID NO: 4, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 18 to 239 of SEQ ID NO: 4.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 6 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 6. In another preferred 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 20 to 258 of SEQ ID NO: 6, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 258 of SEQ ID NO: 6.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 8 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 8. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 8. In another preferred aspect, the polypeptide comprises or consists of amino acids 19 to 226 of SEQ ID NO: 8, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 19 to 226 of SEQ ID NO: 8.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 10 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 10. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 10. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 304 of SEQ ID NO: 10, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 304 of SEQ ID NO: 10.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 12 or an allelic variant thereof; or a fragment thereof having cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 12. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 12. In another preferred aspect, the polypeptide comprises or consists of amino acids 16 to 317 of SEQ ID NO: 12, or an allelic variant thereof; or a fragment thereof having cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 16 to 317 of SEQ ID NO: 12.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 14 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 14. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 14. In another preferred aspect, the polypeptide comprises or consists of amino acids 23 to 250

of SEQ ID NO: 14, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 23 to 250 of SEQ ID NO: 14.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 16 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 16. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 16. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 249 of SEQ ID NO: 16, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 249 of SEQ ID NO: 16.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 18 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 18. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 18. In another preferred aspect, the polypeptide comprises or consists of amino acids 18 to 232 of SEQ ID NO: 18, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 18 to 232 of SEQ ID NO: 18.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 20 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 20. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 20. In another preferred aspect, the polypeptide comprises or consists of amino acids 16 to 235 of SEQ ID NO: 20, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 16 to 235 of SEQ ID NO: 20.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 22 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 22. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 22. In another preferred aspect, the polypeptide comprises or consists of amino acids 19 to 323 of SEQ ID NO: 22, or an allelic variant thereof; or a fragment thereof that has cellulolytic

enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 19 to 323 of SEQ ID NO: 22.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 24 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 24. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 24. In another preferred aspect, the polypeptide comprises or consists of amino acids 16 to 310 of SEQ ID NO: 24, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 16 to 310 of SEQ ID NO: 24.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 26 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 26. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 26. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 246 of SEQ ID NO: 26, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 246 of SEQ ID NO: 26.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 28 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 28. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 28. In another preferred aspect, the polypeptide comprises or consists of amino acids 22 to 354 of SEQ ID NO: 28, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 22 to 354 of SEQ ID NO: 28.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 30 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 30. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 30. In another preferred aspect, the polypeptide comprises or consists of amino acids 22 to 250 of SEQ ID NO: 30, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of

amino acids 22 to 250 of SEQ ID NO: 30.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 32 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 32. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 32. In another preferred aspect, the polypeptide comprises or consists of amino acids 22 to 322 of SEQ ID NO: 32, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 22 to 322 of SEQ ID NO: 32.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 34 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 34. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 34. In another preferred aspect, the polypeptide comprises or consists of amino acids 24 to 444 of SEQ ID NO: 34, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 24 to 444 of SEQ ID NO: 34.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 36 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 36. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 36. In another preferred aspect, the polypeptide comprises or consists of amino acids 26 to 253 of SEQ ID NO: 36, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 26 to 253 of SEQ ID NO: 36.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 38 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 38. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 38. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 223 of SEQ ID NO: 38, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 223 of SEQ ID NO: 38.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 40 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 40. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 40. In another preferred aspect, the polypeptide comprises or consists of amino acids 18 to 246 of SEQ ID NO: 40, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 18 to 246 of SEQ ID NO: 40.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 42 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 42. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 42. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 334 of SEQ ID NO: 42, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 334 of SEQ ID NO: 42.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 44 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 44. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 44. In another preferred aspect, the polypeptide comprises or consists of amino acids 18 to 227 of SEQ ID NO: 44, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 18 to 227 of SEQ ID NO: 44.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 46 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 46. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 46. In another preferred aspect, the polypeptide comprises or consists of amino acids 22 to 368 of SEQ ID NO: 46, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 22 to 368 of SEQ ID NO: 46.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists

of the amino acid sequence of SEQ ID NO: 48 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 48. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 48. In another preferred aspect, the polypeptide comprises or consists of amino acids 25 to 330 of SEQ ID NO: 48, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 25 to 330 of SEQ ID NO: 48.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 50 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 50. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 50. In another preferred aspect, the polypeptide comprises or consists of amino acids 17 to 236 of SEQ ID NO: 50, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 17 to 236 of SEQ ID NO: 50.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 52 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 52. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 52. In another preferred aspect, the polypeptide comprises or consists of amino acids 19 to 250 of SEQ ID NO: 52, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 19 to 250 of SEQ ID NO: 52.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 54 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 54. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 54. In another preferred aspect, the polypeptide comprises or consists of amino acids 23 to 478 of SEQ ID NO: 54, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 23 to 478 of SEQ ID NO: 54.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 56 or an allelic variant thereof; or a fragment

thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 56. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 56. In another preferred aspect, the polypeptide comprises or consists of amino acids 17 to 230 of SEQ ID NO: 56, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 17 to 230 of SEQ ID NO: 56.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 58 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 58. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 58. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 257 of SEQ ID NO: 58, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 257 of SEQ ID NO: 58.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 60 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 60. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 60. In another preferred aspect, the polypeptide comprises or consists of amino acids 23 to 251 of SEQ ID NO: 60, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 23 to 251 of SEQ ID NO: 60.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 62 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 62. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 62. In another preferred aspect, the polypeptide comprises or consists of amino acids 19 to 349 of SEQ ID NO: 62, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 19 to 349 of SEQ ID NO: 62.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 64 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide

comprises or consists of the amino acid sequence of SEQ ID NO: 64. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 64. In another preferred aspect, the polypeptide comprises or consists of amino acids 24 to 436 of SEQ ID NO: 64, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 24 to 436 of SEQ ID NO: 64.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 144 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 144. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 144. In another preferred aspect, the polypeptide comprises or consists of amino acids 21 to 344 of SEQ ID NO: 144, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 21 to 344 of SEQ ID NO: 144.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 146 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 146. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 146. In another preferred aspect, the polypeptide comprises or consists of amino acids 21 to 389 of SEQ ID NO: 146, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 21 to 389 of SEQ ID NO: 146.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 148 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 148. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 148. In another preferred aspect, the polypeptide comprises or consists of amino acids 22 to 406 of SEQ ID NO: 148, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 22 to 406 of SEQ ID NO: 148.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 150 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 150. In another preferred

aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 150. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 427 of SEQ ID NO: 150, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 427 of SEQ ID NO: 150.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 152 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 152. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 152. In another preferred aspect, the polypeptide comprises or consists of amino acids 18 to 267 of SEQ ID NO: 152, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 18 to 267 of SEQ ID NO: 152.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 154 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 154. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 154. In another preferred aspect, the polypeptide comprises or consists of amino acids 21 to 273 of SEQ ID NO: 154, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 21 to 273 of SEQ ID NO: 154.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 156 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 156. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 156. In another preferred aspect, the polypeptide comprises or consists of amino acids 21 to 322 of SEQ ID NO: 156, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 21 to 322 of SEQ ID NO: 156.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 158 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 158. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 158.

In another preferred aspect, the polypeptide comprises or consists of amino acids 18 to 234 of SEQ ID NO: 158, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 18 to 234 of SEQ ID NO: 158.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 160 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 160. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 160. In another preferred aspect, the polypeptide comprises or consists of amino acids 24 to 233 of SEQ ID NO: 160, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 24 to 233 of SEQ ID NO: 160.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 162 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 162. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 162. In another preferred aspect, the polypeptide comprises or consists of amino acids 17 to 237 of SEQ ID NO: 162, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 17 to 237 of SEQ ID NO: 162.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 164 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 164. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 164. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 484 of SEQ ID NO: 164, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 20 to 484 of SEQ ID NO: 164.

A polypeptide having cellulolytic enhancing activity preferably comprises or consists of the amino acid sequence of SEQ ID NO: 166 or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In a preferred aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 166. In another preferred aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 166. In another preferred aspect, the polypeptide comprises or consists of amino acids 22 to 320

of SEQ ID NO: 166, or an allelic variant thereof; or a fragment thereof that has cellulolytic enhancing activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 22 to 320 of SEQ ID NO: 166.

Preferably, a fragment of the mature polypeptide of SEQ ID NO: 2 contains at least 277 amino acid residues, more preferably at least 287 amino acid residues, and most preferably at least 297 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 4 contains at least 185 amino acid residues, more preferably at least 195 amino acid residues, and most preferably at least 205 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 6 contains at least 200 amino acid residues, more preferably at least 212 amino acid residues, and most preferably at least 224 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 8 contains at least 175 amino acid residues, more preferably at least 185 amino acid residues, and most preferably at least 195 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 10 contains at least 240 amino acid residues, more preferably at least 255 amino acid residues, and most preferably at least 270 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 12 contains at least 255 amino acid residues, more preferably at least 270 amino acid residues, and most preferably at least 285 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 14 contains at least 175 amino acid residues, more preferably at least 190 amino acid residues, and most preferably at least 205 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 16 contains at least 200 amino acid residues, more preferably at least 210 amino acid residues, and most preferably at least 220 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 18 contains at least 185 amino acid residues, more preferably at least 195 amino acid residues, and most preferably at least 205 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 20 contains at least 190 amino acid residues, more preferably at least 200 amino acid residues, and most preferably at least 210 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 22 contains at least 260 amino acid residues, more preferably at least 275 amino acid residues, and most preferably at least 290 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 24 contains at least 250 amino acid residues, more preferably at least 265 amino acid residues, and most preferably at least 280 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 26 contains at least 195 amino acid residues, more preferably at least 205 amino acid residues, and most preferably at least 214 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 28 contains at least 285 amino acid residues, more preferably at least 300 amino acid residues, and most preferably at least 315 amino acid residues. Preferably, a fragment

of the mature polypeptide of SEQ ID NO: 30 contains at least 200 amino acid residues, more preferably at least 210 amino acid residues, and most preferably at least 220 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 32 contains at least 255 amino acid residues, more preferably at least 270 amino acid residues, and most preferably at least 285 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 34 contains at least 360 amino acid residues, more preferably at least 380 amino acid residues, and most preferably at least 400 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 36 contains at least 200 amino acid residues, more preferably at least 210 amino acid residues, and most preferably at least 220 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 38 contains at least 170 amino acid residues, more preferably at least 180 amino acid residues, and most preferably at least 190 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 40 contains at least 190 amino acid residues, more preferably at least 200 amino acid residues, and most preferably at least 210 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 42 contains at least 265 amino acid residues, more preferably at least 280 amino acid residues, and most preferably at least 295 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 44 contains at least 180 amino acid residues, more preferably at least 190 amino acid residues, and most preferably at least 200 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 46 contains at least 320 amino acid residues, more preferably at least 335 amino acid residues, and most preferably at least 350 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 48 contains at least 255 amino acid residues, more preferably at least 270 amino acid residues, and most preferably at least 285 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 50 contains at least 190 amino acid residues, more preferably at least 200 amino acid residues, and most preferably at least 210 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 52 contains at least 200 amino acid residues, more preferably at least 210 amino acid residues, and most preferably at least 220 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 54 contains at least 380 amino acid residues, more preferably at least 400 amino acid residues, and most preferably at least 420 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 56 contains at least 180 amino acid residues, more preferably at least 190 amino acid residues, and most preferably at least 200 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 58 contains at least 210 amino acid residues, more preferably at least 220 amino acid residues, and most preferably at least 230 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 60 contains at least 190 amino acid residues, more

preferably at least 200 amino acid residues, and most preferably at least 210 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 62 contains at least 270 amino acid residues, more preferably at least 290 amino acid residues, and most preferably at least 310 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 64 contains at least 340 amino acid residues, more preferably at least 360 amino acid residues, and most preferably at least 380 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 144 contains at least 280 amino acid residues, more preferably at least 295 amino acid residues, and most preferably at least 310 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 146 contains at least 310 amino acid residues, more preferably at least 330 amino acid residues, and most preferably at least 350 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 148 contains at least 320 amino acid residues, more preferably at least 340 amino acid residues, and most preferably at least 360 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 150 contains at least 350 amino acid residues, more preferably at least 370 amino acid residues, and most preferably at least 390 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 152 contains at least 220 amino acid residues, more preferably at least 230 amino acid residues, and most preferably at least 240 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 154 contains at least 220 amino acid residues, more preferably at least 230 amino acid residues, and most preferably at least 240 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 156 contains at least 255 amino acid residues, more preferably at least 270 amino acid residues, and most preferably at least 285 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 158 contains at least 185 amino acid residues, more preferably at least 195 amino acid residues, and most preferably at least 205 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 160 contains at least 180 amino acid residues, more preferably at least 190 amino acid residues, and most preferably at least 200 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 162 contains at least 190 amino acid residues, more preferably at least 200 amino acid residues, and most preferably at least 210 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 164 contains at least 385 amino acid residues, more preferably at least 410 amino acid residues, and most preferably at least 435 amino acid residues. Preferably, a fragment of the mature polypeptide of SEQ ID NO: 166 contains at least 255 amino acid residues, more preferably at least 270 amino acid residues, and most preferably at least 285 amino acid residues.

Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 1 contains at least 831 nucleotides, more preferably at least 861 nucleotides, and most

preferably at least 891 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 3 contains at least 555 nucleotides, more preferably at least 585 nucleotides, and most preferably at least 615 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 5 contains at least 600 nucleotides, more preferably at least 636 nucleotides, and most preferably at least 672 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 7 contains at least 525 nucleotides, more preferably at least 555 nucleotides, and most preferably at least 585 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 9 contains at least 720 nucleotides, more preferably at least 765 nucleotides, and most preferably at least 810 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 11 contains at least 765 nucleotides, more preferably at least 810 nucleotides, and most preferably at least 855 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of nucleotides 67 to 796 of SEQ ID NO: 13 contains at least 525 nucleotides, more preferably at least 570 nucleotides, and most preferably at least 615 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 15 contains at least 600 nucleotides, more preferably at least 630 nucleotides, and most preferably at least 660 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 17 contains at least 555 nucleotides, more preferably at least 585 nucleotides, and most preferably at least 615 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 19 contains at least 570 nucleotides, more preferably at least 600 nucleotides, and most preferably at least 630 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 21 contains at least 780 nucleotides, more preferably at least 825 nucleotides, and most preferably at least 870 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 23 contains at least 750 nucleotides, more preferably at least 795 nucleotides, and most preferably at least 840 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 25 contains at least 585 nucleotides, more preferably at least 615 nucleotides, and most preferably at least 645 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 27 contains at least 855 nucleotides, more preferably at least 900 nucleotides, and most preferably at least 945 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 29 contains at least 600 nucleotides, more preferably at least 630 nucleotides, and most preferably at least 660 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 31 contains at least 765 nucleotides, more preferably at least 810 nucleotides, and most preferably at least 855 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 33 contains at

least 1180 nucleotides, more preferably at least 1140 nucleotides, and most preferably at least 1200 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 35 contains at least 600 nucleotides, more preferably at least 630 nucleotides, and most preferably at least 660 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 37 contains at least 170 amino acid residues, more preferably at least 180 amino acid residues, and most preferably at least 190 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 39 contains at least 570 nucleotides, more preferably at least 600 nucleotides, and most preferably at least 630 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 41 contains at least 795 nucleotides, more preferably at least 840 nucleotides, and most preferably at least 885 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 43 contains at least 540 nucleotides, more preferably at least 570 nucleotides, and most preferably at least 600 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 45 contains at least 960 nucleotides, more preferably at least 1005 nucleotides, and most preferably at least 1050 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 47 contains at least 765 nucleotides, more preferably at least 810 nucleotides, and most preferably at least 855 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 49 contains at least 570 nucleotides, more preferably at least 600 nucleotides, and most preferably at least 630 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 51 contains at least 600 nucleotides, more preferably at least 630 nucleotides, and most preferably at least 660 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 53 contains at least 1140 nucleotides, more preferably at least 1200 nucleotides, and most preferably at least 1260 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 55 contains at least 540 nucleotides, more preferably at least 570 nucleotides, and most preferably at least 600 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 57 contains at least 630 nucleotides, more preferably at least 690 nucleotides, and most preferably at least 720 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 59 contains at least 570 nucleotides, more preferably at least 600 nucleotides, and most preferably at least 630 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 61 contains at least 810 nucleotides, more preferably at least 870 nucleotides, and most preferably at least 930 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 63 contains at least 1020 nucleotides, more preferably at least 1080 nucleotides, and most preferably at least 1140 nucleotides.

Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 143 contains at least 840 nucleotides, more preferably at least 885 nucleotides, and most preferably at least 930 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 145 contains at least 930 nucleotides, more preferably at least 960 nucleotides, and most preferably at least 1050 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 147 contains at least 960 nucleotides, more preferably at least 1020 nucleotides, and most preferably at least 1080 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 149 contains at least 1050 nucleotides, more preferably at least 1110 nucleotides, and most preferably at least 1170 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 151 contains at least 660 nucleotides, more preferably at least 690 nucleotides, and most preferably at least 720 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 153 contains at least 660 nucleotides, more preferably at least 690 nucleotides, and most preferably at least 720 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 155 contains at least 765 nucleotides, more preferably at least 810 nucleotides, and most preferably at least 855 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 157 contains at least 555 nucleotides, more preferably at least 585 nucleotides, and most preferably at least 615 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 159 contains at least 540 nucleotides, more preferably at least 570 nucleotides, and most preferably at least 600 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 161 contains at least 570 nucleotides, more preferably at least 600 nucleotides, and most preferably at least 630 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 163 contains at least 1155 nucleotides, more preferably at least 1230 nucleotides, and most preferably at least 1305 nucleotides. Preferably, a subsequence of the mature polypeptide coding sequence of SEQ ID NO: 165 contains at least 765 nucleotides, more preferably at least 810 nucleotides, and most preferably at least 855 nucleotides.

In a fourth aspect, the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide that hybridizes under at least very low stringency conditions, preferably at least low stringency conditions, more preferably at least medium stringency conditions, more preferably at least medium-high stringency conditions, even more preferably at least high stringency conditions, and most preferably at least very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27,

SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 143, SEQ ID NO: 145, SEQ ID NO: 147, SEQ ID NO: 149, SEQ ID NO: 151, SEQ ID NO: 153, SEQ ID NO: 155, SEQ ID NO: 157, SEQ ID NO: 159, SEQ ID NO: 161, or SEQ ID NO: 163, (ii) the genomic DNA sequence of the mature polypeptide coding sequence of SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 155, SEQ ID NO: 157, or SEQ ID NO: 159, or the cDNA sequence of the mature polypeptide coding sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 13, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 143, SEQ ID NO: 145, SEQ ID NO: 147, SEQ ID NO: 149, SEQ ID NO: 151, SEQ ID NO: 153, SEQ ID NO: 161, SEQ ID NO: 163, or SEQ ID NO: 165, (iii) a subsequence of (i) or (ii), or (iv) a full-length complementary strand of (i), (ii), or (iii) (J. Sambrook, E.F. Fritsch, and T. Maniatus, 1989, *supra*). A subsequence of the mature polypeptide coding sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 143, SEQ ID NO: 145, SEQ ID NO: 147, SEQ ID NO: 149, SEQ ID NO: 151, SEQ ID NO: 153, SEQ ID NO: 155, SEQ ID NO: 157, SEQ ID NO: 159, SEQ ID NO: 161, or SEQ ID NO: 163 contains at least 100 contiguous nucleotides or preferably at least 200 contiguous nucleotides. Moreover, the subsequence may encode a polypeptide fragment that has cellulolytic enhancing activity. In a preferred aspect, the mature polypeptide coding sequence is nucleotides 388 to 1332 of SEQ ID NO: 1, nucleotides 98 to 821 of SEQ ID NO: 3, nucleotides 126 to 978 of SEQ ID NO: 5, nucleotides 55 to 678 of SEQ ID NO: 7, nucleotides 58 to 912 of SEQ ID NO: 9, nucleotides 46 to 951 of SEQ ID NO: 11, nucleotides 67 to 796 of SEQ ID NO: 13, nucleotides 77 to 766 of SEQ ID NO: 15, nucleotides 52 to 921 of SEQ ID NO: 17, nucleotides 46 to 851 of SEQ ID NO: 19, nucleotides 55 to 1239 of SEQ ID NO: 21, nucleotides 46 to 1250 of SEQ ID NO: 23, nucleotides 58 to 811 of SEQ ID NO: 25, nucleotides 64 to 1112 of SEQ ID NO: 27, nucleotides 64 to 859 of SEQ ID NO: 29, nucleotides 64 to 1018 of SEQ ID NO: 31, nucleotides 70 to 1483 of SEQ ID NO: 33,

nucleotides 76 to 832 of SEQ ID NO: 35, nucleotides 58 to 974 of SEQ ID NO: 37, nucleotides 52 to 875 of SEQ ID NO: 39, nucleotides 58 to 1250 of SEQ ID NO: 41, nucleotides 52 to 795 of SEQ ID NO: 43, nucleotides 64 to 1104 of SEQ ID NO: 45, nucleotides 73 to 990 of SEQ ID NO: 47, nucleotides 49 to 1218 of SEQ ID NO: 49, nucleotides 55 to 930 of SEQ ID NO: 51, nucleotides 67 to 1581 of SEQ ID NO: 53, nucleotides 49 to 865 of SEQ ID NO: 55, nucleotides 58 to 1065 of SEQ ID NO: 57, nucleotides 67 to 868 of SEQ ID NO: 59, nucleotides 55 to 1099 of SEQ ID NO: 61, nucleotides 70 to 1483 of SEQ ID NO: 63, nucleotides 61 to 1032 of SEQ ID NO: 143, nucleotides 61 to 1167 of SEQ ID NO: 145, nucleotides 64 to 1218 of SEQ ID NO: 147, nucleotides 58 to 1281 of SEQ ID NO: 149, nucleotides 52 to 801 of SEQ ID NO: 151, nucleotides 61 to 819 of SEQ ID NO: 153, nucleotides 61 to 966 of SEQ ID NO: 155, nucleotides 52 to 702 of SEQ ID NO: 157, nucleotides 70 to 699 of SEQ ID NO: 159, nucleotides 49 to 711 of SEQ ID NO: 161, nucleotides 76 to 1452 of SEQ ID NO: 163, or nucleotides 64 to 1018 of SEQ ID NO: 165.

The nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 143, SEQ ID NO: 145, SEQ ID NO: 147, SEQ ID NO: 149, SEQ ID NO: 151, SEQ ID NO: 153, SEQ ID NO: 155, SEQ ID NO: 157, SEQ ID NO: 159, SEQ ID NO: 161, or SEQ ID NO: 163, or a subsequence thereof; as well as the amino acid sequence of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 144, SEQ ID NO: 146, SEQ ID NO: 148, SEQ ID NO: 150, SEQ ID NO: 152, SEQ ID NO: 154, SEQ ID NO: 156, SEQ ID NO: 158, SEQ ID NO: 160, SEQ ID NO: 162, SEQ ID NO: 164, or SEQ ID NO: 166, or a fragment thereof, may be used to design a nucleic acid probe to identify and clone DNA encoding polypeptides having cellulolytic enhancing activity from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic DNA or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably

shorter than the entire sequence, but should be at least 14, preferably at least 25, more preferably at least 35, and most preferably at least 70 nucleotides in length. It is, however, preferred that the nucleic acid probe is at least 100 nucleotides in length. For example, the nucleic acid probe may be at least 200 nucleotides, preferably at least 300 nucleotides, more preferably at least 400 nucleotides, or most preferably at least 500 nucleotides in length. Even longer probes may be used, *e.g.*, nucleic acid probes that are preferably at least 600 nucleotides, more preferably at least 700 nucleotides, even more preferably at least 800 nucleotides, or most preferably at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with ^{32}P, ^{3}H, ^{35}S, biotin, or avidin). Such probes are encompassed by the present invention.

A genomic DNA or cDNA library prepared from such other strains may, therefore, be screened for DNA that hybridizes with the probes described above and encodes a polypeptide having cellulolytic enhancing activity. Genomic or other DNA from such other strains may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA that is homologous with SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 143, SEQ ID NO: 145, SEQ ID NO: 147, SEQ ID NO: 149, SEQ ID NO: 151, SEQ ID NO: 153, SEQ ID NO: 155, SEQ ID NO: 157, SEQ ID NO: 159, SEQ ID NO: 161, or SEQ ID NO: 163, or a subsequence thereof, the carrier material is preferably used in a Southern blot.

For purposes of the present invention, hybridization indicates that the nucleotide sequence hybridizes to a labeled nucleic acid probe corresponding to the mature polypeptide coding sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 143, SEQ ID NO: 145, SEQ ID NO: 147, SEQ ID NO: 149, SEQ ID NO: 151, SEQ ID NO: 153, SEQ ID NO: 155, SEQ ID NO: 157, SEQ ID NO: 159, SEQ ID NO:

161, or SEQ ID NO: 163; the genomic DNA sequence of the mature polypeptide coding sequence of SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 155, SEQ ID NO: 157, or SEQ ID NO: 159, or the cDNA sequence of the mature polypeptide coding sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 13, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 143, SEQ ID NO: 145, SEQ ID NO: 147, SEQ ID NO: 149, SEQ ID NO: 151, SEQ ID NO: 153, SEQ ID NO: 161, SEQ ID NO: 163, or SEQ ID NO: 165; the full-length complementary strand thereof; or a subsequence thereof, under very low to very high stringency conditions, as described *supra*.

In a preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 1. In another preferred aspect, the nucleic acid probe is nucleotides 388 to 1332 of SEQ ID NO: 1. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 2, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 1. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pEJG120 which is contained in *E. coli* NRRL B-30699, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pEJG120 which is contained in *E. coli* NRRL B-30699.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 3. In another preferred aspect, the nucleic acid probe is nucleotides 98 to 821 of SEQ ID NO: 3. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 4, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 3. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pTter61C which is contained in *E. coli* NRRL B-30813, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pTter61C which is contained in *E. coli* NRRL B-30813.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 5. In another preferred aspect, the nucleic acid probe is nucleotides 126 to 978 of SEQ ID NO: 5. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 6, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 5.

In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pTter61D which is contained in *E. coli* NRRL B-30812, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pTter61D which is contained in *E. coli* NRRL B-30812.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 7. In another preferred aspect, the nucleic acid probe is nucleotides 55 to 678 of SEQ ID NO: 7. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 8, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 7. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pTter61E which is contained in *E. coli* NRRL B-30814, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pTter61E which is contained in *E. coli* NRRL B-30814.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 9. In another preferred aspect, the nucleic acid probe is nucleotides 58 to 912 of SEQ ID NO: 9 In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 10, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 9. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pTter61G which is contained in *E. coli* NRRL B-30811, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pTter61G which is contained in *E. coli* NRRL B-30811.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 11. In another preferred aspect, the nucleic acid probe is nucleotides 46 to 951 of SEQ ID NO: 11. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 12, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 11. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pTter61F which is contained in *E. coli* NRRL B-50044, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding region contained in plasmid pTter61F which is contained in *E. coli* NRRL B-50044.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 13. In another preferred aspect, the nucleic acid probe is

nucleotides 67 to 796 of SEQ ID NO: 13. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 14, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 13. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pDZA2-7 which is contained in *E. coli* NRRL B-30704, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pDZA2-7 which is contained in *E. coli* NRRL B-30704.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 15. In another preferred aspect, the nucleic acid probe is nucleotides 77 to 766 of SEQ ID NO: 15. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 16, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 15. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pTr3337 which is contained in *E. coli* NRRL B-30878, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pTr3337 which is contained in *E. coli* NRRL B-30878.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 17. In another preferred aspect, the nucleic acid probe is nucleotides 52 to 921 of SEQ ID NO: 17. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 18, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 17. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pSMai190 which is contained in *E. coli* NRRL B-50084, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pSMai190 which is contained in *E. coli* NRRL B-50084.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 19. In another preferred aspect, the nucleic acid probe is nucleotides 46 to 851 of SEQ ID NO: 19. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 20, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 19. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pSMai192 which is contained in *E. coli* NRRL B-50086, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding

sequence contained in plasmid pSMai192 which is contained in *E. coli* NRRL B-50086.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 21. In another preferred aspect, the nucleic acid probe is nucleotides 55 to 1239 of SEQ ID NO: 21. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 22, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 21. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pSMai191 which is contained in *E. coli* NRRL B-50085, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pSMai191 which is contained in *E. coli* NRRL B-50085.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 23. In another preferred aspect, the nucleic acid probe is nucleotides 46 to 1250 of SEQ ID NO: 23. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 24, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 23. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pSMai193 which is contained in *E. coli* NRRL B-50087, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pSMai193 which is contained in *E. coli* NRRL B-50087.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 25. In another preferred aspect, the nucleic acid probe is nucleotides 58 to 811 of SEQ ID NO: 25. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 26, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 25. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pSMai187 which is contained in *E. coli* NRRL B-50083, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pSMai187 which is contained in *E. coli* NRRL B-50083.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 27. In another preferred aspect, the nucleic acid probe is nucleotides 64 to 1112 of SEQ ID NO: 27. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 28, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 27. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained

in plasmid pXYZ1473 which is contained in *E. coli* DSM 22075, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pXYZ1473 which is contained in *E. coli* DSM 22075.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 29. In another preferred aspect, the nucleic acid probe is nucleotides 64 to 859 of SEQ ID NO: 29. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 30, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 29.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 31. In another preferred aspect, the nucleic acid probe is nucleotides 64 to 1018 of SEQ ID NO: 31. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 32, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 31. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pGEM-T-Ppin7 which is contained in *E. coli* DSM 22711, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pGEM-T-Ppin7 which is contained in *E. coli* DSM 22711.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 33. In another preferred aspect, the nucleic acid probe is nucleotides 70 to 1483 of SEQ ID NO: 33. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 34, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 33. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pXYZ1483 which is contained in *E. coli* DSM 22600, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pXYZ1483 which is contained in *E. coli* DSM 22600.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 35. In another preferred aspect, the nucleic acid probe is nucleotides 76 to 832 of SEQ ID NO: 35. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 36, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 35. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pGEM-T-GH61D23Y4 which is contained in *E. coli* DSM 22882, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing

activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pGEM-T-GH61D23Y4 which is contained in *E. coli* DSM 22882.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 37. In another preferred aspect, the nucleic acid probe is nucleotides 58 to 974 of SEQ ID NO: 37. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 38, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 37. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pSMai213 which is contained in *E. coli* NRRL B-50300, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pSMai213 which is contained in *E. coli* NRRL B-50300.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 39. In another preferred aspect, the nucleic acid probe is nucleotides 52 to 875 of SEQ ID NO: 39. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 40, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 39. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pSMai216 which is contained in *E. coli* NRRL B-50301, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pSMai216 which is contained in *E. coli* NRRL B-50301.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 41. In another preferred aspect, the nucleic acid probe is nucleotides 58 to 1250 of SEQ ID NO: 41. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 42, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 41. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid p pSMai217 which is contained in *E. coli* NRRL B-50302, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pSMai217 which is contained in *E. coli* NRRL B-50302.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 43. In another preferred aspect, the nucleic acid probe is nucleotides 52 to 795 of SEQ ID NO: 43. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 44, or a

subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 43. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pSMai218 which is contained in *E. coli* NRRL B-50303, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pSMai218 which is contained in *E. coli* NRRL B-50303.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 45. In another preferred aspect, the nucleic acid probe is nucleotides 64 to 1104 of SEQ ID NO: 45. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 46, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 45. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pAG68 which is contained in *E. coli* NRRL B-50320, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pAG68 which is contained in *E. coli* NRRL B-50320.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 47. In another preferred aspect, the nucleic acid probe is nucleotides 73 to 990 of SEQ ID NO: 47. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 48, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 47. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pAG69 which is contained in *E. coli* NRRL B-50321, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pAG69 which is contained in *E. coli* NRRL B-50321.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 49. In another preferred aspect, the nucleic acid probe is nucleotides 49 to 1218 of SEQ ID NO: 49. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 50, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 49. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pAG75 which is contained in *E. coli* NRRL B-50322, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pAG75 which is contained in *E. coli* NRRL B-50322.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding

sequence of SEQ ID NO: 51. In another preferred aspect, the nucleic acid probe is nucleotides 55 to 930 of SEQ ID NO: 51. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 52, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 51. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pAG76 which is contained in *E. coli* NRRL B-50323, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pAG76 which is contained in *E. coli* NRRL B-50323.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 53. In another preferred aspect, the nucleic acid probe is nucleotides 67 to 1581 of SEQ ID NO: 53. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 54, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 53. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pAG77 which is contained in *E. coli* NRRL B-50324, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pAG77 which is contained in *E. coli* NRRL B-50324.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 55. In another preferred aspect, the nucleic acid probe is nucleotides 49 to 865 of SEQ ID NO: 55. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 56, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 55. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pAG78 which is contained in *E. coli* NRRL B-50325, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pAG78 which is contained in *E. coli* NRRL B-50325.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 57. In another preferred aspect, the nucleic acid probe is nucleotides 58 to 1065 of SEQ ID NO: 57. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 58, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 57. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid p pAG79 which is contained in *E. coli* NRRL B-50326, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another

preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pAG79 which is contained in *E. coli* NRRL B-50326.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 59. In another preferred aspect, the nucleic acid probe is nucleotides 67 to 868 of SEQ ID NO: 59. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 60, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 59. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid plasmid pGEM-T-GH61a51486 which is contained in *E. coli* DSM 22656, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid plasmid pGEM-T-GH61a51486 which is contained in *E. coli* DSM 22656.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 61. In another preferred aspect, the nucleic acid probe is nucleotides 55 to 1099 of SEQ ID NO: 61. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 62, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 61. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pGEM-T-GH61DYF which is contained in *E. coli* DSM 22654, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pGEM-T-GH61DYF which is contained in *E. coli* DSM 22654.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 63. In another preferred aspect, the nucleic acid probe is nucleotides 70 to 1483 of SEQ ID NO: 63. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 64, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 63. In another preferred aspect, the nucleic acid probe is the polynucleotide sequence contained in plasmid pGEM-T-GH61D14YH which is contained in *E. coli* DSM 22657, wherein the polynucleotide sequence thereof encodes a polypeptide having cellulolytic enhancing activity. In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pGEM-T-GH61D14YH which is contained in *E. coli* DSM 22657.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 143. In another preferred aspect, the nucleic acid probe is

nucleotides 61 to 1032 of SEQ ID NO: 143. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 143, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 143.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 145. In another preferred aspect, the nucleic acid probe is nucleotides 61 to 1167 of SEQ ID NO: 145. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 145, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 145.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 147. In another preferred aspect, the nucleic acid probe is nucleotides 64 to 1218 of SEQ ID NO: 147. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 147, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 147.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 149. In another preferred aspect, the nucleic acid probe is nucleotides 58 to 1281 of SEQ ID NO: 149. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 149, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 149.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 151. In another preferred aspect, the nucleic acid probe is nucleotides 52 to 801 of SEQ ID NO: 151. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 151, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 151.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 153. In another preferred aspect, the nucleic acid probe is nucleotides 61 to 819 of SEQ ID NO: 153. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 153, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 153.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 155. In another preferred aspect, the nucleic acid probe is nucleotides 61 to 966 of SEQ ID NO: 155. In another preferred aspect, the nucleic acid

probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 155, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 155.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 157. In another preferred aspect, the nucleic acid probe is nucleotides 52 to 702 of SEQ ID NO: 157. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 157, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 157.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 159. In another preferred aspect, the nucleic acid probe is nucleotides 70 to 699 of SEQ ID NO: 159. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 159, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 159.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 161. In another preferred aspect, the nucleic acid probe is nucleotides 49 to 711 of SEQ ID NO: 161. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 161, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 161.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 163. In another preferred aspect, the nucleic acid probe is nucleotides 76 to 1452 of SEQ ID NO: 163. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 163, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 163.

In another preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 165. In another preferred aspect, the nucleic acid probe is nucleotides 64 to 1018 of SEQ ID NO: 165. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 165, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 165.

For long probes of at least 100 nucleotides in length, very low to very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high

stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 45°C (very low stringency), at 50°C (low stringency), at 55°C (medium stringency), at 60°C (medium-high stringency), at 65°C (high stringency), and at 70°C (very high stringency).

For short probes of about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as prehybridization and hybridization at about 5°C to about 10°C below the calculated T_m using the calculation according to Bolton and McCarthy (1962, *Proc. Natl. Acad. Sci. USA* 48:1390) in 0.9 M NaCl, 0.09 M Tris-HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1X Denhardt's solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per ml following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed once in 6X SCC plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6X SSC at 5°C to 10°C below the calculated T_m.

In a fifth aspect, the polypeptide having cellulolytic enhancing activity is encoded by a polynucleotide comprising or consisting of a nucleotide sequence that has a degree of identity to the mature polypeptide coding sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 143, SEQ ID NO: 145, SEQ ID NO: 147, SEQ ID NO: 149, SEQ ID NO: 151, SEQ ID NO: 153, SEQ ID NO: 155, SEQ ID NO: 157, SEQ ID NO: 159, SEQ ID NO: 161, or SEQ ID NO: 163 of preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 91%, at least 92%, at least 93%, at least 94%, or at least 95%, and even most preferably at least 96%, at least 97%, at least 98%, at least 99%, or at least 100%.

In a sixth aspect, the polypeptide having cellulolytic enhancing activity is an artificial variant comprising a substitution, deletion, and/or insertion of one or more (*e.g.*, several) amino acids of the mature polypeptide of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, or SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO:

62, SEQ ID NO: 64, SEQ ID NO: 144, SEQ ID NO: 146, SEQ ID NO: 148, SEQ ID NO: 150, SEQ ID NO: 152, SEQ ID NO: 154, SEQ ID NO: 156, SEQ ID NO: 158, SEQ ID NO: 160, SEQ ID NO: 162, SEQ ID NO: 164, or SEQ ID NO: 166; or a homologous sequence thereof. Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

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

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

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

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

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

The total number of amino acid substitutions, deletions and/or insertions of the mature polypeptide of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, or SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 144, SEQ ID NO: 146, SEQ ID NO: 148, SEQ ID NO: 150, SEQ ID NO: 152, SEQ ID NO: 154, SEQ ID NO: 156, SEQ ID NO: 158, SEQ ID NO: 160, SEQ ID NO: 162, SEQ ID NO: 164, or SEQ ID NO: 166, is not more than 10, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8 or 9.

A polypeptide having cellulolytic enhancing activity may be obtained from microorganisms of any genus. For purposes of the present invention, the term “obtained from” as used herein in connection with a given source shall mean that the polypeptide encoded by a 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.

A polypeptide having cellulolytic enhancing 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 enhancing 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 enhancing activity.

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 having cellulolytic enhancing activity.

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

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

The polypeptide having cellulolytic enhancing 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 enhancing activity; or more preferably a filamentous fungal polypeptide such as aan *Acremonium*, *Agaricus*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Botryospaeria*, *Ceriporiopsis*, *Chaetomidium*, *Chrysosporium*, *Claviceps*, *Cochliobolus*, *Coprinopsis*, *Coptotermes*, *Corynascus*, *Cryphonectria*, *Cryptococcus*, *Diplodia*, *Exidia*, *Filibasidium*, *Fusarium*, *Gibberella*, *Holomastigotoides*, *Humicola*, *Irpex*, *Lentinula*, *Leptospaeria*, *Magnaporthe*, *Melanocarpus*, *Meripilus*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Piromyces*, *Poitrasia*, *Pseudoplectania*, *Pseudotrichonympha*, *Rhizomucor*, *Schizophyllum*, *Scytalidium*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trichoderma*, *Trichophaea*, *Verticillium*, *Volvariella*, or *Xylaria* polypeptide having cellulolytic enhancing activity.

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

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

Fusarium roseum, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola grisea*, *Humicola insolens*, *Humicola lanuginosa*, *Irpex lacteus*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium funiculosum*, *Penicillium pinophilum*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Thielavia achromatica*, *Thielavia albomyces*, *Thielavia albopilosa*, *Thielavia australeinsis*, *Thielavia fimeti*, *Thielavia microspora*, *Thielavia ovispora*, *Thielavia peruviana*, *Thielavia spededonium*, *Thielavia setosa*, *Thielavia subthermophila*, *Thielavia terrestris*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, *Trichoderma viride*, or *Trichophaea saccata* polypeptide having cellulolytic enhancing activity.

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.

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

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

Polynucleotides comprising nucleotide sequences that encode polypeptide having cellulolytic enhancing activity can be isolated and utilized to express the polypeptide having cellulolytic enhancing activity for evaluation in the methods of the present invention.

The techniques used to isolate or clone a polynucleotide encoding a polypeptide are known in the art and include isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the polynucleotides from such genomic DNA can be effected, *e.g.*, by using the well known polymerase chain reaction (PCR) or antibody screening of expression libraries to detect cloned DNA fragments with shared structural features. See, *e.g.*, Innis *et al.*, 1990, *PCR: A Guide to Methods and Application*, Academic

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

The polynucleotides comprise nucleotide sequences that have a degree of identity to the mature polypeptide coding sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 143, SEQ ID NO: 145, SEQ ID NO: 147, SEQ ID NO: 149, SEQ ID NO: 151, SEQ ID NO: 153, SEQ ID NO: 155, SEQ ID NO: 157, SEQ ID NO: 159, SEQ ID NO: 161, or SEQ ID NO: 163 of preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 91%, at least 92%, at least 93%, at least 94%, or at least 95%, and even most preferably at least 96%, at least 97%, at least 98%, or at least 99%, which encode a polypeptide having cellulolytic enhancing activity.

The polynucleotide may also be a polynucleotide encoding a polypeptide having cellulolytic enhancing activity that hybridizes under at least very low stringency conditions, preferably at least low stringency conditions, more preferably at least medium stringency conditions, more preferably at least medium-high stringency conditions, even more preferably at least high stringency conditions, and most preferably at least very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 143, SEQ ID NO: 145, SEQ ID NO: 147, SEQ ID NO: 149, SEQ ID NO: 151, SEQ ID NO: 153, SEQ ID NO: 155, SEQ ID NO: 157, SEQ ID NO: 159, SEQ ID NO: 161, or SEQ ID NO: 163, (ii) the genomic DNA sequence of the mature polypeptide coding sequence of SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 155, SEQ ID NO: 157, or SEQ ID NO: 159 or the cDNA sequence of the mature polypeptide coding sequence of SEQ ID NO: 1, SEQ ID

NO: 3, SEQ ID NO: 5, SEQ ID NO: 13, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 143, SEQ ID NO: 145, SEQ ID NO: 147, SEQ ID NO: 149, SEQ ID NO: 151, SEQ ID NO: 153, SEQ ID NO: 161, SEQ ID NO: 163, or SEQ ID NO: 165, or (iii) a full-length complementary strand of (i) or (ii); or allelic variants and subsequences thereof (Sambrook *et al.*, 1989, *supra*), as defined herein.

As described earlier, the techniques used to isolate or clone a polynucleotide encoding a polypeptide are known in the art and include isolation from genomic DNA, preparation from cDNA, or a combination thereof.

Enzyme Compositions

The enzyme compositions can comprise any protein that is useful in degrading or converting a cellulosic material.

In one aspect, the enzyme composition comprises or further comprises one or more (*e.g.*, several) proteins selected from the group consisting of a cellulase, a hemicellulase, an esterase, an expansin, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin. In another aspect, the cellulase is preferably one or more (*e.g.*, several) enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase. In another aspect, the hemicellulase is preferably one or more (*e.g.*, several) enzymes selected from the group consisting of an acetylmannan esterase, an acetylxylan esterase, an arabinanase, an arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase.

In another aspect, the enzyme composition comprises one or more (*e.g.*, several) cellulolytic enzymes. In another aspect, the enzyme composition comprises or further comprises one or more (*e.g.*, several) hemicellulolytic enzymes. In another aspect, the enzyme composition comprises one or more (*e.g.*, several) cellulolytic enzymes and one or more (*e.g.*, several) hemicellulolytic enzymes. In another aspect, the enzyme composition comprises one or more (*e.g.*, several) enzymes selected from the group of cellulolytic enzymes and hemicellulolytic enzymes. In another aspect, the enzyme composition comprises an endoglucanase. In another aspect, the enzyme composition comprises a cellobiohydrolase. In another aspect, the enzyme composition comprises a beta-glucosidase. In another aspect, the enzyme composition comprises an endoglucanase and a cellobiohydrolase. In another aspect, the enzyme composition comprises an endoglucanase

and a beta-glucosidase. In another aspect, the enzyme composition comprises a cellobiohydrolase and a beta-glucosidase. In another aspect, the enzyme composition comprises an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

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

In another aspect, the enzyme composition comprises an esterase. In another aspect, the enzyme composition comprises an expansin. In another aspect, the enzyme composition comprises a laccase. In another aspect, the enzyme composition comprises a ligninolytic enzyme. In a preferred aspect, the ligninolytic enzyme is a manganese peroxidase. In another preferred aspect, the ligninolytic enzyme is a lignin peroxidase. In another preferred aspect, the ligninolytic enzyme is a H_2O_2-producing enzyme. In another aspect, the enzyme composition comprises a pectinase. In another aspect, the enzyme composition comprises a peroxidase. In another aspect, the enzyme composition comprises a protease. In another aspect, the enzyme composition comprises a swollenin.

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

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

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

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

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

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

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

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

lividans polypeptide having enzyme activity.

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

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

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

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

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

In one aspect, the one or more (*e.g.*, several) cellulolytic enzymes comprise a commercial cellulolytic enzyme preparation. Examples of commercial cellulolytic enzyme preparations suitable for use in the present invention include, for example, CELLIC™ CTec (Novozymes A/S), CELLIC™ CTec2 (Novozymes A/S), CELLUCLAST™ (Novozymes A/S), NOVOZYM™ 188 (Novozymes A/S), CELLUZYME™ (Novozymes A/S), CEREFLO™ (Novozymes A/S), and ULTRAFLO™ (Novozymes A/S), ACCELERASE™ (Genencor Int.), LAMINEX™ (Genencor Int.), SPEZYME™ CP (Genencor Int.), FILTRASE® NL (DSM); METHAPLUS® S/L 100 (DSM), ROHAMENT™ 7069 W (Röhm GmbH), FIBREZYME® LDI (Dyadic International, Inc.), FIBREZYME® LBR (Dyadic International, Inc.), or VISCOSTAR® 150L (Dyadic International, Inc.). The cellulase enzymes are added in amounts effective from about 0.001 to about 5.0 wt % of solids, more preferably from about 0.025 to about 4.0 wt % of solids, and most preferably from about 0.005 to about 2.0 wt % of solids. The cellulase enzymes are added in amounts effective from about 0.001 to about 5.0 wt % of solids, more preferably from about 0.025 to about 4.0 wt % of solids, and most preferably from about 0.005 to about 2.0 wt % of solids.

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

Examples of fungal endoglucanases that can be used in the present invention include, but are not limited to, a *Trichoderma reesei* endoglucanase I (Penttila *et al.*, 1986, *Gene* 45: 253-263; *Trichoderma reesei* Cel7B endoglucanase I; GENBANK™ accession no. M15665; SEQ ID NO: 66); *Trichoderma reesei* endoglucanase II (Saloheimo, *et al.*, 1988, *Gene* 63:11-22; *Trichoderma reesei* Cel5A endoglucanase II; GENBANK™ accession no. M19373; SEQ ID NO: 68); *Trichoderma reesei* endoglucanase III (Okada *et al.*, 1988, *Appl. Environ. Microbiol.* 64: 555-563; GENBANK™ accession no. AB003694; SEQ ID NO: 70); *Trichoderma reesei* endoglucanase V (Saloheimo *et al.*, 1994, *Molecular Microbiology* 13:

219-228; GENBANK™ accession no. Z33381; SEQ ID NO: 72); *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 carotovara* endoglucanase (Saarilahti *et al.*, 1990, *Gene* 90: 9-14); *Fusarium oxysporum* endoglucanase (GENBANK™ accession no. L29381); *Humicola grisea* var. *thermoidea* endoglucanase (GENBANK™ accession no. AB003107); *Melanocarpus albomyces* endoglucanase (GENBANK™ accession no. MAL515703); *Neurospora crassa* endoglucanase (GENBANK™ accession no. XM_324477); *Humicola insolens* endoglucanase V (SEQ ID NO: 74); *Myceliophthora thermophila* CBS 117.65 endoglucanase (SEQ ID NO: 76); basidiomycete CBS 495.95 endoglucanase (SEQ ID NO: 78); basidiomycete CBS 494.95 endoglucanase (SEQ ID NO: 80); *Thielavia terrestris* NRRL 8126 CEL6B endoglucanase (SEQ ID NO: 82); *Thielavia terrestris* NRRL 8126 CEL6C endoglucanase (SEQ ID NO: 84); *Thielavia terrestris* NRRL 8126 CEL7C endoglucanase (SEQ ID NO: 86); *Thielavia terrestris* NRRL 8126 CEL7E endoglucanase (SEQ ID NO: 88); *Thielavia terrestris* NRRL 8126 CEL7F endoglucanase (SEQ ID NO: 90); *Cladorrhinum foecundissimum* ATCC 62373 CEL7A endoglucanase (SEQ ID NO: 92); and *Trichoderma reesei* strain No. VTT-D-80133 endoglucanase (SEQ ID NO: 94; GENBANK™ accession no. M15665). The endoglucanases of SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 88, SEQ ID NO: 90, SEQ ID NO: 92, and SEQ ID NO: 94 described above are encoded by the mature polypeptide coding sequence of SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO: 79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, and SEQ ID NO: 93, respectively.

Examples of cellobiohydrolases useful in the present invention include, but are not limited to, *Trichoderma reesei* cellobiohydrolase I (SEQ ID NO: 96); *Trichoderma reesei* cellobiohydrolase II (SEQ ID NO: 98); *Humicola insolens* cellobiohydrolase I (SEQ ID NO: 100); *Myceliophthora thermophila* cellobiohydrolase II (SEQ ID NO: 102 and SEQ ID NO: 104); *Thielavia terrestris* cellobiohydrolase II (CEL6A) (SEQ ID NO: 106); *Chaetomium thermophilum* cellobiohydrolase I (SEQ ID NO: 108); and *Chaetomium thermophilum* cellobiohydrolase II (SEQ ID NO: 110). The cellobiohydrolases of SEQ ID NO: 96, SEQ ID NO: 98, SEQ ID NO: 100, SEQ ID NO: 102, SEQ ID NO: 104, SEQ ID NO: 106, SEQ ID NO: 108, SEQ ID NO: 110, and SEQ ID NO: 112 described above are encoded by the mature polypeptide coding sequence of SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, and SEQ ID NO: 109, respectively.

Examples of beta-glucosidases useful in the present invention include, but are not

limited to, *Aspergillus oryzae* beta-glucosidase (SEQ ID NO: 112); *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 114); *Penicillium brasilianum* IBT 20888 beta-glucosidase (SEQ ID NO: 116); *Aspergillus niger* beta-glucosidase (SEQ ID NO: 118); and *Aspergillus aculeatus* beta-glucosidase (SEQ ID NO: 120). The beta-glucosidases of SEQ ID NO: 112, SEQ ID NO: 114, SEQ ID NO: 116, SEQ ID NO: 118, and SEQ ID NO: 120 described above are encoded by the mature polypeptide coding sequence of SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, and SEQ ID NO: 119, respectively.

Examples of other beta-glucosidases useful in the present invention include a *Aspergillus oryzae* beta-glucosidase variant fusion protein of SEQ ID NO: 122 or the *Aspergillus oryzae* beta-glucosidase fusion protein of SEQ ID NO: 124. The beta-glucosidase fusion proteins of SEQ ID NO: 122 and SEQ ID NO: 124 are encoded by SEQ ID NO: 121 and SEQ ID NO: 123, respectively.

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

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

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

5,776,757.

In one aspect, the one or more (*e.g.*, several) hemicellulolytic enzymes comprise a commercial hemicellulolytic enzyme preparation. Examples of commercial hemicellulolytic enzyme preparations suitable for use in the present invention include, for example, SHEARZYME™ (Novozymes A/S), CELLIC™ HTec (Novozymes A/S), CELLIC™ HTec2 (Novozymes A/S), VISCOZYME® (Novozymes A/S), ULTRAFLO® (Novozymes A/S), PULPZYME® HC (Novozymes A/S), MULTIFECT® Xylanase (Genencor), ACCELLERASE® XY (Genencor), ACCELLERASE® XC (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 Q0UHJ1), 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 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.

Nucleic Acid Constructs

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

The control sequence may be a promoter sequence, a polynucleotide that is recognized by a host cell for expression of a polynucleotide encoding a polypeptide. The promoter sequence contains transcriptional control sequences that mediate the expression of the polypeptide. The promoter may be any polynucleotide that shows transcriptional

activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

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

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

In a yeast host, useful promoters are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* galactokinase (GAL1),

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

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

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.

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 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.

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

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

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

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

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

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

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

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

polypeptide and the signal peptide sequence is positioned next to the N-terminus of the propeptide sequence.

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

Expression Vectors

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

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

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

Furthermore, a single vector or plasmid or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

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

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 adenyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in an *Aspergillus* cell are the *amdS* and *pyrG* genes of *Aspergillus nidulans* or *Aspergillus oryzae* and the *bar* gene of *Streptomyces hygroscopicus*.

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

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in

a cell. The term “origin of replication” or “plasmid replicator” means a polynucleotide that enables a plasmid or vector to replicate *in vivo*.

Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*, and pUB110, pE194, pTA1060, and pAMß1 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.

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

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

Host Cells

Recombinant host cells comprising a polynucleotide encoding a polypeptide, *e.g.*, a polypeptide having cellulolytic enhancing activity, a cellulolytic enzyme, a hemicellulolytic enzyme, etc., can be advantageously used in the recombinant production of the polypeptide. A construct or vector comprising such a polynucleotide is introduced into a host cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source.

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

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

The bacterial host cell may be any *Bacillus* cell including, but not limited to, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *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, for instance, be effected by protoplast transformation (see, *e.g.*, Chang and Cohen, 1979, *Mol. Gen. Genet.* 168: 111-115), by using competent cells (see, *e.g.*, Young and Spizizen, 1961, *J. Bacteriol.* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *J. Mol. Biol.* 56: 209-221), by electroporation (see, *e.g.*, Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or by conjugation (see, *e.g.*, Koehler and Thorne, 1987, *J. Bacteriol.* 169: 5271-5278). The introduction of DNA into an *E. coli* cell may, for instance, be effected by protoplast transformation (see, *e.g.*, Hanahan, 1983, *J. Mol. Biol.* 166: 557-580) or electroporation (see, *e.g.*, Dower *et al.*, 1988, *Nucleic Acids Res.* 16: 6127-6145). The introduction of DNA into a *Streptomyces* cell may, for instance, be effected by protoplast transformation and electroporation (see, *e.g.*, Gong *et al.*, 2004, *Folia Microbiol. (Praha)* 49: 399-405), by conjugation (see, *e.g.*, Mazodier *et al.*, 1989, *J. Bacteriol.* 171: 3583-3585), or by transduction (see, *e.g.*, Burke *et al.*, 2001, *Proc. Natl. Acad. Sci. USA* 98: 6289-6294). The introduction of DNA into a *Pseudomonas* cell may, for instance, be effected by electroporation (see, *e.g.*, Choi *et al.*, 2006, *J. Microbiol. Methods* 64: 391-397) or by conjugation (see, *e.g.*, Pinedo and Smets, 2005, *Appl. Environ. Microbiol.* 71: 51-57). The introduction of DNA into a *Streptococcus* cell may, for instance, be effected by natural competence (see, *e.g.*, Perry and Kuramitsu, 1981, *Infect. Immun.* 32: 1295-1297), by protoplast transformation (see, *e.g.*, Catt and Jollick, 1991, *Microbios* 68: 189-207), by electroporation (see, *e.g.*, Buckley *et al.*, 1999, *Appl. Environ. Microbiol.* 65:

3800-3804) or by conjugation (see, *e.g.*, Clewell, 1981, *Microbiol. Rev.* 45: 409-436). However, any method known in the art for introducing DNA into a host cell can be used.

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*, *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.*, 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*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, or *Trichoderma* cell.

For example, the filamentous fungal host cell may be an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*,

Ceriporiopsis caregiea, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium 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

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

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

In the production methods, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods well known in the art. For example, the cell may

be cultivated by shake flask cultivation, and small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the 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. The polypeptides having cellulolytic enhancing activity are detected using the methods described herein.

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

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

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

Methods for Processing Cellulosic Material

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

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

The present invention also relates to methods for producing a fermentation product, comprising: (a) saccharifying a cellulosic material with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity and a liquor; (b) fermenting the saccharified cellulosic material with one or more (*e.g.*, 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 (*e.g.*, several) fermenting microorganisms, wherein the cellulosic material is saccharified with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity and a liquor. In one aspect, the fermenting of the cellulosic material produces a fermentation product. In another aspect, the method further comprises recovering the fermentation product from the fermentation.

In one aspect, the liquor is recovered following saccharification or fermentation and recycled back to a new saccharification reaction. Recycling of the liquor can be accomplished using processes conventional in the art.

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 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), also sometimes called consolidated bioprocessing (CBP). SHF uses separate process steps to first enzymatically hydrolyze cellulosic material to fermentable sugars, *e.g.,* glucose, cellobiose, cellotriose, and pentose monomers, and then ferment the fermentable sugars to ethanol. In SSF, the enzymatic hydrolysis of cellulosic material and the fermentation of sugars to ethanol are combined in one step (Philippidis, G. P., 1996, Cellulose bioconversion technology, in *Handbook on Bioethanol: Production and Utilization*, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212). SSCF involves the cofermentation of multiple

sugars (Sheehan, J., and Himmel, M., 1999, Enzymes, energy and the environment: A strategic perspective on the U.S. Department of Energy's research and development activities for bioethanol, *Biotechnol. Prog.* 15: 817-827). HHF involves a separate hydrolysis step, and in addition a simultaneous saccharification and hydrolysis step, which can be carried out in the same reactor. The steps in an HHF process can be carried out at different temperatures, *i.e.*, high temperature enzymatic saccharification followed by SSF at a lower temperature that the fermentation strain can tolerate. DMC combines all three processes (enzyme production, hydrolysis, and fermentation) in one or more (*e.g.*, several) steps where the same organism is used to produce the enzymes for conversion of the cellulosic material to fermentable sugars and to convert the fermentable sugars into a final product (Lynd, L. R., Weimer, P. J., van Zyl, W. H., and Pretorius, I. S., 2002, Microbial cellulose utilization: Fundamentals and biotechnology, *Microbiol. Mol. Biol. Reviews* 66: 506-577). It is understood herein that any method known in the art comprising pretreatment, enzymatic hydrolysis (saccharification), fermentation, or a combination thereof, can be used in the practicing the methods of the present invention.

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

Pretreatment. In practicing the methods of the present invention, any pretreatment process known in the art can be used to disrupt plant cell wall components of cellulosic material (Chandra *et al.*, 2007, Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics? *Adv. Biochem. Engin./Biotechnol.* 108: 67-93; Galbe and Zacchi, 2007, Pretreatment of lignocellulosic materials for efficient bioethanol production, *Adv. Biochem. Engin. / Biotechnol.* 108: 41-65; Hendriks and Zeeman, 2009, Pretreatments to enhance the digestibility of lignocellulosic biomass, *Bioresource Technol.* 100: 10-18; Mosier *et al.*, 2005, Features of promising technologies for pretreatment of lignocellulosic

biomass, *Bioresource Technol.* 96: 673-686; Taherzadeh and Karimi, 2008, Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review, *Int. J. of Mol. Sci.* 9: 1621-1651; Yang and Wyman, 2008, Pretreatment: the key to unlocking low-cost cellulosic ethanol, *Biofuels Bioproducts and Biorefining-Biofpr.* 2: 26-40).

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

Conventional pretreatments include, but are not limited to, steam pretreatment (with or without explosion), dilute acid pretreatment, hot water pretreatment, alkaline pretreatment, lime pretreatment, wet oxidation, wet explosion, ammonia fiber explosion, organosolv pretreatment, and biological pretreatment. Additional pretreatments include ammonia percolation, ultrasound, electroporation, microwave, supercritical CO_2, supercritical H_2O, ozone, and gamma irradiation pretreatments.

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

Steam Pretreatment: In steam pretreatment, cellulosic material is heated to disrupt the plant cell wall components, including lignin, hemicellulose, and cellulose to make the cellulose and other fractions, *e.g.*, hemicellulose, accessible to enzymes. Cellulosic material is passed to or through a reaction vessel where steam is injected to increase the temperature to the required temperature and pressure and is retained therein for the desired reaction time. Steam pretreatment is preferably done at 140-230°C, more preferably 160-200°C, and most preferably 170-190°C, where the optimal temperature range depends on any addition of a chemical catalyst. Residence time for the steam pretreatment is preferably 1-15 minutes, more preferably 3-12 minutes, and most preferably 4-10 minutes, where the optimal residence time depends on temperature range and any addition of a chemical catalyst. Steam pretreatment allows for relatively high solids loadings, so that cellulosic material is generally only moist during the pretreatment. The steam pretreatment is often combined with an explosive discharge of the material after the pretreatment, which is known as steam explosion, that is, rapid flashing to atmospheric pressure and turbulent flow of the material to increase the accessible surface area by fragmentation (Duff and Murray, 1996, *Bioresource Technology* 855: 1-33; Galbe and Zacchi, 2002, *Appl. Microbiol. Biotechnol.* 59: 618-628; U.S. Patent Application No. 20020164730). During steam pretreatment, hemicellulose acetyl groups are cleaved and the resulting acid autocatalyzes partial hydrolysis of the hemicellulose to monosaccharides and oligosaccharides. Lignin is removed to only a limited extent.

A catalyst such as H_2SO_4 or SO_2 (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_2SO_4, 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 85-150ºC 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 180-200ºC 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 90-100ºC 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 160-200ºC 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 160-220ºC, and more preferably 165-195ºC, 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 300ºC, preferably about 140 to about 235ºC. 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 cellulolytic enhancing activity and a liquor. The enzyme and protein components of the compositions can be added sequentially.

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

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

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

In one aspect, an effective amount of cellulolytic or hemicellulolytic enzyme protein 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 aspect, an effective amount of a polypeptide having cellulolytic enhancing activity to cellulosic material is about 0.01 to about 50.0 mg, preferably about 0.01 to about 40 mg, more preferably about 0.01 to about 30 mg, more preferably about 0.01 to about 20 mg, more preferably about 0.01 to about 10 mg, more preferably about 0.01 to about 5 mg, more preferably at about 0.025 to about 1.5 mg, more preferably at about 0.05 to about 1.25

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

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

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

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

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

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

"Fermenting microorganism" refers to any microorganism, including bacterial and fungal organisms, suitable for use in a desired fermentation process to produce a fermentation product. The fermenting organism can be C_6 and/or C_5 fermenting organisms, or a combination thereof. Both C_6 and C_5 fermenting organisms are well known in the art. Suitable fermenting microorganisms are able to ferment, *i.e.*, convert, sugars, such as glucose, xylose, xylulose, arabinose, maltose, mannose, galactose, or oligosaccharides, directly or indirectly into the desired fermentation product.

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

Examples of fermenting microorganisms that can ferment C_6 sugars include bacterial and fungal organisms, such as yeast. Preferred yeast includes strains of the *Saccharomyces* spp., preferably *Saccharomyces cerevisiae*.

Examples of fermenting organisms that can ferment C_5 sugars include bacterial and fungal organisms, such as some yeast. Preferred C_5 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*.

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*; *E. coli*, especially *E. coli* strains that have been genetically modified to improve the yield of ethanol; *Clostridium*, such as *Clostridium acetobutylicum*, *Chlostridium thermocellum*, and *Chlostridium phytofermentans*; *Geobacillus sp.*; *Thermoanaerobacter*, such as *Thermoanaerobacter saccharolyticum*; and *Bacillus*, such as *Bacillus coagulans*.

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*, *Clostridium acetobutylicum*, *Clostridium thermocellum*,

Chlostridium phytofermentans, *Geobacillus* sp., *Thermoanaerobacter saccharolyticum*, and *Bacillus coagulans* (Philippidis, 1996, *supra*).

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

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

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

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

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

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

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

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

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

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

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

Fermentation products: A fermentation product can be any substance derived from the fermentation. The fermentation product can be, without limitation, an alcohol (*e.g.*, arabinitol, n-butanol, isobutanol, ethanol, glycerol, methanol, ethylene glycol, 1,3-propanediol [propylene glycol], butanediol, glycerin, sorbitol, and xylitol); an alkane (*e.g.*, pentane, hexane, heptane, octane, nonane, decane, undecane, and dodecane), a cycloalkane (*e.g.*, cyclopentane, cyclohexane, cycloheptane, and cyclooctane), an alkene (*e.g.* pentene, hexene, heptene, and octene); an amino acid (*e.g.*, aspartic acid, glutamic acid, glycine, lysine, serine, and threonine); a gas (*e.g.*, methane, hydrogen (H_2), carbon dioxide (CO_2), and carbon monoxide (CO)); isoprene; a ketone (*e.g.*, acetone); an organic acid (*e.g.*, acetic acid, acetonic acid, adipic acid, ascorbic acid, citric acid, 2,5-diketo-D-gluconic acid, formic acid, fumaric acid, glucaric acid, gluconic acid, glucuronic acid, glutaric acid, 3-hydroxypropionic acid, itaconic acid, lactic acid, malic acid, malonic acid, oxalic acid, oxaloacetic acid, propionic acid, succinic acid, and xylonic acid); and polyketide. 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 n-butanol. In another more preferred aspect, the alcohol is isobutanol. In another more preferred aspect, the alcohol is ethanol. In another more preferred aspect, the alcohol is methanol. In another more preferred aspect, the alcohol is arabinitol. In another more preferred aspect, the alcohol is butanediol. In another more preferred aspect, the alcohol is ethylene glycol. In another more preferred aspect, the alcohol is glycerin. In another more preferred aspect, the alcohol is glycerol. In another more preferred aspect, the alcohol is 1,3-propanediol. In another more preferred aspect, the alcohol is sorbitol. In another more preferred aspect, the alcohol is xylitol. See, for example, Gong, C. S., Cao, N. J., Du, J., and Tsao, G. T., 1999, Ethanol production from renewable resources, in *Advances in Biochemical Engineering/Biotechnology*, Scheper, T., ed., Springer-Verlag Berlin Heidelberg, Germany, 65: 207-241; Silveira, M. M., and Jonas, R., 2002, The biotechnological production of sorbitol, *Appl. Microbiol. Biotechnol.* 59: 400-408; Nigam, P., and Singh, D., 1995, Processes for fermentative production of xylitol – a sugar substitute, *Process Biochemistry* 30 (2): 117-124; Ezeji, T. C., Qureshi, N. and Blaschek, H. P., 2003, Production of acetone, butanol and ethanol by *Clostridium beijerinckii* BA101 and *in situ* recovery by gas stripping, *World Journal of Microbiology and Biotechnology* 19 (6): 595-603.

In another preferred aspect, the fermentation product is an alkane. The alkane can be an unbranched or a branched alkane. In another more preferred aspect, the alkane is pentane. In another more preferred aspect, the alkane is hexane. In another more preferred aspect, the alkane is heptane. In another more preferred aspect, the alkane is octane. In

another more preferred aspect, the alkane is nonane. In another more preferred aspect, the alkane is decane. In another more preferred aspect, the alkane is undecane. In another more preferred aspect, the alkane is dodecane.

In another preferred aspect, the fermentation product is a cycloalkane. In another more preferred aspect, the cycloalkane is cyclopentane. In another more preferred aspect, the cycloalkane is cyclohexane. In another more preferred aspect, the cycloalkane is cycloheptane. In another more preferred aspect, the cycloalkane is cyclooctane.

In another preferred aspect, the fermentation product is an alkene. The alkene can be an unbranched or a branched alkene. In another more preferred aspect, the alkene is pentene. In another more preferred aspect, the alkene is hexene. In another more preferred aspect, the alkene is heptene. In another more preferred aspect, the alkene is octene.

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

In another preferred aspect, the fermentation product is a gas. In another more preferred aspect, the gas is methane. In another more preferred aspect, the gas is H_2. In another more preferred aspect, the gas is CO_2. In another more preferred aspect, the gas is CO. See, for example, Kataoka, N., A. Miya, and K. Kiriyama, 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.

In another preferred aspect, the fermentation product is isoprene.

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 organic acid. In another more preferred aspect, the organic acid is acetic acid. In another more preferred aspect, the organic acid is acetonic acid. In another more preferred aspect, the organic acid is adipic acid. In another more preferred aspect, the organic acid is ascorbic acid. In another more preferred aspect, the organic acid is citric acid. In another more preferred aspect, the organic

acid is 2,5-diketo-D-gluconic acid. In another more preferred aspect, the organic acid is formic acid. In another more preferred aspect, the organic acid is fumaric acid. In another more preferred aspect, the organic acid is glucaric acid. In another more preferred aspect, the organic acid is gluconic acid. In another more preferred aspect, the organic acid is glucuronic acid. In another more preferred aspect, the organic acid is glutaric acid. In another preferred aspect, the organic acid is 3-hydroxypropionic acid. In another more preferred aspect, the organic acid is itaconic acid. In another more preferred aspect, the organic acid is lactic acid. In another more preferred aspect, the organic acid is malic acid. In another more preferred aspect, the organic acid is malonic acid. In another more preferred aspect, the organic acid is oxalic acid. In another more preferred aspect, the organic acid is propionic acid. In another more preferred aspect, the organic acid is succinic acid. In another more preferred aspect, the organic acid is xylonic acid. See, for example, Chen, R., and Lee, Y. Y., 1997, Membrane-mediated extractive fermentation for lactic acid production from cellulosic biomass, *Appl. Biochem. Biotechnol.* 63-65: 435-448.

In another preferred aspect, the fermentation product is polyketide.

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

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

Examples

Media

2X YT plates were composed of 16 g of tryptone, 10 g of yeast extract, 5 g of NaCl, 15 g of Noble agar, and deionized water to 1 liter.

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

MDU2BP medium was composed of 45 g of maltose, 1 g of $MgSO_4 \cdot 7H_2O$, 1 g of NaCl, 2 g of K_2SO_4, 12 g of KH_2PO_4, 7 g of yeast extract, 2 g of urea, 0.5 ml of AMG trace metals solution, and deionized water to 1 liter; pH 5.0.

AMG trace metals solution was composed of 14.3 g of $ZnSO_4 \cdot 7H_2O$, 2.5 g of

$CuSO_4 \cdot 5H_2O$, 0.5 g of $NiCl_2 \cdot 6H_2O$, 13.8 g of $FeSO_4 \cdot 7H_2O$, 8.5 g of $MnSO_4 \cdot 7H_2O$, 3 g of citric acid, and deionized water to 1 liter.

Example 1: Pretreatment of corn stover

Corn stover was pretreated at the U.S. Department of Energy National Renewable Energy Laboratory (NREL) using 1.4% (w/v) sulfuric acid for 8 minutes at 165ºC and 107 psi. The water-insoluble solids in the pretreated corn stover contained 57.5% cellulose, 4.6% hemicelluloses, and 28.4% lignin. Cellulose and hemicellulose were determined by a two-stage sulfuric acid hydrolysis with subsequent analysis of sugars by high performance liquid chromatography using NREL Standard Analytical Procedure #002. Lignin was determined gravimetrically after hydrolyzing the cellulose and hemicellulose fractions with sulfuric acid using NREL Standard Analytical Procedure #003.

The pretreated corn stover was adjusted to pH 5.0 by repeated addition of 10 N NaOH in aliquots of a few milliliters, followed by thorough mixing and incubation at room temperature for approximately 1 hour. The pH was confirmed after overnight incubation at 4°C, and the pH-adjusted corn stover was autoclaved for 20 minutes at approximately 120°C, and then stored at 4°C to minimize the risk of microbial contamination. The dry weight of the pretreated corn stover was 33% TS (total solids), which was confirmed before each use.

The pretreated corn stover was milled prior to use. Milled pretreated corn stover (initial dry weight 32.35% TS) was prepared by milling in a Cosmos ICMG 40 wet multi-utility grinder (EssEmm Corporation, Tamil Nadu, India). Milled pretreated corn stover was also, in some cases, subsequently washed repeatedly with deionized water followed by decanting off the supernatant fraction. The dry weight of the milled, water-washed pretreated corn stover was 7.114% TS.

Alternatively, milled, water-washed pretreated corn stover was washed extensively with water at 50°C. Approximately 600 ml of water washed pretreated corn stover was diluted with approximately 500 ml of distilled, deionized water and incubated at 50°C with shaking for 7 days. Three to four times per day, the diluted pretreated corn stover was permitted to settle, and the supernatant water was decanted and replaced with 500 ml of fresh deionized water. The dry weight of the milled, hot-water washed pretreated corn stover was 6.74% TS.

Example 2: Separation of pretreated corn stover liquor

Acid pretreated corn stover liquor was obtained by vacuum-filtration of the pH-adjusted NREL pretreated corn stover (Example 1) using Whatman #3 filter paper in a Buchner funnel, or through a 0.22 µm STERICUP® sterile vacuum-filter (Millipore, Bedford,

MA, USA). For the Whatman-filtered liquor, the liquor was additionally sterile-filtered using a 0.22 µm STERICUP® sterile vacuum-filter to minimize the risk of microbial contamination.

In later experiments, pretreated corn stover liquor was obtained by squeezing acid-pretreated corn stover in the following manner. Approximately 20 kg of dilute acid pretreated corn stover was loaded into the cotton sheet lining of a SRL Water Press Model BP40-S/S (Zambelli Enotech, Camisano Vicentino, Italy). Municipal water pressure (approximately 35 psi) was applied for 20 minutes, and the resulting liquid pressed out was captured as liquor. This acidic liquor was stored at 4°C, and was subsequently pH-adjusted to 5.0 by addition of 10 N NaOH, and sterile-filtered using a 0.22 µm STERICUP® sterile vacuum-filter.

Example 3: Hydrolysis of cellulose and assay for GH61 polypeptide enhancement thereof

The hydrolysis of pretreated corn stover was conducted using 2.2 ml, 96-deep well plates (Axygen, Union City, CA, USA) containing a total reaction mass of 1 g. The hydrolysis was performed with 5% total solids of either washed milled pretreated corn stover, unwashed pretreated corn stover, equivalent to 28.75 or 14.75 mg of cellulose per ml, respectively, or with a concentration of microcrystalline cellulose (AVICEL®, EM Science, Gibbstown, NJ, USA) equivalent to 28.75 mg of cellulose per ml. Later hydrolysis reactions were performed with milled, washed or milled, unwashed pretreated corn stover with a cellulose content of 59%, equivalent to 29.5 mg of cellulose per ml, or an equivalent concentration of microcrystalline cellulose (AVICEL®, Sigma-Aldrich, St. Louis, MO, USA). Hydrolysis reactions were performed in 50 mM sodium acetate pH 5.0 containing 1 mM manganese sulfate using a *Trichoderma reesei* cellulase preparation (CELLUCLAST® supplemented with *Aspergillus oryzae* beta-glucosidase available from Novozymes A/S, Bagsvaerd, Denmark; the cellulase composition is designated herein in the Examples as "*Trichoderma reesei* cellulase composition") at 4 mg per g of cellulose. *Thermoascus aurantiacus* GH61A or *Thelavia terrestris* GH61E polypeptide having cellulolytic enhancing activity was added at concentrations between 0 and 50% (w/w) of total protein. Pretreated corn stover liquors, enzymatically- or chemically-treated corn stover liquors, synthetic mixtures containing mono- and disaccharides at equivalent concentrations to corn stover liquors, pretreated biomass component liquors, and post-fermentation residual liquors were added between 0 and 20% (v/v) as indicated. Plates were sealed using an ALPS-300™ plate heat sealer (Abgene, Epsom, United Kingdom) and incubated at 50ºC for 0-168 hours with mixing at 150 rpm. All experiments were performed in duplicate or triplicate. Other hydrolysis reactions were performed similarly, with the following differences: plates were sealed using an ALPS-3000™ plate heat sealer (Abgene, Epsom, United Kingdom), and incubated at 50°C with vigorous initial mixing at each sampling time, but mixing was not

continuous.

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

For HPLC analysis, samples were filtered using a 0.45 µm MULTISCREEN® 96-well filter plate (Millipore, Bedford, MA, USA) and filtrates were analyzed for sugar content as described below. The sugar concentrations of samples diluted in 0.005 M H_2SO_4 were measured using a 4.6 x 250 mm AMINEX® HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, USA) by elution with 0.5% w/w benzoic acid-5 mM H_2SO_4 at a flow rate of 0.6 ml per minute at 65°C for 11 minutes, and quantification by integration of glucose and cellobiose signals from refractive index detection (CHEMSTATION®, AGILENT® 1100 HPLC, Agilent Technologies, Santa Clara, CA, USA) calibrated by pure sugar samples. The resultant equivalents were used to calculate the fraction or percentage of cellulose conversion for each reaction. The extent of each hydrolysis was determined as the fraction of total cellulose converted to cellobiose + glucose, and was not corrected for soluble sugars present in pretreated corn stover liquor, or was corrected for soluble sugars present in liquor as indicated.

All HPLC data processing was performed using KALEIDAGRAPH® software (Synergy software, Reading, PA, USA) or MICROSOFT EXCEL® (Microsoft, Seattle, WA, USA). Measured sugar concentrations were adjusted for the appropriate dilution factor. Glucose and cellobiose were chromatographically separated and integrated and their respective concentrations determined independently. To calculate fractional conversion the glucose and cellobiose values were combined. Fractional hydrolysis is reported as the ratio of the mass corrected concentrations of glucose and cellobiose to the initial concentration of cellulose as given by Equation 1. Triplicate data points were averaged and standard deviation was calculated.

$$\text{fractional hydrolysis} = \frac{(([\text{cellobiose}]\ (\text{mg/ml}) \times 1.053) + ([\text{glucose}](\text{mg/ml}))/1.111)}{[\text{cellulose}]\ (\text{mg/ml})} \qquad \text{(Equation 1)}$$

The concentration-dependence of GH61 polypeptide-dependent enhancement of cellulose hydrolysis by the *T. reesei* cellulase composition was determined by titration of the GH61 polypeptide between 0 and 50% (w/w) total protein added to a constant *T. reesei* cellulase concentration of 4 mg per g cellulose, plotting fractional hydrolysis against GH61 polypeptide concentration, and fitting using a modified saturation-binding model as given by Equation 2.

$$\text{fractional hydrolysis} = \frac{\Delta\text{fractional hydrolysis} \times [\text{GH61}] + \text{fractional hydrolysis}_{(0)}\left(K_{\frac{1}{2}^{\text{apparent}}} + [\text{GH61}]\right)}{K_{\frac{1}{2}^{\text{apparent}}} + [\text{GH61}]} \qquad \text{(Equation 2)}$$

In Equation 2 "fractional hydrolysis$_{(0)}$" was the hydrolysis in the absence of a GH61 polypeptide; Δfractional hydrolysis was the total GH61 polypeptide-dependent enhancement; *i.e.*, the difference between the fractional hydrolysis at apparent "saturating" GH61 polypeptide concentration and the fractional hydrolysis in the absence of the GH61 polypeptide; and $K_{1/2\ apparent}$ was the GH61 polypeptide concentration necessary to observe a half-maximal enhancement of hydrolysis.

Example 4: Preparation of *Thermoascus aurantiacus* GH61A polypeptide having cellulolytic enhancing activity

Thermoascus aurantiacus GH61A polypeptide having cellulolytic enhancing activity (SEQ ID NO: 13 [DNA sequence] and SEQ ID NO: 14 [deduced amino acid sequence]) was recombinantly produced in *Aspergillus oryzae* JaL250 according to WO 2005/074656. The recombinantly produced *Thermoascus aurantiacus* GH61A polypeptide was first concentrated by ultrafiltration using a 10 kDa membrane, buffer exchanged into 20 mM Tris-HCl pH 8.0, and then purified using a 100 ml Q-SEPHAROSE® Big Beads column (GE Healthcare, Piscataway, NJ, USA) with a 600 ml 0-600 mM NaCl linear gradient in the same buffer. Fractions of 10 ml were collected and pooled based on SDS-PAGE.

The pooled fractions (90 ml) were then further purified using a 20 ml MONO Q® column (GE Healthcare, Piscataway, NJ, USA) with a 500 ml 0-500 mM NaCl linear gradient in the same buffer. Fractions of 6 ml were collected and pooled based on SDS-PAGE. The pooled fractions (24 ml) were concentrated by ultrafiltration using a 10 kDa membrane, and chromatographed using a 320 ml SUPERDEX® 200 SEC column (GE Healthcare, Piscataway, NJ, USA) with isocratic elution of approximately 1.3 liter of 150 mM NaCl-20 mM Tris-HCl pH 8.0. Fractions of 5 ml were collected and pooled based on SDS-PAGE. Protein concentration was determined using a Microplate BCA™ Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) in which bovine serum albumin was used as a protein standard.

Example 5: Preparation of *Thielavia terrestris* GH61E polypeptide having cellulolytic enhancing activity

Thielavia terrestris GH61E polypeptide having cellulolytic enhancing activity (SEQ ID NO: 7 [DNA sequence] and SEQ ID NO: 8 [deduced amino acid sequence]) was recombinantly produced in *Aspergillus oryzae* JaL250 according to U.S. Patent No. 7,361,495. The *Thielavia terrestris* GH61E polypeptide was desalted and buffer-exchanged into 20 mM sodium acetate-150 mM NaCl pH 5.0 using a HIPREP® 26/10 desalting column according to the manufacturer's instructions. Protein concentration was determined using a

Microplate BCA™ Protein Assay Kit in which bovine serum albumin was used as a protein standard.

Example 6: Preparation of *Aspergillus fumigatus* GH61B polypeptide having cellulolytic enhancing activity

Aspergillus fumigatus GH61B polypeptide having cellulolytic enhancing activity (SEQ ID NO: 29 [DNA sequence] and SEQ ID NO: 30 [deduced amino acid sequence]) was recombinantly produced using *Aspergillus oryzae* JaL355 as a host according to WO 2010/138754. The recombinantly produced *A. fumigatus* GH61B polypeptide was desalted and concentrated into 20 mM Tris pH 8.0 using a 10 kDa MWCO membrane and purified by size exclusion chromatography using SUPERDEX® S75 (GE Healthcare, Piscataway, NJ, USA). The purification buffer was 150 mM NaCl, 20 mM Tris 8.0. Homogeneity was confirmed by SDS-PAGE.

Example 7: Preparation of *Penicillium pinophilum* GH61A polypeptide having cellulolytic enhancing activity

Penicillium pinophilum GH61A polypeptide having cellulolytic enhancing activity (SEQ ID NO: 31 [DNA sequence] and SEQ ID NO: 32 [deduced amino acid sequence]) was recombinantly produced using *Aspergillus oryzae* HowB101 as a host according to WO 2011/005867. The recombinantly produced *P. pinophilum* GH61A polypeptide was desalted and concentrated into 20 mM Tris pH 8.0 using a 10 kDa MWCO membrane and purified by size exclusion chromatography using SUPERDEX® S75. The purification buffer was 150 mM NaCl, 20 mM Tris 8.0. Homogeneity was confirmed by SDS-PAGE.

Example 8: Preparation of *Trichoderma reesei* CEL7B endoglucanase I

Trichoderma reesei CEL7B endoglucanase I (EGI) (SEQ ID NO: 65 [DNA sequence] and SEQ ID NO: 66 [deduced amino acid sequence]) was cloned and expressed in *Aspergillus oryzae* JaL250 as described in WO 2005/067531. Filtered broth was concentrated and buffer exchanged using a tangential flow concentrator equipped with a 10 kDa polyethersulfone membrane with 20 mM Tris-HCl pH 8.5. The sample was loaded onto a Q SEPHAROSE® High Performance column (GE Healthcare, Piscataway, NJ, USA) equilibrated in 20 mM Tris pH 8.5, and bound proteins were eluted with a linear gradient from 0-600 mM sodium chloride. The fractions were concentrated and desalted into 20 mM Tris pH 8.0, 150 mM NaCl using VIVASPIN 20® 10 kDa MWCO centrifugal concentration devices (GE Healthcare UK limited, Little Chalfont, Buckinghamshire, UK). Protein concentration was determined using a Microplate BCA™ Protein Assay Kit in which bovine serum albumin was used as a protein standard.

Example 9: Preparation of *Trichoderma reesei* CEL5A endoglucanase II

The *Trichoderma reesei* RutC30 Cel5A endoglucanase II gene (SEQ ID NO: 67 [DNA sequence] and SEQ ID NO: 68 [deduced amino acid sequence]) was cloned and expressed in *Aspergillus oryzae* as described below.

Two synthetic oligonucleotide primers, shown below, were designed to PCR amplify the endoglucanase II gene from *Trichoderma reesei* RutC30 genomic DNA. Genomic DNA was isolated using a DNEASY® Plant Maxi Kit (QIAGEN Inc., Valencia, CA, USA). An IN-FUSION™ PCR Cloning Kit (BD Biosciences, Palo Alto, CA, USA) was used to clone the fragment directly into pAILo2 (WO 2004/099228).

Forward primer:

5'-ACTGGATTTACC**ATGAACAAGTCCGTGGCTCCATTGCT**-3' (SEQ ID NO: 125)

Reverse primer:

5'-TCACCTCTAGTTAATTAA**CTACTTTCTTGCGAGACACG**-3' (SEQ ID NO: 126)

Bold letters represent coding sequence. The remaining sequence contains sequence identity to insertion sites of pAILo2.

Fifty picomoles of each of the primers above were used in a PCR reaction containing 200 ng of *Trichoderma reesei* genomic DNA, 1X *Pfx* Amplification Buffer (Invitrogen, Carlsbad, CA, USA), 6 µl of 10 mM blend of dATP, dTTP, dGTP, and dCTP, 2.5 units of PLATINUM® *Pfx* DNA polymerase (Invitrogen Corp., Carlsbad, CA, USA), and 1 µl of 50 mM $MgSO_4$ in a final volume of 50 µl. The amplification reaction was incubated in an EPPENDORF® MASTERCYCLER® 5333 (Eppendorf Scientific, Inc., Westbury, NY, USA) programmed for one cycle at 98°C for 2 minutes; and 35 cycles each at 94°C for 30 seconds, 61°C for 30 seconds, and 68°C for 1.5 minutes. After the 35 cycles, the reaction was incubated at 68°C for 10 minutes and then cooled at 10°C. A 1.5 kb PCR reaction product was isolated on a 0.8% GTG® agarose gel (Cambrex Bioproducts, East Rutherford, NJ, USA) using 40 mM Tris base, 20 mM sodium acetate, 1 mM disodium EDTA (TAE) buffer and 0.1 µg of ethidium bromide per ml. The DNA band was visualized with the aid of a DARKREADER™ Transilluminator (Clare Chemical Research, Dolores, CO, USA). The 1.5 kb DNA band was excised with a disposable razor blade and purified using an ULTRAFREE® DA spin cup (Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

Plasmid pAILo2 was linearized by digestion with *Nco* I and *Pac* I. The plasmid fragment was purified by gel electrophoresis and ultrafiltration as described above. Cloning of the purified PCR fragment into the linearized and purified pAILo2 vector was performed using an IN-FUSION™ PCR Cloning Kit. The reaction (20 µl) contained 1X IN-FUSION™ Buffer (BD Biosciences, Palo Alto, CA, USA), 1X BSA, 1 µl of IN-FUSION™ enzyme (diluted

1:10) (BD Biosciences, Palo Alto, CA, USA), 100 ng of pAlLo2 digested with *Nco* I and *Pac* I, and 100 ng of the *Trichoderma reesei* Cel5A endoglucanase II PCR product. The reaction was incubated at room temperature for 30 minutes. A 2 µl sample of the reaction was used to transform *E. coli* XL10 SOLOPACK® Gold cells (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. After a recovery period, two 100 µl aliquots from the transformation reaction were plated onto 150 mm 2X YT plates supplemented with 100 µg of ampicillin per ml. The plates were incubated overnight at 37°C. A set of 3 putative recombinant clones was recovered from the selection plates and plasmid DNA was prepared from each one using a BIOROBOT® 9600 (QIAGEN Inc., Valencia, CA, USA). Clones were analyzed by *Pci* I/*Bsp* LU11 I restriction digestion. One clone with the expected restriction digestion pattern was then sequenced to confirm that there were no mutations in the cloned insert. Clone #3 was selected and designated pAlLo27.

Aspergillus oryzae JaL250 protoplasts were prepared according to the method of Christensen *et al.*, 1988, *supra*. Five micrograms of pAlLo27 (as well as pAlLo2 as a control) were used to transform *Aspergillus oryzae* JaL250 protoplasts. The transformation of *Aspergillus oryzae* JaL250 with pAlLo27 yielded about 50 transformants. Eleven transformants were isolated to individual PDA plates and incubated for five days at 34ºC.

Confluent spore plates were washed with 3 ml of 0.01% TWEEN® 80 (a nonionic surfactant and emulsifier derived from polyethoxylated sorbitan and oleic acid) and the spore suspension was used to inoculate 25 ml of MDU2BP medium in 125 ml glass shake flasks. Transformant cultures were incubated at 34ºC with constant shaking at 200 rpm. At day five post-inoculation, cultures were centrifuged at 6000 *x g* and their supernatants collected. Five microliters of each supernatant were mixed with an equal volume of 2X loading buffer (10% beta-mercaptoethanol) and loaded onto a 1.5 mm 8%-16% Tris-glycine SDS-PAGE gel and stained with SIMPLYBLUE™ SafeStain (Invitrogen Corp., Carlsbad, CA, USA). SDS-PAGE profiles of the culture broths showed that ten out of eleven transformants produced a new protein band of approximately 45 kDa. Transformant number 1, designated *Aspergillus oryzae* JaL250AlLo27, was cultivated in a fermentor.

One hundred ml of shake flask medium were added to a 500 ml shake flask. The shake flask medium was composed per liter of 50 g of sucrose, 10 g of KH_2PO_4, 0.5 g of $CaCl_2$, 2 g of $MgSO_4 \cdot 7H_2O$, 2 g of K_2SO_4, 2 g of urea, 10 g of yeast extract, 2 g of citric acid, and 0.5 ml of trace metals solution. The trace metals solution was composed per liter of 13.8 g of $FeSO_4 \cdot 7H_2O$, 14.3 g of $ZnSO_4 \cdot 7H_2O$, 8.5 g of $MnSO_4 \cdot H_2O$, 2.5 g of $CuSO_4 \cdot 5H_2O$, and 3 g of citric acid. The shake flask was inoculated with two plugs of *Aspergillus oryzae* JaL250AlLo27 from a PDA plate and incubated at 34ºC on an orbital shaker at 200 rpm for 24 hours. Fifty ml of the shake flask broth was used to inoculate a 3 liter fermentation vessel.

A total of 1.8 liters of the fermentation batch medium was added to a three liter glass

jacketed fermentor (Applikon Biotechnology, Schiedam, Netherlands). The fermentation batch medium was composed per liter of 10 g of yeast extract, 24 g of sucrose, 5 g of $(NH_4)_2SO_4$, 2 g of KH_2PO_4, 0.5 g of $CaCl_2 \cdot 2H_2O$, 2 g of $MgSO_4 \cdot 7H_2O$, 1 g of citric acid, 2 g of K_2SO_4, 0.5 ml of anti-foam, and 0.5 ml of trace metals solution. The trace metals solution was composed per liter of 13.8 g of $FeSO_4 \cdot 7H_2O$, 14.3 g of $ZnSO_4 \cdot 7H_2O$, 8.5 g of $MnSO_4 \cdot H_2O$, 2.5 g of $CuSO_4 \cdot 5H_2O$, and 3 g of citric acid. Fermentation feed medium was composed of maltose, which was. dosed at a rate of 0 to 4.4 g/l/hr for a period of 185 hours. The fermentation vessel was maintained at a temperature of 34ºC and pH was controlled using an Applikon 1030 control system (Applikon Biotechnology, Schiedam, Netherlands) to a set-point of 6.1 +/- 0.1. Air was added to the vessel at a rate of 1 vvm and the broth was agitated by a Rushton impeller rotating at 1100 to 1300 rpm. At the end of the fermentation, whole broth was harvested from the vessel and centrifuged at 3000 *x g* to remove the biomass. The supernatant was sterile filtered and stored at 5 to 10ºC.

The supernatant was desalted and buffer-exchanged into 20 mM Bis-Tris pH 6.0 using a HIPREP® 26/10 desalting column (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions. The buffer exchanged sample was loaded onto a MonoQ® column (GE Healthcare, Piscataway, NJ, USA) equilibrated with 20 mM Bis-Tris pH 6.0, and the bound protein was eluted with a linear gradient from 0 to 1000 mM sodium chloride. Protein fractions were pooled and buffer exchanged into 1.2 M $(NH_4)_2SO_4$-20 mM Tris-HCl pH 8.5. The sample was loaded onto a Phenyl SUPEROSE™ column (HR 16/10) equilibrated with 1.2 M $(NH_4)_2SO_4$-20 mM Tris-HCl pH 8.0. Bound proteins were eluted with a linear gradient over 20 column volumes from 1.2 to 0 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl pH 8.5. The fractions were pooled, concentrated, and loaded onto a SUPERDEX® 75 HR 26/60 column (GE Healthcare, Piscataway, NJ, USA) equilibrated with 20 mM Tris-150 mM sodium chloride pH 8.5. Fractions were pooled and concentrated in 20 mM Tris-150 mM sodium chloride pH 8.5. Protein concentration was determined using a Microplate BCA™ Protein Assay Kit in which bovine serum albumin was used as a protein standard.

Example 10: Preparation of *Trichoderma reesei* CEL7A cellobiohydrolase I

Trichoderma reesei CEL7A cellobiohydrolase I (SEQ ID NO: 95 [DNA sequence] and SEQ ID NO: 96 [deduced amino acid sequence]) was prepared as described by Ding and Xu, 2004, "Productive cellulase adsorption on cellulose" in Lignocellulose Biodegradation (Saha, B. C. ed.), Symposium Series 889, pp.154-169, American Chemical Society, Washington, DC. Protein concentration was determined using a Microplate BCA™ Protein Assay Kit in which bovine serum albumin was used as a protein standard.

Example 11: Preparation of *Trichoderma reesei* CEL6A cellobiohydrolase II

The *Trichoderma reesei* RutC30 CEL6A cellobiohydrolase II gene (SEQ ID NO: 97 [DNA sequence] and SEQ ID NO: 98 [deduced amino acid sequence]) was isolated from *Trichoderma reesei* RutC30 as described in WO 2005/056772. The *Trichoderma reesei* CEL6A cellobiohydrolase II gene was expressed in *Fusarium venenatum* using pEJG61 as an expression vector according to the procedures described in U.S. Published Application No. 20060156437. Fermentation was performed as described in U.S. Published Application No. 20060156437. Filtered broth was desalted and buffer-exchanged into 20 mM sodium acetate-150 mM NaCl pH 5.0 using a HIPREP® 26/10 Desalting Column according to the manufacturer's instructions. Protein concentration was determined using a Microplate BCA™ Protein Assay Kit in which bovine serum albumin was used as a protein standard.

Example 12: Preparation of *Aspergillus oryzae* CEL3A beta-glucosidase

Aspergillus oryzae CEL3A beta-glucosidase (SEQ ID NO: 111 [DNA sequence] and SEQ ID NO: 112 [deduced amino acid sequence]) was recombinantly prepared as described in WO 2004/099228, and purified as described by Langston *et al.*, 2006, *Biochim. Biophys. Acta Proteins Proteomics* 1764: 972-978. Protein concentration was determined using a Microplate BCA™ Protein Assay Kit.

Example 13: Effect of GH61 polypeptides having cellulolytic enhancing activity on hydrolysis of microcrystalline cellulose or PCS by the *Trichoderma reesei* cellulase composition

The effect of the *Thermoascus aurantiacus* GH61A polypeptide on the hydrolysis of AVICEL® or milled washed PCS by the *Trichoderma reesei* cellulase composition was determined using the same experimental conditions and procedures according to Example 3. In general, in experiments performed in subsequent examples, a control reaction was included in which increasing concentrations of GH61 polypeptide were added to the hydrolysis of either AVICEL® or pretreated corn stover with the *T. reesei* cellulase composition in the absence of other liquors, compounds, or variously treated liquors.

The presence of the *T. aurantiacus* GH61A polypeptide did not enhance the hydrolysis of AVICEL® by the *T. reesei* cellulase composition. Conversion of AVICEL® was 0.119 ± 0.00251 and 0.339 ± 0.00222 at 1 and 3 days, respectively, in the absence of the *T. aurantiacus* GH61A polypeptide compared to 0.112 ± 0.00376 and 0.333 ± 0.00328 at 1 and 3, respectively, in the presence of 24% (w/w) of the *T. aurantiacus* GH61A polypeptide.

The presence of the *T. aurantiacus* GH61A polypeptide enhanced the hydrolysis of milled washed PCS by the *T. reesei* cellulase composition. Conversion of milled washed PCS was 0.249 ± 0.00104 and 0.545 ± 0.00656 at 1 and 3 days, respectively, in the

presence of the *T. aurantiacus* GH61A polypeptide compared to 0.222 ± 0.00464 and 0.412 ± 0.0237 at 1 and 3 days, respectively, in the absence of the *T. aurantiacus* GH61A polypeptide.

The presence of the *T. aurantiacus* GH61A polypeptide marginally enhanced the hydrolysis of milled hot-washed PCS by the *T. reesei* cellulase composition. Conversion of hot washed PCS was 0.315 ± 0.00267 and 0.383 ± 0.00498, at 1 and 3 days, respectively, in the absence of the *T. aurantiacus* GH61A polypeptide compared to 0.331 ± 0.0115 and 0.409 ± 0.0145 at 1 and 3, respectively, in the presence of 8% (w/w) of the *T. aurantiacus* GH61A polypeptide.

The presence of the *Thelavia terrestris* GH61E polypeptide did not enhance the hydrolysis of AVICEL® by the *T. reesei* cellulase composition. Conversion of AVICEL® was 0.122 ± 0.00426, 0.242 ± 0.00813 and 0.315 ± 0.00814, at 1, 3, and 5 days, respectively, in the absence of the *T. terrestris* GH61E polypeptide compared with 0.121 ± 0.000824, 0.228 ± 0.000978 and 0.307 ± 0.00348 at 1, 3, and 5 days respectively, in the presence of 24% (w/w) of the *T. terrestris* GH61E polypeptide.

The presence of the *Aspergillus fumigatus* GH61B polypeptide did not significantly enhance the hydrolysis of AVICEL® by the *T. reesei* cellulase composition. Conversion of AVICEL® was 0.150 ± 0.009, 0.31 ± 0.001, and 0.48 ± 0.001 at 1, 3, and 7 days, respectively, in the absence of the *A. fumigatus* GH61B polypeptide compared to 0.148 ± 0.002%, 0.311 ± 0.001%, and 0.54 ± 0.02% at 1, 3, and 7 days, respectively, in the presence of the *A. fumigatus* GH61B polypeptide.

Example 14: Effect of *Thermoascus aurantiacus* GH61A polypeptide on cellulolysis of unwashed and washed acid-pretreated corn stover

The effect of *Thermoascus aurantiacus* GH61A polypeptide on hydrolysis of both milled washed and milled unwashed NREL pretreated corn stover by the *Trichoderma reesei* cellulase composition were assayed for comparison. To an equivalent, fixed concentration of the *T. reesei* cellulase composition at 4 mg per gram of cellulose, increasing concentrations of *T. aurantiacus* GH61A polypeptide between 0 and 24% (w/w) were added to equivalent dry masses of milled unwashed pretreated corn stover or milled washed pretreated corn stover, and the fractional hydrolysis was assayed according to Example 3.

Figure 1 shows the fractional hydrolysis of variously washed pretreated corn stover substrates. The total enhancement in total conversion from the *T. aurantiacus* GH61A polypeptide was larger in magnitude and was apparent at earlier stages of hydrolysis for unwashed corn stover in comparison to washed pretreated corn stover. Over the range of concentrations tested, there was no GH61 polypeptide concentration-dependence of

hydrolysis after 1 day of hydrolysis for washed milled pretreated corn stover (open squares), whereas a slight but significant increase of hydrolysis as GH61 polypeptide concentration increased was observed for unwashed milled corn stover (open circles). After 3 days of hydrolysis, washed pretreated corn stover showed a sharp, saturable enhancement of hydrolysis with GH61 polypeptide addition, well-fitted by a square hyperbolic binding function (closed squares). Conversely, there was a linear increase in hydrolysis with GH61 polypeptide concentration for the unwashed pretreated corn stover that extrapolated to a higher overall conversion at GH61 polypeptide concentrations greater than 50% (w/w) (closed circles). For hot-water washed pretreated corn stover prepared according to Example 1, this trend was even more apparent; after 3 days of hydrolysis, no GH61 polypeptide enhancement was observed (open triangles), and GH61 polypeptide-dependent enhancement of cellulolysis was not apparent until 5 days of hydrolysis (closed triangles). The total magnitude of GH61 polypeptide-dependent enhancement for hot-water washed pretreated corn stover was only 5% at 7 days, whereas for the GH61 polypeptide enhancement for unwashed pretreated corn stover was at least 15% at 7 days of hydrolysis (data not shown). In each case, the conversion by the *T. reesei* cellulase composition in the absence of the GH61 polypeptide (y-intercept) was higher for the more extensively washed substrates, indicating the removal of soluble cellulase inhibitors by washing.

Example 15: The effect of addition of acid-pretreated corn stover liquor to washed milled pretreated corn stover

Acid-pretreated corn stover liquor, fractionated according to Example 2, was added at concentrations between 0 and 15% (v/v) to milled, water-washed acid-pretreated corn stover and hydrolyzed by 4 mg of the *Trichoderma reesei* cellulase composition per g cellulose plus increasing concentrations of the *Thermoascus aurantiacus* GH61A polypeptide according to Example 3.

Figure 2A shows the extent of hydrolysis for the various additions of the *T. aurantiacus* GH61A polypeptide and NREL acid-pretreated corn stover liquor to milled, water-washed acid-pretreated corn stover at 1 day (white bars) and 3 days (gray bars) of hydrolysis.

Figure 2B shows a replot of the data presented in Figure 2A with non-linear least square fits to Equation 2 according to Example 3 or with linear least square fits. The Figure demonstrates that the functional dependence of the extent of hydrolysis was square hyperbolic, *i.e.*, saturating, for concentrations of liquor <10% (v/v), and became linear or exponential at concentrations of liquor ≥10% (v/v) at 3 days of hydrolysis. The extent of hydrolysis at the highest added concentration of the *T. aurantiacus* GH61A polypeptide was greater than that observed in the absence of liquor, and extrapolation of the trends to predict

fractional hydrolysis levels at higher GH61 polypeptide concentrations indicated a greater conversion at higher liquor concentrations. From fits of Equation 2, the total enhancement from the GH61 polypeptide, Δfractional hydrolysis, was 0.150±0.000550 in the absence of added liquor, 0.166±0.0155 with 2% liquor, 0.227±0.0827 with 5% liquor, and 0.417±0.240 with 10% liquor, and the increase was linear with GH61 polypeptide concentration at 15% liquor.

Example 16: Effect of addition of acid-pretreated corn stover liquor on *Thermoascus aurantiacus* GH61A polypeptide during hydrolysis of microcrystalline cellulose

Pretreated corn stover liquor, fractionated according to Example 2, was added to a saccharification reaction of microcrystalline cellulose by the *Trichoderma reesei* cellulase composition according to Example 3 at concentrations between 0 and 20% (v/v). The *Thermoascus aurantiacus* GH61A polypeptide was titrated between 0 and 24% (w/w) of total protein.

Figure 3 shows that the *T. aurantiacus* GH61A polypeptide did not enhance on hydrolysis of microcrystalline cellulose by the *T. reesei* cellulase composition in the absence of liquor. However, in the presence of NREL pretreated corn stover liquor, the *T. aurantiacus* GH61A polypeptide enhanced cellulolysis. Figure 3A shows fractional hydrolysis at various GH61 polypeptide concentrations for increasing concentrations of NREL pretreated corn stover liquor at 1 day (open circles) and 3 days of hydrolysis (closed circles). As pretreated corn stover liquor was added from 0% v/v (circles) to 5% (diamonds), 10% (triangles), and 15% (inverted triangles), in the absence of the *T. aurantiacus* GH61A polypeptide, there was increasing inhibition of the *T. reesei* cellulase composition, as was apparent from the reduction in fractional hydrolysis. As the concentration of the *T. aurantiacus* GH61A polypeptide was increased, for those samples containing acid-pretreated corn stover liquor, the extent of hydrolysis increased to an extent beyond the level observed for hydrolysis in the absence of liquor. These data were corrected for saccharides present in the added liquor, and the possibility that either the *T. aurantiacus* GH61A polypeptide or the *T. reesei* cellulase composition in combination with the *T. aurantiacus* GH61A polypeptide were converting some substrate in pretreated corn stover liquor to glucose was unlikely, as the extent of enhancement from the *T. aurantiacus* GH61A polypeptide at 5% liquor addition would correspond to a conversion of 55 g/L glucose equivalents from the added liquor.

Figure 3B shows the results of addition of a synthetic mixture of the major sugar components of pretreated corn stover liquor; glucose, cellobiose, xylose, and arabinose with or without added phenol to mimic the phenolic lignin degradation compounds present in pretreated corn stover liquor, at the concentrations present in actual liquor. The synthetic

sugar mixture was added between 0% and 15%, (symbols as in Figure 3A), and the synthetic sugar mixture containing phenol was added at either 5% (squares) or 15% (right triangles). For this example, added liquor sugar concentrations were not subtracted from the overall apparent hydrolysis, as was evident from the upward shift in apparent hydrolysis with increasing liquor addition. From Figure 3B, fractional hydrolysis was independent of GH61 polypeptide concentration, thus there was no apparent GH61 polypeptide cellulolytic enhancing activity in the presence of the synthetic liquor mixtures on microcrystalline cellulose. These data indicated that the GH61 polypeptide cellulolytic enhancing activity derives from the minor components of NREL pretreated corn stover liquor.

Example 17: Effect of preconditioning acid-pretreated corn stover liquor with *Thelavia terrestris* GH61E polypeptide

Pretreated corn stover liquor, extracted according to Example 2, was added to a saccharification reaction of microcrystalline cellulose by the *Trichoderma reesei* cellulase composition according to Example 3 at concentrations between 0 and 20% (v/v). Alternatively, 10 ml of liquor were incubated at 50°C overnight with 0.43 mg protein of the *T. reesei* cellulase composition per ml, or with 20 µg protein of the *Thielavia terrestris* GH61E polypeptide per ml, or both (pre-conditioned liquor). The enzyme was removed from these samples using a 3 kDa MWCO AMICON® centrifuge filter (Millipore, Bedford, MA, USA), and the filtered flow-through liquor was added to saccharifications of microcrystalline cellulose at concentrations between 0 and 20% (v/v). The *T. terrestris* GH61E polypeptide was titrated between concentrations of 0 and 24% (w/w) of total protein.

Figure 4 shows that, like the *T. aurantiacus* GH61A polypeptide demonstrated in Example 11, the *Thelavia terrestris* GH61E polypeptide had cellulolytic enhancing activity on microcrystalline cellulose in the presence of NREL pretreated corn stover liquor and did not enhance cellulolysis in the absence of liquor. Figure 4 (all panels, circles) shows that increasing *T. terrestris* GH61E polypeptide concentration on microcrystalline cellulose in the absence of liquor did not enhance hydrolysis. As liquor was added from 0% (v/v) (circles) to 5% (squares), 10% (diamonds), and 15% (triangles) in the absence of the *T. terrestris* GH61E polypeptide, there was increasing inhibition of the *T. reesei* cellulase composition, as was apparent from the reduction in fractional hydrolysis (all panels, y-intercepts). As the *T. terrestris* GH61E polypeptide concentration was increased, for those samples containing either liquor or enzymatically pre-conditioned liquor, the extent of hydrolysis increased. In most cases, despite the inhibition of cellulolysis arising from the liquor addition, the increase in hydrolysis led to an extent of saccharification beyond the level observed for hydrolysis in the absence of liquor at high *Thelavia terrestris* GH61E polypeptide concentrations. Figure 4A shows the effects of liquor that had not been enzymatically treated, Figure 4B shows the

effects of liquor that had been saccharified using the *T. reesei* cellulase composition, Figure 4C shows the effects of liquor that had been incubated with both the *T. reesei* cellulase composition and the *T. terrestris* GH61E polypeptide. In each case, GH61 polypeptide cellulolytic enhancing activity was apparent in the presence of pretreated corn stover liquor. Control reactions containing identical concentrations of liquor or pretreated liquor and enzyme in identical concentrations to those present in the cellulose-containing samples were performed in parallel, and sugars produced from these control reactions were subtracted from the saccharification totals. In each case the conversion of pretreated corn stover liquor to glucose equivalents produced minimal total sugar, and far less than was necessary to account for the large cellulolytic enhancement observed in saccharifications containing both the *Thielavia terrestris* GH61E polypeptide and liquor.

Example 18: Effect of acid-pretreated corn stover liquor or steam-pretreated corn stover liquor on *Thelavia terrestris* GH61E polypeptide activity

Pretreated corn stover liquor was extracted according to Example 2 with the following exceptions: 0.6 kg of steam explosion-pretreated corn stover or NREL acid-pretreated corn stover were suspended in 900 ml of deionized water and mixed for 2 hours. The solids were filtered using filter paper and sterile filtered with a 0.45 µm filter. Two hundred ml of each was ultracentrifuged using a 44.5 mM diameter 1 kDa MWCO centrifuge filter (Millipore, Bedford, MA, USA). The concentrated retentate was restored to the original volume by addition of water. The original liquors and the molecular weight separated fractions of each were added to saccharification reactions of microcrystalline cellulose with the *Trichoderma reesei* cellulase composition according to Example 3 at concentrations of 5% and 15% (v/v). The *Thelavia terrestris* GH61E polypeptide was titrated at concentrations between 0 and 24% (w/w) total protein.

Figure 5A shows that increasing concentrations of the *Thelavia terrestris* GH61E polypeptide increased the hydrolysis of microcrystalline cellulose by the *T. reesei* cellulase composition in the presence of the acid-pretreated corn stover liquor. Thus in the presence of the acid-pretreated corn stover liquor, the *T. terrestris* GH61E polypeptide significantly enhanced cellulolysis. Figure 5B shows that increasing concentrations of the *Thelavia terrestris* GH61E polypeptide marginally increased hydrolysis in the presence of steam explosion-pretreated corn stover, thus demonstrating minimal enhancement of cellulolysis by *Thelavia terrestris* GH61E under similar conditions.

Overall, the results indicated that conditions of pretreatment are critical for the production of liquor components necessary for GH61 cellulolytic enhancing activity on microcrystalline cellulose. More severe pretreatments, exemplified herein by the acid-pretreated corn stover (*e.g.*, NREL PCS), generate soluble components or higher

concentrations of the soluble components than do milder pretreatments, as illustrated by steam-pretreated corn stover above.

Example 19: Separation of GH61 polypeptide-enhancing liquor components from non-enhancing components and inhibitory components in NREL pretreated corn stover liquor

Pretreated corn stover liquor was extracted and separated by ultrafiltration into nominal molecular weight fractions above and below 1 kDa according to Example 18. The molecular weight separated fractions were added to a saccharification reaction of microcrystalline cellulose with the *T. reesei* cellulase composition at concentrations between 0 and 15% (v/v) according to Example 3. The *Thelavia terrestris* GH61E polypeptide was titrated at concentrations between 0 and 24% (w/w) total protein.

As shown in Figure 5A, at zero *T. terrestris* GH61E polypeptide concentration, both the whole liquor and the lower molecular weight fractions showed inhibition at higher liquor concentrations (circles, squares). The inhibition from liquor has been discussed previously in Examples 10, 12 and 13. Conversely, there was no difference in hydrolysis at either 5 or 15% (v/v) of the higher molecular weight fraction (triangles). Increasing concentrations of the *T. terrestris* GH61E polypeptide with all these liquor molecular weight fractions increased cellulose hydrolysis. The unfractionated pretreated corn stover liquor yielded the highest GH61 polypeptide-dependent enhancement, however the high molecular weight pretreated corn stover liquor fraction also yielded substantial GH61 polypeptide-dependent enhancement.

The efficacy of molecular weight-based filtration was confirmed by fractionation of PCS liquor using AMICON® 30, 10, and 3 kDa MWCO centrifuge filters (Millipore, Bedford, MA, USA) and assaying for GH61 cellulolytic enhancing activity. NREL acid-pretreated corn stover liquor was extracted according to Example 2, and then filtered through successively smaller MWCO filters. The retentates were repeatedly washed with 3 to 5 volumes of water. The retentates for each molecular weight filter were assayed for GH61 cellulolytic enhancing activity according to Example 3 with the following exceptions: 5% (v/v) of each retentate was added to saccharification reactions of microcrystalline cellulose (AVICEL®), and the extent of hydrolysis was determined at 1 and 6 days of saccharification. Figure 6 shows the fractional hydrolysis of AVICEL® by the *T. reesei* cellulase composition with added *T. aurantiacus* GH61A polypeptide as indicated, in the presence of the filter rententates indicated. Figure 6 shows that the GH61 polypeptide-enhancing factor was not retained, or was only marginally retained by a 3 kDa filter. Increasing concentrations of GH61 polypeptide increased the hydrolysis of AVICEL® in the presence of flow-through from the 3 kDa MWCO filter, whereas a decrease, or no change in hydrolysis with GH61 polypeptide

concentration was observed when incubated in the presence of retentates from molecular weight filters of 3 kDa and greater. These data, combined with the data presented in Figure 5A, indicated that the nominal molecular weight of the compounds facilitating GH61 polypeptide cellulolytic enhancing activity may lie between 1 and 3 kDa, and that molecular weight separation of the NREL acid-pretreated corn stover liquor can separate enhancing from inhibitory factors.

Example 20: Effect of *Thermoascus aurantiacus* GH61 polypeptide and pretreated corn stover liquor enhances cellulolytic activity of individual cellulases on AVICEL®

Individual monocomponent cellulases Cel5A endoglucanase II, Cel6A cellobiohydrolase, Cel7A cellobiohydrolase, and Cel7B endoglucanase I from *Trichoderma reesei* and beta-glucosidase from *Aspergillus oryzae* were assayed for enhancement of their ability to hydrolyze microcrystalline cellulose when incubated with the *Thermoascus aurantiacus* GH61A polypeptide with or without 5% NREL acid-pretreated corn stover liquor. AVICEL® hydrolyses were performed according to Example 3, with the following exceptions. Instead of the *Trichoderma reesei* cellulase composition, purified *T. reesei* monocomponents and purified *Aspergillus oryzae* beta-glucosidase were used at a concentration of 10 mg of enzyme protein per g cellulose. Alternatively, mixtures of monocomponent cellulases were used, with each cellulase dosed at 10 mg of enzyme protein per g cellulose. Saccharification reactions were performed with or without 1 mg of the *T. aurantiacus* GH61A polypeptide per g cellulose and with or without 5% NREL pretreated corn stover liquor at 50°C for 7 days.

Figure 7A shows the fractional hydrolysis achieved by each monocomponent and monocomponent mixture at 3, 5, and 7 days of hydrolysis, with and without the *T. aurantiacus* GH61A polypeptide in the presence of acid-pretreated corn stover liquor. In each case, in the absence of liquor, no GH61 polypeptide-dependent enhancement was observed (data not shown). Conversely, when acid-pretreated corn stover liquor was present, the *T. aurantiacus* GH61A polypeptide enhanced the cellulase activity of each monocomponent or monocomponent mixture by 5% or more at 7 days of hydrolysis.

Figure 7B shows the enhancement of cellulase activity for each monocomponent arising from the *T. aurantiacus* GH61A polypeptide in the presence of NREL acid-pretreated corn stover liquor at 3, 5, and 7 days of hydrolysis, which is given by the ratio of fractional hydrolysis in the presence to the absence of the GH61 polypeptide:

$$GH61\ effect = \frac{\%\ \text{conversion}_{(+\ GH61+\ liquor)}}{\%\ \text{conversion}_{(no\ GH61+\ liquor)}} \qquad \text{(Equation 3)}$$

Enhancement of hydrolysis by the GH61 polypeptide yields a ratio >1; inhibition of hydrolysis yields a ratio <1, and no effect on hydrolysis yields a ratio = 1

In each case at 5 and 7 days of hydrolysis, every monocomponent and every

monocomponent mixture had enhanced cellulolytic activity, indicated by a *GH61 effect* > 1. The mixtures containing 3 to 5 cellulase components had the highest overall cellulase concentration in the reactions, thus had the highest fractional hydrolysis, and the lowest apparent *GH61 effect*. Despite this, the *T. aurantiacus* GH61A polypeptide still provided an enhancement of 1.05 ± 0.00604 for the 5-component cellulose mixture (Cel5A endoglucanase II, Cel6A cellobiohydrolase, Cel7A cellobiohydrolase, and Cel7B endoglucanase I from *T. reesei* and beta-glucosidase from *Aspergillus oryzae*) at 7 days of hydrolysis. The largest relative enhancements by the *T. aurantiacus* GH61A polypeptide to AVICEL® saccharification by cellulose monocomponents or mixtures thereof were observed for GH61 polypeptide enhancement of *T. reesei* Cel7A cellobiohydrolase (1.33 ± 0.127), or mixtures containing the *T. reesei* Cel7A cellobiohydrolase and *T. reesei* Cel5A endoglucanase II (1.33 ± 0.00719).

Example 21: Enrichment of NREL acid-pretreated corn stover liquor components

NREL acid-pretreated corn stover liquor was fractionated by adsorption to microcrystalline cellulose as described below. NREL acid-pretreated corn stover liquor (1.2 liters) was incubated with 10 g of AVICEL® overnight at room temperature. The supernatant fraction was removed by vacuum filtration through Whatman #3 filter paper. The AVICEL® and adsorbed liquor components were washed 6 times with 500 ml of acetonitrile (occasionally acetone was used), followed by elution with 2 liters of water in 300-400 ml fractions. The liquor components that in combination with a GH61 polypeptide demonstrated a GH61 polypeptide-dependent enhancement of cellulolysis by the *Trichoderma reesei* cellulase composition were largely eluted in the first 2 water elution fractions (Figure 8B), though later elution fractions contained some small amount of residual liquor. Water-eluted liquor components were concentrated approximately 10-fold using a Macrosep 1kD Omega centrifuge filter (Pall Corporation, East Hills, NY, USA) and applied to an 8.0 mm x 300 mm Shodex Sugar SP0810 HPLC chromatography column (Showa Denka America, Inc., NY, USA) using an AGILENT® 1100 HPLC and CHEMSTATION® software (Agilent Technologies, Santa Clara, CA, USA) and separated by isocratic elution with water at a flow rate of 0.5 ml per minute at 80°C for 50 minutes, and collecting 250 µl fractions in 2.2 ml, 96-deep well plates (Axygen, Union City, CA, USA). Repeated 50 µl injections of the liquor followed by repeated fraction collection yielded approximately 8 ml of each fraction. Peaks were identified by diode array detection at 210, 280 and 340 nm using a CHEMSTATION®, AGILENT® 1100 HPLC (Agilent Technologies, Santa Clara, CA, USA) (Figure 8, dashed lines).

Peak fractions were assayed according to Example 3 with the following exceptions.

400 µl of each fraction were added to the hydrolysis reactions containing 4 mg of the *T. reesei* cellulase composition and 1 mg of *Thermoascus aurantiacus* GH61A polypeptide. Aliquots were removed and analyzed for sugar content at 1, 3, and 10 days of saccharification. Control reactions containing the original NREL acid-pretreated corn stover liquor and control reactions containing no liquor and no GH61 polypeptide were run in parallel. Figure 8B shows that addition of the various fractions resulted in fluctuation between fractions around a mean fractional hydrolysis value of 0.617 (Figure 8, solid lines). Several peaks of activity consisting of multiple sequential fractions with high cellulolytic activity in the presence of the *T. aurantiacus* GH61A polypeptide were observed, including one broad peak of activity centered at fraction 7C. This peak corresponded with a peak in $A_{(280)}$. The extent of the cellulolytic enhancement by the fraction appeared small, indicating that the amount fractionated was very small. Absorbance at 280 nm was consistent with contents of the fractions possessing aromatic characteristics.

Example 22: Effect of NREL pretreated corn stover liquor fractions and the *Thermoascus aurantiacus* GH61A polypeptide on hydrolysis of microcrystalline cellulose by the *Trichoderma reesei* cellulase composition

Pooled, lyophilized fractions of the NREL acid-pretreated corn stover liquor were each added to approximately 25 mg per ml of AVICEL® in 700 µl of 50 mM sodium acetate pH 5.0 in the presence of 3 mM calcium chloride with 37.5 µg of the *Thermoascus aurantiacus* GH61A polypeptide per ml and were incubated in 1.7 ml microcentrifuge tubes at 50°C with shaking at 1500 rpm for 48 hours in a Thermomixer (Eppendorf, Hamburg, Germany). Following the incubation, the samples were centrifuged at 31,000 rpm in a microcentrifuge for 5 minutes. A series of AVICEL® masses were weighed out and suspended in 700 µl of equivalent buffer, incubated for an equivalent length of time, and pelleted equivalently. The height of the AVICEL® was measured using a transparent ruler, and assuming the microcentrifuge tubes were roughly equivalent in volume, the volume of the conical portion of the tube was given by the following equation:

$$V = \frac{1}{3} \pi r^2 h \qquad \text{(Equation 4)}$$

Thus the volume of the AVICEL® scales proportionally with the height of the pellet, and a measurement of pellet height could therefore be used to approximate the volume.

Figure 9 shows a standard curve of AVICEL® height vs. mass of AVICEL®. The measurement of the pellet heights for various masses of AVICEL® scaled linearly within a volume region spanning the conical portion of the tube.

Figure 10 shows the height of the AVICEL® for several of the pooled NREL acid-pretreated corn stover liquor HPLC fractions incubated with the *T. aurantiacus* GH61A

polypeptide. One of these pooled fractions (denoted 6H-7A) incubated with the *T. aurantiacus* GH61A polypeptide was notably higher, 9 mM compared with an average of all other samples of 5.2 mM, a 1.7-fold larger volume. The AVICEL® in this sample had swollen to a volume equivalent to an AVICEL® mass of approximately 90 mg. Pooled fractions 6H-7A along with the *T. aurantiacus* GH61A polypeptide clearly induced swelling of the AVICEL®, whereas inclusion of other liquor fractions with the GH61 polypeptide did not induce swelling.

The supernatants from the AVICEL® incubations were then decanted off and the pellet dried using a GeneVac EZ-2 Plus® vacuum concentrator (Genevac Inc., Gardiner, NY, USA). The dried residues were dissolved in deuterated water and were analyzed by ^{1}H NMR using a VARIAN® MercuryVx 400 MHz NMR (Varian, Palo Alto, CA, USA). While the components of the liquor could not be identified by NMR spectroscopy, the NREL liquor fractions that produced swelling of the AVICEL® on incubation with the *T. aurantiacus* GH61 polypeptide generated NMR peaks corresponding to saccharide chemical shifts.

The solid residual cellulose was then tested for reducing end content using a method modified from Zhang and Lynd, 2005, *Biomacromolecules* 6: 1510-151. The cellulose was washed in 1 ml of 1.1% sodium dodecyl sulfate (SDS) and incubated at 95°C for 10 minutes with shaking, and then the suspension was pelleted by centrifugation at 31,000 rpm in a microcentrifuge. The SDS solution was decanted off and the pellet was washed by repeated resuspension in 1.5 ml of 70% ethanol and pelleting. The pellets were finally incubated with a 1:1 solution of H_2O and BCA working solution (1:1 mixtures of 0.624 g of $CuSO_4 \cdot 5H_2O$, and 0.631 g of L-serine per 500 ml of H_2O with 0.971 g of disodium 2,2'-bichinchoninate, 27.12 g of Na_2CO_3, and 12.1 g of $NaHCO_3$ per 500 ml of H_2O) at 65°C for 30 minutes. The concentration of reducing ends was determined by comparison to a standard curve generated by serial dilution of glucose in the same BCA working solution and measurement of $A_{(600nm)}$ in a 96-well microtiter plate using a POWERWAVE X™ microplate spectrophotometer (Biotek Instruments, Winooski, VT USA).

Figure 11A shows the glucose reducing end standard curve of $A_{600\ nm}$ vs. glucose concentration. The concentration of reducing ends in the *T. aurantiacus* GH61A polypeptide incubated samples was calculated from this standard curve. Figure 11B shows the number of reducing end equivalents for AVICEL® incubated with the GH61 polypeptide and the indicated NREL acid-pretreated corn stover HPLC fractions. The same NREL acid-pretreated corn stover fractions that induced GH61 polypeptide-dependent swelling of the AVICEL®, showed a much higher apparent concentration of reducing end equivalents in the insoluble, washed residual cellulose suggesting that hydration of these reducing ends was the likely cause of the swelling of the cellulose.

Example 23: Enrichment of acid-pretreated xylan components

Xylan was acid-pretreated and then fractionated by HPLC chromatography according to Example 21 for NREL acid-pretreated corn stover liquor with the following exceptions. Acid pretreated xylan was generated by incubating 45 g of beechwood xylan with 400 g of 1.1% H_2SO_4 at 190°C for 2 minutes in a a 1 gallon, high-pressure horizontal stirred reactor (Parr Instrument Company, Moline, IL, USA). No preincubation with cellulose and organic phase wash was performed. The acid-pretreated xylan was filtered with a 3 kDa MWCO VIVASPIN 20® centrifuge filter (GE Healthcare, Piscataway, NJ, USA), retained on a Macrosep 1kD Omega centrifuge filter (Pall, Ann Arbor, MI), washed with 3-fold volumes of deionized water, and applied to a 8.0 mm x 300 mm Shodex Sugar SP0810 chromatography column (Showa Denka America, Inc., NY, USA) using an AGILENT® 1100 HPLC and CHEMSTATION® software, and separated by isocratic elution with water at a flow rate of 0.5 ml per minute at 80°C for 50 minutes, collecting 250 µl fractions in 2.0 ml, 96-deep well plates (Axygen, Union City, CA, USA). Repeated 100 µl injections of the liquor followed by repeated fraction collection yielded approximately 5 ml of each fraction, which were pooled and lyophilized to dryness. Later separations included an overnight incubation of the acid-pretreated xylan with endoxylanase (SHEARZYME™, Novozymes A/S, Bagsvaerd, Denmark) in 50 mM sodium acetate pH 5.0 at 50°C to hydrolyze xylo-oligomers, followed by removal of the enzyme with a 3 kDa MWCO VIVASPIN 20® centrifuge filter prior to chromatography. *Thermoascus aurantiacus* GH61A polypeptide titrations to AVICEL® hydrolysis reactions containing either the endoxylanase-treated or the untreated xylan liquor were performed according to Example 3 and confirmed that endoxylanase treatment had not altered the effects of acid-pretreated xylan on GH61 polypeptide enhancement of cellulolysis in these samples. Absorbance of the eluted fractions was not determined. Fractions were assayed by dissolution of the lyophilized fractions in 200 µl of water, and addition of 100 µl to saccharification reactions according to Example 3, containing 4 mg of the *T. reesei* cellulase composition and 1 mg of the *T. aurantiacus* GH61A polypeptide, or no GH61A.

Figure 12 shows the hydrolysis of AVICEL® by the *T. reesei* cellulase composition with supplemented *T. aurantiacus* GH61A polypeptide and the HPLC fractions as indicated. This elution profile demonstrated a broad elution peak of fractions that in the presence of the GH61 polypeptide resulted in higher hydrolysis by the *T. reesei* cellulase composition. Thus, these fractions contained compounds that in the presence of GH61 polypeptide promoted GH61 polypeptide-dependent cellulolytic enhancement. Several inhibitory fractions were also observed, as is evident in Figure 12, fraction 5D. The elution of the components that facilitate GH61 polypeptide cellulolytic enhancement centered on the same fractions that had previously been shown to facilitate GH61 polypeptide cellulolytic enhancement in chromatography of NREL pretreated corn stover liquor (Example 15). It is likely that the

width of the elution was due to overloading of the HPLC column and concomitant loss of theoretical plate. This hypothesis was further supported by the magnitude of the enhancement in cellulolysis by the HPLC fractions examined, which was much higher than observed following NREL acid-pretreated corn stover liquor.

The fractions that had higher fractional hydrolysis in the presence of the *Thermoascus aurantiacus* GH61A polypeptide were analyzed by LC-MS in the following manner. Samples were diluted in 0.1% formic acid to 20 µg per ml approximate concentrations. Samples were then either filtered using Ultrafree-MC centrifugal filter devices (Millipore Corporation, Billerica, MA, USA) or were centrifuged for 10 minutes at 21,000 x *g* and then stored at 4°C if analysis was not performed immediately. UPLC® tandem mass spectrometry, was performed using a Q-Tof *micro*™ hybrid orthogonal quadrupole time-of-flight mass spectrometer (Waters Micromass MS Technologies, Milford, MA, USA) using MASSLYNX™ software version 4.1 (Waters Micromass MS Technologies, Milford, MA, USA). The Q-TOF *MICRO*™ was fitted with an ACQUITY UPLC® using a 1.0 x 50 mm, C18, 1.7 µm, BEH Acquity column (Waters Corp, Milford, MA, USA) to permit chromatographic separation of analytes. The following elution gradient was applied over a 37 minute interval at a flow rate of 100 µl per minute: 0-15 minutes from 1-15% acetonitrile with 0.1% formic acid, 15-20 minutes from 15-40% acetonitrile with 0.1% formic acid, 20–25 minutes from 40–80% acetonitrile with 0.1% formic acid, 25-30 minutes with 80% acetonitrile with 0.1% formic acid, 30-31 minutes from 80-1 % acetonitrile with 0.1% formic acid, and 31-37 minutes with 1% acetonitrile with 0.1% formic acid. Elution was monitored at 280 nm through a diode array detector and eluents from the column were introduced directly into the Q-TOF *MICRO*™ via electrospray ion source. A cone voltage of 20 volts was typically used and the collision energy was varied in the range of 5–15 volts. Data were acquired in survey scan mode from a mass range of m/z 50 to 1000 with switching criteria for MS to MS/MS that included an ion intensity of greater than 50.0 counts per second. The acquired spectra were combined, smoothed, and centered in an automated fashion. Analytes were identified by comparison to spectra of standard compounds when possible. Standard compounds were analyzed before and after the samples to confirm the mass accuracy and retention time stability of the sample analyses.

Figure 13 shows a representative LC-MS chromatogram of a *T. aurantiacus* GH61A polypeptide cellulolytic enhancing acid-pretreated xylan HPLC fraction. It is clear from the number of peaks that significant heterogeneity was observed. The bulk of the components were eluted from the LC in the first 6-minutes, suggesting a highly polar or charged composition.

Example 24: Using GH61 polypeptide-binding affinity to enrich NREL acid-pretreated corn stover liquor for components that are functional in GH61 polypeptide-dependent cellulolytic enhancement

Thermoascus aurantiacus GH61A polypeptide affinity was used as a means to enrich NREL acid-pretreated corn stover liquor components that bind to the GH61 polypeptide. Seventy mg of the *T. aurantiacus* GH61A polypeptide were incubated overnight with 50 ml of liquor. The GH61 polypeptide-bound liquor components were separated from the free components by ultracentrifugation using a 10 kDa MWCO VIVASPIN 20® centrifuge filter, concentrating the protein and protein-bound fraction 10-fold, followed by washing with an equivalent volume of water to the starting material (50 ml total volume), and repeating 5-times. Finally, the protein was denatured by incubation at 90°C for 30 minutes, and centrifuged using a 10 kDa MWCO VIVASPIN 20® centrifuge filter. The flow-through from the filter was analyzed using liquid chromatography mass spectrometry according to Example 23, with electrospray mass spectrometry performed in both positive and negative ionization modes.

Figure 14 shows the LC-MS chromatogram of the NREL acid-pretreated corn stover liquor components that bound to the *T. aurantiacus* GH61A polypeptide and were eluted by denaturation. Both positive and negative mode ion chromatographs were combined, yielding a small set of unique mass ions, many of which could be excluded as dimers, fragments of larger parent ions or contaminants derived from the buffer or from the centrifuge columns, leading to identification of approximately 30 unique mass ions of reasonable intensity. Based on the monoisotopic masses obtained, chemical formulae were determined and a database search of chemical compounds (ChemSpider, Royal Society of Chemistry) yielded a list of putative compounds for GH61 polypeptide assay. In the chromatograms, 3 broadly classified sets of compounds could be identified: the first set eluted in the first 2-minutes, had smaller mass and were likely small polar or charged compounds; a second lower abundance set, corresponding to a broadly varied set of molecular weights but likely to be less polar, eluted with intermediate retention times; and finally a series of larger molecular weight ions eluted between 12 and 20 minutes, corresponding to less polar or higher molecular weight compounds. It was observed that the search of monoisotopic masses frequently yielded compounds consistent with plant flavonols, flavanols, oxidized flavonoids, oxidized flavanols and similar compounds, thus a large set of these compounds were tested for GH61 cellulolytic enhancing activity.

Example 25: Effect of NREL pretreated corn stover liquor and the *Thermoascus aurantiacus* GH61A polypeptide on generating soluble cellodextrin from microcrystalline cellulose or phosphoric acid-swollen cellulose

Monosaccharide, disaccharide, and aldonic and uronic acid standards (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in water at 1 mg per ml and were diluted to 50 µg per ml in 10 mM NaOH. Cellooligosaccharides (Sigma-Aldrich, St, Louis, MO, USA) were dissolved in water at 25 to 30 mg per ml and were diluted as above. Xylooligosaccharides (Megazyme Bray, Co. Wicklow, Ireland) were dissolved in water at 10 to 30 mg per ml and were diluted as above. Lactobionic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA), dissolved in water at 2.5 mg per ml. The aldonic acids of each cellooligosaccharide were generated by incubation of 17 µg of *Humicola insolens* cellobiose dehydrogenase (WO 2010/080527) per ml with 2.5 mg per ml of each cellooligosaccharide in 50 mM sodium acetate pH 5.0 at 50°C and 10 mM dichloroindophenol (DCIP; Sigma-Aldrich, St. Louis, MO, USA) was added incrementally. The equivalence point was achieved when DCIP was not reduced by the solution, and the color remained purple. Formation of the aldonate products was confirmed by LC-MS according to Example 25. Cellobiose dehydrogenase was then removed by ultrafiltration through a VIVASPIN 20® 10 kDa MWCO centrifuge filter.

The *Thermoascus aurantiacus* GH61A polypeptide was incubated at 37.5 µg/ml with either 29.5 mg per ml microcrystalline cellulose (AVICEL®™, Sigma Aldrich, St. Louis, MO, USA) or 1.2% (w/w) phosphoric acid-swollen cellulose (PASC) in 50 mM sodium acetate, 1 mM $MnSO_4$ for 5 days at 50°C with or without 10% (v/v) NREL pretreated corn stover liquor. NREL pretreated corn stover liquor was extracted according to Example 2. Phosphoric acid swollen cellulose was prepared from AVICEL® PH101 using the protocol described by Zhang *et al.*, 2006, *Biomacromolecules 7*: 644–648. Control incubations containing AVICEL® alone, PASC alone, AVICEL® or PASC with NREL acid-pretreated corn stover liquor in the absence of the *T. aurantiacus* GH61A polypeptide were performed in the same manner. The samples were then filtered through a 0.22 µm centrifuge filter, and the filtrates were diluted 1:10 in 1 ml final volume of 10 mM NaOH and analyzed by DIONEX® Ion Chromatography with pulsed amperometry detection (IC-PAD, Dionex Corporation, Sunnyvale, CA) using DIONEX® Chromeleon or PeakNet Software. Ten µl of 2.5 mg per ml lactobionic acid was added as an external loading control. Chromatographic separation was obtained using a PA-20 column (Dionex Corporation, Sunnyvale, CA, USA), with relevant guard, borate and amine-trap pre-columns, and elution was achieved with an isocratic gradient of 13 mM sodium hydroxide, 2.5 mM sodium acetate for 20 minutes, followed by a linear gradient from 13 to 50 mM NaOH, isocratic gradient for 10 minutes and linear gradient from 0.5 to 40 mM sodium acetate in 50 mM NaOH.

Figure 15A shows the chromatograms of AVICEL® and PASC incubations with GH61 polypeptide, with GH61 polypeptide and pretreated corn stover liquor, and control incubations. The chromatograms indicated that soluble oligosaccharide products were evolved from the incubations containing the GH61 polypeptide, liquor, buffer, and cellulose

in both PASC and microcrystalline forms. The arrows indicate unique elution peaks that were present only in the incubations containing the full set of components. Figure 15B shows a comparison of chromatogram of AVICEL® incubated with the *T. aurantiacus* GH61A polypeptide, acid-pretreated corn stover liquor, and buffer to the chromatograms of standard compounds. Figure 15B indicated that the retention times of the novel products were consistent with those of cellopentaose, cellotetraose, and cellotriose. Three additional elution peaks were also evident; a large peak with retention time of 49 minutes matched no available standard but was likely cellohexaose based on its retention time in comparison to the cellooligosaccharides and aldonic acid standards. The relative peak intensities indicated that the major product may be cellotetraose, though the sparing solubility of cello-oligosaccharides of DP ≥ 6 may have understated the actual production of these oligomers.

Example 26: Effect of water-extracted and alkaline pretreated corn stover liquors in combination with the *Thermoascus aurantiacus* GH61A on cellulolysis by the *T. reesei* cellulase composition

The *Thermoascus aurantiacus* GH61A polypeptide was assayed for cellulolytic enhancing activity according to Example 3 with 10% (v/v) added liquor from either alkaline pretreated corn stover or 10% (v/v) added liquor from water-extracted acid-pretreated corn stover according to Example 2. Alkaline pretreated corn stover was prepared in the following manner. Milled and washed, raw PCS was treated with 4%, 6%, 8% or 10% (w/w) NaOH for 1 hr at 90°C in a 2-L stirred tank reactor (IKA Works, Inc., Wilmington, NC, USA). Water extracted corn stover liquors were generated in the following manner. Raw corn stover was milled in a WILEY® Mill (Model 4; Thomas Scientific, Swedesboro, NJ, USA) with a nominal screen size of 2 mm. The milled stover was sieved through a #40 mesh screen and the fines were discarded. A Dionex Accelerated Solvent Extractor (ASE) 350 (Dionex Corporation, Sunnyvale, CA, USA) instrument was used for all pretreatments with two cycles for each pretreatment. The first cycle of each pretreatment was a pressurized hot water extraction run in standard flow mode of operation. The second cycle of the pretreatment was the same for each experimental sample, and was a mid-severity dilute acid pretreatment run in pressure solvent saver mode of operation. Approximately 20.0 g of the sieved corn stover were packed into a 100 ml stainless steel extraction cell. During the pressurized hot water extraction the stover was extracted at temperatures of 100-170°C in 10°C increments for a static reaction time of 7 minutes. After extraction the cell was purged with nitrogen for 60 seconds, and all extraction liquor collected. Static pretreatment time was defined from the time the internal temperature of the cell reached equilibrium with the heating chamber. Liquors from the first, water-extraction step were filtered using a 10 kDa MWCO VIVASPIN 20® centrifuge filter and were pH adjusted to 5.0 by addition of NaOH or HCl, prior to use.

As shown previously, GH61 polypeptide titrations in the absence of added liquors did not significantly enhance microcrystalline cellulose hydrolysis by the *T. reesei* cellulase composition (Examples 15, 16, and 18). Figure 16A shows the fractional hydrolysis of AVICEL® by the *T. reesei* cellulase composition with increasing concentrations of the *T. aurantiacus* GH61A polypeptide in the presence of liquors from various severities of alkaline pretreated corn stover. Addition of alkaline corn stover liquors increasing in severity from 4% NaOH to 10% NaOH showed reduced fractional hydrolysis, thus increasing inhibition in the absence of the GH61 polypeptide. Unlike acid-pretreated corn stover liquors, however, addition of the GH61 polypeptide in the presence of alkaline liquors provided no substantial improvement over saccharifications without liquor.

The higher severity alkaline pretreated corn stover liquors in combination with the *T. aurantiacus* GH61A polypeptide did provide a small amount of cellulolytic enhancing activity at 7 days of hydrolysis. Comparing zero to 50% GH61 polypeptide additions, hydrolysis in the presence of 6% NaOH liquor was 0.473 ± 0.00483 and 0.488 ± 0.00724, 8% NaOH was 0.454 ± 0.00352 and 0.485 ± 0.000119, and 10% NaOH was 0.445 ± 0.00625 and 0.476 ± 0.00732 The addition of the *T. aurantiacus* GH61A polypeptide was sufficient to partially mitigate the inhibition arising from the liquor additions; as the hydrolysis in the absence of liquor was 0.502 ± 0.0109 and 0.499 ± 0.00642 at zero and 50% (w/w) GH61A polypeptide, respectively.

Figure 16B shows the fractional hydrolysis of AVICEL® by the *T. reesei* cellulase composition with increasing concentrations of the *T. aurantiacus* GH61A polypeptide in the presence of corn stover liquors extracted with water at the indicated temperatures. Pressurized water-extracted corn stover liquors did not appreciably enhance fractional hydrolysis in the presence of the GH61 polypeptide. Water-extracted liquors generated at 150°C and above show some inhibition, both in the absence and presence of the GH61 polypeptide. These data indicated that pressurized water-extraction did not extract the liquor components required to observe GH61 polypeptide cellulolytic enhancing activity on microcrystalline cellulose.

Example 27: Effect of acid-pretreated xylan, acid-pretreated biomass component mixtures containing xylan, or acid-pretreated monosaccharide components of xylan including xylose and arabinose in combination with *Thermascus aurantiacus* GH61A polypeptide on cellulose hydrolysis by the *Trichoderma reesei* cellulase composition

The cellulolytic enhancing effect of the *T. aurantiacus* GH61A polypeptide on microcrystalline cellulose was assayed with components of biomass that had been acid-pretreated.

The *Thermoascus aurantiacus* GH61A polypeptide was assayed for cellulolytic enhancing activity according to the procedure described in Example 3 with the following exceptions: hydrolysis reactions were performed with 10% (v/v) added liquor from acid-pretreated corn stover (Example 2) or with 10% (v/v) added liquor from acid-pretreated biomass components including xylan, cellulose, protein, lipid, monosaccharide, and combinations thereof. AVICEL® (1.8 g), oatspelt xylan (1.1 g; TCI), lignin (0.9 g; MeadWestvaco), corn gluten (0.25 g; Sigma Aldrich, St. Louis, MO, USA), gallic acid (0.25 g; Sigma Aldrich, St. Louis, MO, USA), ferulic acid (0.25 g; Sigma Aldrich, St. Louis, MO, USA), or corn oil (0.25 g; Sigma Aldrich, St. Louis, MO, USA) were pretreated by dissolution in 25 ml of 1% H_2SO_4 and incubation in an SBL-2 fluidized sand bath reactor with TC-8D Temperature Controller (Techne Inc., Burlington, NJ, USA) for a 5 minutes temperature ramp followed by 5 minutes at 190°C. Aliquots from saccharification reaction were removed for analysis at 1 and 5 days. Liquors were filtered using a 10 kDa MWCO VIVASPIN 20® centrifuge filter and pH adjusted to 5.0 by addition of NaOH or HCl, prior to use.

Figure 17A and 17B show fractional hydrolysis of AVICEL® by the *T. reesei* cellulase composition with various concentrations of the *T. aurantiacus* GH61A polypeptide in the presence of liquors derived from acid-pretreatment of various biomass components. Titration of the *T. aurantiacus* GH61A polypeptide from 0 to 50% (w/w) addition to the *T. reesei* cellulase composition yielded, in the absence of liquor, no enhancement of AVICEL® hydrolysis (Example 15, 16, 18, and Figure 19B). Increasing concentrations of the *T. aurantiacus* GH61A polypeptide increases fractional hydrolysis in the presence of a subset of the liquors examined. Small GH61 polypeptide concentration-dependent hydrolysis enhancements were observed in the presence of liquors of acid-pretreated lignin, lipid, protein, cellulose, lignin model compounds such as gallic acid, and hemicellulase components such as ferulic acid. The extent of GH61 polypeptide enhancement of fractional hydrolysis in the presence of acid-liquors was from 0.390 ± 0.007 to 0.435 ± 0.010 in the presence of lignin liquor, from 0.305 ± 0.013 to 0.341 ± 0.016 in the presence of corn oil liquor, 0.493 ± 0.007 to 0.524 ± 0.004 in the presence of corn gluten liquor, 0.134 ± 0.021 to 0.184 ± 0.023 in the presence of gallic acid liquor, 0.352 ±.011 to 0.397 ± 0.029 in the presence of ferulic acid liquor at 7 days of hydrolysis. Conversely, liquor derived from acid-pretreatment of xylan yielded a substantial GH61 polypeptide concentration-dependent enhancement of cellulolysis, from 0.390 ± 0.009 to 0.540 ± 0.013 at 7 days of hydrolysis. Where possible, biomass components had been acid pretreated at their respective concentrations in NREL acid-pretreated corn stover, thus the observed GH61 cellulolytic enhancements should scale proportionally with their contributions to enhancements in pretreated corn stover. The bulk of the apparent GH61 polypeptide concentration-dependent

cellulolytic enhancements on cellulose are thus derived from the xylan components.

Example 28: Effect of residual liquors post-fermentation in combination with the *Thermoascus aurantiacus* GH61A polypeptide on cellulose hydrolysis by the *T. reesei* cellulase composition

The *Thermoascus aurantiacus* GH61A polypeptide was assayed for cellulolytic-enhancing activity according to Example 3 with the following exceptions: 10% (v/v) added liquor from post-fermentation residues was added, and aliquots were removed at 1, 3, and 7 days of saccharification. Liquors were filtered using a 10 kDa MWCO VIVASPIN 20® centrifuge filter and pH adjusted to 5.0 by addition of NaOH or HCl, prior to use. Fermentation liquors were obtained as follows: 20% total solids NREL unwashed, unmilled pretreated corn stover was hydrolyzed by 5 mg per gram cellulose of a *T. reesei* cellulase composition for 5-days. The hydrolysate was fermented for 2 days using a *Saccharomyces cerevisiae* strain comprising a xylose isomerase gene (WO 2003/062430) at 3% cell density for 2 days. Fifty ml of the fermentation liquor was filtered as described and assayed for GH61 polypeptide concentration-dependent cellulolytic enhancement activity.

Figure 18 shows the fractional hydrolysis of AVICEL® by the *T. reesei* cellulase composition with various concentrations of the *T. aurantiacus* GH61A polypeptide in the presence of post-fermentation liquors. The addition of increasing *T. aurantiacus* GH61A polypeptide concentrations significantly enhanced hydrolysis of AVICEL® in the presence of the post-fermentation liquors from 0.246 ± 0.007 to 0.265 ± 0.007 at 1 day of hydrolysis and from 0.616 ± 0.006 to 0.895 ± 0.002 at 7 days of hydrolysis.

Example 29: Effect of corn stover liquors from increasing severity of acid pretreatments in combination with the *Thermoascus aurantiacus* GH61A polypeptide on cellulose hydrolysis by the *T. reesei* cellulase composition

The *Thermoascus aurantiacus* GH61A polypeptide was assayed for cellulolytic enhancing activity according to Example 3 with 10% (v/v) added liquor from acid-pretreated corn stover or microcrystalline cellulose (AVICEL®) of various severity pretreatments. Liquors were filtered using a 10 kDa MWCO VIVASPIN 20® centrifuge filter and pH adjusted to 5.0 by addition of NaOH or HCl, prior to use. Liquors were generated using an SBL-2 fluidized sand bath reactor with TC-8D Temperature Controller, incubating corn stover with 1% H_2SO_4 in 25 ml and incubating at temperatures between 140°C and 170°C for 1 to 5 minutes, or by incubating AVICEL® with 1% H_2SO_4 in 25 ml at temperatures between 110°C and 190°C for 5 minutes.

Figures 19A and 19B show the fractional hydrolysis of AVICEL® containing various severity acid-treatments of corn stover by the *T. reesei* cellulase composition and various

concentrations of the *T. aurantiacus* GH61A polypeptide. Liquors were generated by systematically varying both temperature between 140°C and 170°C and varying time at each temperature between 1 and 5 minutes. For saccharification reactions containing liquors produced by acid-pretreatment at temperatures greater than 140°C and 5 minutes, increasing *T. aurantiacus* GH61A polypeptide concentrations increased fractional hydrolysis. The higher severity pretreatments, particularly those of 160°C and above, showed a greater increase in fractional hydrolysis from GH61 polypeptide addition than did the lower severity pretreatments of 150°C and less. Additionally, higher severity pretreatment liquors were increasingly inhibitory to cellulolysis, thus the greatest overall cellulose conversion was obtained by addition of high GH61 polypeptide concentrations (50% w/w) at intermediate pretreatment severity, specifically 150°C for 5 minutes, 160°C for 3-5 minutes, or 170°C for 1 minute. These data demonstrated that pretreatment of a biomass can be tailored to maximize hydrolysis for a given GH61 polypeptide concentration in a cellulase composition. There is an optimum pretreatment condition that balances GH61 polypeptide cellulolytic-enhancing activity with production of soluble inhibitor compounds.

Figure 19C shows the fractional hydrolysis of AVICEL® containing various severity acid-treatments of AVICEL® by the *T. reesei* cellulase composition and various concentrations of the *T. aurantiacus* GH61A polypeptide. In calculating the fractional hydrolysis, sugars derived from liquor addition were not subtracted, and contributed up to 3% of conversion. From Figure 19C it is clear that liquors derived from acid-treatment of microcrystalline cellulose in combination with the *T. aurantiacus* GH61A polypeptide enhanced hydrolysis only marginally.

Example 30: Effect of xylan liquors from increasing severity of acid pretreatments in combination with the *Thermoascus aurantiacus* GH61A polypeptide on cellulose hydrolysis by the *T. reesei* cellulase composition

The *Thermoascus aurantiacus* GH61A polypeptide was assayed for cellulolytic enhancing activity according to Example 3 with 10% (v/v) added liquor from xylan pretreated with varying severity. Liquors were generated by incubation of 1.1 g of either oatspelt xylan or wheat flour with 1% H_2SO_4 or 3% HCl in 25 ml total volume in an SBL-2 fluidized sand bath reactor with TC-8D Temperature Controller for 10 minutes total; a 5 minute ramp period followed by a 5 minute incubation at the indicated temperature. Incubation temperatures were varied between 60°C and 180°C. Pretreatment liquors were filtered using a 10 kDa MWCO VIVASPIN 20® centrifuge filter and pH adjusted to 5.0 by addition of NaOH or HCl, prior to use.

Figures 20A and 20B show the effect of acid-pretreated xylan liquors added to the *T. reesei* cellulase compositions with increasing concentrations of the *T. aurantiacus* GH61A

polypeptide. In the absence of the GH61 polypeptide, liquors of increasing pretreatment severity were increasingly inhibitory to the cellulolysis of AVICEL®, particularly those generated with 3% HCl. Titration of increasing GH61 polypeptide concentration in the presence of xylan acid-pretreated at temperatures greater than 140°C led to increasing cellulolysis, and the net change in hydrolysis with GH61 polypeptide addition was higher for higher severity pretreatments. Hydrolysis was not improved by the GH61 polypeptide on addition of xylan pretreated at 60°C to 120°C. Hydrolysis was improved by 0.057, from 0.349 ± 0.00510 to 0.406 ± 0.00394 with 160°C liquor, by 0.089 from 0.287 ± 0.00233 to 0.376 ± 0.00118 with 180°C liquor, and by 0.0740, from 0.216 ± 0.00225 to 0.293 ± 0.00843 with 180°C HCl derived liquor. The effect of increasing pretreatment severity on *T. aurantiacus* GH61A polypeptide cellulolytic enhancing activity with xylan liquors was similar to those of corn stover pretreatment severity (Example 26), and again suggested a maximal overall hydrolysis was obtained by high GH61 polypeptide concentrations in the presence of liquors of intermediate severity. In this case, 50% (w/w) *T. aurantiacus* GH61A polypeptide in the presence of 160°C acid-pretreated xylan produced the highest cellulose conversion. Additionally, as previously observed for corn stover liquors, there appeared to be an optimum balance between GH61 polypeptide cellulolytic enhancing activity and the production of soluble inhibitors. The addition of a specific concentration and tailoring of pretreatment severity can be accomplished for a given GH61 polypeptide concentration within a cellulase composition.

Figure 20C shows the fractional hydrolysis of AVICEL® containing various severity acid-treatments of beechwood xylan by the *T. reesei* cellulase composition and various concentrations of the *T. aurantiacus* GH61A polypeptide. From Figure 20C, it was clear that the maximum conversion was achieved in the presence of a high concentration of the *T. aurantiacus* GH61A polypeptide (50%) and liquor produced at 170°C. In the presence of liquor generated at 190°C, the observed hydrolysis was lower for all concentrations of the *T. aurantiacus* GH61A polypeptide tested, and in the presence of liquors generated below 170°C; the conversion levels reached were not as great. These data suggest that treatment severity of some substrates may be optimized to generate liquors for a given GH61 polypeptide concentration within a cellulase composition.

Example 31: Effect of solid-phase extracted acid pretreated corn stover liquors in combination with the *Thermoascus aurantiacus* GH61A polypeptide on cellulose hydrolysis by the *T. reesei* cellulase composition

The *Thermoascus aurantiacus* GH61A polypeptide was assayed for cellulolytic enhancing activity according to Example 3 with the following exceptions. *T. aurantiacus*

GH61 polypeptide was either not added or was added at 50% (w/w) of total protein and 10% (v/v) solid phase extraction resin-eluted liquor was added to each hydrolysis reaction. Both NREL acid-pretreated corn stover liquor prepared according to Example 2 and acid-pretreated xylan prepared according to Example 23, and then pH adjusted to 5.0, were applied to one of two solid phase extraction cartridges, either a BOND ELUT™ C-18 column (Varian, Palo Alto, CA, USA) or a STRATA™-X Polymeric column (Phenomenex, Torrance, CA, USA) equilibrated in water. The samples were washed 3-times with 1 ml of water and then eluted with two aliquots of 600 µl methanol. The eluted samples were diluted back to their original volumes with water and assayed for GH61 polypeptide cellulolytic enhancing activity.

Figure 21 shows the fractional hydrolysis of AVICEL® by the *T. reesei* cellulase composition with and without 50% (w/w) *T. aurantiacus* GH61A polypeptide addition in the presence of solid-phase extracted liquors as indicated. The fractional hydrolysis was equivalent within error for all samples, indicating that no enhancement of cellulolysis from GH61 polypeptide addition was observed in the presence of solid-phase extracted liquors from acid-pretreated corn stover or from acid-pretreated xylan. A cellulolytic enhancement of the *T. reesei* cellulase composition was previously demonstrated in the presence of these liquors in combination with addition of the GH61 polypeptide, prior to solid-phase extraction. It was therefore concluded that the compounds present in these liquors that were correlated with the observed GH61 polypeptide cellulolytic enhancing activity were eluted during the wash steps.

Example 32: Effect of electrodialyzed acid-pretreated corn stover liquors on *Thermoascus aurantiacus* GH61A polypeptide enhancing the *Trichoderma reesei* cellulase composition

The *Thermoascus aurantiacus* GH61A polypeptide was assayed for cellulolytic-enhancing activity according to Example 3 with the following exceptions: 10% (v/v) NREL pretreated corn stover liquor, acid-pretreated xylan (Example 23) or electrodialyzed NREL pretreated corn stover liquor was added to each hydrolysis reaction. Electrodialyzed NREL pretreated corn stover liquor was generated in the following manner. Two kg of NREL pretreated corn stover liquor was filtered and. the pH was adjusted to 5.0 using concentrated NaOH. The pretreatment liquor was subjected to concentrating electrodialysis using a EUR2B pilot scale electrodialysis unit (Ameridia, Somerset, NJ, USA) equipped with a EUR2B-10 stack Ameridia, Somerset, NJ, USA). The solution of pH adjusted liquor had a conductance of 16.5 mS/cm and a pH of 5.0 and was charged to the diluate tank. A solution of potassium nitrate (20 mS/cm, 2.0 kg) was charged to the electrode rinse tank. A dilute solution of NREL pretreatment liquor (0.657 mS/cm) was charged to the concentrate tank.

The solutions described above were circulated through the EUR2B-10 stack at a flow rate of approximately 0.9 gallon per minute (gpm) using a DC power supply set to 14 volts. After 55 minutes, the conductivity change in the diluate and concentrate tanks remained stable and the run was ended. The final conductance in the concentrate tank was 14.01 mS/cm.

Figure 22 shows that increasing the *T. aurantiacus* GH61A polypeptide concentration in the presence of NREL pretreated corn stover liquor, electrodialyzed NREL acid-pretreated corn stover liquor, and acid-pretreated xylan increased the fractional hydrolysis of AVICEL®. At the highest concentrations of GH61 polypeptide, the electrodialyzed NREL liquor showed slightly lower conversion than the undialyzed sample, 0.541 ± 0.0006 compared with 0.524 ± 0.0118, though at intermediate GH61 polypeptide concentrations the conversion was higher for the electrodialyzed liquor, 0.467 ± 0.00671 compared with 0.502 ± 0.00215.

Example 33: Effect of addition of acid-pretreated corn stover liquors and *Thermoascus aurantiacus* GH61A polypeptide to non-acid pretreated biomass feedstocks

The *Thermoascus aurantiacus* GH61A polypeptide was assayed for cellulolytic-enhancing activity according to Example 3 with the following exceptions: 5% (v/v) added NREL acid-pretreated corn stover liquor was added to either 3% or 5% total solids of various milled biomass feedstocks, and aliquots were removed for analysis at 1, 3, and 7 days of saccharification. Control incubations of each biomass containing either no *T. reesei* cellulase composition and no GH61 polypeptide, or containing GH61 polypeptide in the absence of the *T. reesei* cellulase composition with and without liquor, and control incubations containing an equivalent concentration of liquor with the *T. reesei* cellulase composition and GH61 polypeptide without biomass were performed in parallel. The composition of total accessible cellulose in each biomass was determined by measurement of glucose equivalents produced by saccharification by 50 mg of Cellic CTec™ (available Novozymes A/S, Bagsvaerd, Denmark) over 7 days of hydrolysis. Liquors were filtered using a 10 kDa MWCO VIVASPIN 20® centrifuge filter and pH adjusted to 5.0 by addition of NaOH or HCl, prior to use. Biomass feedstocks included raw sugarcane bagasse and corn stovers pretreated in the following manners: organosolv low severity ethanol, organosolv medium severity ethanol, organosolv high severity ethanol, and organosolv glycerol.

Figures 23A, 23B, 23C and 23D show the fractional hydrolysis of various biomass feedstocks by the *T. reesei* cellulase composition with varying *T. aurantiacus* GH61A polypeptide concentrations, with and without supplemented NREL acid-pretreated corn stover liquor, as indicated. For each biomass, addition of 5% (v/v) NREL acid-pretreated corn stover liquor in the absence of the *T. reesei* cellulase composition added a background

sugar concentration equivalent to approximately 0.05 increase in apparent hydrolysis, depending on the relative amount of cellulose present in each substrate. This was not substantially changed by addition of the *T. reesei* cellulase composition or the GH61 polypeptide. Since these liquor-derived sugars are not subtracted from the fractional hydrolysis values, for each biomass the presence of liquor shifts the apparent hydrolysis to a higher value.

For each biomass, a higher fractional hydrolysis was obtained by addition of higher *T. aurantiacus* GH61A polypeptide concentrations from 0 to 50% (w/w) total protein to the *T. reesei* cellulase composition. For some of these biomasses, the presence of the NREL acid-pretreated corn stover liquor increased the total cellulolytic enhancement by the *T. aurantiacus* GH61A polypeptide. This was particularly apparent at 3 days of hydrolysis (gray bars, Figure 24), as exemplified by the enhancement of low and medium severity ethanol organosolv pretreatments. In the absence of GH61 polypeptide, fractional hydrolysis of low severity ethanol organosolv corn stover was 0.148 ± 0.00447 without liquor and 0.165 ± 0.00126 with liquor, whereas at 50% GH61, fractional hydrolysis was increased from 0.237 ± 0.00737 to 0.273 ± 0.00113. Similarly, in the absence of GH61 polypeptide, fractional hydrolysis of medium severity ethanol corn stover was 0.179 ± 0.000737 without liquor and 0.190 ± 0.00401 with liquor, whereas at 50% GH61 polypeptide, fractional hydrolysis was increased from 0.281 ± 0.0136 to 0.328 ± 0.00543. Substrates that showed this augmentation of the GH61 polypeptide cellulolytic enhancing effect included organosolv low and medium severity ethanol and sugarcane bagasse. It appeared that cellulolysis of lower severity pretreatment biomass feedstocks were better enhanced by the *T. aurantiacus* GH61A polypeptide in the presence of supplemented acid-pretreated biomass liquor. The augmentation of the GH61 polypeptide cellulolytic enhancing activity on the low severity pretreatment by supplemented liquor appeared most dramatic at the early stages of saccharification.

Example 34: Effect of addition of acid-pretreated monosaccharides on *Thermoascus aurantiacus* GH61A enhancement of *Trichoderma reesei* cellulase composition cellulolysis of microcrystalline cellulose

The *Thermoascus aurantiacus* GH61A polypeptide was assayed for cellulolytic enhancing activity as described in Example 3 with the following exceptions. Saccharification reactions were performed with 5 mM $MnSO_4$, and either 5% or 10% (v/v) liquor derived from acid-pretreatment of monosaccharides was added. Liquors were generated in the following manner: 20 mg per ml of each monosaccharide was incubated with 1% H_2SO_4 in an SBL-2 fluidized sand bath reactor with TC-8D Temperature Controller at 190°C for 5 minutes, and

the resulting liquors were then filtered using a 10 kDa MWCO VIVASPIN 20® centrifuge filter and pH adjusted to 5.0 by addition of NaOH or HCl prior to use. Ozonolysis was performed in the following manner: NREL acid-pretreated corn stover was slurried at 15% total solids, and the liquor extracted by vacuum filtration using a glass fiber filter. This liquor was incubated with a low concentration of ozone for 30 minutes prior to pH adjustment.

Figure 24 shows the fractional hydrolysis of AVICEL® by the *T. reesei* cellulase composition with either no GH61A polypeptide or 24% (w/w) *T. aurantiacus* GH61A polypeptide in the presence of 5% or 10% (v/v) of various acid-pretreated monosaccharides, and with ozone-treated NREL pretreated corn stover liquor. Figure 25 demonstrates that acid-pretreated monosaccharides were strongly inhibitory to cellulolysis by the *T. reesei* cellulase composition in the absence of GH61 polypeptide. In the presence of acid-pretreated hemicellulose-derived sugars, *e.g.*, C5 sugars, including arabinose and xylose, the fractional hydrolysis was increased by addition of the *T. aurantiacus* GH61A polypeptide. In the presence of 5% acid-pretreated arabinose, addition of the GH61 polypeptide increased fractional hydrolysis from 0.183 ± 0.00372 to 0.223 ± 0.00548 at 3 days of hydrolysis, and from 0.229 ± 0.00379 to 0.328 ± 0.00272 at 7 days of hydrolysis. Similarly, in the presence of 5% acid-pretreated xylose, addition of the GH61 polypeptide increased fractional hydrolysis from 0.113 ± 0.0148 to 0.140 ± 0.00356 at 3 days of hydrolysis, and from 0.117 ± 0.00252 to 0.177 ± 0.00602 at 7 days of hydrolysis. Conversely, addition of the *T. aurantiacus* GH61A polypeptide in the presence of acid-pretreated glucose increased fractional hydrolysis to a much smaller extent, even at the higher concentration of 10% (v/v), from 0.172 ± 0.00658 to 0.206 ± 0.00108. Ozone treatment of NREL acid-pretreated corn stover liquor altered the liquor components in such a manner as to disrupt the ability of the liquor in combination with the GH61 polypeptide to enhance cellulolysis of AVICEL® by the *T. reesei* cellulase composition. In the presence of liquor, the GH61 polypeptide increased fractional hydrolysis from 0.522 ± 0.00612 to 0.679 ± 0.00807, whereas in the presence of the ozone-treated liquor, the fractional hydrolysis was increased only from 0.512 ± 0.00347 to 0.540 ± 0.00956.

Example 35: Enhancement of AVICEL® **cellulolysis by *T. reesei* cellulases using NREL PCS liquor and various GH61 polypeptides**

Microcrystalline cellulose saccharification reactions were performed as described (Example 3), using 29.5 mg of microcrystalline cellulose (AVICEL®) per ml and 4 mg of the *T. reesei* cellulase composition per g cellulose in 50 mM sodium acetate, 1 mM manganese sulfate at pH 5.0 in the presence of 10% (v/v) NREL PCS liquor prepared as described (Example 2). GH61 polypeptides including *Thermoascus aurantiacus* GH61A polypeptide,

Aspergillus fumigatus GH61B polypeptide and *Penicillium pinophilum* GH61 polypeptide were added between 0 and 2 mg per g cellulose (0 and 50% (w/w) of the *T. reesei* cellulase composition concentration). Alternatively, liquor was added at 10% (v/v) to saccharifications containing either no GH61 polypeptide or 2 mg of the various GH61 polypeptides per g cellulose.

Figure 25 shows the fractional hydrolysis of AVICEL® by the *T. reesei* cellulase composition with various concentrations of the indicated GH61 polypeptides with 10% (v/v) NREL PCS liquor. Fractional hydrolysis is shown for the *T. reesei* cellulase composition with *Thermoascus aurantiacus* GH61A (circles), *Aspergillus fumigatus* GH61B polypeptide (diamonds) and *Penicillium pinophilum* GH61 polypeptide (squares) at 1 day (open symbols) and 3 days of hydrolysis (closed symbols). Addition of liquor in combination with all the GH61 polypeptides showed an apparent increase in hydrolysis of the cellulose. *Thermoascus aurantiacus* GH61A polypeptide produced the greatest enhancement of cellulolysis in the presence of liquor, 0.098 ± at 7 days of hydrolysis. Conversely, addition of the highest concentration of each of the GH61 polypeptides did not enhance cellulolysis in the absence of supplemented liquor. Multiple GH61 polypeptides are thus shown to enhance cellulolysis by *T. reesei* cellulases in the presence of biomass liquor.

Example 36: Effect of addition of Kraft (indulin) lignin or oxidized Kraft (indulin) lignin on the *Thermoascus aurantiacus* GH61A enhancement of *Trichoderma reesei* cellulase composition cellulolysis of microcrystalline cellulose

Microcrystalline cellulose saccharification were performed as described (Example 3), using 25 mg of microcrystalline cellulose (AVICEL®) per ml and 4 mg per g cellulose of a composition containing a blend of an *Aspergillus aculeatus* GH10 xylanase (WO 94/021785) and a *Trichoderma reesei* cellulase preparation containing *Aspergillus fumigatus* beta-glucosidase (WO 2005/047499) and *Thermoascus aurantiacus* GH61A polypeptide (WO 2005/074656) in 50 mM sodium acetate, 1 mM manganese sulfate at pH 5.0 in the presence of zero or 0.1% (w/w) Kraft (Indulin) lignin or oxidized Kraft (Indulin) lignin. Oxidized Kraft lignin was generated by overnight incubation of a 20% total solids slurry of lignin with sodium periodate at 4°C at a concentration of 5 g of sodium periodate per 100 g of slurry. The oxidized lignin was washed extensively with water and then freeze dried. The *T. reesei* cellulase composition was either supplemented with an additional 15% (w/w) *T. aurantiacus* GH61A polypeptide or was not supplemented.

Figure 26 shows the concentration of glucose produced by saccharification at various saccharification times as indicated. Addition of lignin or oxidized lignin enhanced the saccharification of AVICEL® by the *T. reesei* cellulase composition containing GH61A polypeptide. Addition of 15% (w/w) supplemental GH61A polypeptide to a *T. reesei* cellulase

composition containing *T. aurantiacus* GH61A polypeptide further increased the extent of saccharification of a hydrolysis reaction containing Kraft lignin. Addition of 15% supplemental GH61A polypeptide to a cellulase composition containing GH61A polypeptide resulted in lower glucose conversion than comparable reactions without supplemental GH61A polypeptide in hydrolyses of AVICEL® containing no Kraft lignin or containing oxidized Kraft lignin. These data indicate that products of biomass pretreatment derived from specific lignins or the lignins themselves, in combination with GH61A polypeptide, enhance the conversion of cellulose by a *T. reesei* cellulase composition.

Example 37: *Thermoascus aurantiacus* GH61A enhancement of cellulolysis of high total solids alkaline pretreated corn stover by a *Trichoderma reesei* cellulase composition

Raw, washed and milled corn stover was pretreated with 8% (w/w of dry weight) sodium hydroxide at 90°C for 1 hour. The resulting whole slurry was transferred to a vacuum filtration apparatus and washed exhaustively with tap water until the pH of the filtrate was less than or equal to 8.6. The washed solids were then transferred to hydrolysis reactors to give final solids concentrations of 10%. A composition containing a blend of an *Aspergillus aculeatus* GH10 xylanase (WO 94/021785) and a *Trichoderma reesei* cellulase preparation containing *Aspergillus fumigatus* beta-glucosidase (WO 2005/047499) and *Thermoascus aurantiacus* GH61A polypeptide (WO 2005/074656) was replaced with increasing concentrations of GH61A polypeptide, maintaining a fixed total protein concentration of 4 mg protein per gram cellulose. The washed, milled, alkaline pretreated corn stover was hydrolyzed at 50°C for 120 hours in 10-20 g Oak Ridge tubes (Nalge Nunc International Corporation, Rochester, NY, USA) containing ¼ inch steel balls for agitation, using a FINEPCR Hybridization Oven (Daigger, Inc. Vernon Hills, IL, USA), rotating at 12 rpm.

Figure 27 shows that glucose concentration increased with increasing replacement of the cellulase composition with *T. aurantiacus* GH61A polypeptide, indicating that cellulose hydrolysis was enhanced with 5-10% additional GH61A polypeptide. Thus at high total solids, supplementation of a *T. reesei* cellulase composition with higher relative concentrations of GH61 polypeptide resulted in higher cellulose conversion of alkaline pretreated corn stovers.

Example 38: *Thermoascus aurantiacus* GH61A enhancement of cellulolysis of high total solids pretreated corn stover of various pretreatment severities by a *Trichoderma reesei* cellulase composition

Raw corn stover was milled in a Thomas Model 10 Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA) with a nominal screen size of 2 mm and then thoroughly washed

with tap water. The washed corn stover was allowed to dry in a 45ºC convection oven until the dry solids content was above 90%. The dried solids were then sieved through a #40 mesh screen to ensure a uniform size distribution. An Accelerated Solvent Extractor (ASE) 350 instrument (Dionex Corporation, Bannockburn, IL, USA) was used for all pretreatments. Approximately 15.0 g of the washed, milled and sieved corn stover was packed into a 100 ml stainless steel extraction cell to ensure consistent solids loading during pretreatment. The extraction cell was loaded into the heating chamber and filled with sulfuric acid solution of various concentrations until the back-pressure reached 1500 psi. The heating chamber then heated the filled cell to the desired temperature. At the end of the heating phase, the pretreatment was stopped by immediately releasing the pressure in the extraction cell, purging with nitrogen gas, and collecting the pretreatment liquor in a designated glass vial. The extraction cell was then immediately removed from the ASE 350 instrument and quenched in ice. After cooling to room temperature, the pretreated solids were removed from the cell and re-slurried with the pretreatment liquor. The following pretreatment conditions were varied: temperature from 150-190°C, static residence times between 1 and 15 minutes, and acid concentrations between 0.4%-1.0% (w/w) H_2SO_4. The range for combined severity factor ranged from 0.5 to 2.0.

Pretreated corn stover at each pretreatment severity was adjusted to pH 5.0 and a final TS of 15%. Hydrolysis was initiated by adding to each PCS batch either 2 mg of a composition containing a blend of an *Aspergillus aculeatus* GH10 xylanase (WO 94/021785) and a *Trichoderma reesei* cellulase preparation containing *Aspergillus fumigatus* beta-glucosidase (WO 2005/047499) and *Thermoascus aurantiacus* GH61A polypeptide (WO 2005/074656) with 0.4 mg of *T. aurantiacus* GH61A polypeptide per gram cellulose or 1.6 mg of the composition containing a blend of an *Aspergillus aculeatus* GH10 xylanase (WO 94/021785) and a *Trichoderma reesei* cellulase preparation containing *Aspergillus fumigatus* beta-glucosidase (WO 2005/047499) and *Thermoascus aurantiacus* GH61A polypeptide (WO 2005/074656) with 0.4 mg of *T. aurantiacus* GH61A polypeptide per gram cellulose and incubating at 50°C for up to 216 hrs in 10-20 g Oak Ridge tubes containing ¼ inch steel balls for agitation, using a FINEPCR Hybridization Oven, rotating at 12 rpm.

Figure 28 shows the cellulose conversion of high total solids (15% dry weight) corn stover of various severity acid pretreatments. At each set of pretreatment conditions, except for a single, low severity pretreatment, replacement of the cellulase composition with 20% additional *T. aurantiacus* GH61A polypeptide resulted in greater cellulose conversion, particularly at 216 hours of hydrolysis.. Gray bars: 120 hours of saccharification; black bars: 216 hours of saccharification. Thus at high total solids, supplementation of a *T. reesei* cellulase composition with higher relative concentrations of GH61 polypeptide resulted in higher cellulose conversion when the pretreatment severity was sufficient to generate

suitable biomass liquor.

Example 39: *Thermoascus aurantiacus* GH61A enhancement of cellulolysis of high total solids pretreated giant cane (*Arundo donax*) of various pretreatment severities by a *Trichoderma reesei* cellulase composition

Raw *Arundo donax* was milled in a Thomas Model 10 Wiley Mill with a nominal screen size of 2 mm and then thoroughly washed with tap water. The washed *A. donax* biomass was allowed to dry in a 45ºC convection oven until the dry solids content was above 90%. The dried solids were then sieved through a #40 mesh screen to ensure a uniform size distribution. An Accelerated Solvent Extractor (ASE) 350 instrument was used for all pretreatments. Approximately 20.0 g of the washed, milled and sieved *Arundo donax* was packed into a 100 ml stainless steel extraction cell to ensure consistent solids loading during pretreatment. The extraction cell was loaded into the heating chamber and filled with sulfuric acid solution of various concentrations until the back-pressure reached 1500 psi. The heating chamber then heated the filled cell to the desired temperature. At the end of the heating phase, the pretreatment was stopped by immediately releasing the pressure in the extraction cell, purging with nitrogen gas and collecting the pretreatment liquor in a designated glass vial. The extraction cell was then immediately removed from the ASE 350 instrument and quenched in ice. After cooling to room temperature, the pretreated solids were removed from the cell and re-slurried with the pretreatment liquor. The following pretreatment conditions were varied: temperature from 170-190°C, static residence times between 1 and 5 minutes, and acid concentrations between 0.5%-1.0% (w/w) H_2SO_4. The range for combined severity factor ranged from 0.35 to 2.13.

Arundo donax biomass of each pretreatment severity was adjusted to pH 5.0 and a final TS of 15%. Hydrolysis was initiated by adding either 4 mg of a composition containing a blend of an *Aspergillus aculeatus* GH10 xylanase (WO 94/021785) and a *Trichoderma reesei* cellulase preparation containing *Aspergillus fumigatus* beta-glucosidase (WO 2005/047499) and *Thermoascus aurantiacus* GH61A polypeptide (WO 2005/074656) per gram cellulose or 3.4 mg of the a composition containing a blend of an *Aspergillus aculeatus* GH10 xylanase (WO 94/021785) and a *Trichoderma reesei* cellulase preparation containing *Aspergillus fumigatus* beta-glucosidase (WO 2005/047499) and *Thermoascus aurantiacus* GH61A polypeptide (WO 2005/074656) and 0.6 mg of *T. aurantiacus* GH61A per gram cellulose and incubating at 50°C for up to 120 hours in 10-20 g Oak Ridge tubes containing ¼ inch steel balls for agitation, using a FINEPCR Hybridization Oven, rotating at 12 rpm.

Figure 29 shows the cellulose conversion of high total solids (15% dry weight) *Arundo donax* of various severity acid pretreatments. At each set of pretreatment conditions, replacement of the cellulase composition with 15% additional *T. aurantiacus* GH61A

polypeptide resulted in greater cellulose conversion, particularly at 216 hours of hydrolysis. Gray bars: 72 hours of saccharification; black bars: 120 hours of saccharification. Thus at high total solids, supplementation of a *T. reesei* cellulase composition with higher relative concentrations of GH61 polypeptide resulted in higher cellulose conversion of *A. donax* biomass.

The present invention is further described by the following numbered paragraphs:

[1] A method for degrading or converting a cellulosic material, comprising: treating the cellulosic material with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity and a liquor, wherein the combination of the polypeptide having cellulolytic enhancing activity and the liquor enhances hydrolysis of the cellulosic material by the enzyme composition.

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

[3] The method of paragraph 1 or 2, further comprising recovering the degraded cellulosic material.

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

[5] The method of paragraph 4, wherein the cellulase one or more (several) enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

[6] The method of paragraph 4, wherein the hemicellulase is one or more (several) enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase.

[7] The method of any of paragraphs 1-6, wherein the degraded cellulosic material is a sugar.

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

[9] A method for producing a fermentation product, comprising:

(a) saccharifying a cellulosic material with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity and a liquor, wherein the combination of the polypeptide having cellulolytic enhancing activity and the liquor enhances hydrolysis of the cellulosic material by the enzyme composition;

(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.

[10] The method of paragraph 9, wherein the cellulosic material is pretreated.

[11] The method of paragraph 9 or 10, wherein the enzyme composition comprises one or more (several) enzymes selected from the group consisting of a cellulase, a hemicellulase, an expansin, an esterase, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.

[12] The method of paragraph 11, wherein the cellulase is one or more (several) enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

[13] The method of paragraph 11, wherein the hemicellulase is one or more (several) enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase.

[14] The method of any of paragraphs 9-13, wherein steps (a) and (b) are performed simultaneously in a simultaneous saccharification and fermentation.

[15] The method of any of paragraphs 9-14, wherein the fermentation product is an alcohol, an alkane, a cycloalkane, an alkene, an amino acid, a gas, isoprene, a ketone, an organic acid, or polyketide.

[16] 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 cellulolytic enhancing activity and a liquor, wherein the combination of the polypeptide having cellulolytic enhancing activity and the liquor enhances hydrolysis of the cellulosic material by the enzyme composition.

[17] The method of paragraph 16, wherein the cellulosic material is pretreated before saccharification.

[18] The method of paragraph 16 or 17, wherein the enzyme composition comprises one or more (several) enzymes selected from the group consisting of a cellulase, a hemicellulase, an expansin, an esterase, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.

[19] The method of paragraph 18, wherein the cellulase is one or more (several) enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

[20] The method of paragraph 18, wherein the hemicellulase is one or more (several) enzymes selected from the group consisting of a xylanase, an acetyxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase.

[21] The method of any of paragraphs 16-20, wherein the fermenting of the cellulosic material produces a fermentation product.

[22] The method of paragraph 21, further comprising recovering the fermentation

product from the fermentation.

[23] The method of any of paragraphs 16-22, wherein the fermentation product is an alcohol, an alkane, a cycloalkane, an alkene, an amino acid, a gas, isoprene, a ketone, an organic acid, or polyketide.

[24] The method of any of paragraphs 1-23, wherein the liquor is obtained from a material that is the same as the cellulosic material subjected to saccharification by the enzyme composition.

[25] The method of any of paragraphs 1-23, wherein the liquor is obtained from a material that is different than the cellulosic material subjected to saccharification by the enzyme composition.

[26] The method of any of paragraphs 1-23, wherein the liquor is obtained from a material that is the same as the cellulosic material subjected to saccharification by the enzyme composition, and the treatment conditions used to produce the liquor are different from the pretreatment conditions of the cellulosic material.

[27] The method of any of paragraphs 1-23, wherein the liquor is obtained from a material that is the same as the cellulosic material subjected to saccharification by a cellulase composition, and the treatment conditions used to produce the liquor are the same as the pretreatment conditions of the cellulosic material.

[28] The method of any of paragraphs 1-23, wherein the liquor is obtained from a material that is the same as the cellulosic material subjected to saccharification by the enzyme composition, and the treatment conditions used to produce the liquor are the same as the pretreatment conditions of the cellulosic material, and the liquor is further processed to remove cellulose inhibitors.

[39] The method of any of paragraphs 1-23, wherein the liquor is obtained from a material that is different than the cellulosic material subjected to saccharification by the enzyme composition, and the treatment conditions used to produce the liquor are different from the pretreatment conditions of the cellulosic material.

[30] The method of any of paragraphs 1-23, wherein the liquor is obtained from a material that is different than the cellulosic material subjected to saccharification by the enzyme composition, and the treatment conditions used to produce the liquor are the same as the pretreatment conditions of the cellulosic material.

[31] The method of any of paragraphs 1-23, wherein the liquor is obtained from a material that is different than the cellulosic material subjected to saccharification by the enzyme composition, and the treatment conditions used to produce the liquor are the same as the pretreatment conditions of the cellulosic material, and the liquor is further processed to remove cellulose inhibitors.

[32] The method of any of paragraphs 1-31, wherein the liquor optimizes the

cellulolytic enhancing activity of a GH61 polypeptide with a *GH61 effect* of preferably at least 1.05, more preferably at least 1.10, more preferably at least 1.15, more prefereably at least 1.2, more preferably at least 1.25, more preferably at least 1.3, more preferably at least 1.35, more preferably at least 1.4, more preferably at least 1.45, more preferably at least 1.5, more preferably at least 1.55, more preferably at least 1.6, more preferably at least 1.65, more preferably at least 1.7, more preferably at least 1.75, more preferably at least 1.8, more preferably at least 1.85, most preferably at least 1.9, most preferably at least 1.95, and even most preferably at least 2..

[33] The method of any of paragraphs 1-31, wherein an effective amount of the liquor to cellulose is about 10^{-6} to about 10 g per g of cellulose, *e.g.*, about 10^{-6} to about 7.5 g, about 10^{-6} to about 5, about 10^{-6} to about 2.5 g, about 10^{-6} to about 1 g, about 10^{-5} to about 1 g, about 10^{-5} to about 10^{-1} g, about 10^{-4} to about 10^{-1} g, about 10^{-3} to about 10^{-1} g, or about 10^{-3} to about 10^{-2} g per g of cellulose.

[34] The method of any of paragraphs 1-31, wherein the liquor is present in an amount that minimizes inhibition of a cellulase composition of about 1 to about 20% (v/v), *e.g.*, about 1 to about 15%, about 1 to about 10%, about 2 to about 7%, about 2 to about 5%, or about 3 to about 5%.

[35] The method of any of paragraphs 1-31, wherein the liquor is obtained from a lignocellulose material, a hemicellulose material, a lignacious material, monosaccharides of the lignocellulose material, monosaccharides of the hemicellulose material, or a combination thereof

[36] The method of any of paragraphs 1-35, wherein the liquor is further processed to remove inhibitors of a cellulase, a hemicellulase, or a combination thereof.

[37] An isolated liquor, which in combination with a polypeptide having cellulolytic enhancing activity enhances hydrolysis of a cellulosic material by a cellulolytic enzyme.

[38] A composition comprising a polypeptide having cellulolytic enhancing activity and a liquor, wherein the combination of the polypeptide having cellulolytic enhancing activity and the liquor enhances hydrolysis of a cellulosic material by a cellulolytic enzyme.

[39] The composition of paragraph 38, which further comprises one or more (several) enzymes selected from the group consisting of a cellulase, a hemicellulase, an esterase, an expansin, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.

[40] The composition of paragraph 39, wherein the cellulase one or more (several) enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

[41] The composition of paragraph 39, wherein the hemicellulase is one or more (several) enzymes selected from the group consisting of a xylanase, an acetyxylan esterase,

a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase.

[42] The composition of any of paragraphs 38-41, wherein the liquor optimizes the cellulolytic enhancing activity of a GH61 polypeptide with a *GH61 effect* of preferably at least 1.05, more preferably at least 1.10, more preferably at least 1.15, more prefereably at least 1.2, more preferably at least 1.25, more preferably at least 1.3, more preferably at least 1.35, more preferably at least 1.4, more preferably at least 1.45, more preferably at least 1.5, more preferably at least 1.55, more preferably at least 1.6, more preferably at least 1.65, more preferably at least 1.7, more preferably at least 1.75, more preferably at least 1.8, more preferably at least 1.85, most preferably at least 1.9, most preferably at least 1.95, and even most preferably at least 2.

[43] The composition of any of paragraphs 38-41, wherein an effective amount of the liquor to cellulose is about 10^{-6} to about 10 g per g of cellulose, *e.g.*, about 10^{-6} to about 7.5 g, about 10^{-6} to about 5, about 10^{-6} to about 2.5 g, about 10^{-6} to about 1 g, about 10^{-5} to about 1 g, about 10^{-5} to about 10^{-1} g, about 10^{-4} to about 10^{-1} g, about 10^{-3} to about 10^{-1} g, or about 10^{-3} to about 10^{-2} g per g of cellulose.

[44] The composition of any of paragraphs 38-41, wherein the liquor is obtained from a lignocellulose material, a hemicellulose material, a lignacious material, monosaccharides of the lignocellulose material, monosaccharides of the hemicellulose material, or a combination thereof.

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

Claims

What is claimed is:

1. A method for degrading or converting a cellulosic material, comprising: treating the cellulosic material with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity and a liquor, wherein the combination of the polypeptide having cellulolytic enhancing activity and the liquor enhances hydrolysis of the cellulosic material by the enzyme composition.

2. The method of claim 1, further comprising recovering the degraded cellulosic material.

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

(a) saccharifying a cellulosic material with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity and a liquor, wherein the combination of the polypeptide having cellulolytic enhancing activity and the liquor enhances hydrolysis of the cellulosic material by the enzyme composition;

(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.

4. 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 cellulolytic enhancing activity and a liquor, wherein the combination of the polypeptide having cellulolytic enhancing activity and the liquor enhances hydrolysis of the cellulosic material by the enzyme composition.

5. The method of claim 4, wherein the fermenting of the cellulosic material produces a fermentation product.

6. The method of claim 5, further comprising recovering the fermentation product from the fermentation.

7. The method of any of claims 1-6, wherein the liquor is obtained from a material that is the same as the cellulosic material subjected to saccharification by the enzyme composition.

8. The method of any of claims 1-6, wherein the liquor is obtained from a material that is

different than the cellulosic material subjected to saccharification by the enzyme composition.

9. The method of any of claims 1-6, wherein the liquor is obtained from a material that is the same as the cellulosic material subjected to saccharification by the enzyme composition, and the treatment conditions used to produce the liquor are different from the pretreatment conditions of the cellulosic material.

10. The method of any of claims 1-6, wherein the liquor is obtained from a material that is the same as the cellulosic material subjected to saccharification by a cellulase composition, and the treatment conditions used to produce the liquor are the same as the pretreatment conditions of the cellulosic material.

11. The method of any of claims 1-6, wherein the liquor is obtained from a material that is the same as the cellulosic material subjected to saccharification by the enzyme composition, and the treatment conditions used to produce the liquor are the same as the pretreatment conditions of the cellulosic material, and the liquor is further processed to remove cellulose inhibitors.

12. The method of any of claims 1-6, wherein the liquor is obtained from a material that is different than the cellulosic material subjected to saccharification by the enzyme composition, and the treatment conditions used to produce the liquor are different from the pretreatment conditions of the cellulosic material.

13. The method of any of claims 1-6, wherein the liquor is obtained from a material that is different than the cellulosic material subjected to saccharification by the enzyme composition, and the treatment conditions used to produce the liquor are the same as the pretreatment conditions of the cellulosic material.

14. The method of any of claims 1-6, wherein the liquor is obtained from a material that is different than the cellulosic material subjected to saccharification by the enzyme composition, and the treatment conditions used to produce the liquor are the same as the pretreatment conditions of the cellulosic material, and the liquor is further processed to remove cellulose inhibitors.

15. The method of any of claims 1-14, wherein the liquor is obtained from a lignocellulose material, a hemicellulose material, a lignacious material, monosaccharides of the lignocellulose material, monosaccharides of the hemicellulose material, or a combination thereof.

16. The method of any of claims 1-15, wherein the liquor is further processed to remove inhibitors of a cellulase, a hemicellulase, or a combination thereof.

17. The method of any of claims 1-16, wherein the enzyme composition comprises one or more enzymes selected from the group consisting of a cellulase, a hemicellulase, an esterase, an expansin, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.

18. An isolated liquor, which in combination with a polypeptide having cellulolytic enhancing activity enhances hydrolysis of a cellulosic material by a cellulolytic enzyme.

19. A composition comprising a polypeptide having cellulolytic enhancing activity and a liquor, wherein the combination of the polypeptide having cellulolytic enhancing activity and the liquor enhances hydrolysis of a cellulosic material by a cellulolytic enzyme.

20. The composition of claim 19, which further comprises one or more enzymes selected from the group consisting of a cellulase, a hemicellulase, an esterase, an expansin, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.

21. The composition of claim 19 or 20, wherein the liquor is obtained from a lignocellulose material, a hemicellulose material, a lignacious material, monosaccharides of the lignocellulose material, monosaccharides of the hemicellulose material, or a combination thereof.

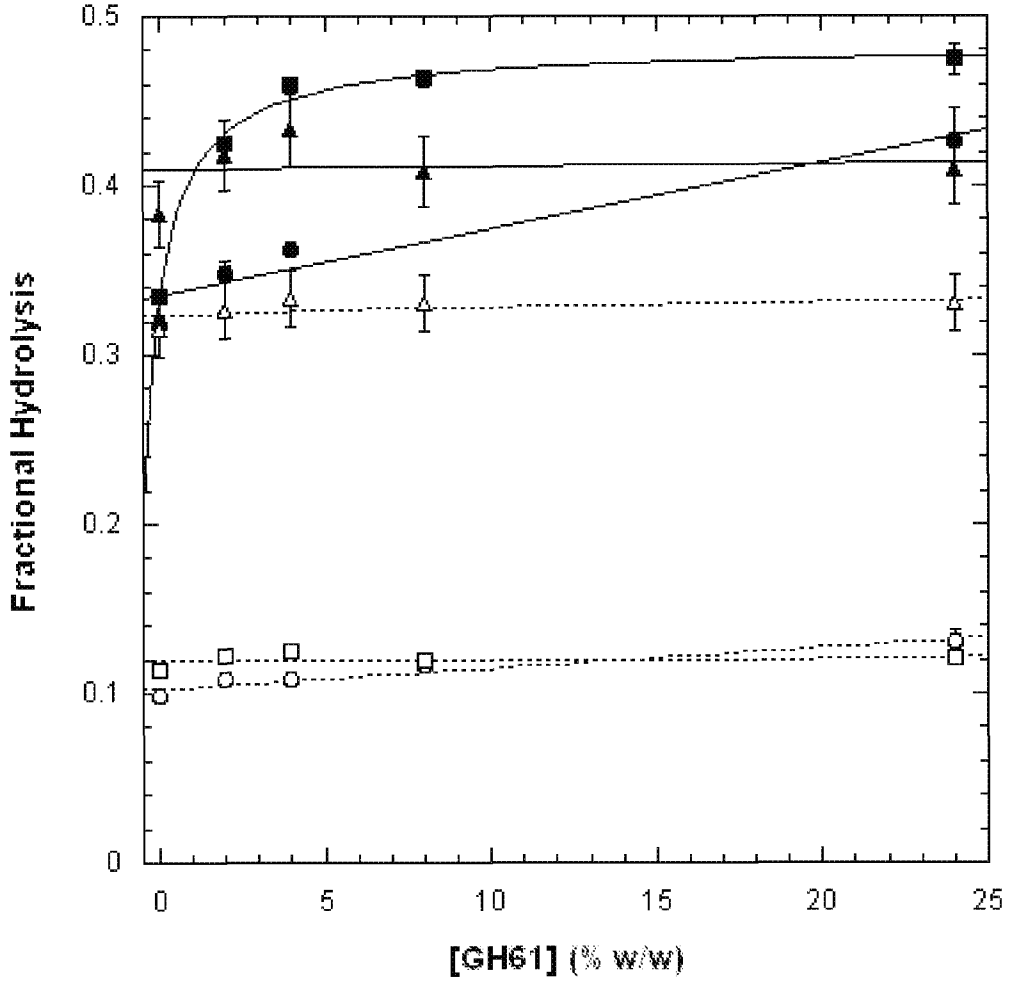

Fig. 1

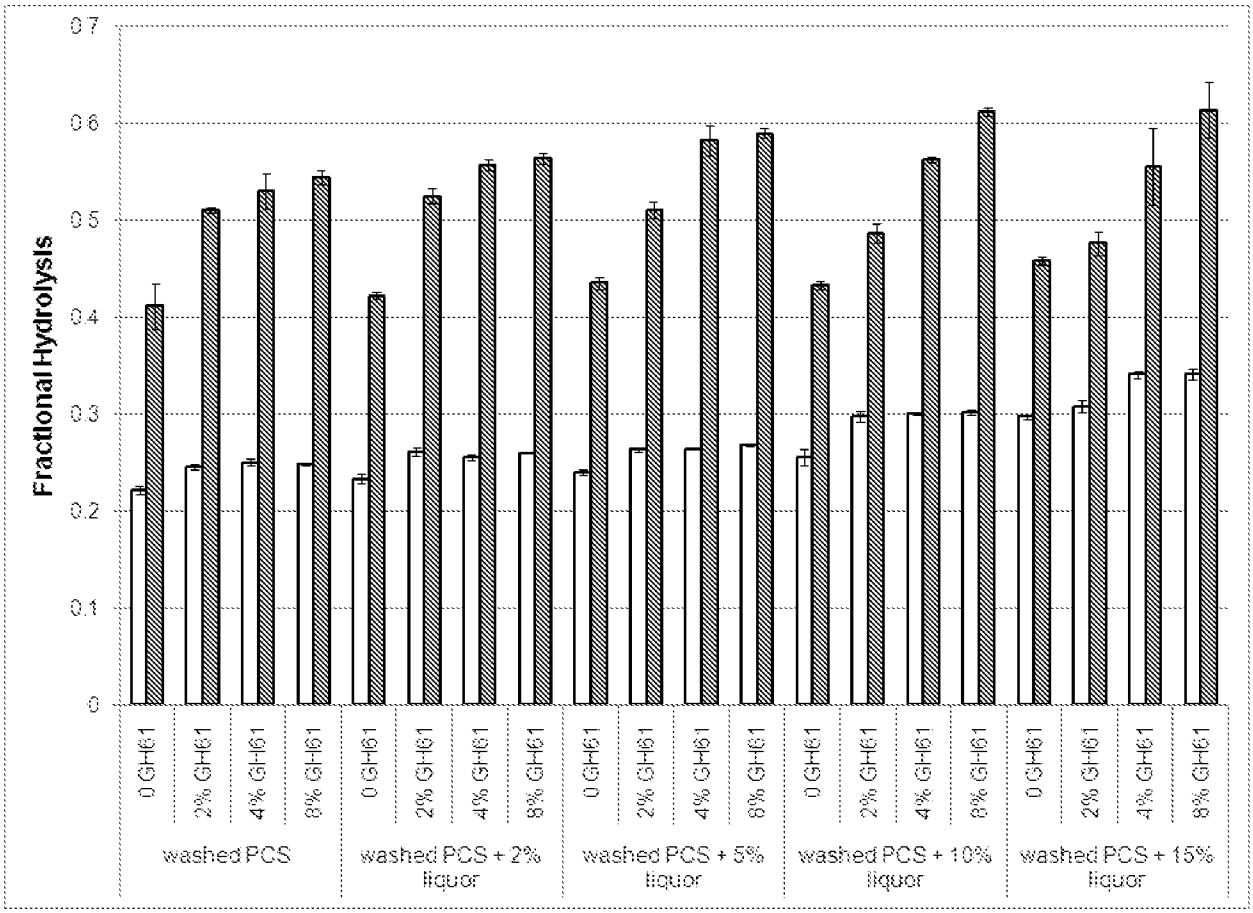
Fractional Hydrolysis
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 GH61
2% GH61
4% GH61
8% GH61
washed PCS
washed PCS + 2% liquor
washed PCS + 5% liquor
washed PCS + 10% liquor
washed PCS + 15% liquor

Fig. 2A

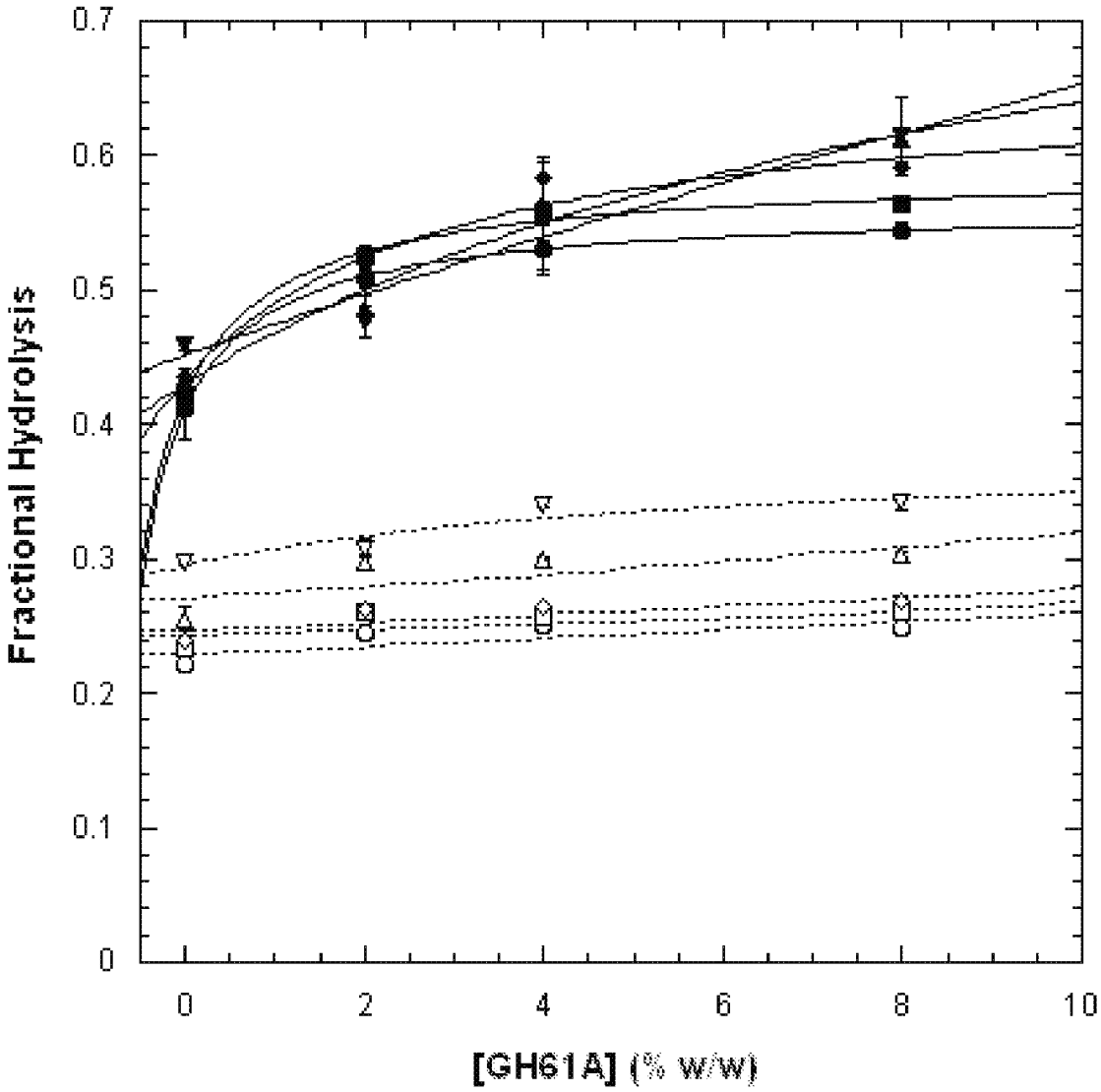

Fig. 2B

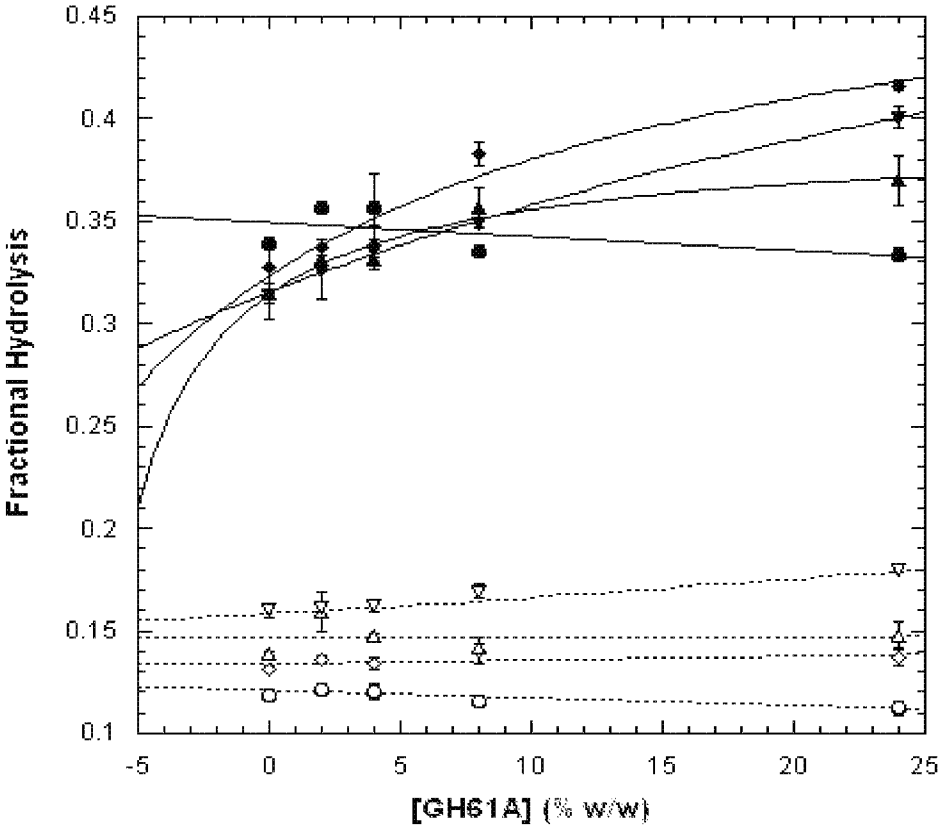
Fractional Hydrolysis
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
-5
0
5
10
15
20
25
[GH61A] (% w/w)

Fig. 3A

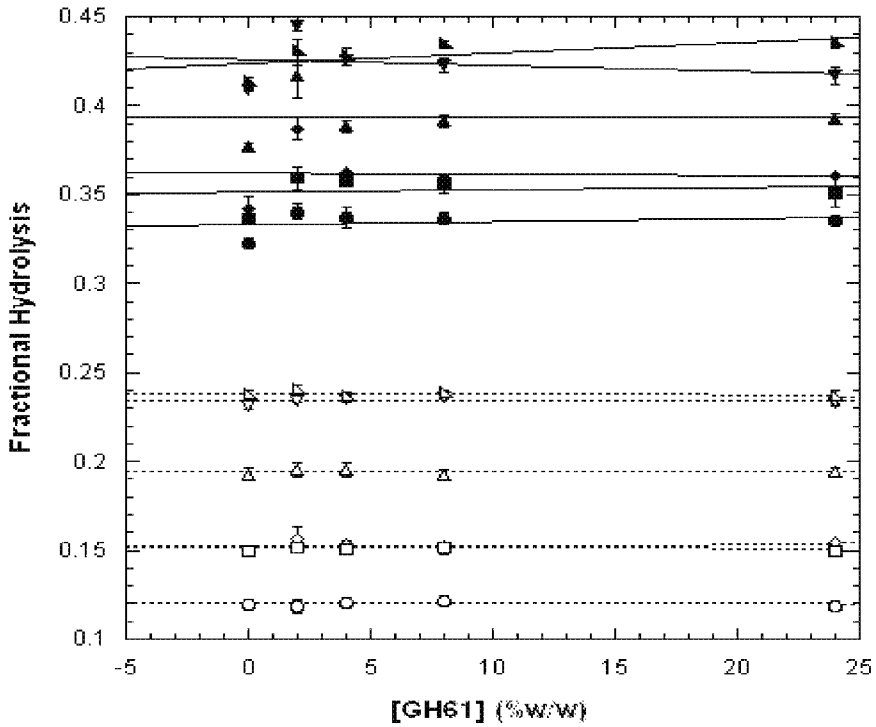
Fractional Hydrolysis
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
-5
0
5
10
15
20
25
[GH61] (%w/w)

Fig. 3B

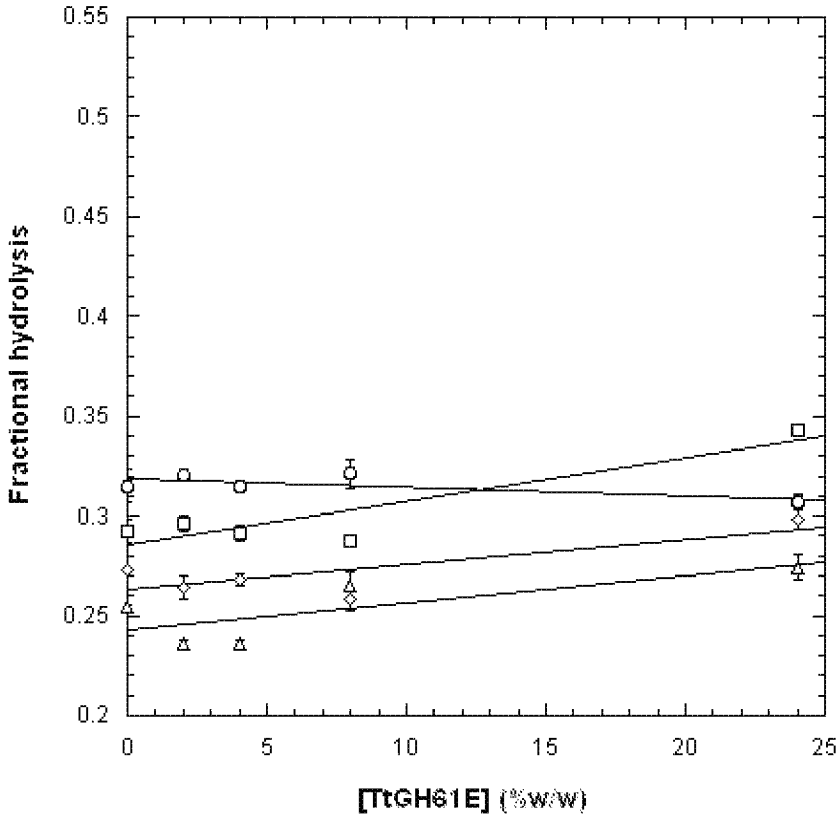
Fractional hydrolysis
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0
5
10
15
20
25
[TtGH61E] (%w/w)

Fig. 4A

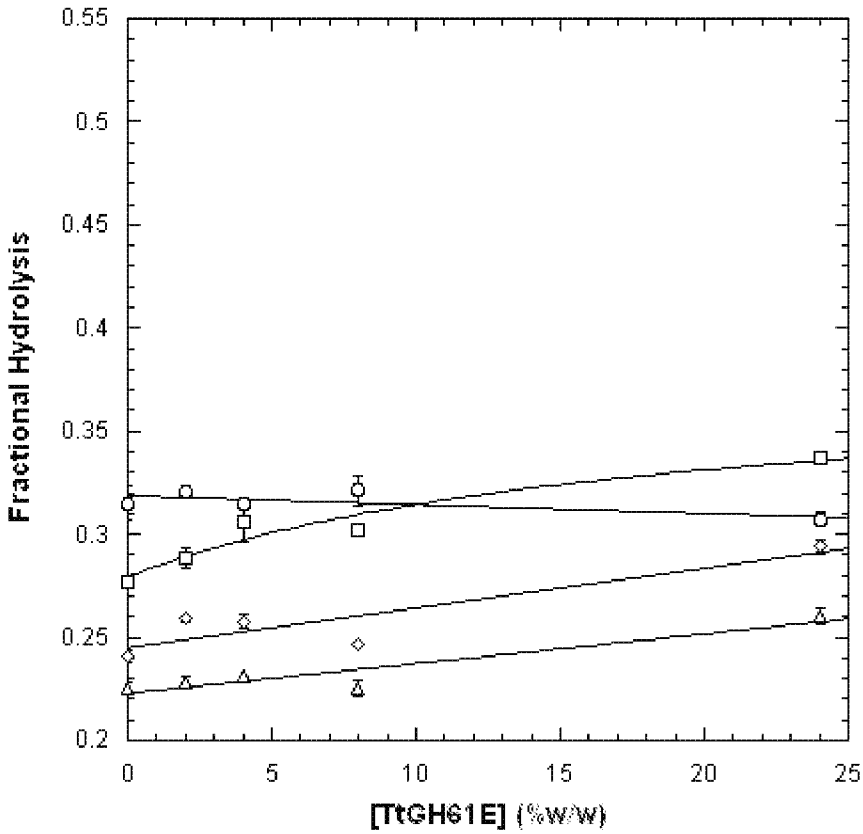
Fractional Hydrolysis
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0
5
10
15
20
25
[TtGH61E] (%w/w)

Fig. 4B

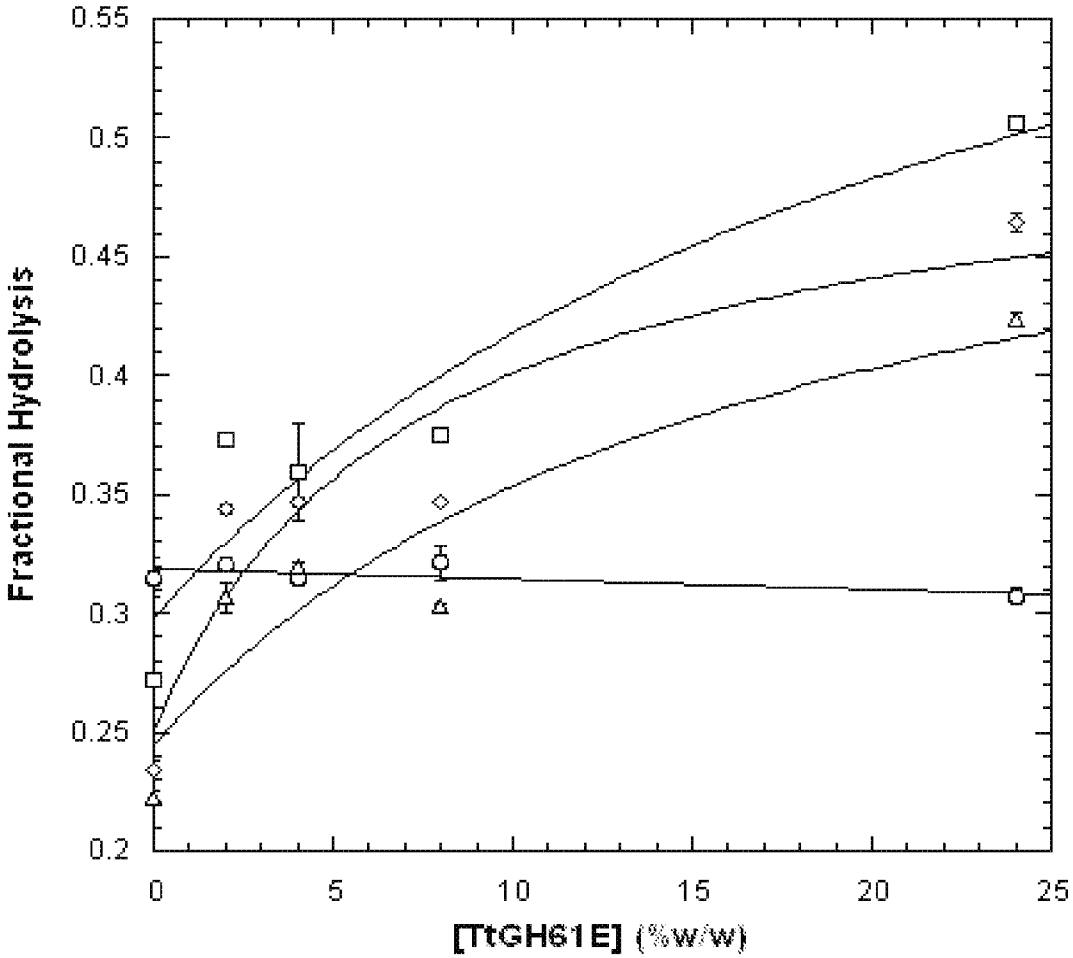
Fractional Hydrolysis
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0
5
10
15
20
25
[TtGH61E] (%w/w)

Fig. 4C

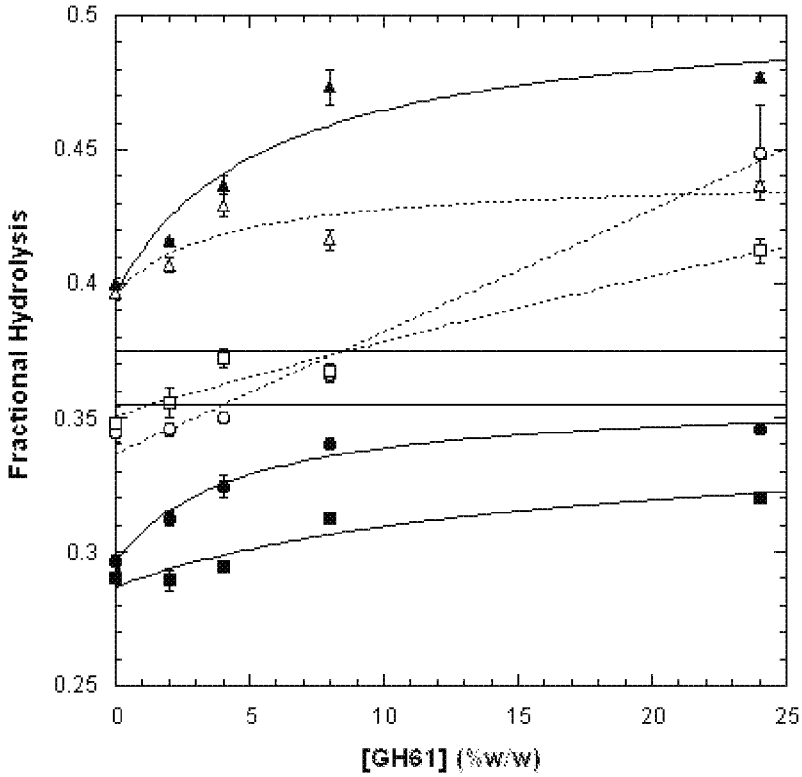
Fractional Hydrolysis
0.5
0.45
0.4
0.35
0.3
0.25
0
5
10
15
20
25
[GH61] (%w/w)

Fig. 5A

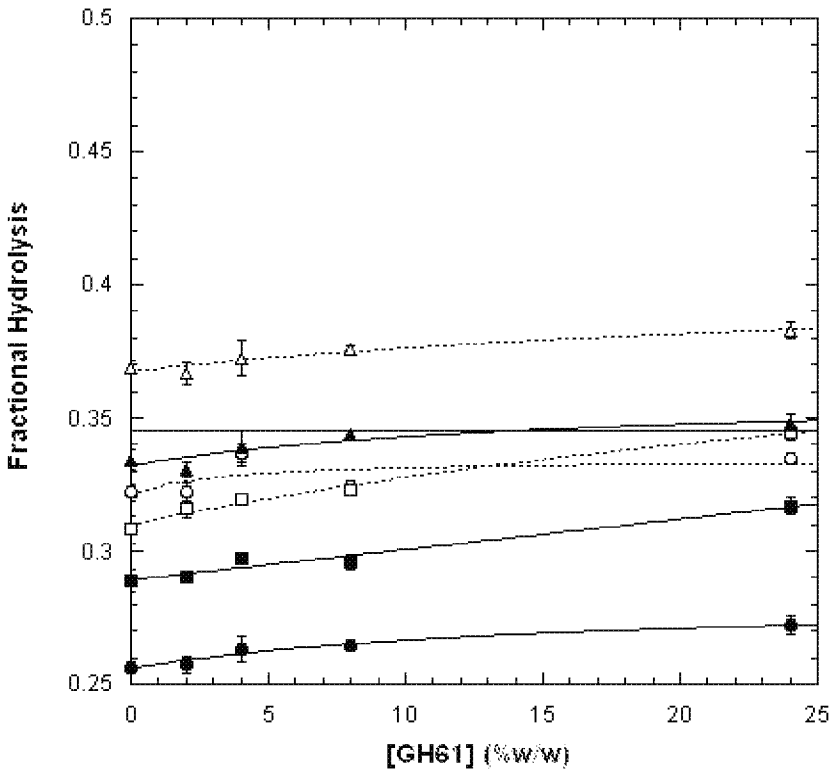
Fractional Hydrolysis
0.5
0.45
0.4
0.35
0.3
0.25
0
5
10
15
20
25
[GH61] (%w/w)

Fig. 5B

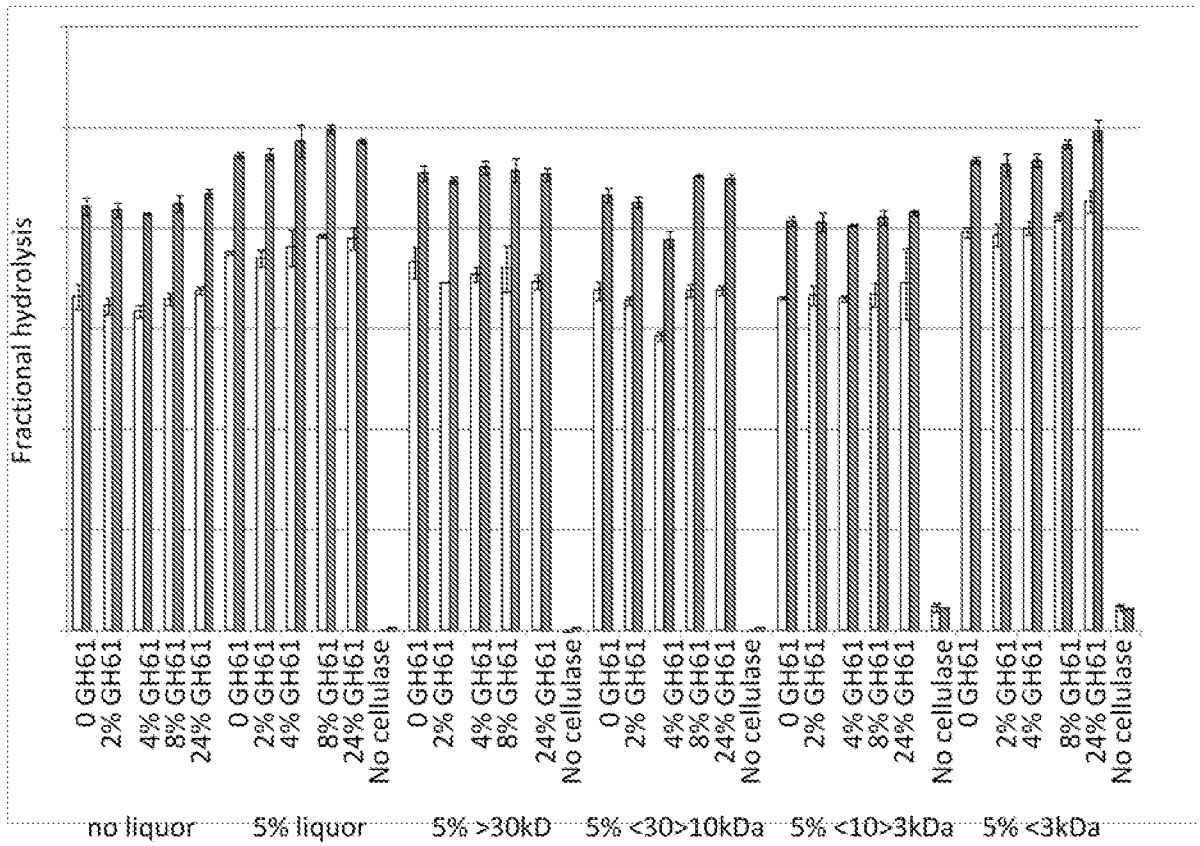
Fractional hydrolysis
0 GH61
2% GH61
4% GH61
8% GH61
24% GH61
No cellulase
no liquor
5% liquor
5% >30kD
5% <30>10kDa
5% <10>3kDa
5% <3kDa

Fig. 6

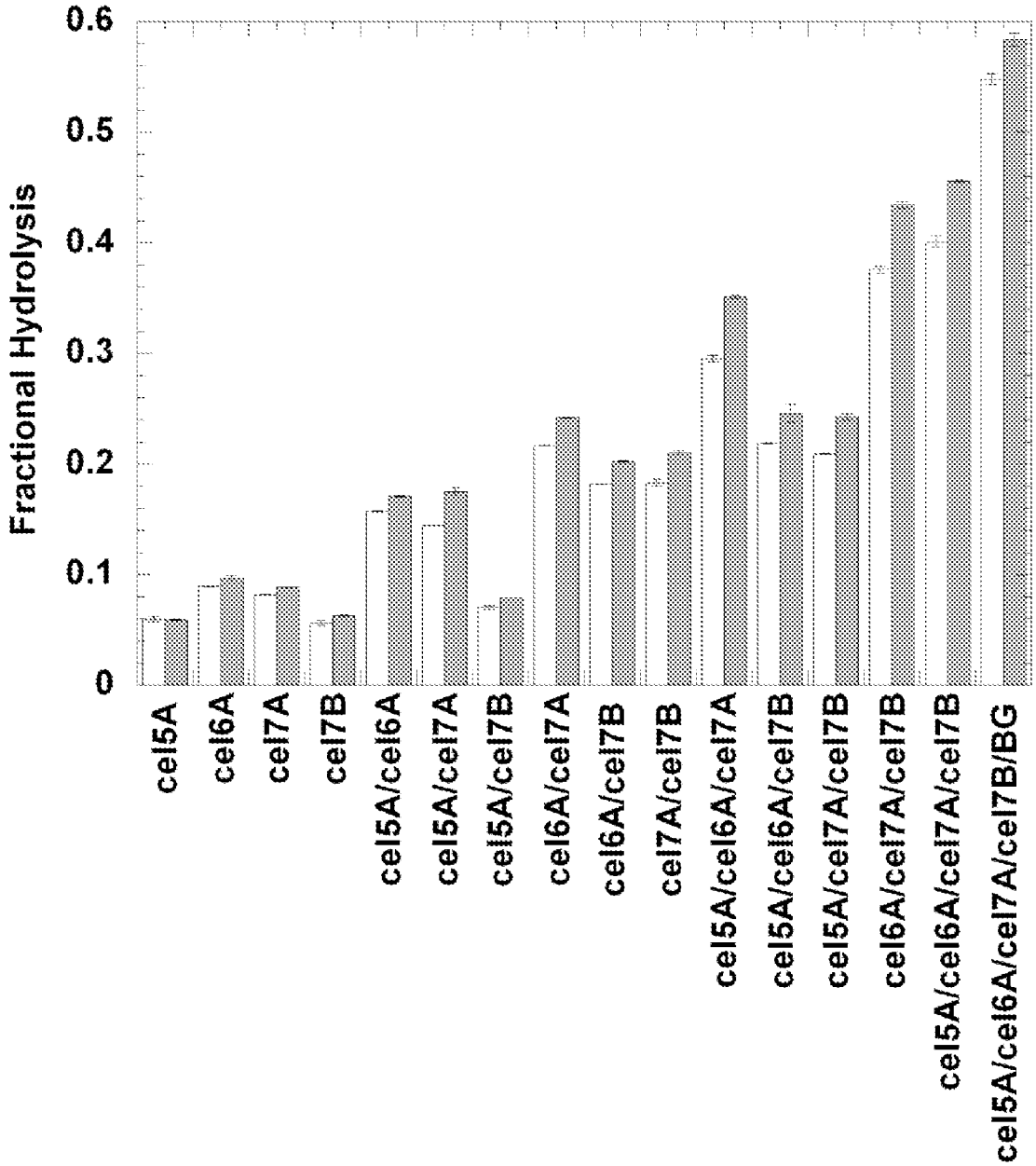
Fractional Hydrolysis
0.6
0.5
0.4
0.3
0.2
0.1
0
cel5A
cel6A
cel7A
cel7B
cel5A/cel6A
cel5A/cel7A
cel5A/cel7B
cel6A/cel7A
cel6A/cel7B
cel7A/cel7B
cel5A/cel6A/cel7A
cel5A/cel6A/cel7B
cel5A/cel7A/cel7B
cel6A/cel7A/cel7B
cel5A/cel6A/cel7A/cel7B
cel5A/cel6A/cel7A/cel7B/BG

Fig. 7A

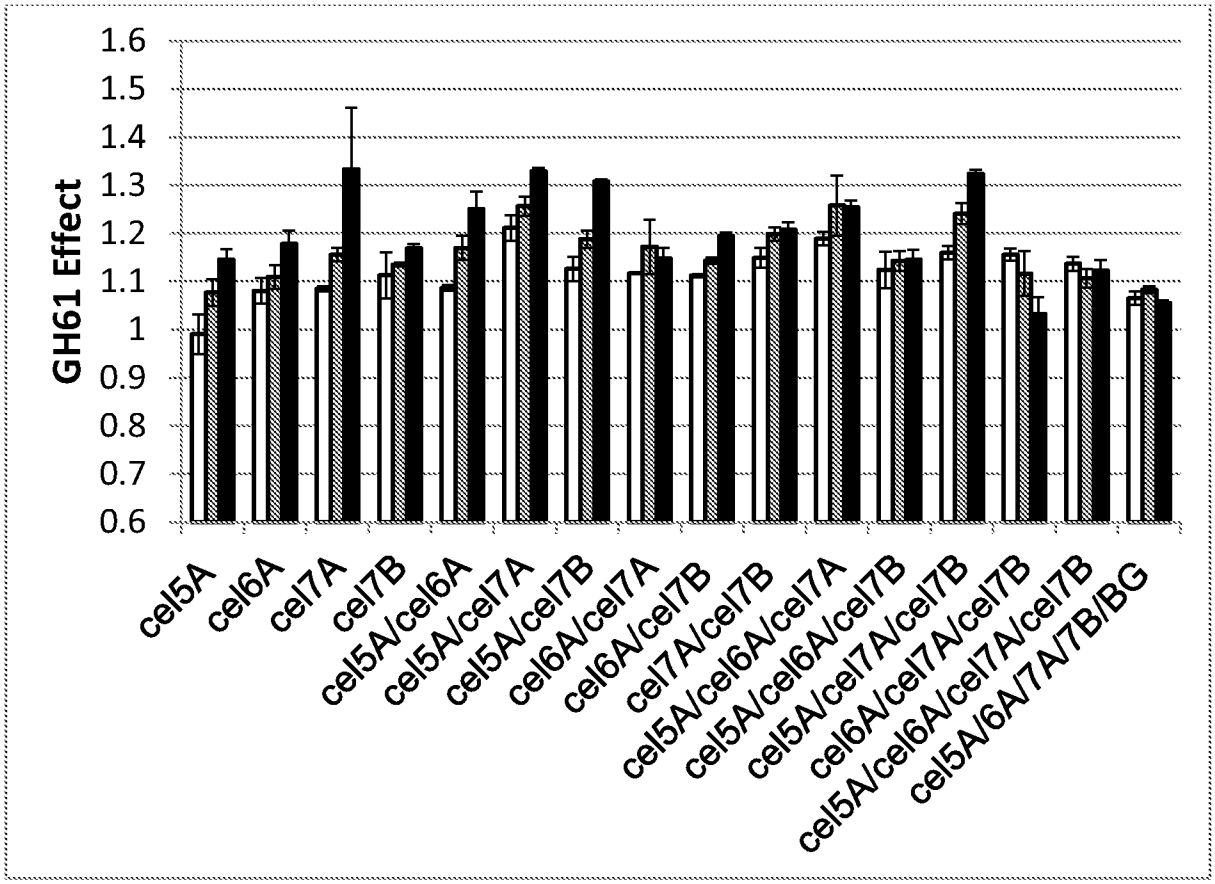
GH61 Effect
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
cel5A
cel6A
cel7A
cel7B
cel5A/cel6A
cel5A/cel7A
cel5A/cel7B
cel6A/cel7A
cel6A/cel7B
cel7A/cel7B
cel5A/cel6A/cel7A
cel5A/cel6A/cel7B
cel5A/cel7A/cel7B
cel6A/cel7A/cel7B
cel5A/cel6A/cel7A/cel7B
cel5A/6A/7A/7B/BG

Fig. 7B

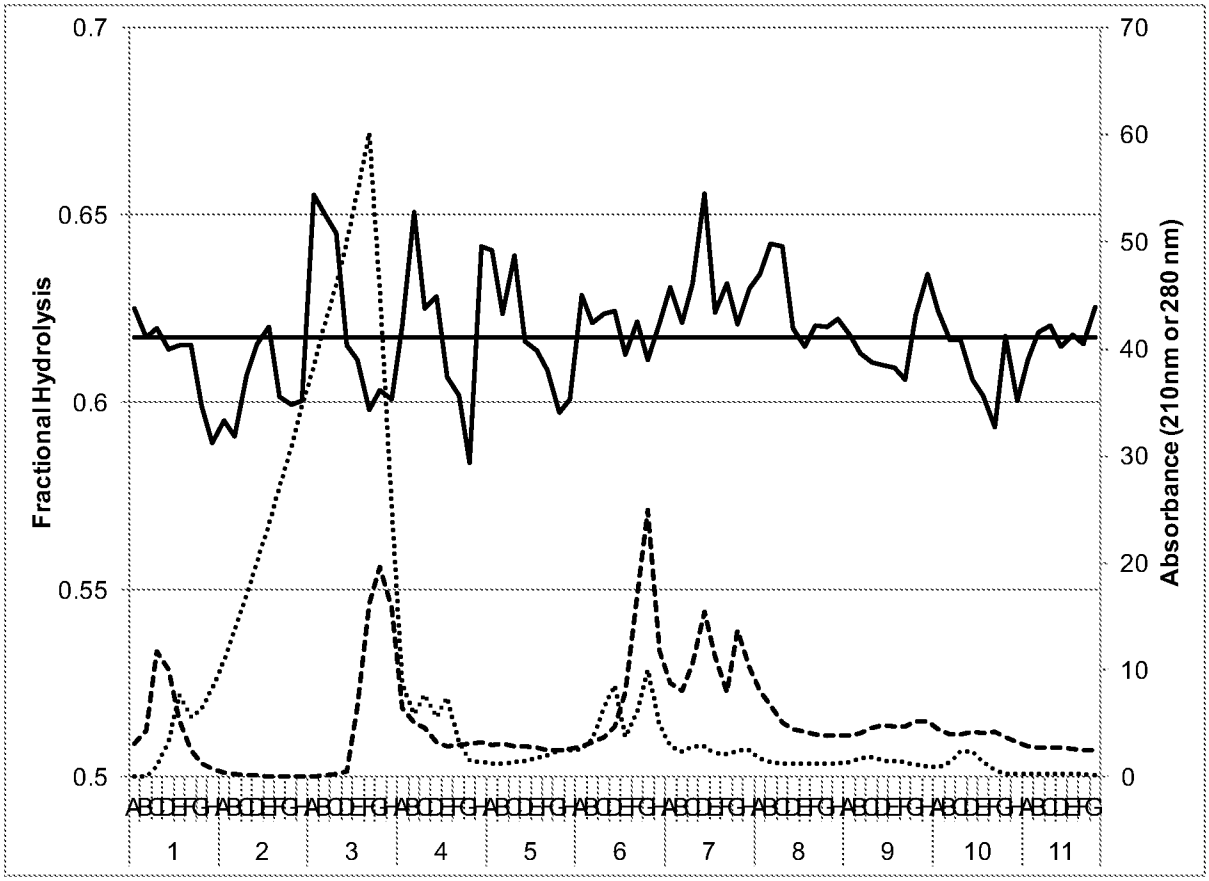
0.7
0.65
0.6
0.55
0.5
Fractional Hydrolysis
70
60
50
40
30
20
10
0
Absorbance (210nm or 280 nm)
ABCDEFG
1
2
3
4
5
6
7
8
9
10
11

Fig. 8A

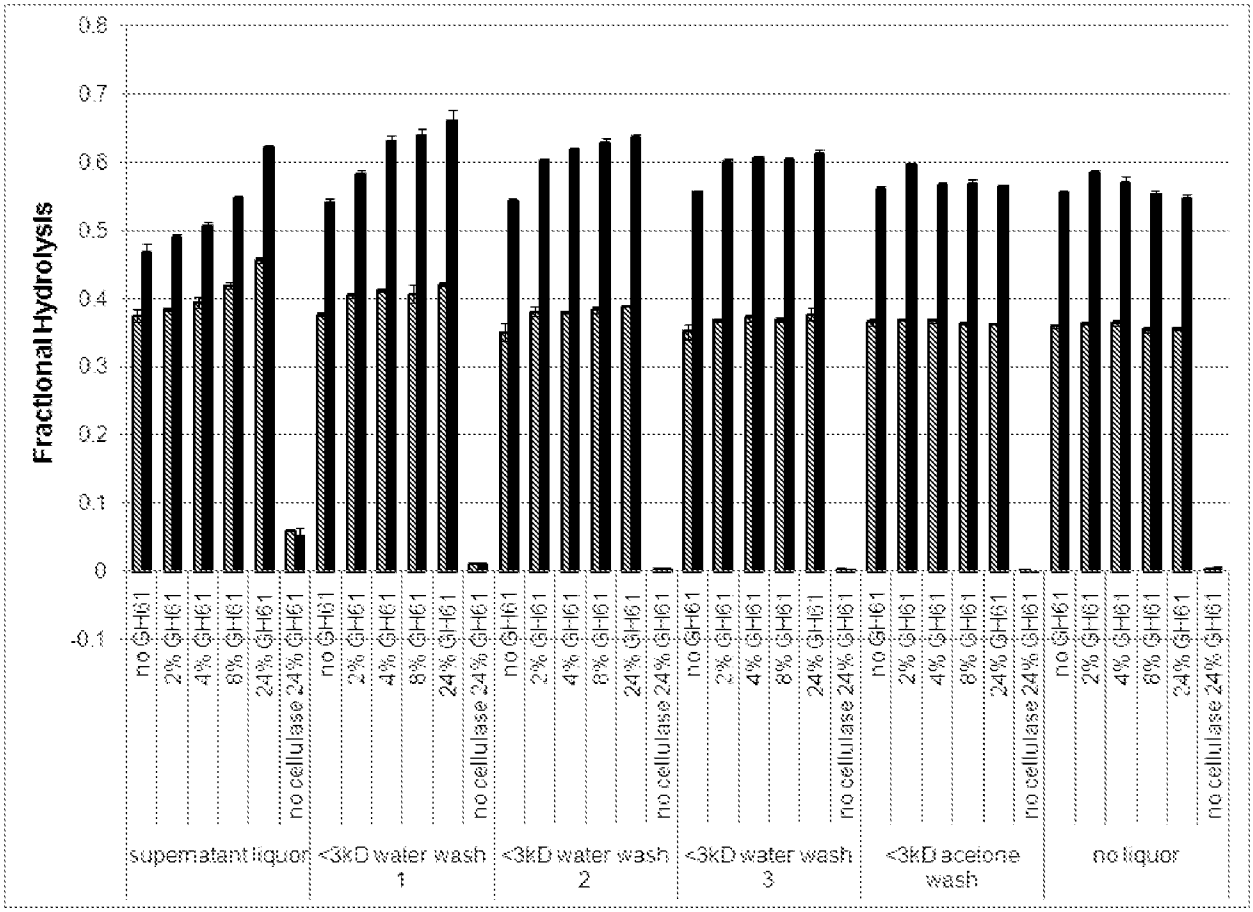
Fractional Hydrolysis
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
no GH61
2% GH61
4% GH61
8% GH61
24% GH61
no cellulase 24% GH61
supernatant liquor
<3kD water wash 1
<3kD water wash 2
<3kD water wash 3
<3kD acetone wash
no liquor

Fig. 8B

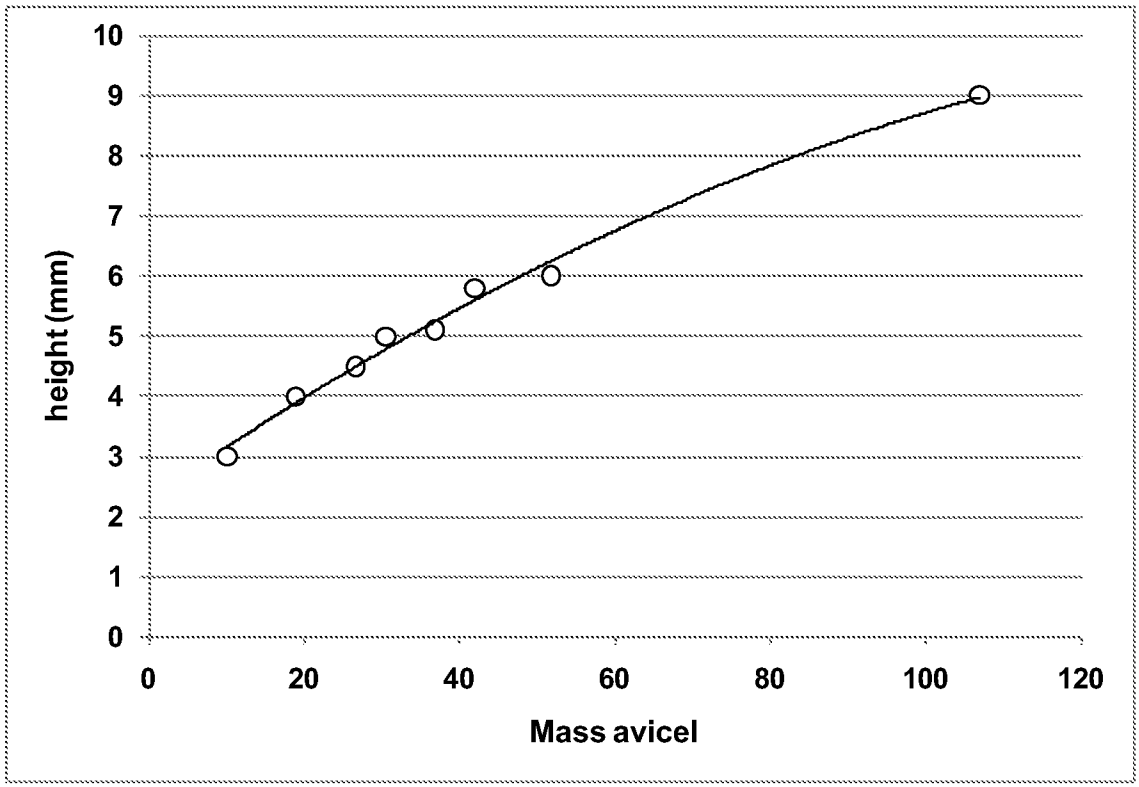

Fig. 9

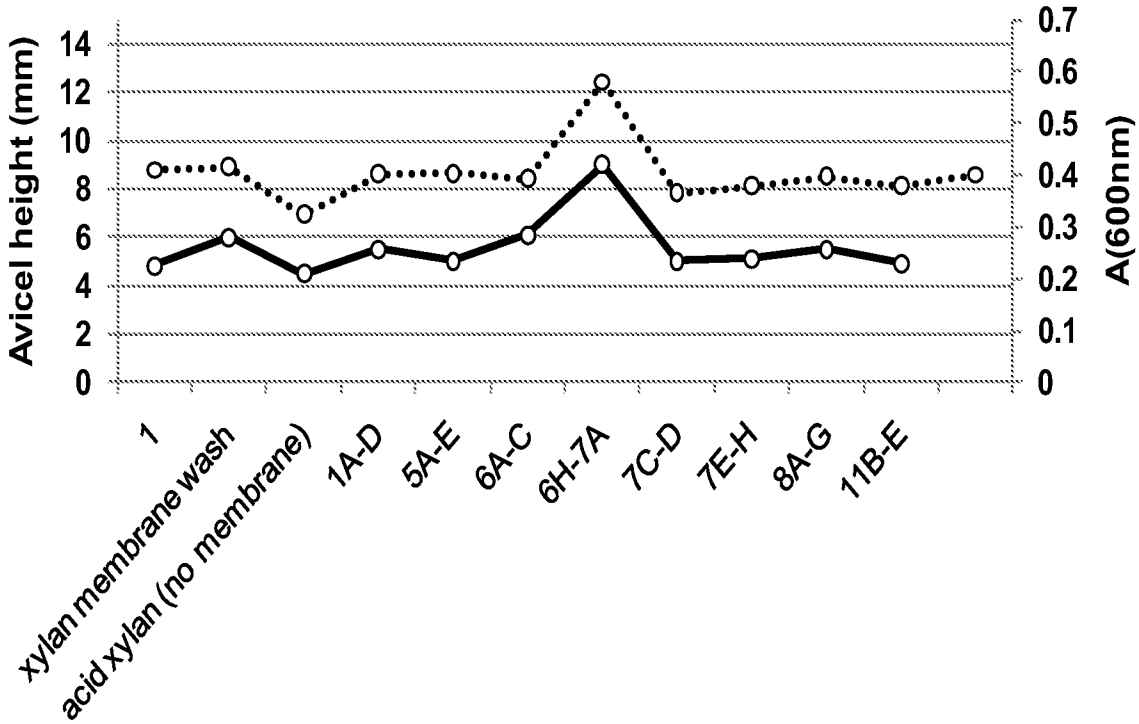
Avicel height (mm)
A(600nm)
0
2
4
6
8
10
12
14
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
1
xylan membrane wash
acid xylan (no membrane)
1A-D
5A-E
6A-C
6H-7A
7C-D
7E-H
8A-G
11B-E

Fig. 10

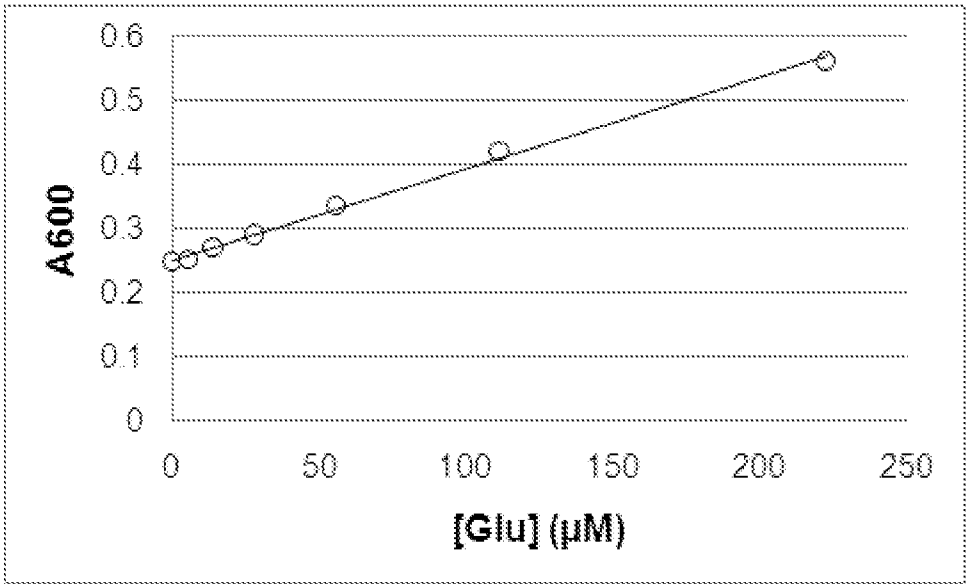

Fig. 11A

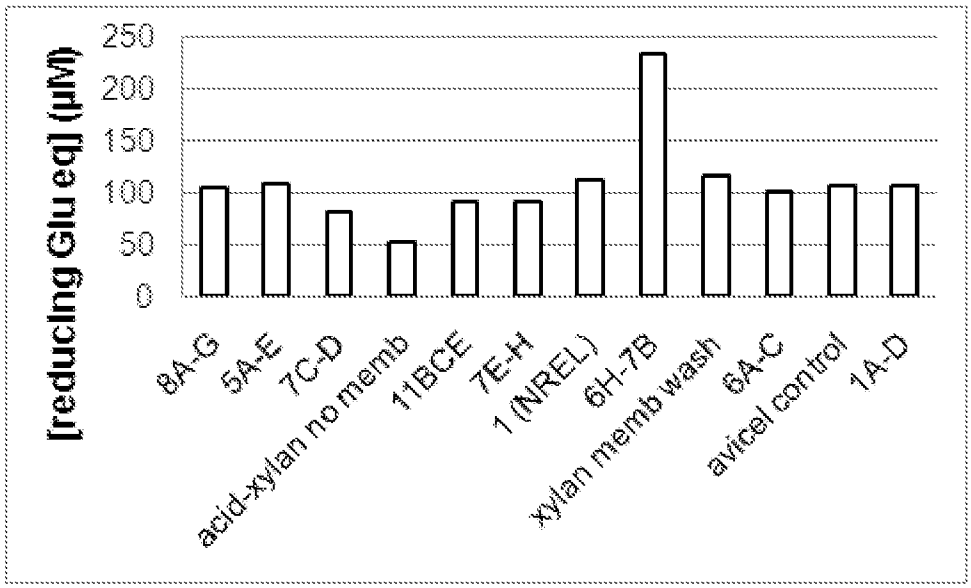

Fig. 11B

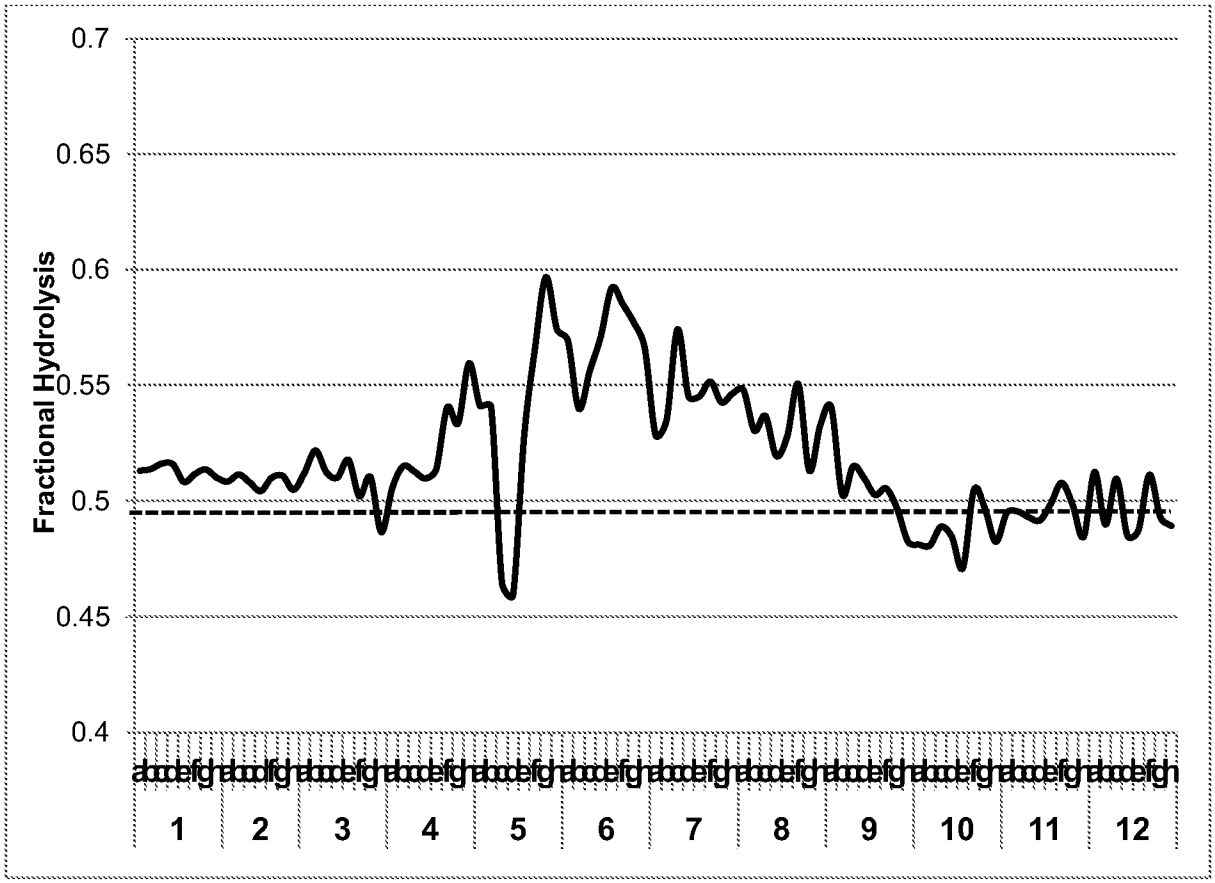
0.7
0.65
0.6
0.55
0.5
0.45
0.4
Fractional Hydrolysis
abcdefgh
1
2
3
4
5
6
7
8
9
10
11
12

Fig. 12

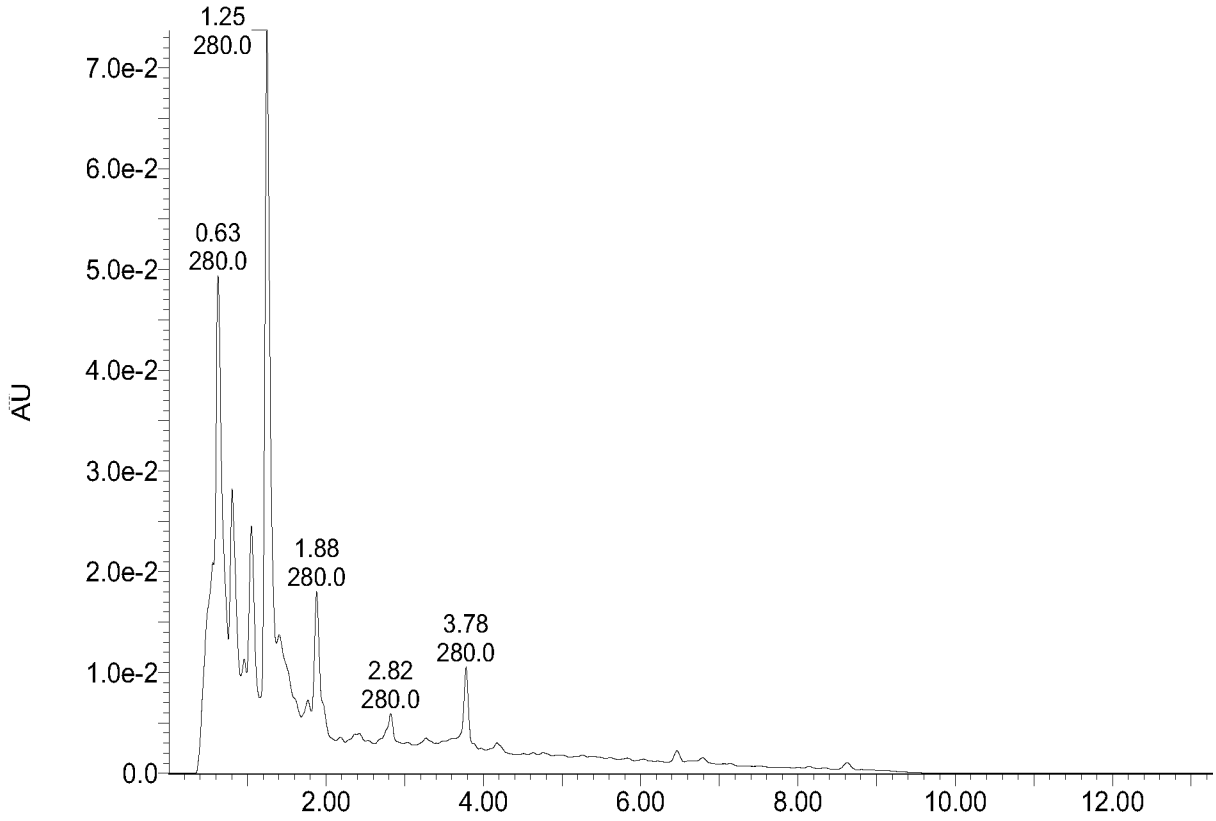
1.25
280.0
0.63
280.0
1.88
280.0
2.82
280.0
3.78
280.0
AU
7.0e-2
6.0e-2
5.0e-2
4.0e-2
3.0e-2
2.0e-2
1.0e-2
0.0
2.00
4.00
6.00
8.00
10.00
12.00

Fig. 13

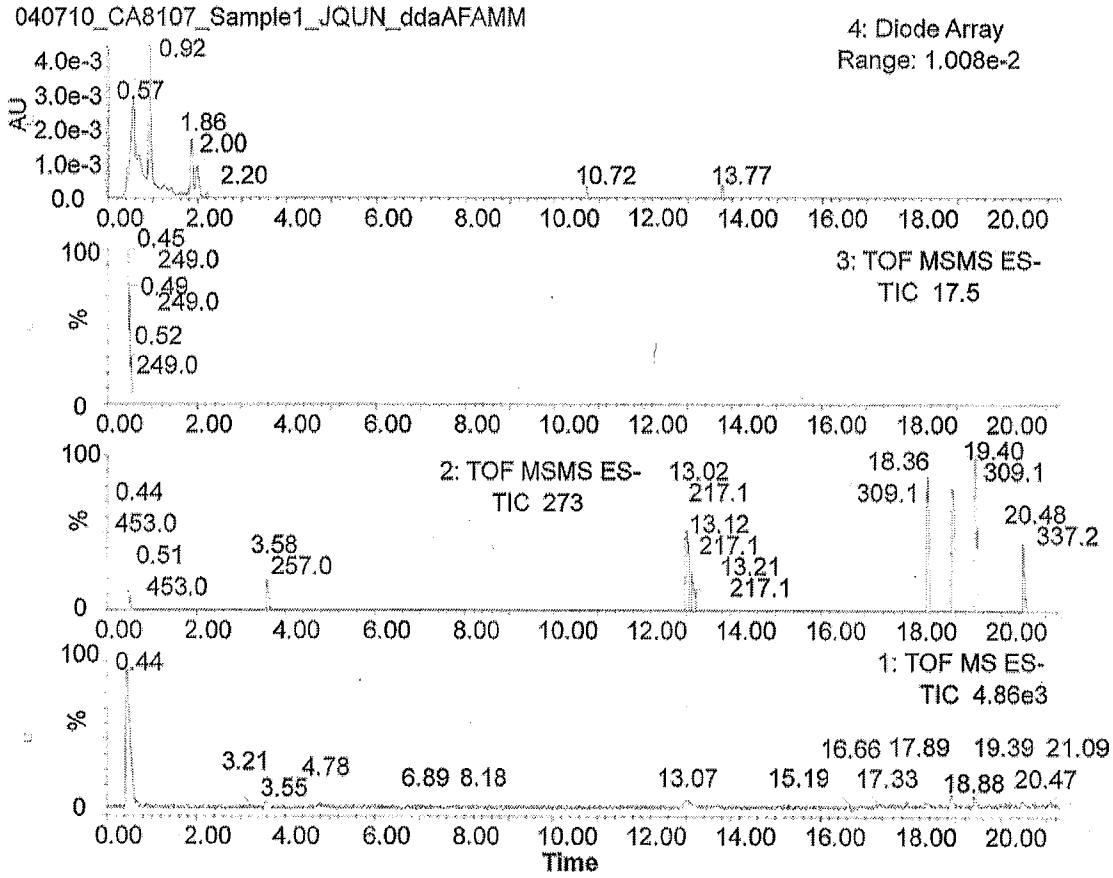
040710_CA8107_Sample1_JQUN_ddaAFAMM
4: Diode Array
Range: 1.008e-2
AU
4.0e-3
3.0e-3
2.0e-3
1.0e-3
0.0
0.92
0.57
1.86
2.00
2.20
10.72
13.77
0.00 2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00 20.00
100
%
0
0.45
249.0
0.49
249.0
0.52
249.0
3: TOF MSMS ES-
TIC 17.5
2: TOF MSMS ES-
TIC 273
0.44
453.0
0.51
453.0
3.58
257.0
13.02
217.1
13.12
217.1
13.21
217.1
18.36
309.1
19.40
309.1
20.48
337.2
1: TOF MS ES-
TIC 4.86e3
0.44
3.21
3.55
4.78
6.89
8.18
13.07
15.19
16.66
17.33
17.89
18.88
19.39
20.47
21.09
Time

Fig. 14

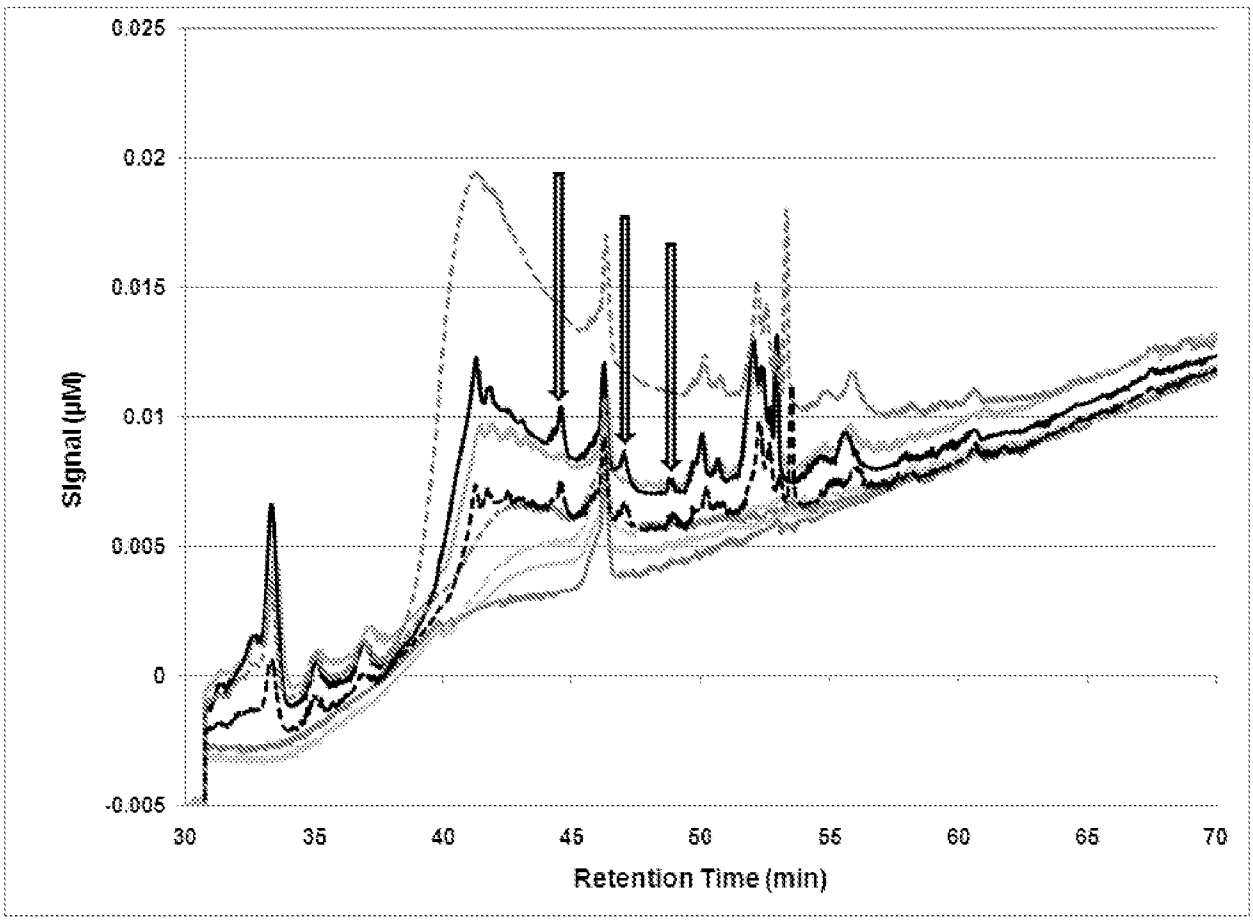
0.025
0.02
0.015
0.01
0.005
0
-0.005
Signal (μM)
30
35
40
45
50
55
60
65
70
Retention Time (min)

Fig. 15A

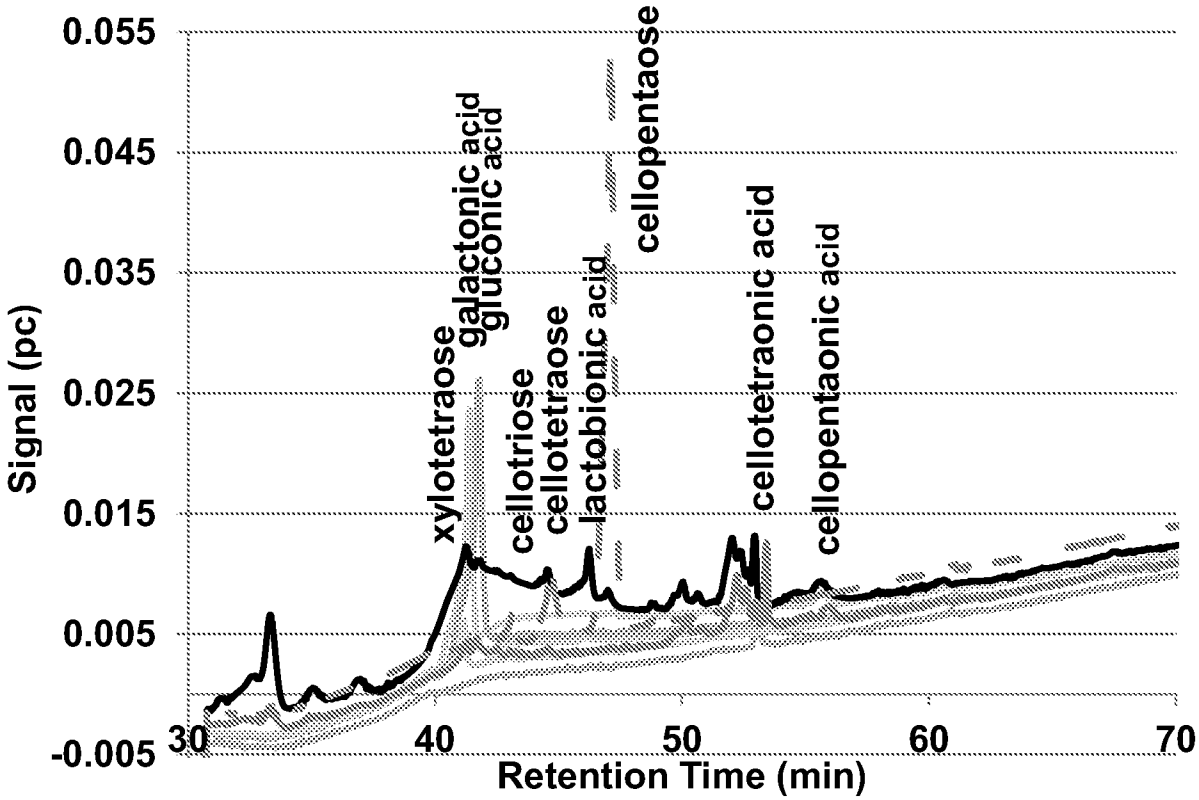
0.055
0.045
0.035
0.025
0.015
0.005
-0.005
Signal (pc)
30
40
50
60
70
Retention Time (min)
xylotetraose
galactonic acid
gluconic acid
cellotriose
cellotetraose
lactobionic acid
cellopentaose
cellotetraonic acid
cellopentaonic acid

Fig. 15B

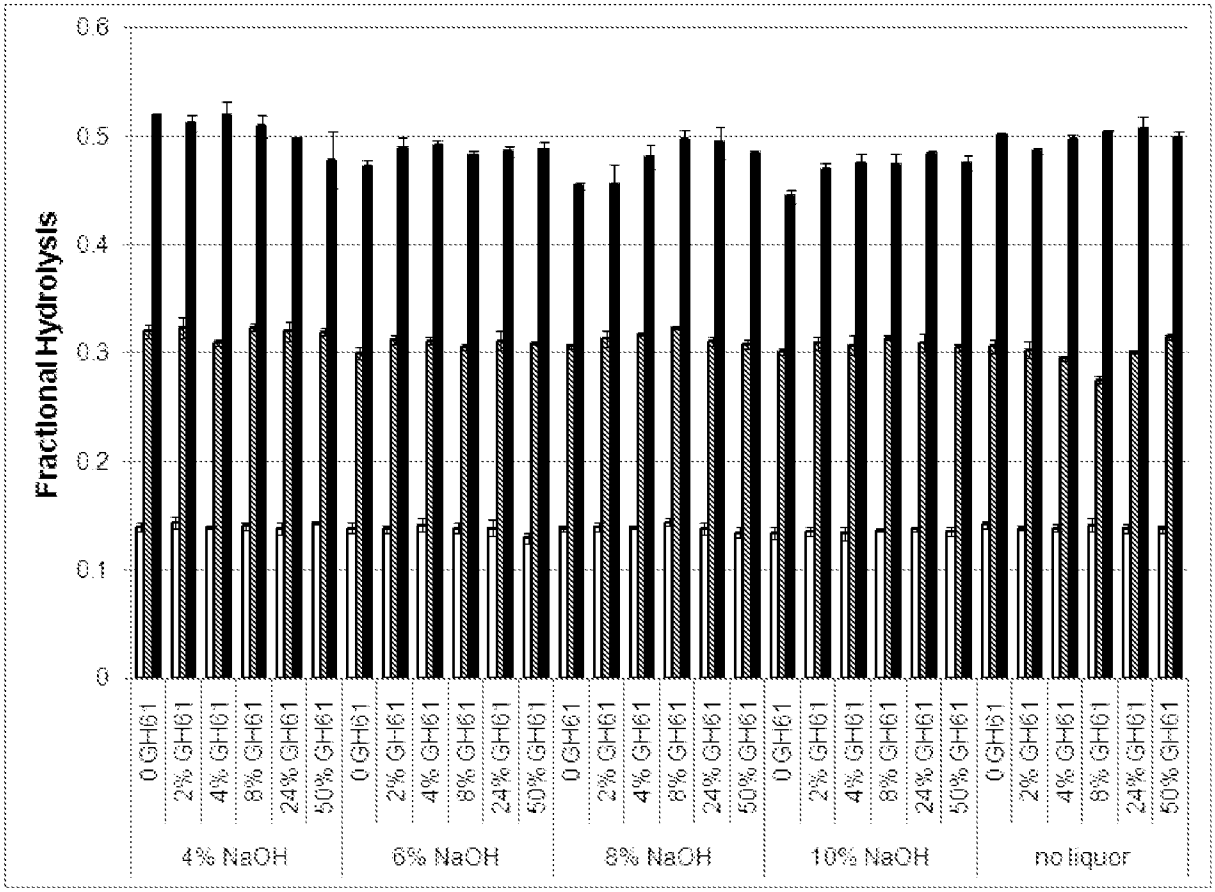
Fractional Hydrolysis
0.6
0.5
0.4
0.3
0.2
0.1
0
0 GH61
2% GH61
4% GH61
8% GH61
24% GH61
50% GH61
4% NaOH
6% NaOH
8% NaOH
10% NaOH
no liquor

Fig. 16A

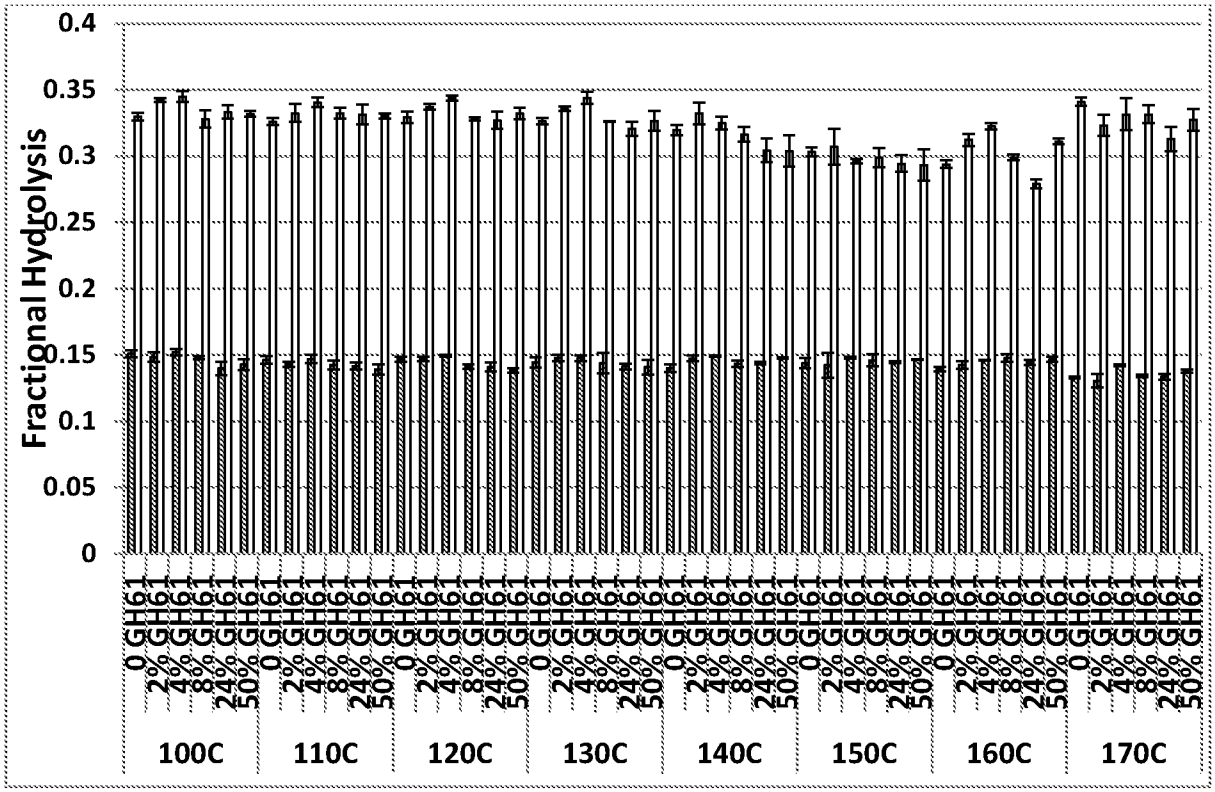
Fractional Hydrolysis
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
100C
110C
120C
130C
140C
150C
160C
170C

Fig. 16B

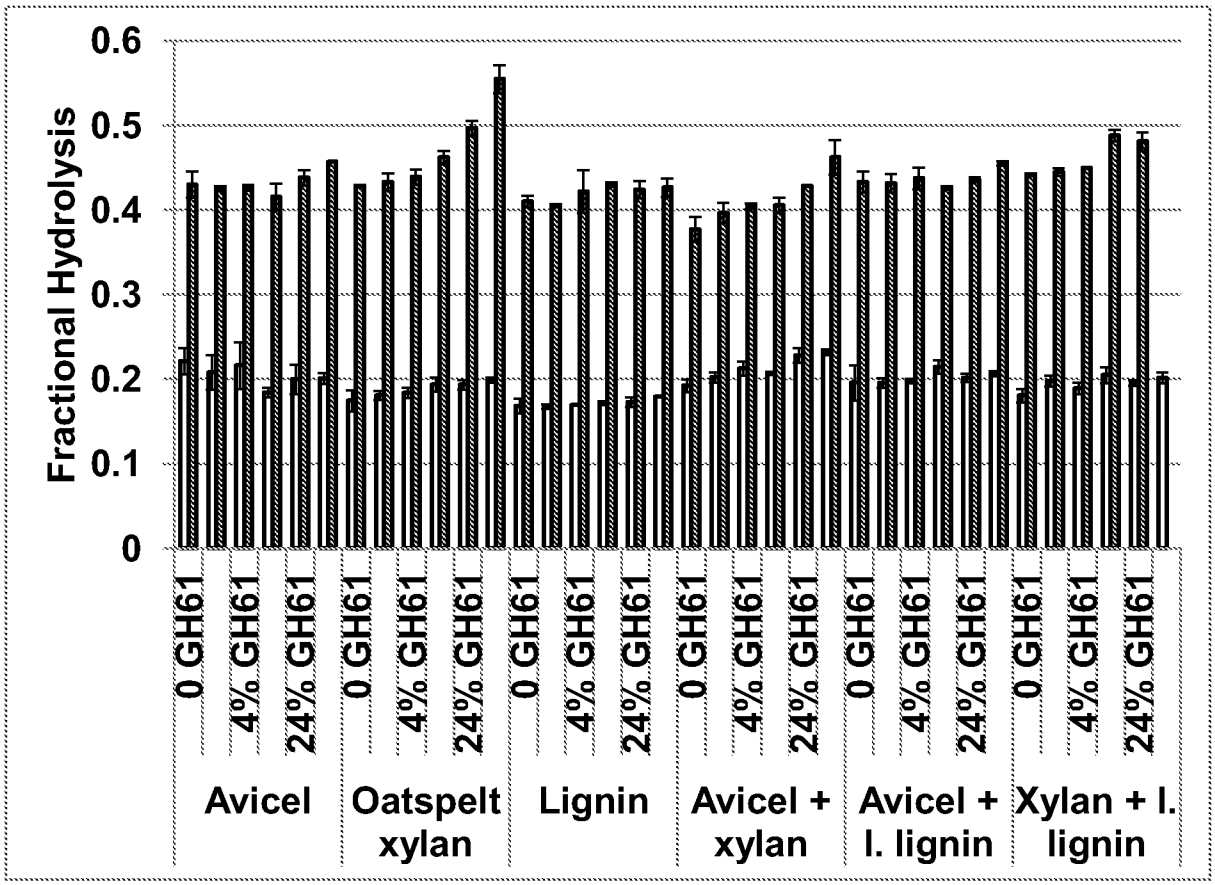
Fractional Hydrolysis
0.6
0.5
0.4
0.3
0.2
0.1
0
0 GH61
4% GH61
24% GH61
Avicel
Oatspelt xylan
Lignin
Avicel + xylan
Avicel + l. lignin
Xylan + l. lignin

Fig. 17A

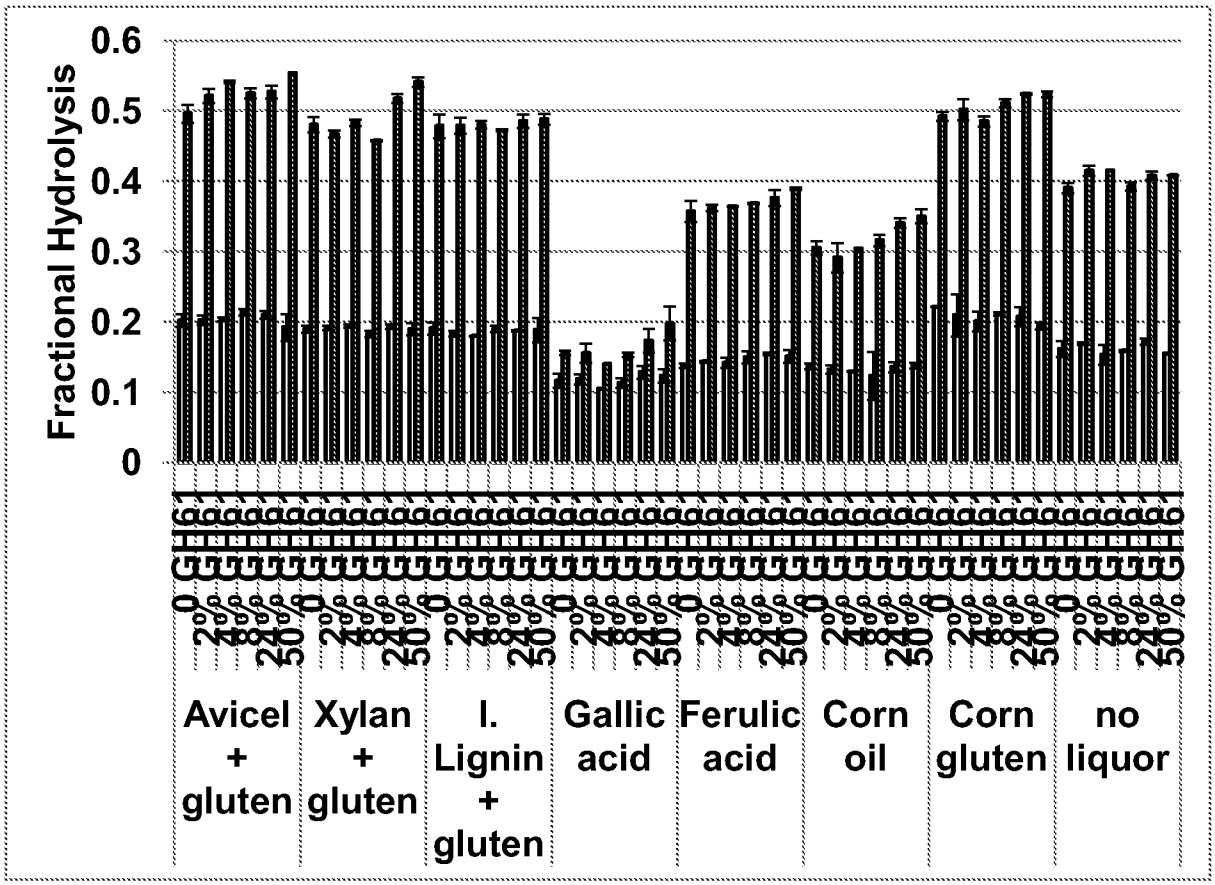
Fractional Hydrolysis
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
8
24
50
Avicel + gluten
Xylan + gluten
l. Lignin + gluten
Gallic acid
Ferulic acid
Corn oil
Corn gluten
no liquor

Fig. 17B

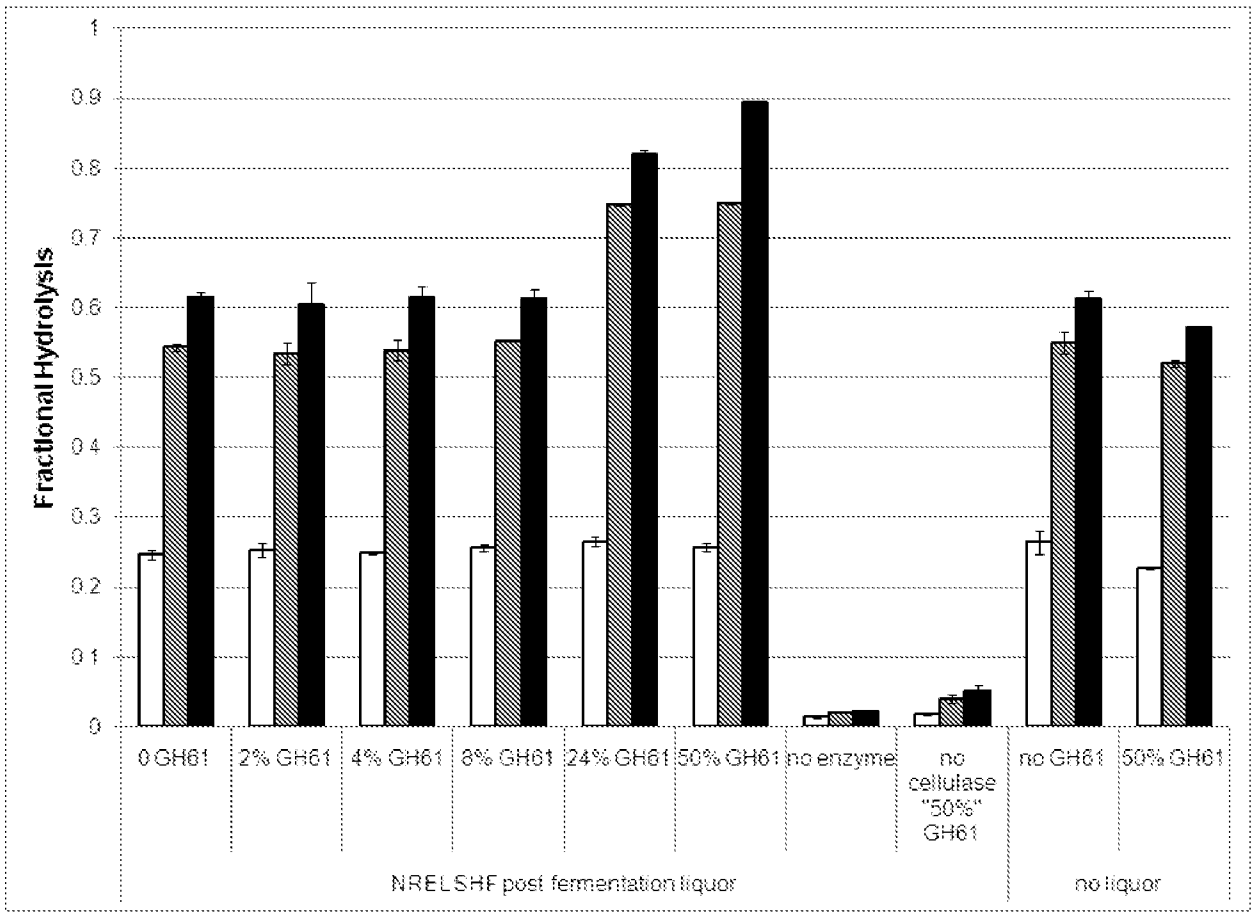
Fractional Hydrolysis
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 GH61
2% GH61
4% GH61
8% GH61
24% GH61
50% GH61
no enzyme
no cellulase "50%" GH61
no GH61
50% GH61
NRELSHF post fermentation liquor
no liquor

Fig. 18

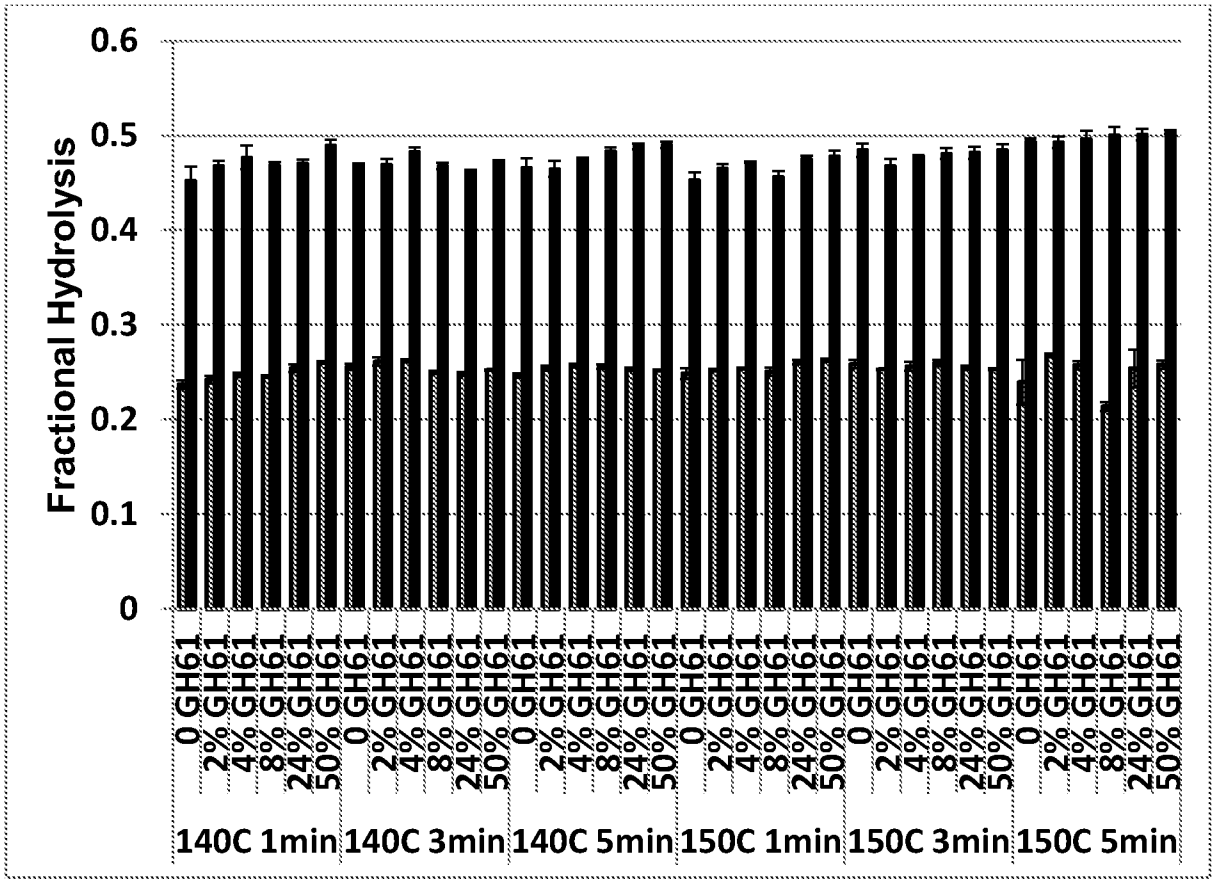
Fractional Hydrolysis
0.6
0.5
0.4
0.3
0.2
0.1
0
0 GH61
2% GH61
4% GH61
8% GH61
24% GH61
50% GH61
140C 1min
140C 3min
140C 5min
150C 1min
150C 3min
150C 5min

Fig. 19A

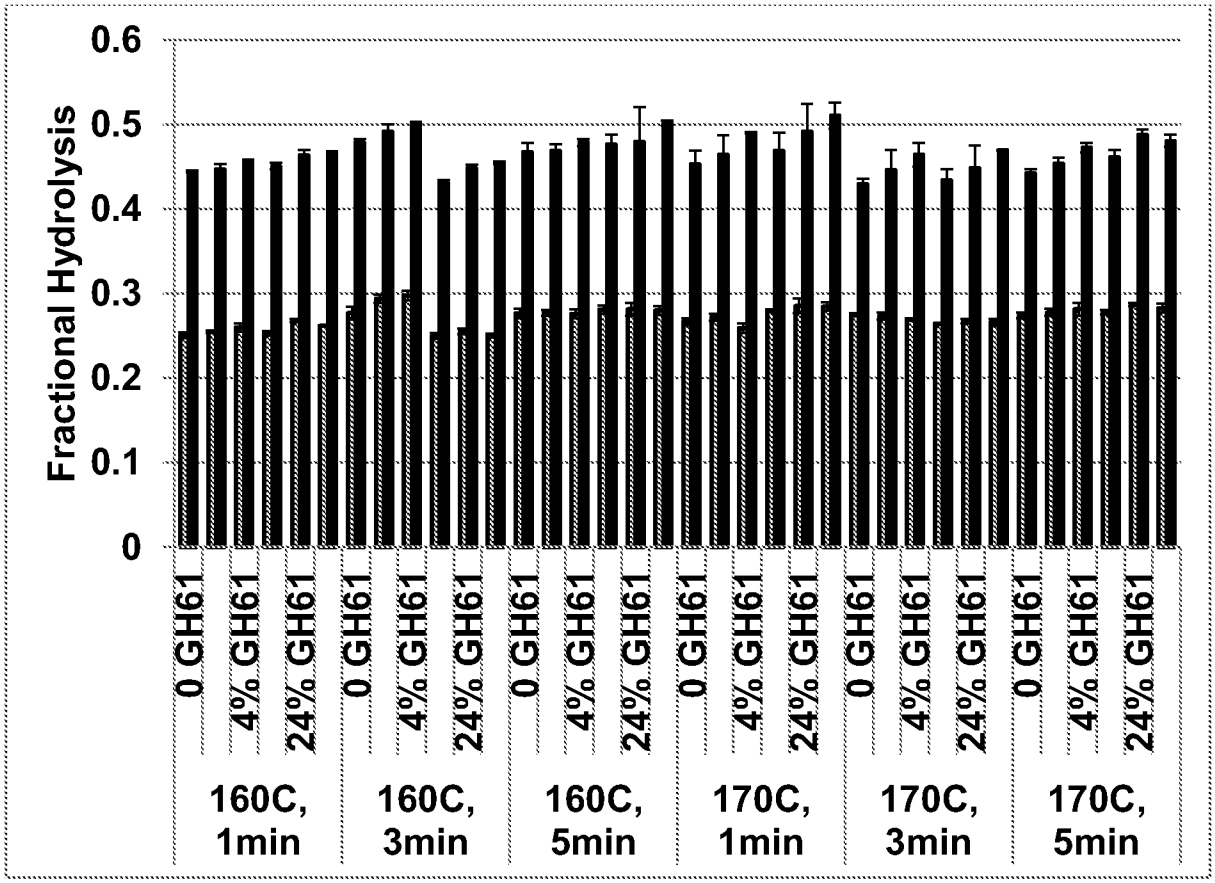
Fractional Hydrolysis
0.6
0.5
0.4
0.3
0.2
0.1
0
0 GH61
4% GH61
24% GH61
160C, 1min
160C, 3min
160C, 5min
170C, 1min
170C, 3min
170C, 5min

Fig. 19B

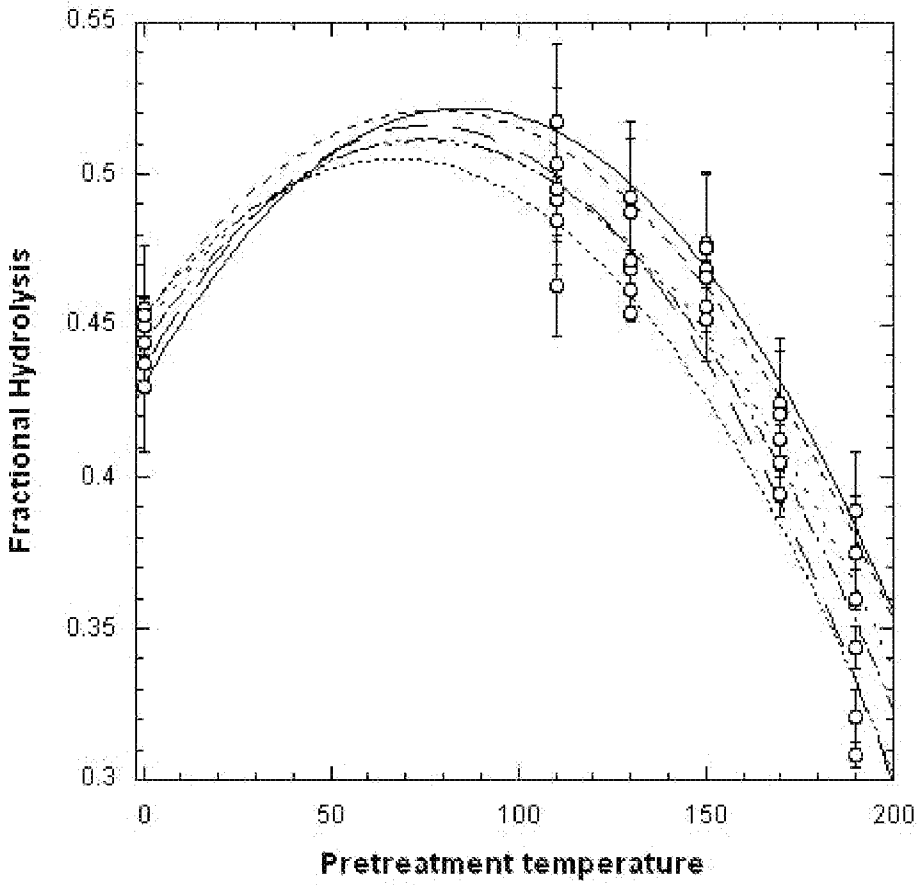
0.55
0.5
0.45
0.4
0.35
0.3
Fractional Hydrolysis
0
50
100
150
200
Pretreatment temperature

Fig. 19C

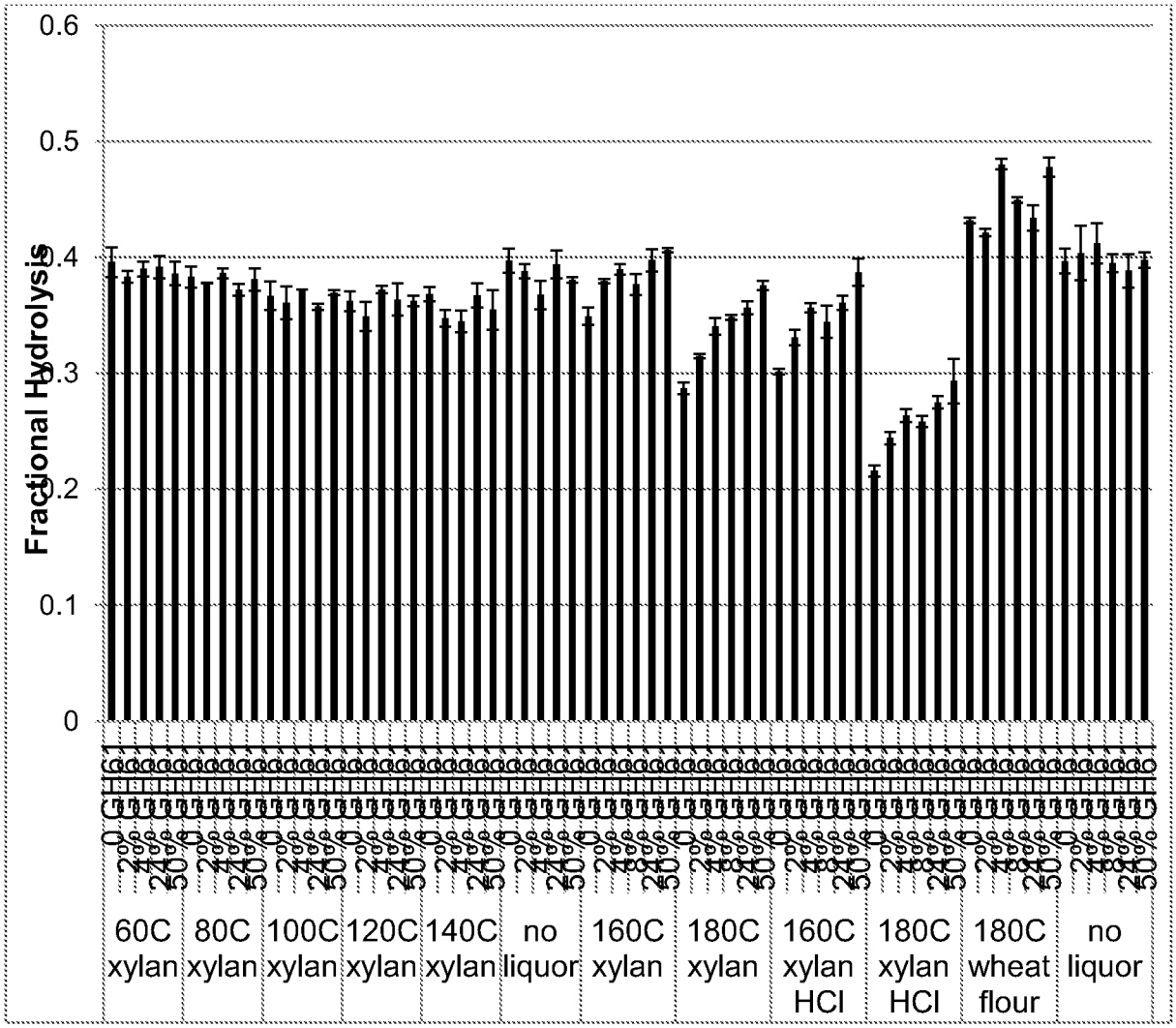
Fractional Hydrolysis
0.6
0.5
0.4
0.3
0.2
0.1
0
60C xylan
80C xylan
100C xylan
120C xylan
140C xylan
no liquor
160C xylan
180C xylan
160C xylan HCl
180C xylan HCl
180C wheat flour
no liquor

Fig. 20A

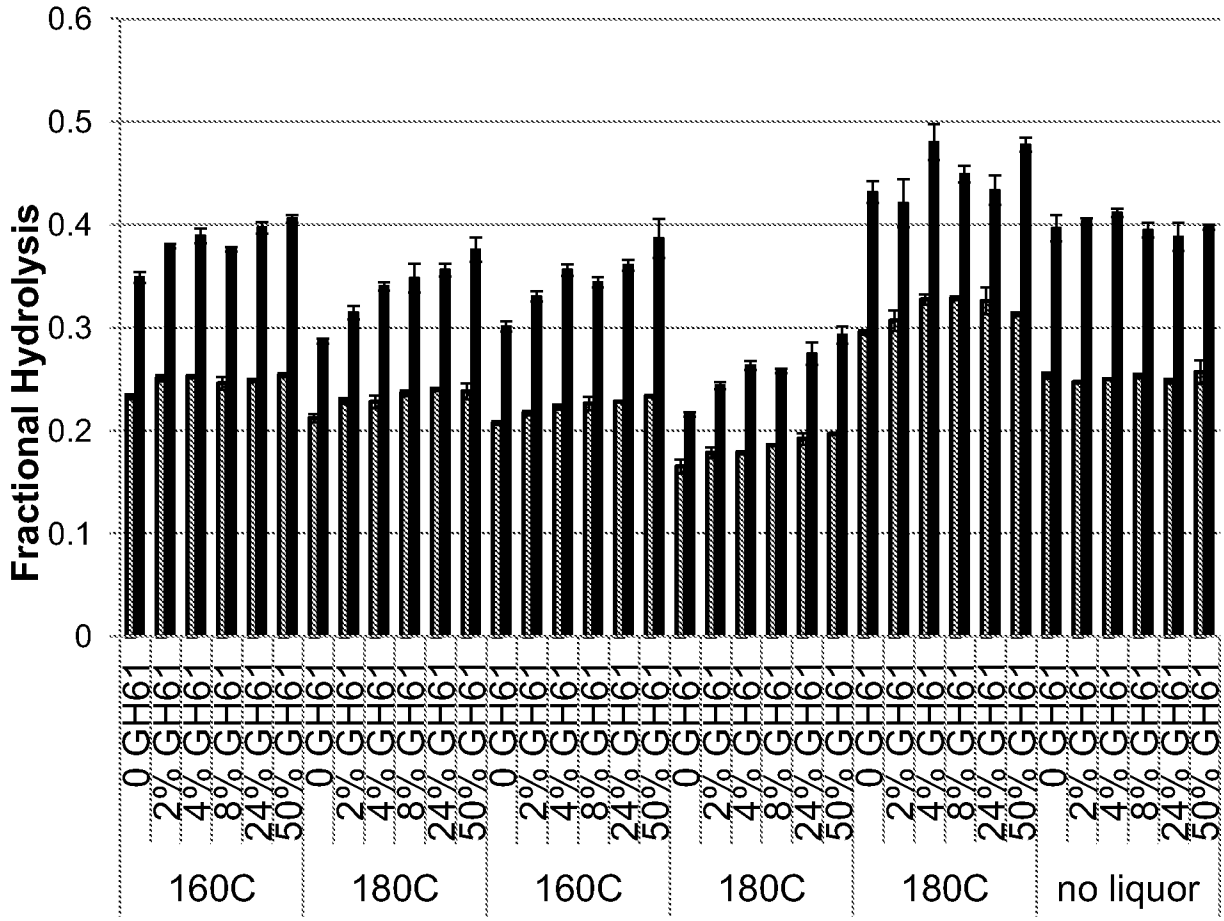
Fractional Hydrolysis
0.6
0.5
0.4
0.3
0.2
0.1
0
0 GH61
2% GH61
4% GH61
8% GH61
24% GH61
50% GH61
160C
180C
160C
180C
180C
no liquor

Fig. 20B

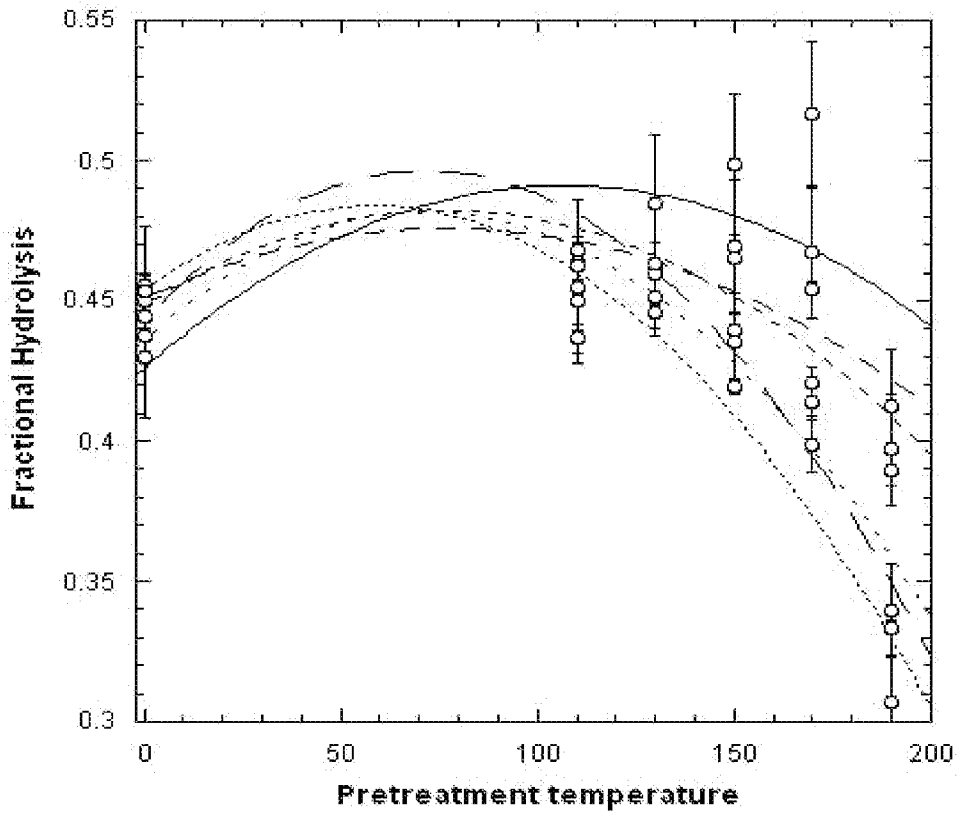
0.55
0.5
0.45
0.4
0.35
0.3
Fractional Hydrolysis
0
50
100
150
200
Pretreatment temperature

Fig. 20C

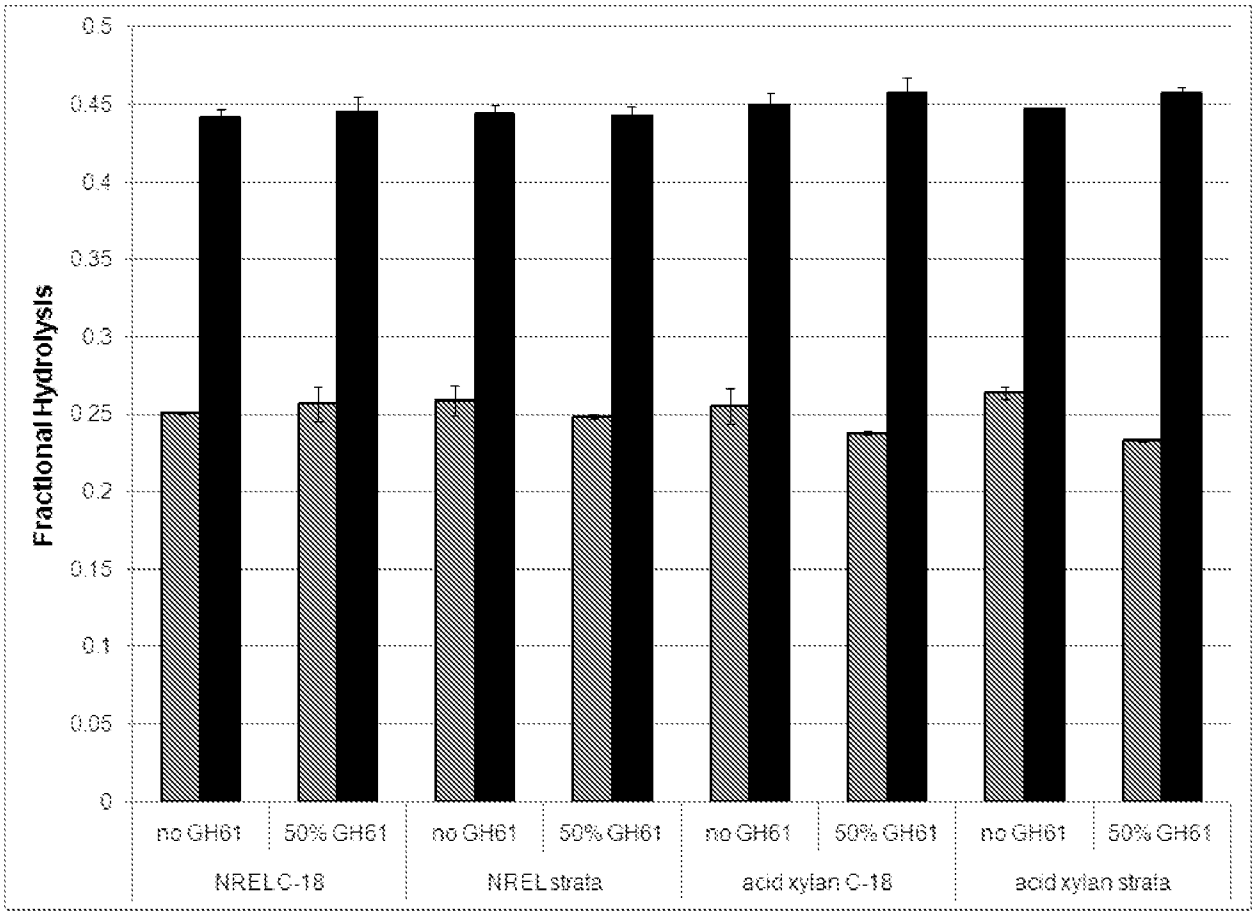
Fractional Hydrolysis
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
no GH61
50% GH61
no GH61
50% GH61
no GH61
50% GH61
no GH61
50% GH61
NREL C-18
NREL strata
acid xylan C-18
acid xylan strata

Fig. 21

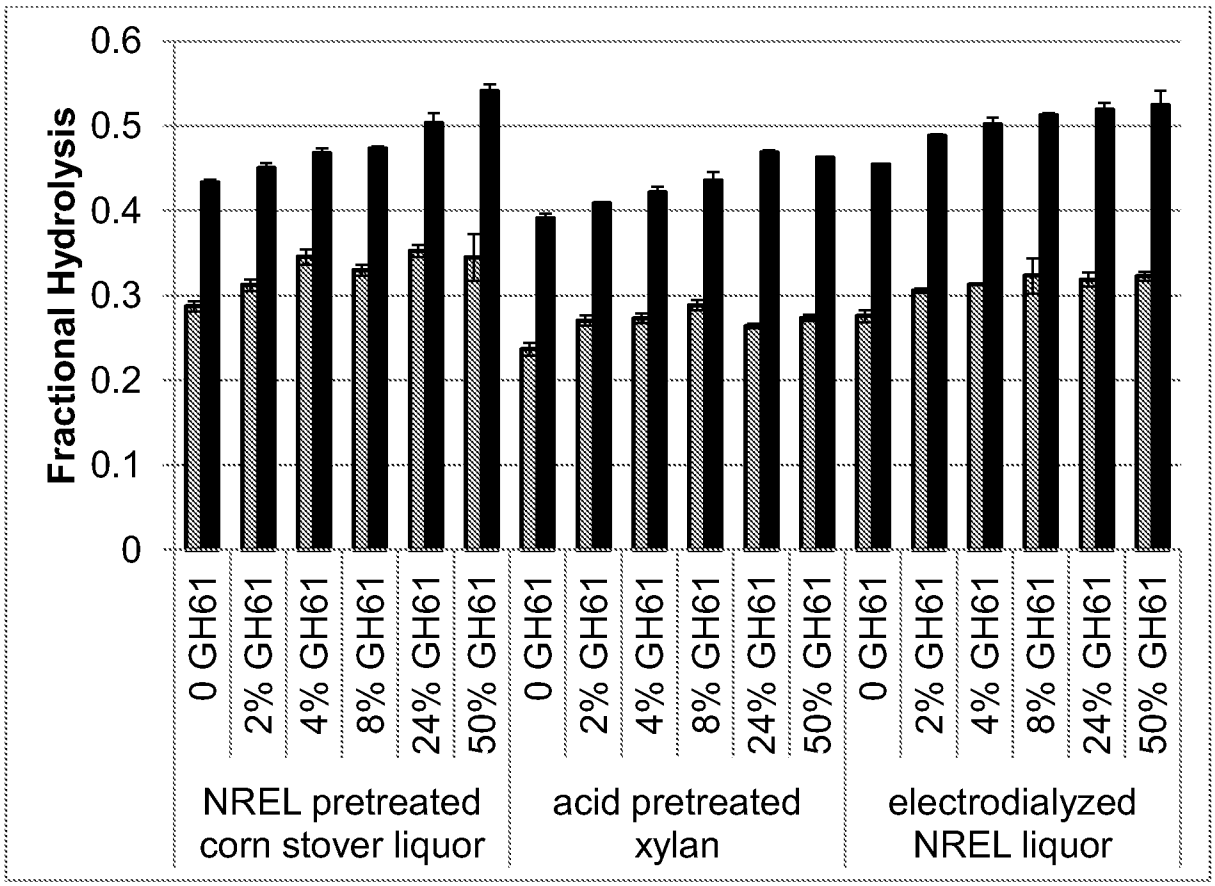
Fractional Hydrolysis
0.6
0.5
0.4
0.3
0.2
0.1
0
0 GH61
2% GH61
4% GH61
8% GH61
24% GH61
50% GH61
NREL pretreated corn stover liquor
acid pretreated xylan
electrodialyzed NREL liquor

Fig. 22

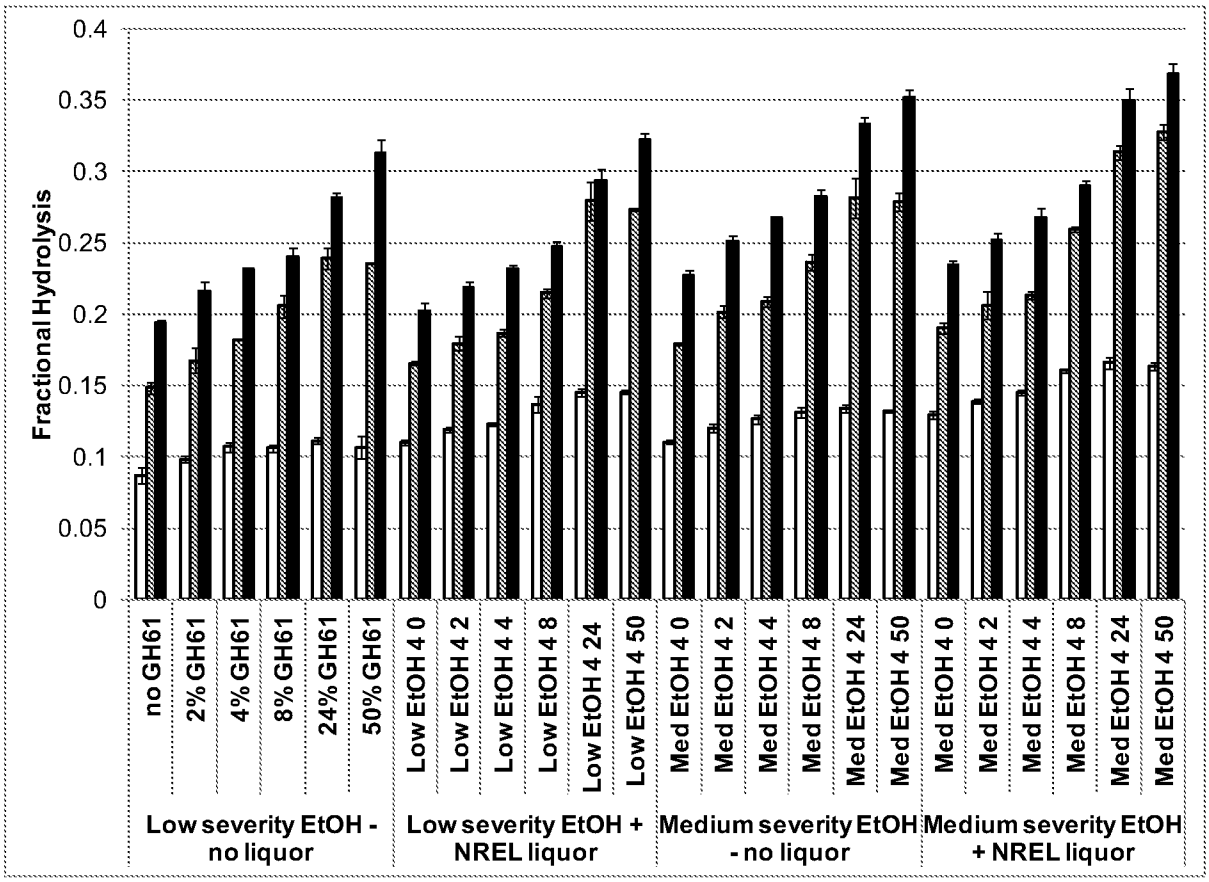
Fractional Hydrolysis
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
no GH61
2% GH61
4% GH61
8% GH61
24% GH61
50% GH61
Low EtOH 4 0
Low EtOH 4 2
Low EtOH 4 4
Low EtOH 4 8
Low EtOH 4 24
Low EtOH 4 50
Med EtOH 4 0
Med EtOH 4 2
Med EtOH 4 4
Med EtOH 4 8
Med EtOH 4 24
Med EtOH 4 50
Med EtOH 4 0
Med EtOH 4 2
Med EtOH 4 4
Med EtOH 4 8
Med EtOH 4 24
Med EtOH 4 50
Low severity EtOH - no liquor
Low severity EtOH + NREL liquor
Medium severity EtOH - no liquor
Medium severity EtOH + NREL liquor

Fig. 23A

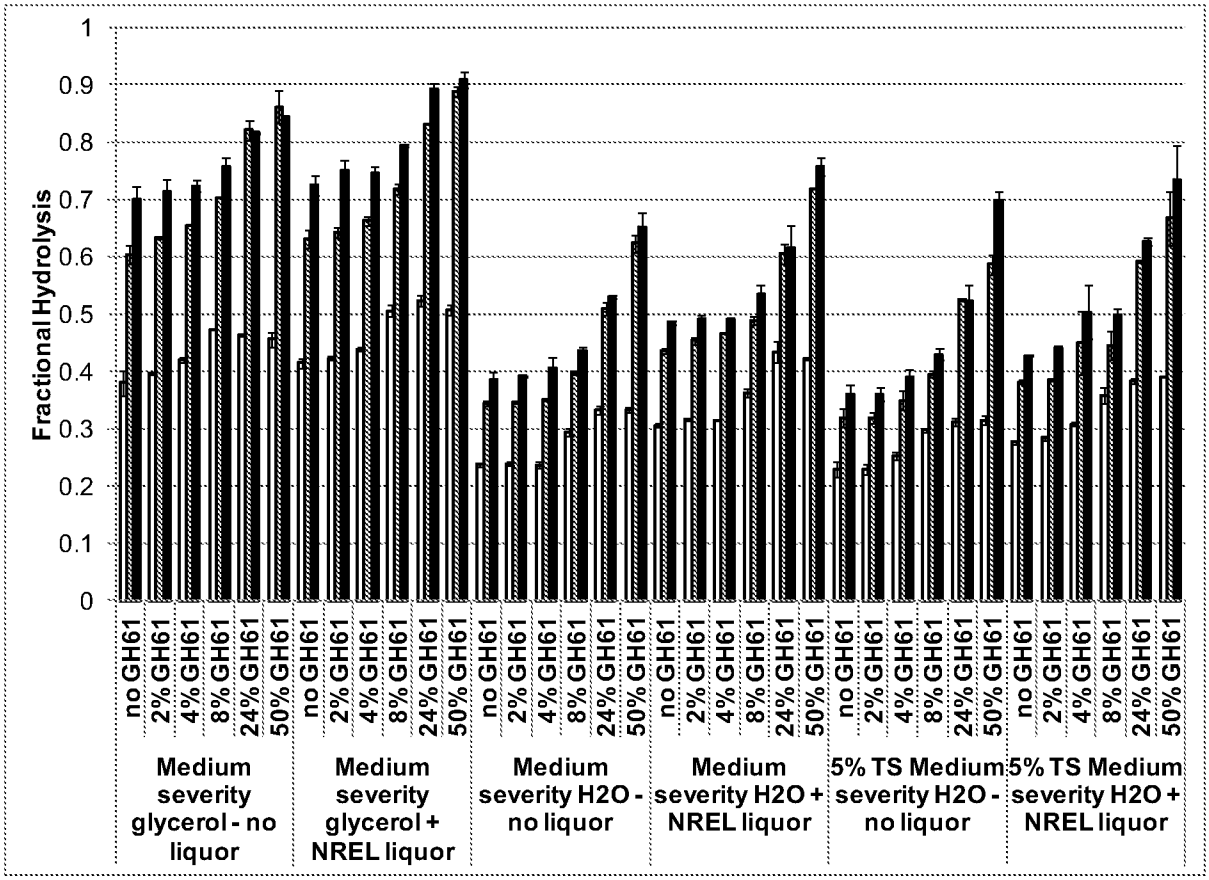
Fractional Hydrolysis
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
no GH61
2% GH61
4% GH61
8% GH61
24% GH61
50% GH61
Medium severity glycerol - no liquor
Medium severity glycerol + NREL liquor
Medium severity H2O - no liquor
Medium severity H2O + NREL liquor
5% TS Medium severity H2O - no liquor
5% TS Medium severity H2O + NREL liquor

Fig. 23B

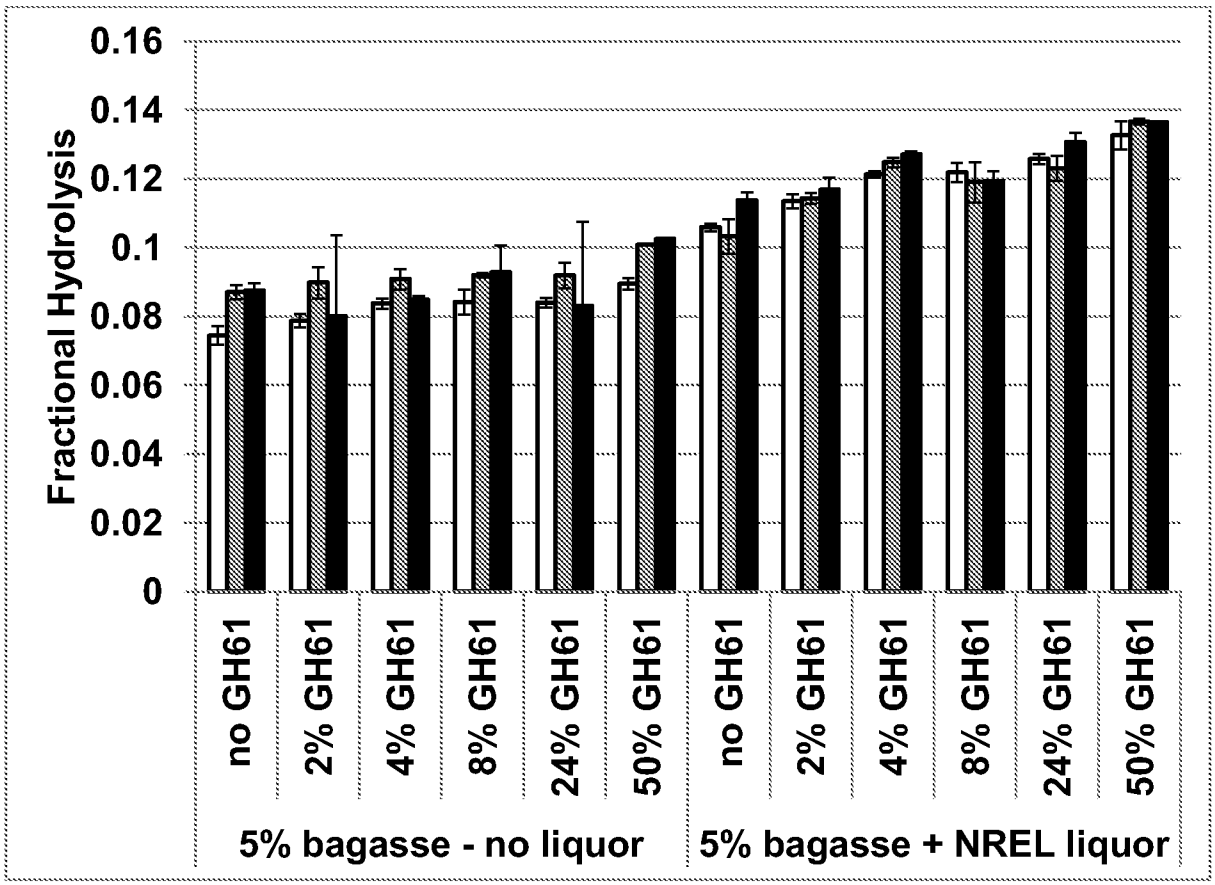
Fractional Hydrolysis
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
no GH61
2% GH61
4% GH61
8% GH61
24% GH61
50% GH61
no GH61
2% GH61
4% GH61
8% GH61
24% GH61
50% GH61
5% bagasse - no liquor
5% bagasse + NREL liquor

Fig. 23C

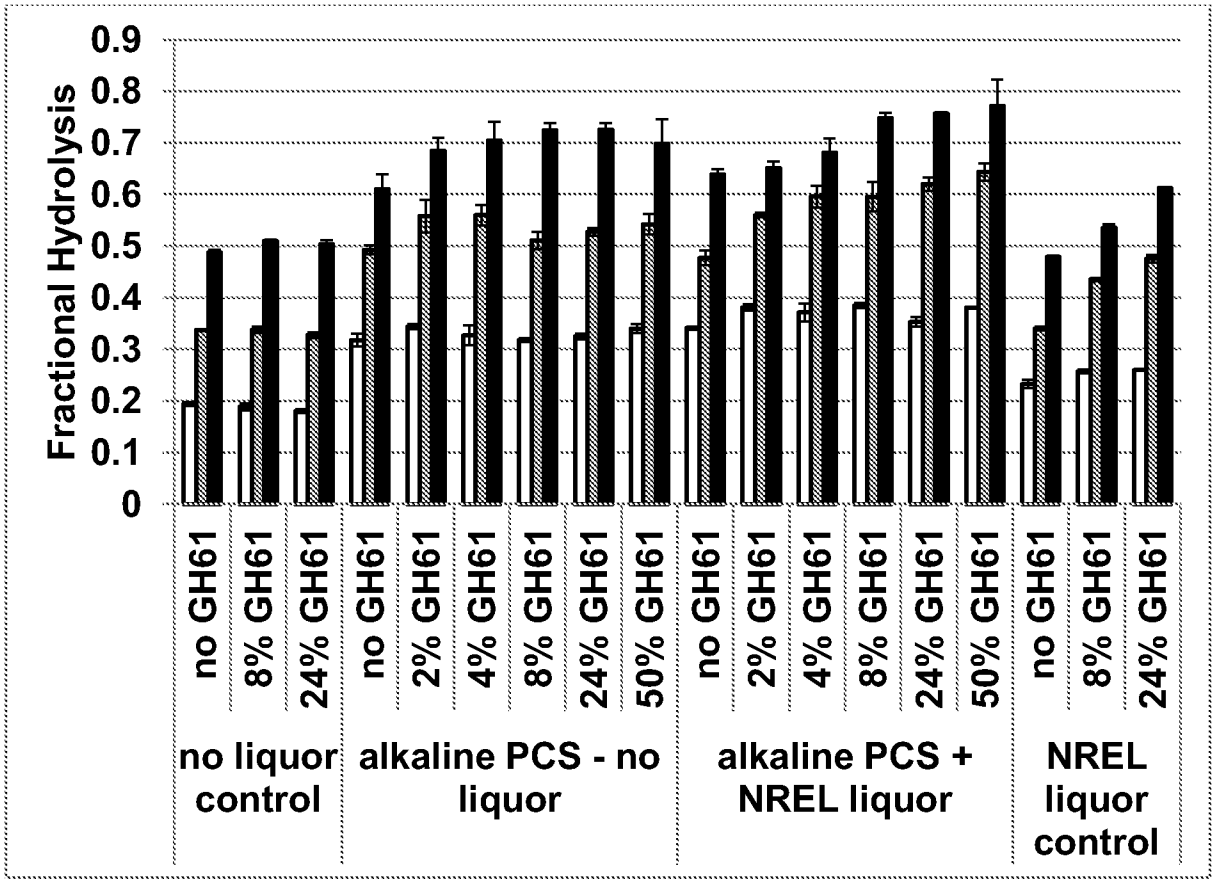
Fractional Hydrolysis
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
no GH61
8% GH61
24% GH61
no GH61
2% GH61
4% GH61
8% GH61
24% GH61
50% GH61
no GH61
2% GH61
4% GH61
8% GH61
24% GH61
50% GH61
no GH61
8% GH61
24% GH61
no liquor control
alkaline PCS - no liquor
alkaline PCS + NREL liquor
NREL liquor control

Fig. 23D

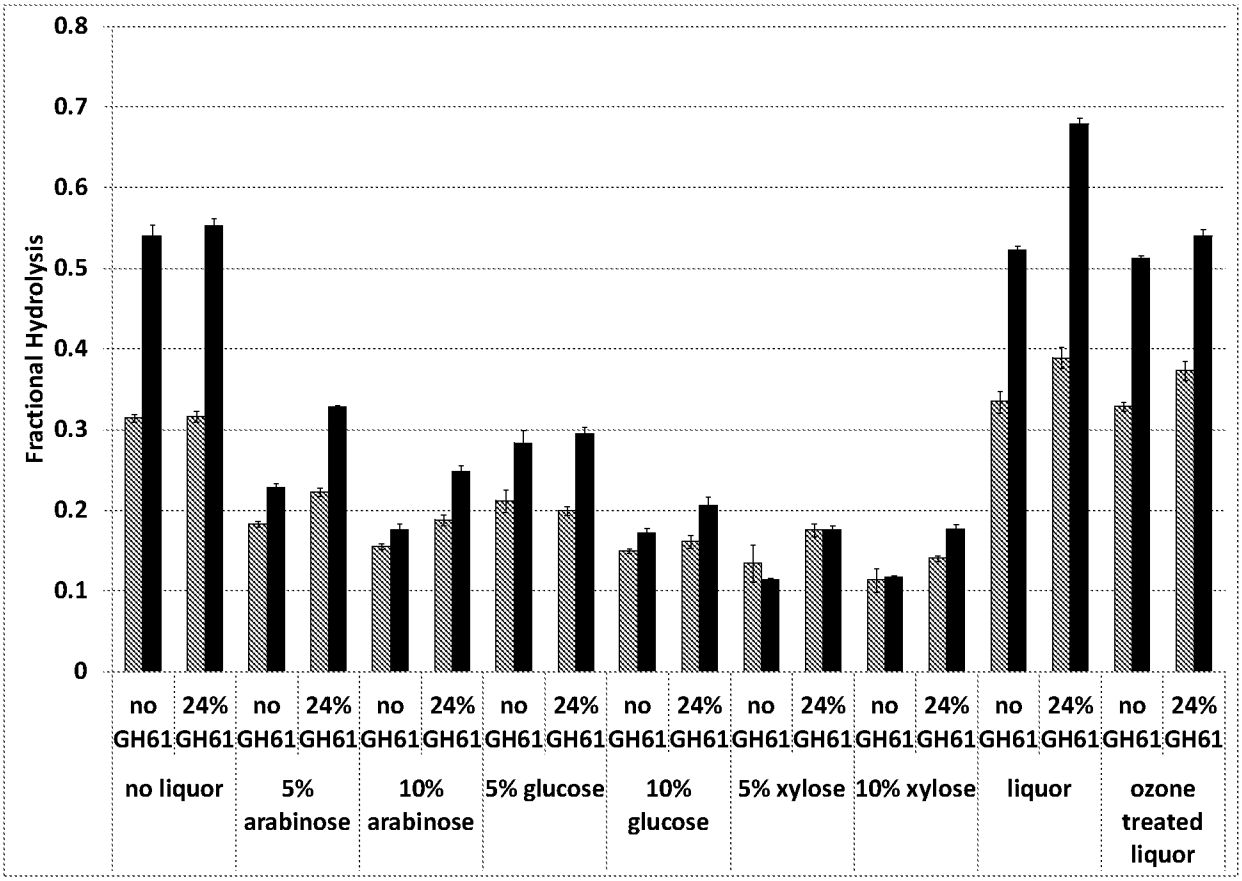
Fractional Hydrolysis
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
no GH61
24% GH61
no liquor
5% arabinose
10% arabinose
5% glucose
10% glucose
5% xylose
10% xylose
liquor
ozone treated liquor

Fig. 24

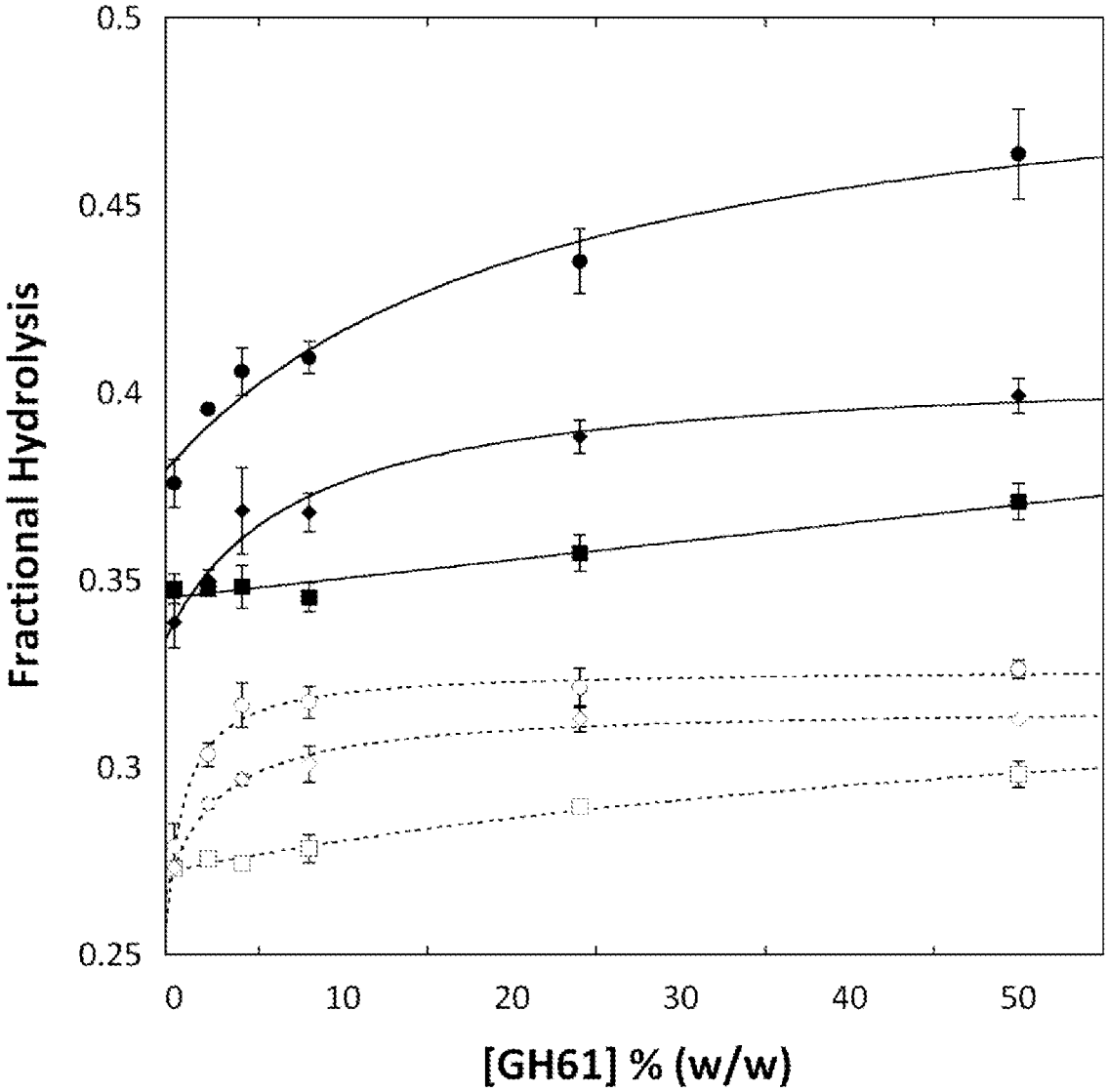
0.5
0.45
0.4
0.35
0.3
0.25
Fractional Hydrolysis
0
10
20
30
40
50
[GH61] % (w/w)

Fig. 25

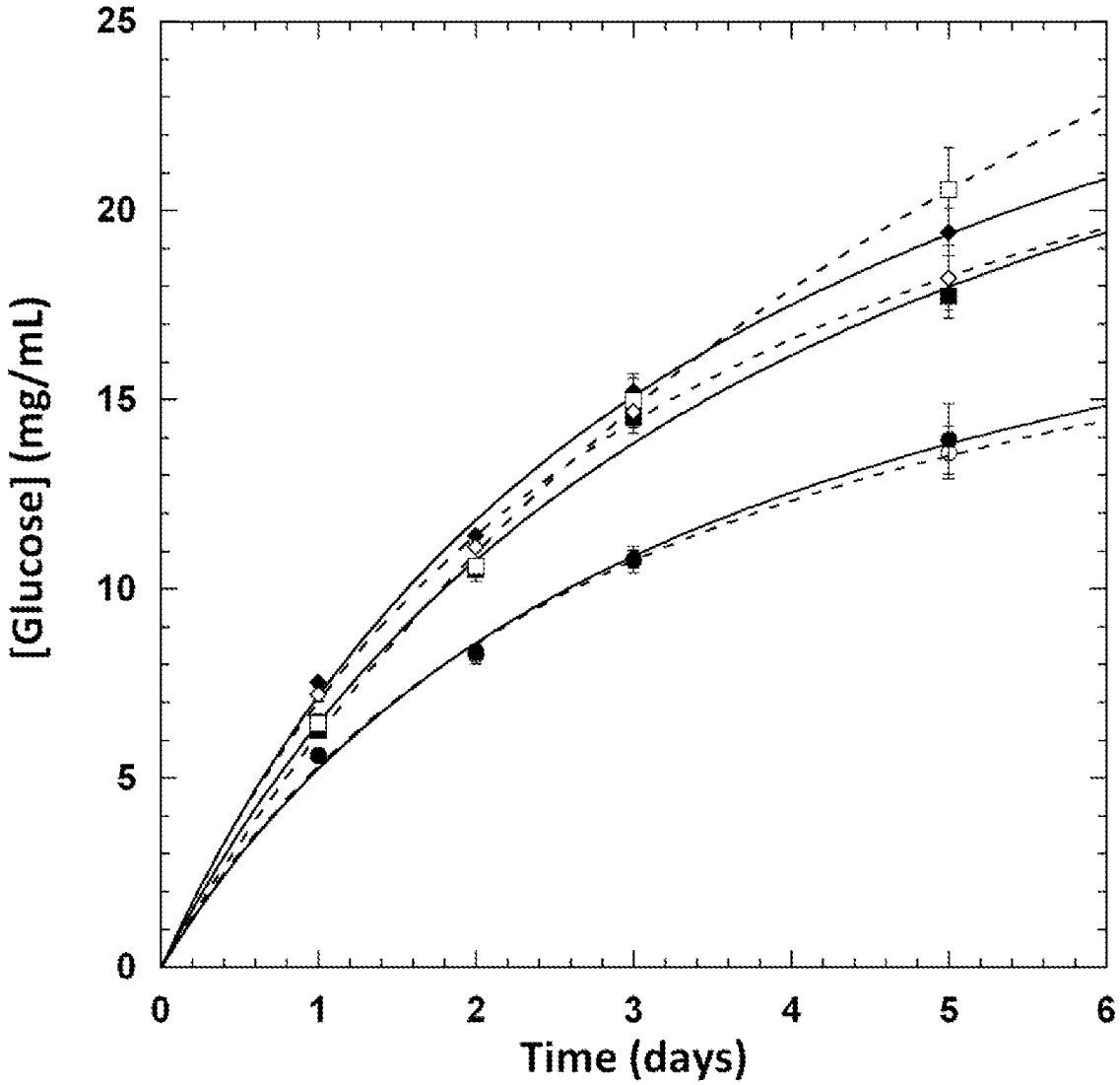

Fig. 26

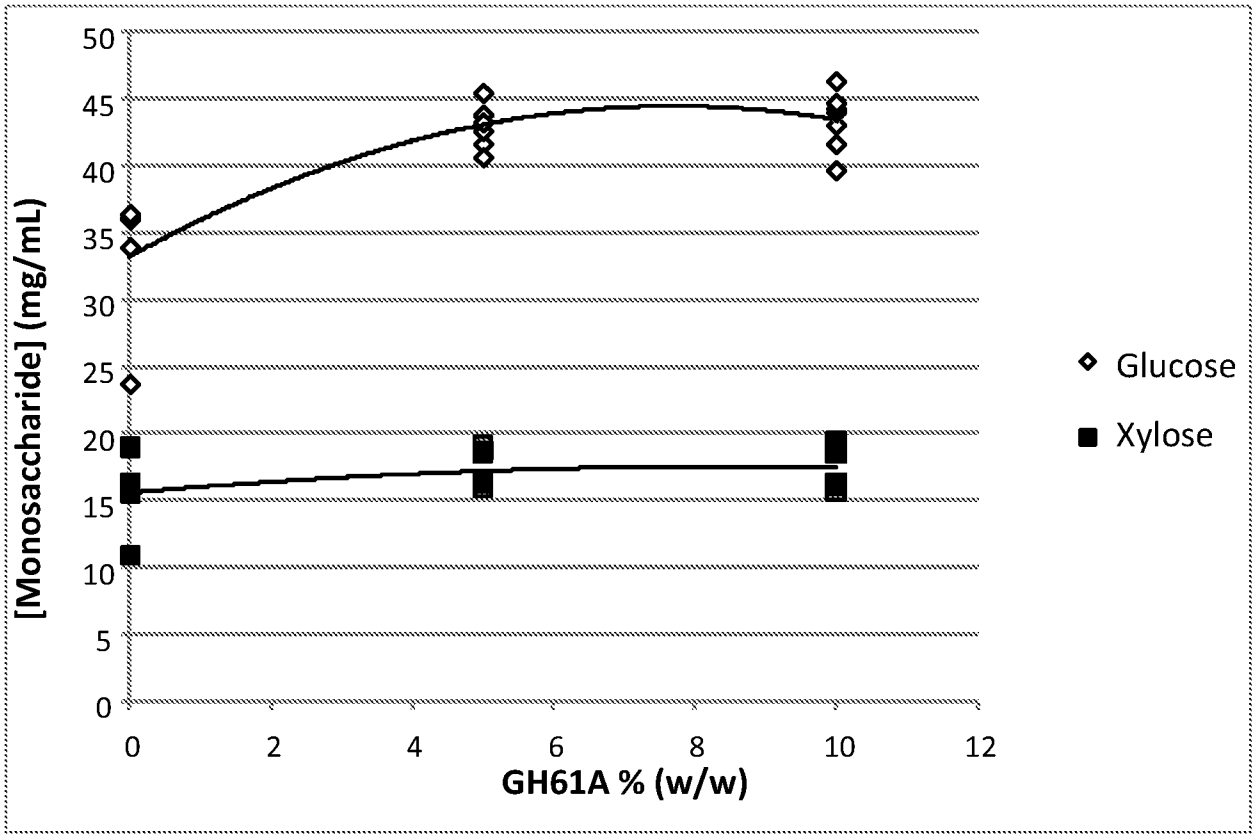
50
45
40
35
30
25
20
15
10
5
0
[Monosaccharide] (mg/mL)
0
2
4
6
8
10
12
GH61A % (w/w)
Glucose
Xylose

Fig. 27

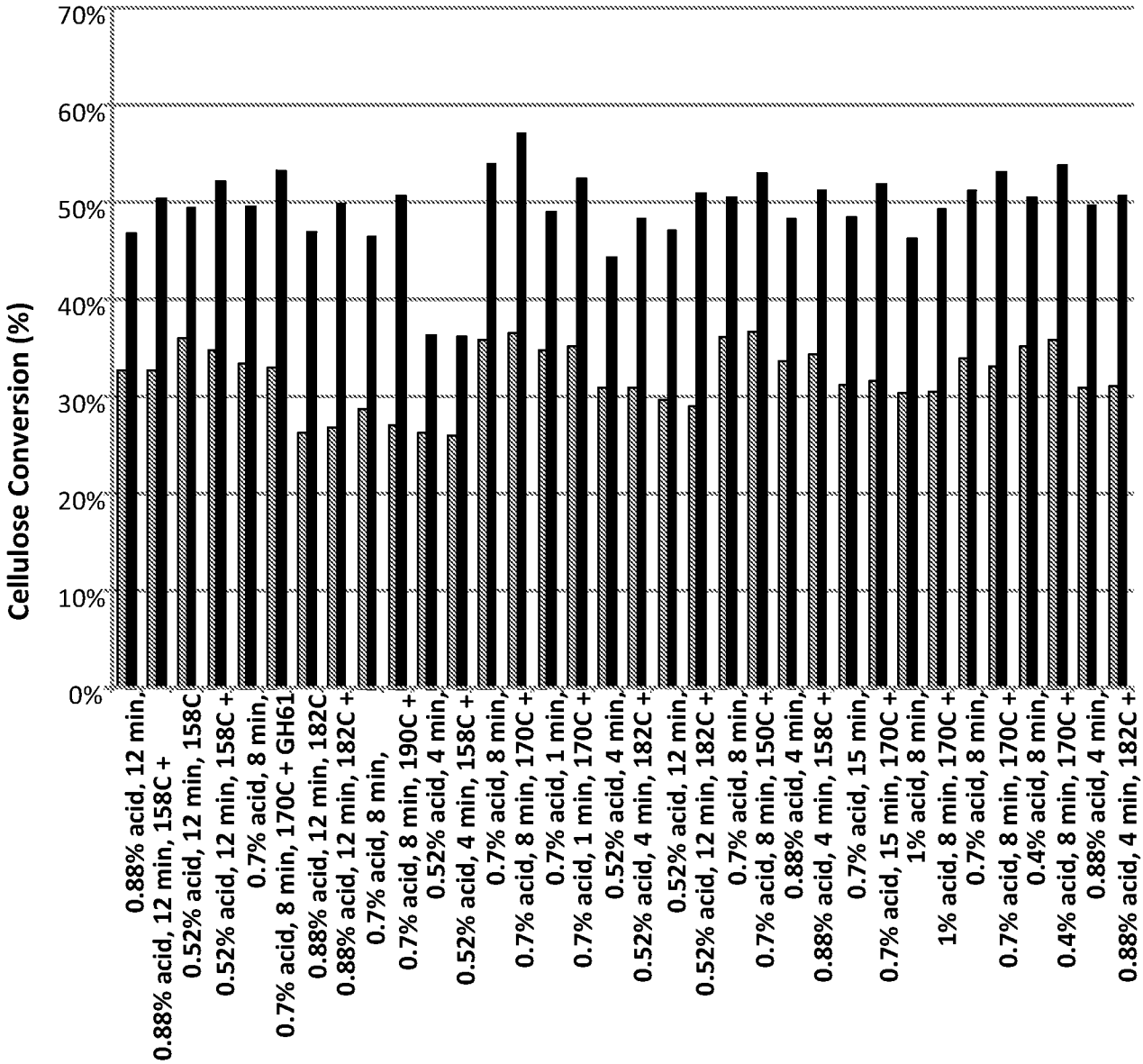
Cellulose Conversion (%)
70%
60%
50%
40%
30%
20%
10%
0%
0.88% acid, 12 min,
0.88% acid, 12 min, 158C +
0.52% acid, 12 min, 158C
0.52% acid, 12 min, 158C +
0.7% acid, 8 min,
0.7% acid, 8 min, 170C + GH61
0.88% acid, 12 min, 182C
0.88% acid, 12 min, 182C +
0.7% acid, 8 min,
0.7% acid, 8 min, 190C +
0.52% acid, 4 min,
0.52% acid, 4 min, 158C +
0.7% acid, 8 min,
0.7% acid, 8 min, 170C +
0.7% acid, 1 min,
0.7% acid, 1 min, 170C +
0.52% acid, 4 min,
0.52% acid, 4 min, 182C +
0.52% acid, 12 min,
0.52% acid, 12 min, 182C +
0.7% acid, 8 min,
0.7% acid, 8 min, 150C +
0.88% acid, 4 min,
0.88% acid, 4 min, 158C +
0.7% acid, 15 min,
0.7% acid, 15 min, 170C +
1% acid, 8 min,
1% acid, 8 min, 170C +
0.7% acid, 8 min,
0.7% acid, 8 min, 170C +
0.4% acid, 8 min,
0.4% acid, 8 min, 170C +
0.88% acid, 4 min,
0.88% acid, 4 min, 182C +

Fig. 28

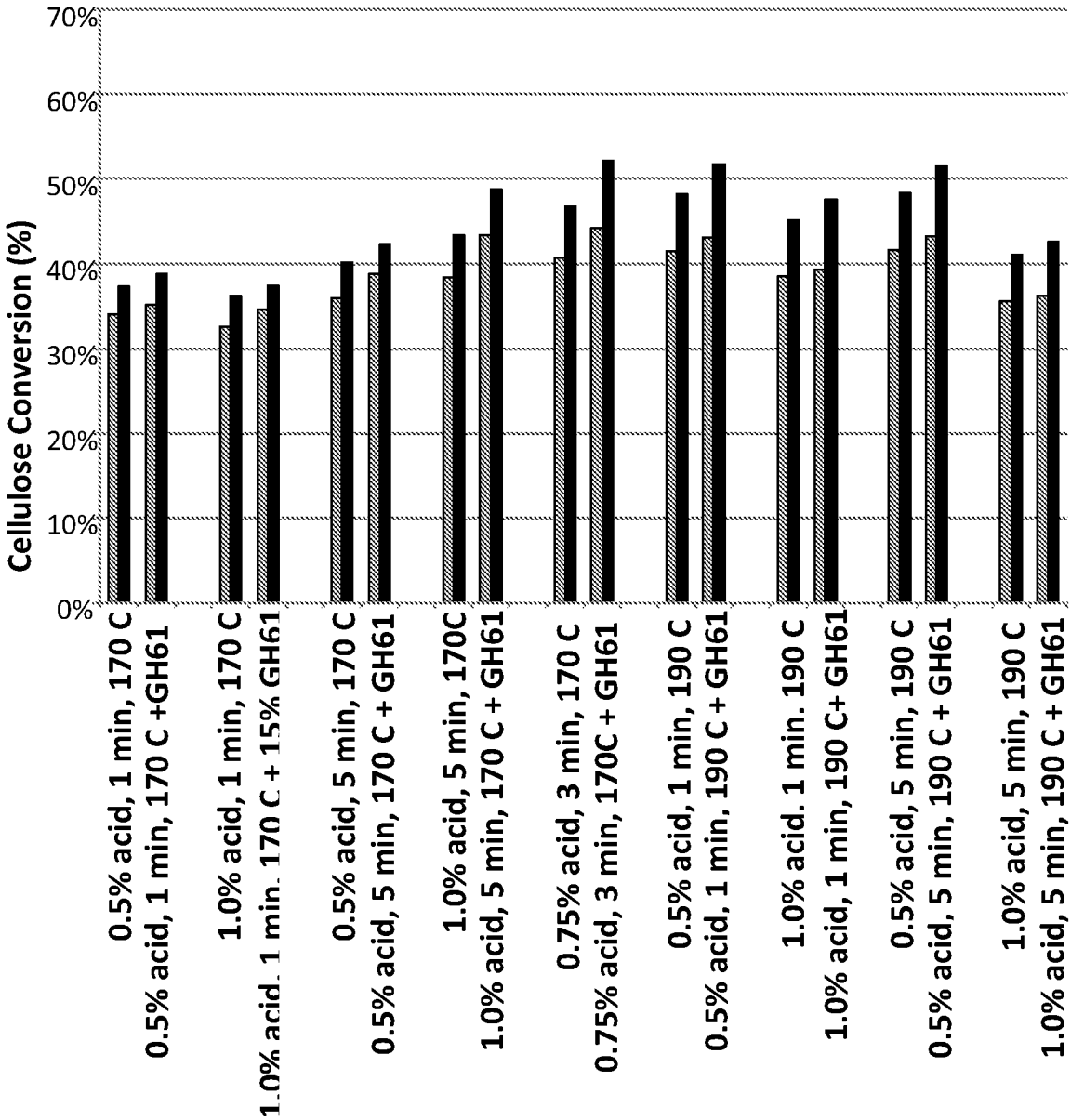
Cellulose Conversion (%)
70%
60%
50%
40%
30%
20%
10%
0%
0.5% acid, 1 min, 170 C
0.5% acid, 1 min, 170 C +GH61
1.0% acid, 1 min, 170 C
1.0% acid, 1 min, 170 C + 15% GH61
0.5% acid, 5 min, 170 C
0.5% acid, 5 min, 170 C + GH61
1.0% acid, 5 min, 170C
1.0% acid, 5 min, 170 C + GH61
0.75% acid, 3 min, 170 C
0.75% acid, 3 min, 170C + GH61
0.5% acid, 1 min, 190 C
0.5% acid, 1 min, 190 C + GH61
1.0% acid. 1 min. 190 C
1.0% acid, 1 min, 190 C+ GH61
0.5% acid, 5 min, 190 C
0.5% acid, 5 min, 190 C + GH61
1.0% acid, 5 min, 190 C
1.0% acid, 5 min, 190 C + GH61

Fig. 29

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/046795

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/10 C12P19/14 C12N9/42 C12P19/02
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P C12N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, COMPENDEX, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2009/056889 A1 (REN HAIYU [CN] ET AL) 5 March 2009 (2009-03-05) abstract paragraph [0012] paragraph [0074] paragraphs [0122] - [0127]; examples 1-2; tables 1-4 ----- -/--	1-7,10, 11,15-21

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 November 2011	29/11/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schröder, Gunnar

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2011/046795

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [X] on paper

   [ ] in electronic form

   b. (time)

   [ ] in the international application as filed

   [X] together with the international application in electronic form

   [X] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2011/046795

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/046795

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HARRIS PAUL V ET AL: "Stimulation of Lignocellulosic Biomass Hydrolysis by Proteins of Glycoside Hydrolase Family 61: Structure and Function of a Large, Enigmatic Family", BIOCHEMISTRY, AMERICAN CHEMICAL SOCIETY, US, vol. 49, no. 15, 1 April 2010 (2010-04-01) , pages 3305-3316, XP002608645, ISSN: 0006-2960, DOI: 10.1021/BI100009P [retrieved on 2010-03-15] abstract; figures 2-4 page 3306, left-hand column, last paragraph - right-hand column, paragraph 1	1,2,7, 10,15, 17-21
X	US 2010/129860 A1 (MCFARLAND KEITH [US] ET AL) 27 May 2010 (2010-05-27) abstract paragraphs [0276], [0441], [0442]	1-7,10, 15,17-21
X	WO 2009/090480 A2 (INBICON AS [DK]; LARSEN JAN [DK]) 23 July 2009 (2009-07-23)	18
A	abstract page 15, lines 20-26	1-17, 19-21
X,P	WO 2011/002832 A1 (NOVOZYMES AS [DK]; NOVOZYMES NORTH AMERICA INC [US]; REN HAIYU [CN]; H) 6 January 2011 (2011-01-06) abstract page 1, line 29 - page 2, line 2 page 11, lines 7-8	1-7,10, 11,15-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2011/046795

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2009056889 A1	05-03-2009	CN 101796247 A	04-08-2010
		EP 2191061 A1	02-06-2010
		US 2009056889 A1	05-03-2009
		WO 2009030713 A1	12-03-2009
US 2010129860 A1	27-05-2010	AU 2008259937 A1	11-12-2008
		AU 2008259986 A1	11-12-2008
		CA 2689261 A1	11-12-2008
		CA 2689910 A1	11-12-2008
		CN 101809150 A	18-08-2010
		EP 2064323 A1	03-06-2009
		EP 2069492 A2	17-06-2009
		JP 2010528621 A	26-08-2010
		KR 20100020977 A	23-02-2010
		RU 2009149467 A	10-07-2011
		US 2010129860 A1	27-05-2010
		WO 2008151043 A1	11-12-2008
		WO 2008151079 A2	11-12-2008
WO 2009090480 A2	23-07-2009	CA 2708962 A1	23-07-2009
		EP 2227551 A2	15-09-2010
		US 2011065785 A1	17-03-2011
		US 2011171708 A1	14-07-2011
		WO 2009090480 A2	23-07-2009
WO 2011002832 A1	06-01-2011	US 2011020873 A1	27-01-2011
		WO 2011002832 A1	06-01-2011

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 2(completely); 7-17(partially)

   Method for degrading or converting a cellulosic material, comprising treating the material with an enzyme composition in the presence of a polypeptide having cellulolytic enhancing activity and a liquor, wherein the combination of the polypeptide and the liquor enhances the enzymatic hydrolysis of the cellulosic material

1.1. claims: 7, 9-11, 15-17(all partially)

   The liquor is obtained from a material that is the SAME as the material which is degraded by the enzyme composition

1.2. claims: 8, 12-17(all partially)

   The liquor is obtained from a material that is DIFFERENT than the material which is degraded by the enzyme composition

---

2. claims: 3(completely); 7-17(partially)

   A method for producing a fermentation product, comprising saccharifying a cellulosic material by the method of invention 1

---

3. claims: 4-6(completely); 7-17(partially)

   A method of fermenting a cellulosic material, comprising saccharifying a cellulosic material by the method of invention 1

---

4. claim: 18

   Liquor, which in combination with a polypeptide having cellulolytic enhancing activity enhances hydrolysis of a cellulosic material

---

5. claims: 19-21

   Composition comprising a polypeptide having cellulolytic enhancing activity and a liquor

---