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(54) Title: METHODS OF DEGRADING OR HYDROLYZING A POLYSACCHARIDE

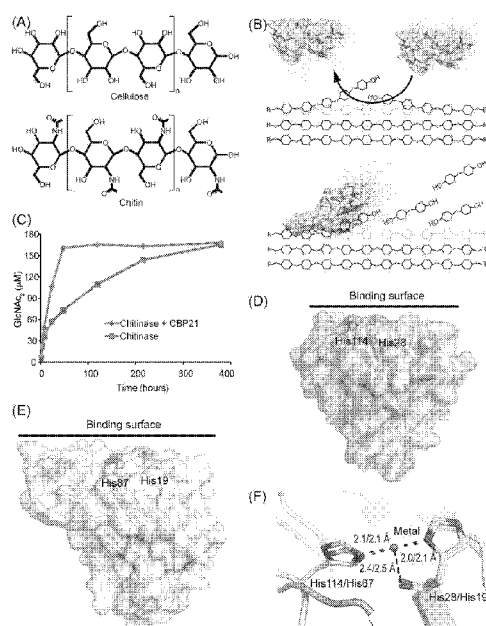


Figure 1

(57) Abstract: The invention provides a method of degrading or hydrolyzing a polysaccharide, preferably cellulose or chitin, comprising contacting said polysaccharide with one or more oxido-hydrolytic enzymes, preferably a CBM33 family protein (preferably CBP21) or a GH61 family protein, wherein said degradation or hydrolysis is carried out in the presence of at least one reducing agent and at least one divalent metal ion. A method of producing an organic substance comprising said method is also provided.



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METHODS OF DEGRADING OR HYDROLYZING A POLYSACCHARIDE

Reference to a Sequence Listing

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

Background of the Invention

Field of the Invention

The present invention relates to methods of degrading or hydrolyzing a polysaccharide, such as chitin or cellulose, comprising contacting said polysaccharide with an oxidohydrolytic enzyme, such as CBP21 or a GH61 protein, wherein said degradation or hydrolysis is carried out in the presence of at least one reducing agent and at least one divalent metal ion. The invention also extends to the use of additional saccharolytic enzymes such as hydrolases and beta-glucosidases to increase the level or extent of degradation and to fermentation of the resulting sugars to generate an organic substance such as an alcohol, preferably ethanol, which may be used as a biofuel.

Description of the Related Art

Efficient enzymatic conversion of crystalline polysaccharides is crucial for an economically and environmentally sustainable bioeconomy, but remains unfavourably inefficient.

The transition to a more environmental friendly economy has spurred research on enzymes capable of efficiently degrading recalcitrant carbohydrates, such as cellulose and chitin (Fig. 1A), for the production of biofuels (Himmel *et al.*, 2007, *Science* 315: 804). Cellulose is the most abundant organic molecule on the earth and offers a renewable and seemingly inexhaustible feedstock for the production of fuels and chemicals. Chitin is a common constituent of fungal cell walls, shells of crustaceans and exoskeletons of insects. It is the second most abundant polymer in nature and each year more than one billion tons of chitin are produced in the biosphere, mainly by insects, fungi, crustaceans and other marine organisms. Chitin is abundantly available as a by-product from aquaculture, one of the fastest growing bioproduction industries on earth.

The conversion of cellulosic 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 cleanness 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 the non-polysaccharide lignin. Once the cellulose is converted to glucose, the glucose is easily fermented by yeast into ethanol.

A variety of microorganisms exist for fermenting the products of hydrolysis of polysaccharides to yield desirable end products such as alcohol. Selection of appropriate microorganisms allows the products of hydrolysis of cellulose, chitin and other polysaccharides to be fermented to yield useful products, such as alcohol.

The predominant polysaccharide in the primary cell wall of biomass is cellulose, the second most abundant is hemi-cellulose 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.

Bacteria and fungi have evolved complex enzymatic systems enabling their growth on plant material rich in cellulose, but these organisms typically require weeks, months or even years to decompose a fallen log or a tilled corn stalk. Likewise, microorganisms contain enzymatic systems for degrading chitin. Bacterial chitinase helps to provide a carbon source for bacterial growth. Insects produce chitinase to digest their cuticle at each molt. In plants, chitinase is thought to provide a protective role against parasitic fungi. For chemical or fuel production from these same cellulose- and chitin-containing materials, industry requires affordable chemical or enzymatic systems that can do the job in hours or in days.

Traditionally, enzyme systems capable of degrading such carbohydrates were considered to consist of two types of hydrolytic enzymes called glycoside hydrolases: endo-acting enzymes that cut randomly in the carbohydrate chain and processive exo-acting enzymes (chito- or cellobiohydrolases), which degrade the polymers from chain ends (Fig. 1B). Although this model is generally accepted, it remains difficult to understand how, e.g., an endoglucanase would be capable of pulling a single polysaccharide chain out of its crystalline environment and forcing the chain productively into its active site cleft (Fig. 1B).

Ever since cellulases caught the interest of biochemists, there have been speculations about the possible existence of a substrate-disrupting factor that could make the crystalline substrate more accessible to hydrolytic enzyme (Reese *et al.*, 1950, *J Bacteriol.* 59: 485). Recently, it was discovered that microorganisms that breakdown chitin indeed produce a protein that increases substrate accessibility and potentiates hydrolytic enzymes (Vaaje-Kolstad *et al.*, 2005, *J. Biol. Chem.* 280: 28492; Figs. 1C and D). The first example was a single-domain

protein called CBP21 (CBP for Chitin-Binding Protein) produced by the chitinolytic bacterium *Serratia marcescens*. This protein has been classified as carbohydrate-binding module (CBM) and belongs to family CBM33 as defined by the CAZy nomenclature (Boraston *et al.*, 2004, *Biochem. J.* 382: 769, Henrissat, 1991, *Biochem. J.* 280 (Pt 2): 309). Another example
5 concerns two CBM33-containing proteins from *Thermobifida fusca*, called E7 and E8, which potentiate chitin hydrolysis by chitinase and cellulose hydrolysis by cellulases (Moser *et al.*, 2008, *Biotechnol. Bioeng.* 100(6): 1066-77). Like CBP21, E7 is a single domain protein only comprising a CBM33 domain. E8 is a three-domain protein, meaning that it carries two domains in addition to a CBM33 domain.

10 It has recently been shown that proteins presently classified as family 61 glycoside hydrolases (GH61) in the CAZy nomenclature act synergistically with cellulases (Harris *et al.*, 2010, *Biochemistry* 49: 3305) and are structurally similar to CBM33 proteins (Harris *et al.*, 2010, *supra*; Karkehabadi *et al.*, 2008, *J. Mol. Biol.* 383: 144; Fig. 1E). While CBM33 and GH61 have little sequence similarity, the structural similarity is evident (Figs. 1D and 1E), including a
15 diagnostic fully conserved arrangement of the N-terminal amino group, an N-terminal histidine and one other histidine residue (Fig. 1F; Karkehabadi *et al.*, 2008, *supra*) forming a promiscuous metal-binding site (see below). On the basis of available literature data, including a recent comprehensive study of several GH61 proteins, it seems highly unlikely that GH61
20 proteins are endoglucanases, as originally thought (Harris *et al.*, 2010, *supra*). Like CBM33 proteins, GH61 proteins do not have a substrate-binding cleft or pocket, nor do they possess a characteristic arrangement of acidic amino acids that could indicate a glycoside hydrolase activity. Instead, both types of proteins show an almost flat substrate-binding surface (Figs. 1D and 1E; Harris *et al.*, 2010, *supra*; Vaaje-Kolstad *et al.*, 2005, *J. Biol. Chem.* 280: 11313). All in
25 all, these results and observations show that CBM33 and GH61 proteins have similar functions, *i.e.*, potentiating the efficacy of known hydrolytic enzymes (glycoside hydrolases) acting on crystalline polysaccharides. Furthermore, these results and observations strongly suggest that these proteins do so employing the same type of mechanism. So far, this mechanism has remained elusive.

30 The present invention provides methods of degrading or hydrolyzing a polysaccharide, such as chitin or cellulose, comprising contacting said polysaccharide with an oxidohydrolytic enzyme in the presence of at least one reducing agent and at least one divalent metal ion.

Summary of the Invention

35 The present invention relates to methods of degrading or hydrolyzing a polysaccharide comprising contacting said polysaccharide with one or more oxidohydrolytic enzymes, wherein said degradation or hydrolysis is carried out in the presence of at least one reducing agent and at least one divalent metal ion.

The present invention also relates to methods of producing soluble saccharides, wherein said method comprises degrading or hydrolyzing a polysaccharide by the method defined above, wherein said degradation or hydrolysis releases said soluble saccharides and optionally isolating said soluble saccharides.

5 The present invention also relates to methods of producing an organic substance, comprising the steps of: (i) degrading or hydrolyzing a polysaccharide by the method defined above to produce a solution comprising soluble saccharides; (ii) fermenting said soluble saccharides, to produce said organic substance as the fermentation product; and optionally, (iii) recovering said organic substance.

10 The present invention also relates to a process for producing a fermentation product, comprising: (a) saccharifying a cellulosic material with an enzyme composition comprising an endoglucanase, a cellobiohydrolase, a beta-glucosidase, a GH61 polypeptide having cellulolytic enhancing activity, and a CBM33; (b) fermenting the saccharified cellulosic material with one or more fermenting microorganisms to produce the fermentation product; and (c) recovering the
15 fermentation product from the fermentation.

Brief Description of the Figures

Figure 1 shows (A) Repetitive disaccharide units in cellulose and chitin. (B) A schematic representation of the degradation of chitin or cellulose by endo-acting (top) and (processive) exo-acting glycoside hydrolases (bottom). (C) The effect of CBP21 on chitinase efficiency; the
20 chitinase is chitinase C (ChiC), a family 18 endochitinase from *S. marcescens* (data from Vaaje-Kolstad *et al.*, 2005, *J. Biol. Chem.* 280: 28492). (D) The crystal structure of CBP21 (Vaaje-Kolstad *et al.*, 2005, *J. Biol. Chem.* 280: 11313). The side chains of the conserved histidine residues on the flat binding surface are shown in stick representation. (E) The crystal
25 structure of a GH61E from *Thielavia terrestris* (Harris *et al.*, 2010, *Biochemistry* 49: 3305). The side chains of the conserved histidine residues on the binding surface are shown in stick representation. (F) A detailed view of the conserved arrangement of the two histidines and the N-terminal amino group in CBP21 (dark grey) and GH61E (light grey), superimposed using PyMol. Figs. D, E and F were generated using PyMol.

30 Figure 2 shows (A) A MALDI-TOF MS spectrum showing oxidized soluble chitooligosaccharides generated by CBP21 acting on beta-chitin whiskers. This represents the very first time that such oxidized products were detected. (B) A chitin chain with a GlcNAcA unit. (C) 2.0 mg/mL beta-chitin treated with 1.0 μ M CBP21 in the presence (left vial) or absence (right vial) of 1.0 mM ascorbic acid. After the incubation with or without ascorbic acid, the tubes
35 were treated in an identical manner: They were shaken and then left for one minute for the chitin material to settle/sediment. The picture shows that the disrupted material in the left vial sediments much more slowly. This picture shows the enormous potential of CBP21 in

disrupting crystalline chitin when applied under optimal conditions, *i.e.*, in the presence of a reducing agent and a divalent metal ion. **(D)** A MALDI-TOF MS spectrum of products obtained after incubating beta-chitin (2.0 mg/mL) with 1.0 μ M AnCDA (an overexpressed and purified chitin deacetylase from *Aspergillus nidulans*), 1.0 μ M CBP21 and 1.0 mM ascorbic acid for 16h, showing oxidized deacetylated chitooligosaccharides (all major products contain two acetylated sugars). A control reaction without CBP21 did not yield soluble products (results not shown). **(E)** A MALDI-TOF MS spectrum of products obtained after incubating beta-chitin (2.0 mg/mL) with 1.0 μ M AnCDA and 0.5 μ M ChiC for 8 hours, showing deacetylated chitooligosaccharides (all major products contain one acetylated sugar). MS peaks are labelled by observed atomic mass and the degree of polymerization (DP) of the oligosaccharide. Labels always refer to the peak of highest intensity in the respective cluster (which comprises several adducts; see Fig. 3). "ox" indicates the presence of a GlcNAcA at the chain end opposite to the non-reducing end. Asterisks indicate a deacetylated product. 100% relative intensity represents 2.5×10^4 , 1.1×10^3 and 5.4×10^4 arbitrary units (a.u.), in panels A, D and E, respectively. See Fig. 3 for additional experiments for product verification.

Figure 3 shows controls of product identities by MALDI-TOF. **(A)** GlcNAcA containing products of CBP21 degraded beta-chitin are in equilibrium with the 1,5 δ -lactone and at low pH this equilibrium should become observable (Pocker & Green, 1973, *J. Am. Chem. Soc.* 95: 113). A sample with hexameric products was adjusted to pH $\sim$ 3.0 with acetic acid and incubated for four hours at room temperature. The sample was then analyzed by MALDI-TOF MS. The spectrum clearly shows both the acid form and the 1,5 δ -lactone which differ by 18 atomic mass units (amu), representing a water molecule. The equilibrium between the two forms will eventually lead to exchange of both oxygens added during the reaction with oxygens from solvent water. Indeed when product samples obtained after carrying out the reaction in H_2^{18}O (see Examples) were adjusted to low pH, we observed not only a lactone and an acid showing a mass increase of 2 amu compared to reactions performed in H_2^{16}O , but also, after some time, an acid showing a mass increase of 4 amu (results not shown). **(B)** Details of the mass spectrum for DP6_{ox}. The fact that the mass difference between Na^+ and K^+ corresponds to the mass of an oxygen atom could complicate interpretation. However, the similar ratios between the single sodium- and potassium adducts and their corresponding sodium salt of sodium/potassium adduct (often observed for sugar acids) indicate that the product indeed is an oxidized hexamer. A second verification of the identity of the products observed by MALDI-TOF MS was done by adding LiCl to a final concentration of 33 mM to the sample before MALDI-TOF MS analysis to generate new diagnostic adducts. Products corresponding to the Li-adduct of the acid and the Li-adduct of the Li-salt of the acid were observed **(C)**, thus confirming the identity of Na- and K- adducts observed in (B). Peaks in all MALDI-TOF MS spectra are labelled according to their observed atomic mass and the degree of polymerization

(DP) of the oligosaccharide. "ox" stands for oxidized. **(D)** Superposed PSD spectra for the hexamer fraction obtained upon CBP21 treatment of beta-chitin in H_2^{16}O (black) and H_2^{18}O (grey). Inserts provide detailed views of parts of the spectrum. The data show that the Y-ions differ by 2 amu, whereas the B ions show identical masses regardless of the type of water used. The data are fully compatible with the chemical structure shown above the mass spectrum and show that the heavy oxygen atom is introduced at the oxidized reducing end. Note that the chemical structure shown has been simplified for the purpose of clarity: most hydroxyl groups as well as C6 are lacking. 100% relative intensity represents 4.6×10^4 , 1.1×10^4 , 0.8×10^3 and 1.8×10^4 a.u. in panels A, B, C and D, respectively.

Figure 4 shows the effect of ascorbic acid (a reductant) on the potentiating effect of CBP21 on chitinase efficiency. The efficiency of beta-chitin (0.1 mg/mL) degradation by 0.5 μM ChiC was analyzed in the presence (top grey line on filled triangles) or absence (bottom grey line on filled diamonds) of 1.0 μM CBP21 and 1.0 mM ascorbic acid at pH 8.0. A parallel reaction containing beta-chitin, ChiC and CBP21 (same concentrations as above) but no ascorbic acid was run as a control (central dark grey line on filled squares). The results show that CBP21 promotes chitin degradation by ChiC considerably better in the presence of ascorbic acid (all chitin is degraded after 2.5 hours), compared to conditions where ascorbic acid is absent. A parallel reaction containing beta-chitin and ChiC (same concentrations as above), in the absence of ascorbic acid, would yield a result similar to that represented by the grey line on filled diamonds (results not shown, but the experiment is included and shown in Fig. 10B), meaning that ascorbic acid alone had no effect on ChiC efficiency.

Figure 5 shows the analysis of soluble reaction products from CBP21 catalysis. **(A)** After 4 days of incubation of 2 mg/mL beta-chitin with 1.0 μM CBP21 and 1.0 mM ascorbic acid at pH 8.0, soluble products are dominated by even numbered short oligosaccharides with acidic reducing ends. Due to the low solubility of chitin oligosaccharides longer oligosaccharides are not observed (see Examples for the detection of longer products). **(B)** Each acidic oligosaccharide species was observed as H-, Na- and K-adducts and Na- and K-adducts of the acidic oligosaccharide Na-salt. 100% relative intensity represents 3.5×10^3 a.u.

Figure 6 shows UHPLC separation of oxidized oligosaccharides with acidic (GlcNAcA) ends. A semi-quantitative analysis of soluble products generated by CBP21 was performed by separating the oxidized oligosaccharides by UHPLC. **(A)** Typical product spectrum showing the periodicity of even and odd numbered products that was also observed in the MALDI-TOF MS analysis (e.g., Figs 2D and 5). Note that the alpha- and beta-anomers of non-oxidized oligosaccharides would be separated under these chromatographic conditions. The fact that only single peaks are observed confirms the modification of the reducing end. Peak identities were determined using MALDI-TOF MS (results not shown). **(B)** Initial reaction velocities of CBP21 mediated chitin solubilization into hexameric products (full lines) or pentameric products

(dashed lines) in the presence of 5.0 (grey lines, circular data points), 1.0 (dark grey lines, square data points) and 0.2 mM (black lines, triangular data points) ascorbic acid. These results show that the velocity of the reaction depends on the ascorbic acid concentration and that odd-numbered products are generated at a slower rate than even-numbered products.

5 Figure 7 shows MALDI-TOF MS analysis of products detected after treating 2.0 mg/mL beta-chitin with 1.0 μ M CBP21 and 1.0 mM ascorbic acid in Tris buffered H_2^{18}O pH 8.0 (**A**) or in Tris buffered H_2^{16}O pH 8.0 saturated with $^{18}\text{O}_2$ (**B**). All major products show a mass increase of 2 amu compared to reactions performed in solutions not containing isotope labeled water or molecular oxygen (see Figs. 2A and 5). (**C**) Adducts of the oxidized hexameric product shown
10 in panel (B). Note the small amount of non-isotope labeled product (indicated by the arrow), most likely resulting from the initial stage of the reaction where $^{16}\text{O}_2$ was still present (prior to $^{18}\text{O}_2$ saturation; see Materials and Methods). 100% relative intensity represents 6.8×10^3 and 2.0×10^3 a.u. in panels A and B and C, respectively. (**D**) Scheme for the enzymatic reaction catalyzed by CBP21. In the final oxidized product, one oxygen comes from molecular oxygen
15 and one from water.

Figure 8 shows the effect of various potentially inhibiting factors on CBP21 activity. Sodium dithionite (SD) is a well known oxygen scavenger routinely used to create a oxygen free (anaerobic) environments for enzyme reactions. (**A**) MALDI-TOF MS spectrum of a
20 reaction mixture containing 2.0 mg/mL beta-chitin, 1.0 μ M CBP21, 1.0 mM ascorbic acid and 10 mM sodium dithionite, incubated for 16 hours at 37°C under anaerobic conditions (all vials were degassed with a Schlenk line). The spectrum shows baseline noise and no peaks of noteworthy intensity; in other words, there are no detectable amounts of CBP21 generated products. 100% relative intensity represents 0.9×10^2 a.u. Panel (**B**) shows that the potentiating effect of CBP21 on chitinase (ChiC) is abolished by adding sodium dithionite (SD). Reaction conditions: 0.1
25 mg/mL beta-chitin, 0.1 μ M ChiC, 1.0 mM ascorbic acid, 10 mM sodium dithionite, 1.0 μ M CBP21, incubated for 16 hours at 37°C under anaerobic conditions (all vials were degassed with a Schlenk line). End product quantities were analyzed by HPLC. Panel (**C**) shows additional control experiments, showing product development during incubation of 0.45 mg/mL beta-chitin with 0.5 μ M ChiC and 1 mM reduced glutathione (which has the same effect as ascorbic acid; see Fig. 11) in 20 mM Tris-HCl, pH 8.0. Further additions/ conditions were 1.0 M
30 CBP21 (standard conditions for maximum activity; dark line on filled diamonds), 1.0 μ M CBP21 in a solution with reduced oxygen concentration obtained by gas exchange (line on plusses; note that the results displayed in panel C of Fig. 7, showing the presence of $^{16}\text{O}_2$ even after extensive gas exchange in the Schlenk line, show that truly anaerobic conditions were not
35 obtained), 1.0 μ M CBP21 and 2 mM potassium cyanide, a well known O_2 mimic (line on filled squares) and no CBP21, no further additions (standard control experiment; line on filled circles). Addition of 2.0 mM sodium azide, a known inhibitor of heam proteins, did not inhibit

CBP21 activity and the curves were similar to the curve on filled diamonds. CBP21 was also fully inhibited by oxyrase (Oxyrase Inc., Mansfield, OH), a bacterial oxidase that uses lactate as hydrogen donor in order to create an anaerobic environment (results not shown). Panel (D) shows the production of GlcNAc₃GlcNAcA (*i.e.*, an oxidized tetramer) during a reaction of 0.45 mg/mL beta-chitin, 1.0 μ M CBP21 and 1.0 mM reduced glutathione in 20 mM Tris-HCl pH 8.0 in the absence (line on filled diamonds) or presence (line on filled squares) of 2.0 mM potassium cyanide. This result shows that cyanide inhibits the oxidation reaction. Data shown in panels B, C and D are mean $\pm$ SD ($N=3$); error bars (not visible for every point) indicate SD.

Figure 9 shows the effect of divalent cations on CBP21 activity. The picture shows ChiC activity, monitored by measuring the concentration of produced (GlcNAc)₂ by HPLC. Reaction mixtures contained 2.0 mg/mL beta-chitin in 1.0 mM ascorbic acid, 5 mM EDTA, 0.5 μ M ChiC in 20 mM Tris pH 8.0, in the presence or absence of 1.0 μ M metal-free CBP21. Solid lines indicate reactions with metal-free CBP21; dashed lines indicate reactions without CBP21. Half way through the reaction (indicated by the arrow) MgCl₂ (final concentration 25 mM), ZnCl₂ (25 mM) or buffer was added to one of two (parallel) reaction mixtures. Squares, MgCl₂ added; circles, ZnCl₂ added; triangles, buffer added. The results clearly demonstrate that divalent cations are essential for the function of CBP21; the controls show that the cations do not affect ChiC activity.

Figure 10 shows the importance of the conserved His114. CBP21-like and GH61 proteins contain two highly conserved histidines (H28 and H114 in CBP21), one of which is the N-terminal histidine of the secreted mature protein. In order to probe the importance of His114 the H114A mutant variant of CBP21 was analyzed. Panel (A) shows that incubation of 1.0 μ M CBP21^{H114A} with 2.0 mg/mL beta-chitin, 1.0 mM ascorbic acid in 20 mM Tris, pH 8.0 for 16 hours at 37°C did not lead to production of soluble oxidized oligosaccharides. 100% relative intensity represents 3.5×10^4 a.u. Panel (B) shows product release upon degradation of 0.1 mg/mL beta-chitin with 0.5 μ M ChiC in 1.0 mM ascorbic acid, 20 mM Tris, pH 8.0, in the presence or absence of CBP21^{WT} (top grey line on filled triangles and dark grey line on filled diamonds, respectively) and in the presence of CBP21^{H114A} (grey line on filled squares). The dark grey lines on filled circles show a reaction without CBP21 and without ascorbic acid. The data clearly show that His114 is essential for the function of CBP21 as CBP21^{H114A} is unable to boost chitinase activity, even in the presence of ascorbic acid.

Figure 11 shows the effect of other reductants on CBP21 activity. Reaction products obtained upon incubating 2.0 mg/mL beta-chitin for 16 hours at 37°C in the presence of 1.0 μ M CBP21 and (A) 1.0 mM reduced glutathione or (B) in 1.0 mM Fe(II)SO₄, in 20 mM Tris pH 8.0. Note that the observed product profiles are highly similar to those obtained in the presence of ascorbic acid (Fig. 5). Figs. 5 and 6 show that the activity of CBP21 is increased in the presence of ascorbic acid. Reductants such as reduced glutathione and Fe(II)SO₄ had similar

effects on the kinetics of the degradation reaction (results not shown). 100% relative intensity represents 1.5×10^4 and 0.8×10^3 a.u. in panels A and B, respectively.

Figure 12 shows the effect of ascorbic acid on beta-chitin. It is well established that reductants undergo autooxidation in oxygenated solutions, thus generating reactive oxygen species that may putatively oxidize biomolecules present in the surrounding medium. The picture shows MALDI-TOF MS analysis of a reaction mixture obtained after incubating a sample containing 2.0 mg/mL beta-chitin and 1 mM ascorbic acid in 20 mM Tris pH 8.0 for four days at 37°C. The spectrum does not show any sign of soluble oxidized oligosaccharides. 100% relative intensity represents 4.4×10^4 a.u.

Figure 13 shows the effect of CBP21 on soluble native oligosaccharides. Earlier studies (Vaaje-Kolstad *et al.*, 2005, *J. Biol. Chem.* 280: 11313; Suzuki *et al.*, 1998, *Biosci. Biotechnol. Biochem.* 62: 128) have shown that CBP21 binds specifically to beta-chitin and not to soluble or amorphous forms of chitin. With the results presented here in mind, the ability of CBP21 to oxidize and/or hydrolyze native oligosaccharides of chitin was investigated. A solution containing 100 μ M GlcNAc₆ in 20 mM Tris pH 8.0 was incubated for 16 hours in the presence of buffer only, 1.0 mM ascorbic acid (AA) or 1.0 mM ascorbic acid and 1.0 μ M CBP21 and reaction products were analyzed by MALDI-TOF MS (panels **A**, **B** and **C**, respectively) and quantified by HPLC (panel **D**). No degradation or oxidation of GlcNAc₆ was observed. Similar experiments were performed with the soluble polymeric chitin-derivative chitosan; again, no degradation or oxidation products could be detected (results not shown). 100% relative intensity represents 5.7×10^3 , 1.4×10^4 and 4.0×10^4 a.u. for panels A, B and C, respectively.

Figure 14 shows the possible effect of Fenton chemistry on beta-chitin solubilization. It could be envisaged that the observed CBP21 mediated oxidohydrolytic cleavage of beta-chitin is related to generation of conditions leading to Fenton type chemistry (analogous to what has been proposed for cellulose degradation by cellobiose dehydrogenases (Hammel *et al.*, 2002, *Enzyme. Microb. Tech.* 30: 445; Henriksson *et al.*, 2000, *J. Biotechnol.* 78: 93; Hyde & Wood, 1997, *Microbiol-Uk* 143: 259). Although, Fenton chemistry is not particularly effective at the pH used in this study (8.0), we did check whether Fenton-like processes might occur. Fenton chemistry is well known to depolymerize polysaccharides and could yield oxidized products similar to those generated by CBP21. 2.0 mg/mL beta-chitin was incubated for 16 hours with 0.03% H₂O₂ and 5.0 mM Fe(II)SO₄ in 20 mM Tris pH 8.0, at 37°C. The soluble phase of the reaction was analyzed by MALDI-TOF MS. The mass spectrum shown in the picture did not reveal formation of soluble products. As controls, MALDI-TOF experiments were repeated using various dilutions of the original sample; samples from reactions where the H₂O₂ concentration was 0.3% or 0.003% were also analyzed. Soluble products were never observed (results not shown). 100% relative intensity represents 1.2×10^4 a.u.

Figure 15 shows: **(A)** Crystals of EfCBM33 (Uniprot ID:Q838S1; EF0362; uniprot.org/uniprot/Q838S1), obtained by hanging drop vapor diffusion experiments that have been used to collect 0.95Å data. **(B)** The side chain of the solvent exposed Trp modeled in the 2Fo-Fc map. **(C)** Superposition of the main chains of CBP21 and EfCBM33; the side chains of the conserved histidines and a surface exposed aromatic amino acid (Tyr in CBP21, Trp in EfCBM33) are shown. **(D)** MALDI-TOF MS spectrum of soluble products obtained after treating 2.0 mg/ml beta-chitin with “washed” EfCBM33 crystals dissolved in 20 mM Tris pH 8.0; as for CBP21, the product spectrum is dominated by even-numbered oxidized chitooligosaccharides, clearly demonstrating oxidohydrolytic activity. **(E)** Degradation of 2.0 mg/ml alpha-chitin (from shrimp shells) by 0.3 µM of the chitinase from *Enterococcus faecalis* (protein name (EF0361) in the presence or absence of 0.3 µM EfCBM33 and 1.0 mM reductant (R: reduced glutathione) incubated at 37°C with agitation at 900 rpm. A boost of the chitinase activity is clearly observed in the presence of EfCBM33 and reductant.

Figure 16 shows the speed and extent of oxidative cleavage by CBP21 activity. Panel **(A)** shows the production of non-oxidized dimers (top line with diamonds), oxidized trimers (line on triangles) and oxidized tetramers (obscured line on squares) produced during incubation of 0.45 mg/mL beta-chitin, 1.0 µM CBP21, 0.5 µM ChiC and 1.0 mM reduced glutathione in 20 mM Tris-HCl pH 8.0 (hydrolysis of oxidized oligosaccharides by ChiC produces only minimal amounts of oxidized dimers; the three products shown represent the large majority of oligomers produced under these conditions). After five hours, approximately 4.9% of the total sugars (theoretical number based on chitin concentration) are oxidized*. Panel **(B)** shows production of oxidized sugars during incubation of 0.45 mg/mL beta-chitin, 1.0 µM CBP21 and 1.0 mM reduced glutathione in 20 mM Tris-HCl pH 8.0. The degree of oxidation was determined by rapid conversion of the treated chitin with a large dose of a chitinase cocktail followed by UHPLC detection of oxidized dimers (filled diamonds) and trimers (filled triangles), as described in the materials and methods section. The linear part of the reaction represents an oxidation rate of approximately 1 per minute**. Maximum levels are reached after 2-3 hours and represent a degree of oxidation of 7.6% of the total sugars (theoretical number based on chitin concentration***. Data in both panels are mean +/- SD (N=3); error bars (not visible for every point) indicate SD.

* The end concentrations of oxidized trimer and tetramer are 53 and 55 µM, respectively, giving rise to a total of 108 µM GlcNAcA. The molar concentration of GlcNAc in the solution is 2217 µM. Thus the degree of oxidation is 108/2217; in other words 4.9% of the sugars are GlcNAcA. ** The rates calculated for oxidized dimer and oxidized trimer are 0.68 and 0.60 µM/min. When added, the rate of oxidized products generated is 1.28 µM/min and when taking the CBP21 concentration into account (1.0 µM), the rate of oxidohydrolysis is 1.28 per minute. *** At the maximum levels reached, CBP21 has produced 93 µM and 75 µM

oxidized dimer and oxidized trimer, respectively, which adds up to 168 μM GlcNAcA. The molar concentration of GlcNAc in the solution is 2217 μM . Thus the degree of oxidation is 168/2217; in other words 7.6% of the sugars are GlcNAcA.

Figure 17. MALDI-TOF MS analysis of soluble products generated by a CBM33 protein (1 μM CelS2) incubated with microcrystalline cellulose (2.0 mg/ml AVICEL®) in 20 mM Tris-HCl buffer pH 8.0 in the presence of 1.0 mM ascorbic acid (external electron donor), incubated for 20 hours, at 50°C with horizontal agitation at 250 rpm. The main peaks are annotated with molecular weight and degree of polymerization (DP). All annotated peaks are Na-adducts of oxidized cellooligosaccharides (hence the subscript "ox"). More detailed analysis of the adduct clusters is shown in Fig. 18.

Figure 18. MALDI-TOF MS analysis of the adduct cluster of the oxidized cellooligosaccharide hexamer generated by CelS2 (from the same sample as described in Fig. 17 legend). Peaks are annotated with molecular weight, degree of polymerization (DP); "ox" indicates oxidation. The spectrum shows small quantities of the native hexameric oligosaccharide ($m/z = 1013.14$). The native oligosaccharides may arise from CelS2 substrate cleavage near the reducing end or liberation due to substrate disruption (see also Fig. 19). The dominant peak represents the Na-adduct of the oxidized hexamer ($m/z = 1029.14$). Additionally, peaks representing the K-adduct of the oxidized hexamer ($m/z = 1045.08$) and the Na-adduct of the Na-salt of the oxidized hexamer ($m/z = 1051.13$) can also be observed.

Figure 19. HPAEC analysis of soluble products generated after incubation of 1.0 μM CelS2 with 10 mg/ml AVICEL® in 50 mM Tris-HCl pH 8.0, 1 mM MgCl_2 , at 50°C, 900 rpm (horizontal agitation) in the presence ("pink", upper line) or absence ("dark purple", lower line) of 0.5 mM reduced glutathione (external electron donor). In the absence of reduced glutathione only small amounts of native cellooligosaccharides are observed (Glc3-Glc6) whereas larger amounts of both native cellooligosaccharides and oxidized cellooligosaccharides are observed when reduced glutathione is present. The DP of the oxidized cellooligosaccharides is indicated by n, where $n=3$ (the shortest labelled oxidized cellooligosaccharide has a DP of three ($=n$)). The presence of small amounts of native cellooligosaccharides was also seen in the control reaction without added CelS2 (10 mg/ml AVICEL® in 50 mM Tris-HCl pH 8.0 in the presence and absence of 0.5 mM reduced glutathione; results not shown). Thus, the substrate itself contains some shorter soluble non-oxidized cello-oligomers that are released upon incubation, even without CelS2. It is well known from the literature that AVICEL® has a relatively low degree of polymerization (Wallis et al., 1992, *Carbohydrate Polymers* 17: 103-110). Additionally, chain cleavage by CelS2 near the reducing end of a cellulose chain will give rise to such products. Peak annotation was performed by comparing the chromatogram with the chromatogram obtained for a chemically prepared mixture of oxidized cellooligosaccharides

with lengths varying from DP1 to DP10. Note the periodicity of the oxidized celooligosaccharides also observed by MALDI-TOF MS analysis (Fig. 17).

Figure 20. Total sugar (g/l) ([Glc] + cellobiose which is reported as [Glc]) after incubation of 1 μ M CelS2 or E7 and 0.05 μ l/ml CELLUCLAST™ ("CC") with 10 mg/ml filter paper for 24 or 120 hours in the presence or absence of 1 mM reduced glutathione ("RG"). Reactions were run in 50 mM Bis-tris/HCl pH 6.5, 1 mM MgCl₂, at 50°C and 900 rpm (horizontal agitation). Control reactions containing the cellulosic substrate suspended in the same buffer as used for the enzyme assays gave no detectable signal for either glucose or cellobiose.

Figure 21. Cellobiose concentration (g/l) after 18 or 40 hours incubation of 1.0 μ M CelS2 and 5 μ g/ml Cel7A ("Cel7A") with 10 mg/ml filter paper in the presence or absence of 1 mM reduced glutathione ("RG"). These reactions were conducted in 50 mM sodium acetate pH 5.5, 1 mM MgCl₂, at 50°C, 900 rpm (horizontal incubation). Only the cellobiose concentration was quantified as the amount of glucose was minor (less than 5% of the total sugar). Control reactions containing the cellulosic substrate suspended in the same buffer as used for the enzyme assays gave no detectable signal for either glucose or cellobiose.

Figure 22. HPAEC profiles showing products obtained after incubating CelS2 (1.0 μ M) with microcrystalline cellulose (10 mg/ml AVICEL®) in 20 mM Tris-HCl buffer pH 8.0 in the presence of 1.0 mM ascorbic acid (external electron donor), in the presence (line labelled "pink", lower line) or absence (line labelled "green" line, upper line) of 2.0 mM potassium cyanide. Products were analyzed after incubation for 24 hours at 50°C with vertical agitation at 900 rpm. The two remaining (lower) lines represent the same conditions as noted above (CelS2+cyanide; "blue" line, buffer+cyanide; "brown" line), in the absence of ascorbic acid. The DP of the oxidized celooligosaccharides is indicated (n=3), *i.e.*, the shortest visible oxidized celooligosaccharide has a DP of three (GlcNAc-GlcNAc-GlcNAcA).

Figure 23. MALDI-TOF MS analysis of soluble products generated by 1 μ M CelS2-WT (panel A) or 1 μ M CelS2-H144 (panel B) upon incubation with microcrystalline cellulose (10 mg/ml AVICEL®) in 20 mM Tris-HCl buffer pH 8.0 in the presence of 1.0 mM ascorbic acid (external electron donor), for 24 hours at 50°C with horizontal agitation at 250 rpm. The main peaks are annotated with molecular weight and degree of polymerization (DP). All annotated peaks are Na adducts of native celooligosaccharides, oxidized celooligosaccharides (denoted "ox") or the sodium salts of oxidized celooligosaccharides. Neither native nor oxidized celooligosaccharides can be observed for the reaction with CelS2-H144 (panel B). The low intensity peak observed at 1199.55 m/z does not correspond to any oligosaccharide resulting from cellulose oxidation or hydrolysis and is likely to be a background component. The presence of native celooligosaccharides is likely to be the result of the low mean DP of the substrate (AVICEL®; Wallis *et al.*, 1992, *Carbohydrate Polymers* 17: 103-110); chain cleavage

by CelS2 near the reducing end of a cellulose chain will also give rise to such products. See legend to Fig. 19 for additional discussion.

Figure 24. Effect of CelS2 (1.0 μ M) on the degradation of steam exploded saw dust from poplar (SEP; 2.0 mg/ml) by CELLUCLAST™ (0.016 μ l/ml) in 20 mM sodium acetate buffer pH 5.5, 1.0 mM MgCl_2 , 1.0 mM reduced glutathione (RG; external electron donor) incubated for 20 hours at 50°C, 250 rpm (horizontal agitation). The figure shows cellobiose release after 20 hours.

Figure 25. Degradation of high molecular weight filter paper cellulose (10 mg/ml) in 20 mM sodium acetate buffer pH 5.5 by 0.8 μ g/ml CELLUCLAST™ (CC), in the presence or absence of 40 μ g/ml CelS2 and 0.5 mM reduced glutathione (RG) as shown by the increase of soluble cellooligosaccharides (Glc and Glc₂; converted to total Glc) over time. In reactions where no CelS2 was present, 40 μ g/ml purified BSA was added in order to maintain an identical protein load. Under these conditions, reactions with only CelS2 did not yield detectable amounts of Glc or Glc₂ (not shown). The chitin-active CBM33, CBP21, did not affect CC efficiency (not shown). RG had no effect in reactions with only CC; only one of the two overlapping curves is shown. Data are mean $\pm$ SD ($N=3$); error bars indicate SD.

Figure 26. Degradation of cellulose in a reaction of 10 mg/ml filter paper with a combination of 40 μ g/ml CelS2 (or 40 μ g/ml BSA to compensate for the protein load in reactions without CelS2) and 5 μ g/ml purified HjCel7A in 1 mM MgCl_2 , 20 mM sodium acetate buffer, pH 5.5. By far the major products of these reactions are cellooligosaccharides released by the cellulases (whose activity is boosted by CelS2). In the case of HjCel7A (Panel C), the main product is cellobiose and formation of this product is shown. RG, indicates the presence of a reductant, in this case reduced glutathione. The label “+/- RG” indicates that production curves for Cel7A alone (no CelS2 present) with and without RG were essentially identical. The reactions were run at 50°C.

Figure 27. HPAEC profiles of products obtained after incubating AVICEL® with the N-terminal CBM33 domain of CelS2 in the absence of reductant (bottom line) and in the presence of reduced glutathione (2nd from bottom line), gallic acid (darker of upper lines) or ascorbic acid (lighter of upper lines), which all serve as external electron donor. 1 μ M of the N-terminal CBM33 domain of CelS2 was incubated with 10 mg/ml AVICEL® in the presence of 0.8 mM of one of the reductants, or in the absence of any reductant. Reactions were run in 50 mM succinate buffer pH 5.5, 1 mM MgCl_2 , at 50°C and 900 rpm (vertical agitation) for 20 hours.

Figure 28. Total sugar (g/l) [Glc] (glucose and cellobiose reported as [Glc]) released after incubation of 0.08 μ l/ml CELLUCLAST™ (CC) in the presence or absence of 1 μ M E7 and 2 mM reduced glutathione (RG) as external electron donor with 2 mg/ml filter paper cellulose. Reactions were incubated in 20 mM sodium acetate buffer pH 5.5, 1 mM MgCl_2 , at 50°C and 900 rpm (horizontal agitation) for up to 50 hours. RG had no effect in reactions with only CC;

only one of the two overlapping curves is shown. Control reactions with filter paper suspended in the same buffer as used for the enzyme assays, or E7 in absence of cellulases did not yield detectable amounts of Glc or Glc₂ (not shown).

Figure 29. MALDI-TOF MS analysis of soluble products generated by 1 μ M E7 incubated with 10 mg/ml AVICEL® in 20 mM Tris-HCl buffer pH 8.0 and 1 mM MgCl₂ in the presence of 1 mM ascorbic acid as external electron donor, incubated for 20 hours, at 50°C with vertical agitation at 900 rpm. The oxidized oligosaccharides (Glc₃₋₇GlcA) are observed as sodium adducts, and as sodium adducts of the oligosaccharide sodium salts, and annotated with their molecular weights (m/z); native cello-oligosaccharides are also present but have been excluded from annotation. Such native oligosaccharides might be the result of CBM33 cleavage near the reducing end of the substrate.

Figure 30. HPAEC profiles of products generated by 1 μ M full length CelS2 in the presence (top grey line) or the absence (bottom dark grey line) of 1 mM ascorbic acid (AA), or by the N-terminal CBM33 domain of CelS2 in the presence of AA (second top, dark line), or by the C-terminal CBM2 domain of CelS2 in the presence of AA (second bottom, dark line) upon incubation with 10 mg/ml AVICEL®. Products were analyzed after 20 hours of incubation in 20 mM ammonium acetate buffer pH 5.0, 1 mM MgCl₂ at 50°C and 900 rpm (vertical agitation). The chromatogram is enlarged to emphasize the oxidized cello-oligosaccharides produced by the full length CelS2 and the N-terminal CBM33 domain of CelS2.

Figure 31. Reactivation of activity by adding metals to EDTA treated N-terminal CBM33 domain of CelS2. 0.8 mg/ml CBM33 was incubated with 400 μ M EDTA for 3 hours at 20°C. This EDTA treated enzyme (40 μ g/ml) was incubated with 10 mg/ml AVICEL® in 50 mM MES buffer pH 6.6 containing 1.7 mM reduced glutathione and one of 6 different metal ions (10 μ M; Mg²⁺, Fe³⁺, Zn²⁺, Co²⁺, Ca²⁺, Cu²⁺). The remaining concentration of EDTA in these reaction mixtures was 20 μ M. The superimposed HPAEC chromatograms show products released after 20 hours of incubation at 50°C. Only Cu²⁺ (top, grey line) reactivated the enzyme under these conditions and when using these low metal concentrations.

Figure 32. A) Alignment of GH61 sequences using ClustalW. The HjGH61A sequences were aligned to a profile created by structurally aligning TtGH61E and HjGH61B. N-terminal signal peptides present in the natural primary gene products were removed from the sequences prior to producing the alignment. B) The structural superposition of TtGH61E (green) and HjGH61B (orange) is seen. The side chains of six conserved active site and surface residues coloured red in the alignment (A) are shown in magenta in (B). An insertion present in HjGH61B is coloured yellow in (A) (shaded section) and (B) (3 turn helix).

Figure 33. Structure of CBP21 (A) compared with a structural model of E7 (B) and the structure of TtGH61E (C). Note the similarity between E7 and TtGH61E (Met and Val at positions 207 and 161 respectively are both hydrophobic residues; the corresponding residue in

CBP21, T183, is more polar). CBP21 lacks a histidine (CBP21 has D182), a tyrosine (CBP21 has F187) and a glutamine (CBP21 has E60) that are conserved in the two GH61s shown. Residues are numbered as they appear in the primary gene product, *i.e.*, a protein with an N-terminal signal peptide. Mature correctly processed proteins start with a histidine, which would then be histidine 1 (such as shown in the alignment of (A)). H28, H37 and H19 correspond to this histidine 1 in panels A, B and C, respectively.

Figure 34. HPAEC chromatograms showing production of oligosaccharides from microcrystalline cellulose (AVICEL®) by TtGH61E (upper curve, with higher peaks) and TaGH61A (lower curve). GH61 proteins (140 µg/ml) were incubated at 50°C with 10 mg/ml Avicel in 50 mM MES buffer pH 6.6 containing 2.4 mM ascorbic acid (a reductant) for 24 hours with horizontal agitation at 700 rpm.

Figure 35. Chromatograms (Rezex RFQ-Fast Fruit H+ column) showing production of glucose from AVICEL® and Filter Paper by Cellic™ CTec2 (final concentration of added protein was 1.1 µg/mL) in the presence or absence of ascorbic acid. The enzymes were incubated at 50°C with 10 mg/ml substrate (AVICEL® or filter paper) in 50 mM MES buffer pH 6.6 with or without 0.5 mM ascorbic acid for 24 hours with horizontal agitation at 700 rpm.

Detailed Description of the Invention

Surprisingly, the inventors have now found that CBM33 and GH61 proteins as oxidohydrolases exploit molecular oxygen and water to introduce chain breaks on the surfaces of crystalline polysaccharides, *i.e.*, on the surface of a solid phase, to open up the inaccessible polysaccharide material for hydrolysis by normal glycoside hydrolases. Although not wishing to be bound by theory it is believed that the carbohydrate chain is oxidized by molecular oxygen and chain cleavage is accomplished by a concomitant hydrolysis (Fig. 7D). In view of this mechanism we refer to the enzymes as oxidohydrolases. However, these enzymes may alternatively be referred to herein as polysaccharide degrading enzymes (or simply enzymes) in view of their ability to effect or assist cleavage of glycosidic bonds in polysaccharides. Enzymatic cleavage of crystalline polysaccharides in a reaction dependent on reactive oxygen species has so far not been described.

These enzymes have flat surfaces that bind to the flat, solid, well-ordered surfaces of crystalline material and catalyze chain breaks. The chain break will result in disruption of crystalline packing and increased substrate accessibility, an effect that may be augmented by the modification of one of the new chain ends. At the cleavage point one of the new ends is a normal non-reducing end (indicated by R-OH in Fig. 7D). The other new end would have been a new reducing end if the cleavage had been performed by a normal glycoside hydrolase. However, in this case the product is different and the last sugar is oxidized to become 2-(acetylamino)-2-deoxy-D-gluconic acid (Fig. 7D). This novel "acidic chain end" will interfere with

normal crystal packing because it will not have the normal chair conformation of the sugar ring and because it carries a charge. Effects on non-crystalline substrates are also possible.

Genes encoding these oxidohydrolases (such as genes encoding members of the CBM33 or GH61 families) are abundant in chitin- and cellulose-degrading microorganisms. As assessed by gene sequences, CBM33 and GH61 proteins are found both as single-domain proteins (*i.e.*, consisting of a CBM33 or GH61 domain only) and as multi-domain proteins (*i.e.*, consisting at least one more domain, often a domain that is putatively involved in substrate binding). When the CBM33 or GH61 domain containing proteins have more than one domain, the additional domains are usually coupled to the C-terminus of the CBM33 or GH61 domain because the N-terminus of the CBM33 or GH61 domain is essential for oxidohydrolytic activity. Knowledge of the mode of action of these enzymes has allowed their catalytic efficiency to be optimized to achieve more efficient enzymatic conversion of biomass into sugars which may be used for fermentation.

In view of the identification of the role of molecular oxygen in catalysis, it has now been found that the efficiency of the reaction can be improved by the addition of reductants that can act as electron donor and/or generate reactive oxygen species. In the presence of divalent metal ions reductants improve enzymatic conversion of recalcitrant polysaccharides.

Thus, in a first aspect, the present invention provides a method of degrading or hydrolyzing a polysaccharide comprising contacting said polysaccharide with one or more oxidohydrolytic enzymes, wherein said degradation or hydrolysis is carried out in the presence of at least one reducing agent and at least one divalent metal ion.

As referred to herein "degrading" said polysaccharide refers to degradation by disruption of the glycosidic bonds connecting the sugar monomers in the polysaccharide polymer.

The degradation of said polysaccharide is enhanced by the use of said reducing agents and metals relative to performance of said method without those means, thus the rate or degree of disruption of the glycosidic bonds that connect the sugar monomers is increased. This may readily be determined by measuring the product formation, *e.g.*, at certain defined time points or by measuring the amount of undegraded polysaccharide substrate which remains, *e.g.*, at certain defined time points. This can be carried out using methods that are well known in the art, based on, *e.g.*, determination of liberated reducing sugars (Horn *et al.*, 2004, *Carbohydrate Polymers* 56(1): 35-39 and references therein) or determination of liberated fragments, *e.g.*, cellulose or chitin fragments, *e.g.*, by quantitative analysis of chromatograms obtained upon High Performance Liquid Chromatography (Hoell *et al.*, 2005, *Biochim. Biophys. Acta* 1748(2): 180-190). Preferably said measure of degradation is assessed in the presence of one or more relevant saccharolytic enzymes as described hereinafter.

If the rate of degradation (*e.g.*, hydrolysis), *i.e.*, the number of bonds disrupted (*e.g.*, hydrolyzed) in a certain time period is greater when the substrate has been exposed to the oxidohydrolytic enzyme in the presence rather than absence of reducing agents and metal ions, then the rate of degradation is considered to be enhanced. Preferably the use of reducing agents and metal ions reduces the time taken for degradation (either complete or to the same level of partial degradation, *e.g.*, when additional saccharolytic enzymes are used, see hereinafter) by at least 2, 3, 4, 5, 6, 7, 8, 9 or 10 fold. Alternatively expressed, the use of reducing agents and metal ions increases the rate of degradation by at least 2, 3, 4, 5, 6, 7, 8, 9 or 10 fold.

"Hydrolyzing" refers to the chemical reaction in which water reacts with a compound to produce other compounds and involves the splitting of a bond and the addition of the hydrogen cation and the hydroxide anion from the water. In the case of hydrolysis of polysaccharides glycosidic bonds are cleaved by hydrolysis. The hydrolysis of polysaccharides to soluble sugars is referred to as "saccharification". Hydrolysis of polysaccharides as referred to herein results in degradation of the polysaccharide into smaller polysaccharides, including oligosaccharides and saccharide monomers such as glucose.

Hydrolysis of the polysaccharide may be partial or complete. In the case of complete hydrolysis, complete saccharification is achieved, *i.e.*, only soluble sugars (*e.g.*, mono- and di-saccharides) remain. In partial hydrolysis, in addition to soluble sugars, larger oligosaccharides and polysaccharides remain. As described herein methods of the invention include methods in which only oxidohydrolytic enzymes are used for degradation or in which both oxidohydrolytic enzymes and saccharolytic enzymes are used for degradation. In the former case, preferably at least 0.05-10%, *e.g.*, 0.05 to 5%, preferably 0.1 to 1% of the glycosidic bonds of the starting polysaccharide are degraded (*i.e.*, disrupted, *e.g.*, hydrolyzed) into oligosaccharides which may be separate from the polysaccharide substrate or may remain associated despite cleavage. In the latter case in which saccharolytic enzymes are also used, preferably at least 50% (especially preferably 60, 70, 80, 90, 95, 96, 97, 98, 99 or 100%) of the glycosidic bonds of the starting polysaccharide are degraded, *e.g.*, hydrolyzed. Alternatively expressed, in the latter case, preferably at least 50% (especially preferably 60, 70, 80, 90, 95, 96, 97, 98, 99 or 100%) of the starting polysaccharide is hydrolyzed into mono- or di-saccharides.

In relation to cellulose, the level of degradation may be assessed by determining the increase in the level of cellobiose and/or glucose.

As referred to herein said "polysaccharide" is a polymeric carbohydrate structure, formed of repeating units (either mono- or di-saccharides) joined together by glycosidic bonds and having the general formula $(C_6H_{10}O_5)_n$, *e.g.*, in which $40 \leq n \leq 3000$. Preferably said polysaccharide is at least partially crystalline, *i.e.*, is in a crystalline form or has crystalline

portions, *i.e.*, a form or portion which shows a repeating, three-dimensional pattern of atoms, ions or molecules having fixed distances between the constituent parts.

Preferably said polysaccharide is cellulose, hemicellulose or chitin and may be in isolated form or may be present in impure form, *e.g.*, in a cellulose-, hemicellulose- or chitin-containing material (*i.e.*, a polysaccharide-containing material), which optionally may contain other polysaccharides, *e.g.*, in the case of cellulose, hemicellulose and/or pectin may also be present.

By way of example, the cellulose-containing material may be stems, leaves, hulls, husks and cobs of plants or leaves, branches and wood of trees. The cellulose-containing material can be, but is not limited to, herbaceous material, agricultural residues, forestry residues, municipal solid wastes, waste paper and pulp and paper mill residues. The cellulose-containing material can be any type of biomass including, but not limited to, wood resources, municipal solid waste, wastepaper, crops and crop residues (see, for example, Wiseloge *et al.*, 1995, in "Handbook on Bioethanol" (Charles E. Wyman, editor), pp. 105-118). Preferably the cellulose-containing material is in the form of lignocellulose, *e.g.*, a plant cell wall material containing lignin, cellulose and hemicellulose in a mixed matrix.

In a preferred aspect, the cellulose-containing material is corn stover. In another preferred aspect, the cellulose-containing material is corn fiber, corn cobs, switch grass or rice straw. In another preferred aspect, the cellulose-containing material is paper and pulp processing waste. In another preferred aspect, the cellulose-containing material is woody or herbaceous plants. In another preferred aspect, the cellulose-containing material is bagasse.

"Cellulose" is a polymer of the simple sugar glucose covalently bonded by beta-1,4-linkages. Cellulose is a straight chain polymer: unlike starch, no coiling or branching occurs and the molecule adopts an extended and rather stiff rod-like conformation, aided by the equatorial conformation of the glucose residues. The multiple hydroxyl groups on the glucose from one chain form hydrogen bonds with oxygen molecules on the same or on a neighbour chain, holding the chains firmly together side-by-side and forming microfibrils with high tensile strength.

Compared to starch, cellulose is also much more crystalline. Whereas starch undergoes a crystalline to amorphous transition when heated beyond 60-70°C in water (as in cooking), cellulose requires a temperature of 320°C and pressure of 25 MPa to become amorphous in water.

Several different crystalline structures of cellulose are known, corresponding to the location of hydrogen bonds between and within strands. Natural cellulose is cellulose I, with structures I_α and I_β . Cellulose produced by bacteria and algae is enriched in I_α while cellulose of higher plants consists mainly of I_β . Cellulose in regenerated cellulose fibers is cellulose II. The conversion of cellulose I to cellulose II is not reversible, suggesting that cellulose I is metastable

and cellulose II is stable. With various chemical treatments it is possible to produce the structures cellulose III and cellulose IV.

"Hemicellulose" is derived from several sugars in addition to glucose, especially xylose but also including mannose, galactose, rhamnose and arabinose. Hemicellulose consists of shorter chains than cellulose; around 200 sugar units. Furthermore, hemicellulose is branched, whereas cellulose is unbranched.

"Chitin" is defined herein as any polymer containing beta-(1-4) linked *N*-acetylglucosamine residues that are linked in a linear fashion. Crystalline chitin in the alpha form (where the chains run anti-parallel), beta form (where the chains run parallel) or gamma form (where there is a mixture of parallel and antiparallel chains), amorphous chitin, colloidal chitin, chitin forms in which part (e.g., up to 5, 10, 15 or 20%) of the *N*-acetylglucosamine sugars are deacetylated are all included within the definition of this term.

Other forms of chitin that are found in nature include copolymers with proteins and these copolymers, which include protein chitin matrices that are found in insect and crustacean shells and any other naturally occurring or synthetic copolymers comprising chitin molecules as defined herein, are also included within the definition of "chitin".

The term "chitin" thus includes purified crystalline alpha, beta and gamma preparations, or chitin obtained or prepared from natural sources, or chitin that is present in natural sources. Examples of such natural sources include squid pen, shrimp shells, crab shells, insect cuticles and fungal cell walls. Examples of commercially available chitins are those available from sources such as France Chitin, Hov-Bio, Sigma, Sekagaku Corp, amongst others.

As referred to herein "contacting" said polysaccharide with an oxidohydrolytic enzyme refers to bringing the two entities together in an appropriate manner to allow the catalytic properties of the enzyme to be effective.

The precise kinetics of the reaction between the oxidohydrolytic enzyme and the polysaccharide will depend on many factors, such as the type of polysaccharide to be degraded, the amount of enzyme present, the temperature and the pH. The type of polysaccharide and its degree of amorphousness will vary with the substrate source and isolation/purification process, but can be assessed, for example, by measuring the degree of crystallinity of the substrate (which is a method known in the art).

Taking these considerations into account one can determine appropriate incubation times and conditions to maximize degradation (e.g., hydrolysis with glycoside hydrolases). Exemplary methods are discussed below.

Thus, the polysaccharide and oxidohydrolytic enzyme are mixed together or contacted with one another to allow their interaction. This may simply involve directly mixing solutions of the different components or applying the enzyme to the polysaccharide-containing material.

As referred to herein "one or more" preferably denotes 2, 3, 4, 5 or 6 or more of the recited enzymes. When more than one of the enzymes is used they may be selected in line with the substrate to be used, e.g., to provide complementary or synergistic action. Thus, for example, oxidohydrolytic enzymes may be combined which are effective on different regions of the substrate, e.g., different crystal faces. Preferred combinations are described hereinafter.

As used herein an "oxidohydrolytic enzyme" is an enzyme which uses molecular oxygen or an activated form thereof ("reactive oxygen species") for cleavage of glucoside bonds in polysaccharides, preferably chitin or cellulose. The newly generated chain ends are one normal non-reducing end and an oxidized "acidic" end that, in the case of chitin is a 2-(acetylamino)-2-deoxy-D-gluconic acid and in the case of cellulose is a gluconic acid.

Preferably said enzyme has a metal binding site and requires the presence of a divalent metal ion for full activity. The structural environment of this metal ion is diagnostic (and unifying) for the CBM33 and GH61 enzymes. The metal is bound by at least three ligands that are fully conserved in both families: (1) a histidine that is in position 1 of the mature protein (*i.e.*, the N-terminal residue of the protein after the signal peptide for secretion has been cleaved off); (2) the N-terminal amino group of the mature protein; and (3) another histidine residue that is fully conserved.

Oxidohydrolases belonging to the CBM33 or GH61 family can be identified by analysis of gene sequences (and the corresponding predicted amino acid sequences of the gene products), using standard bioinformatic methods. For example one can use an existing multiple sequence alignment of CBM33 or GH61 enzymes, for example represented by a Hidden Markov Model, to search for homologous sequences in sequence databases. Sequences retrieved by such searches would be highly likely to be active oxidohydrolases. More certainty may be obtained by (1) checking that the gene encodes a protein with a signal peptide for secretion, using, e.g., the programme SignalP; (2) checking that the N-terminal residue after cleavage of the signal peptide (cleavage site to be predicted using, e.g., SignalP) is a histidine; (3) checking that there is another histidine in the protein sequence that aligns with a fully or almost fully (>90%) conserved histidine in the multiple sequence alignment; (4) using model-building by homology, using automated servers such as Swiss-Model, to check that this second histidine is likely to be located close to the N-terminus and the N-terminal histidine.

The skilled person can readily determine by experiment whether a protein is an oxidohydrolytic enzyme according to the above described definition by determining if it can cleave glucoside bonds and if this process becomes more effective in the presence of molecular oxygen, a reductant and a divalent metal ion. In addition, one may test whether the putative oxidohydrolase works synergistically with known saccharolytic enzyme preparations and whether the magnitude of this synergistic effect depends on the presence of reductants

and divalent metal ions. Experiments such as those conducted in the examples may be used, thus the effect of reductants and metal ions on enzymatic activity may be assessed.

Preferably said oxidohydrolytic enzyme contains at least one domain that on the basis of sequence similarity as analyzed in, e.g., the current Pfam or CAZy databases is classified as a CBM33 or GH61 family protein. When the CBM33 or GH61 containing proteins have more than one domain, the additional domains are usually coupled to the C-terminus of the CBM33 or GH61 domain because the N-terminus of the CBM33 or GH61 domain is essential for oxidohydrolytic activity (see below). The CBM33 family has been classified by the CAZy (CArbohydrate-Active EnZymes) system as a carbohydrate-binding module family implying the absence of enzymatic activity. GH61 proteins have been classified as glycoside hydrolases. However, neither classification is correct in view of the results presented by the inventors and clearly the CAZy classification of these two protein families needs to be corrected. The CAZy classification is based on sequence similarity, grouping protein domains that share a certain minimal level of sequence similarity into one family. The CBM33 and GH61 domains share similar functions and they share a similar structural fold, the core of which being a twisted beta-sheet sandwich like fold, similar to that seen for fibronectin type-III domains (Figs 1D,E). They also share a fully conserved, diagnostic surface-located structural element (Fig. 1F) consisting of a histidine in position 1 of the mature protein (*i.e.*, the protein after the signal peptide driving secretion has been cleaved off), another fully conserved histidine residue that is known to be crucial for catalytic activity and an N-terminal amino group that acts with these two histidines in binding a metal ion. Despite these many unifying and diagnostic characteristics, the two families share little sequence identity and will therefore most likely remain in two different families even after correction of the CAZy classification.

The oxidohydrolytic enzyme is preferably a class GH61 protein. Thus the oxidohydrolytic enzyme of the invention may contain, consist or consist essentially of a GH61 domain or GH61 protein or a biologically active fragment thereof. In this context, "consists essentially of" indicates that additional amino acids may be present in the protein, in addition to those that make up the GH61 domain or protein. Preferably there are 1-3, 1-5, 1-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90 or 90-100 or more additional amino acids present. These additional amino acids are in general present C terminal to the GH61 domain.

As mentioned above, the oxidohydrolytic enzyme can comprise a GH61 domain or protein. Additional modules or domains may thus be present in the protein, which, when present are preferably at the C-terminus.

In a preferred feature a native GH61 domain or protein or a biologically active fragment thereof is used though variants of the native form may be used, some of which are described hereinafter.

Oxidohydrolytic enzymes which comprise or consist of a GH61 domain or protein or its fragments or variants are referred to herein, collectively, as GH61 proteins or GH61 family members or proteins.

Examples of suitable native proteins in this family are provided in the table below which provides relevant database accession numbers which are hereby incorporated by reference.

5

Protein Name	Organism	GenBank	Uniprot
Cel1	<i>Agaricus bisporus</i> D649	AAA53434.1	Q00023
AfA5C5.025	<i>Aspergillus fumigatus</i>	CAF31975.1	Q6MYM8
endo-beta-1,4-glucanase B (EglB) (Cel61A)	<i>Aspergillus kawachii</i>	BAB62318.1	Q96WQ9
AN1041.2	<i>Aspergillus nidulans</i> FGSC A4	EAA65609.1	C8VTW9
AN3511.2	<i>Aspergillus nidulans</i> FGSC A4	EAA59072.1	
AN9524.2	<i>Aspergillus nidulans</i> FGSC A4	CBF83171.1 EAA66740.1	C8VI93
AN7891.2	<i>Aspergillus nidulans</i> FGSC A4	EAA59545.1	
AN6428.2	<i>Aspergillus nidulans</i> FGSC A4	EAA58450.1	C8V0F9
AN3046.2	<i>Aspergillus nidulans</i> FGSC A4	EAA63617.1	C8VIS7
AN3860.2	<i>Aspergillus nidulans</i> FGSC A4	EAA59125.1	C8V6H2
endo-beta-1,4-glucanase (AN1602.2)	<i>Aspergillus nidulans</i> FGSC A4	ABF50850.1 EAA64722.1	
AN2388.2	<i>Aspergillus nidulans</i> FGSC A4	EAA64499.1	C8VNP4
An14g02670	<i>Aspergillus niger</i> CBS 513.88	CAK46515.1	A2R313
An15g04570	<i>Aspergillus niger</i> CBS 513.88	CAK97324.1	A2R5J9
An15g04900	<i>Aspergillus niger</i> CBS 513.88	CAK42466.1	A2R5N0
An04g08550	<i>Aspergillus niger</i> CBS 513.88	CAK38942.1	A2QJX0

An08g05230	<i>Aspergillus niger</i> CBS 513.88	CAK45495.1	A2QR94
An12g02540	<i>Aspergillus niger</i> CBS 513.88	CAK41095.1	A2QYU6
An12g04610	<i>Aspergillus niger</i> CBS 513.88	CAK97151.1	A2QZE1
AO090005000531	<i>Aspergillus oryzae</i> RIB40	BAE55582.1	
AO090001000221	<i>Aspergillus oryzae</i> RIB40	BAE56764.1	
AO090138000004	<i>Aspergillus oryzae</i> RIB40	BAE64395.1	
AO090023000056	<i>Aspergillus oryzae</i> RIB40	BAE58643.1	
AO090023000159	<i>Aspergillus oryzae</i> RIB40	BAE58735.1	
AO090023000787	<i>Aspergillus oryzae</i> RIB40	BAE59290.1	
AO090103000087	<i>Aspergillus oryzae</i> RIB40	BAE65561.1	
AO090012000090	<i>Aspergillus oryzae</i> RIB40	BAE60320.1	
GH61A	<i>Botryosphaeria rhodina</i> CBS 247.96	CAJ81215.1	
GH61B	<i>Botryosphaeria rhodina</i> CBS 247.96	CAJ81216.1	
GH61C	<i>Botryosphaeria rhodina</i> CBS 247.96	CAJ81217.1	
GH61D	<i>Botryosphaeria rhodina</i> CBS 247.96	CAJ81218.1	
Cel6	<i>Cochliobolus heterostrophus</i> C4	AAM76663.1	Q8J0H7
unnamed protein product	<i>Coprinopsis cinerea</i>	CAG27578.1	
Cel1	<i>Cryptococcus neoformans</i> var. <i>neoformans</i>	AAC39449.1	O59899

CNA05840 (Cel1)	<i>Cryptococcus neoformans</i> var. <i>neoformans</i> JEC21	AAW41121.1	
xylanase II (peptide fragment)	<i>Fusarium oxysporum</i> F3		
Sequence 122805 from U.S. Patent No. 7,214,786	<i>Gibberella zeae</i>	ABT35335.1	
FG03695.1 (Cel61E)	<i>Gibberella zeae</i> PH-1	XP_383871.1	
ORF (possible fragment)	<i>Glomerella graminicola</i> M2	CAQ16278.1	B5WYD8
ORF	<i>Glomerella graminicola</i> M2	CAQ16206.1	B5WY66
ORF	<i>Glomerella graminicola</i> M2	CAQ16208.1	B5WY68
ORF	<i>Glomerella graminicola</i> M2	CAQ16217.1	B5WY77
unnamed protein product	<i>Humicola insolens</i>	CAG27577.1	
cellulase-enhancing factor (Cel61B)	<i>Hypocrea jecorina</i> QM6A	AAP57753.1 ABH82048.1 ACK19226.1 ACR92640.1	Q7Z9M7
endo-beta-1,4-glucanase IV (EGIV;Egl4) (Cel61A)	<i>Hypocrea jecorina</i> RUTC-30	CAA71999.1	O14405.1
MG05364.4	<i>Magnaporthe grisea</i> 70-15	EAA54572.1 XP_359989.1	
MG07686.4	<i>Magnaporthe grisea</i> 70-15	EAA53409.1 XP_367775.1	
MG07300.4	<i>Magnaporthe grisea</i> 70-15	EAA56945.1 XP_367375.1	
MG07575.4	<i>Magnaporthe grisea</i> 70-15	EAA53298.1 XP_367664.1	
MG08020.4	<i>Magnaporthe grisea</i> 70-15	EAA57051.1 XP_362437.1	
MG02502.4	<i>Magnaporthe grisea</i> 70-15	EAA54517.1 XP_365800.1	
MG08254.4	<i>Magnaporthe grisea</i> 70-15	EAA57285.1 XP_362794.1	

MG08066.4	<i>Magnaporthe grisea</i> 70-15	EAA57097.1 XP_362483.1	
MG04547.4	<i>Magnaporthe grisea</i> 70-15	EAA50788.1 XP_362102.1	
MG08409.4	<i>Magnaporthe grisea</i> 70-15	EAA57439.1 XP_362640.1	
MG09709.4	<i>Magnaporthe grisea</i> 70-15	EAA49718.1 XP_364864.1	
MG04057.4	<i>Magnaporthe grisea</i> 70-15	EAA50298.1 XP_361583.1	
MG06069.4	<i>Magnaporthe grisea</i> 70-15	EAA52941.1 XP_369395.1	
MG09439.4	<i>Magnaporthe grisea</i> 70-15	EAA51422.1 XP_364487.1	
MG06229.4	<i>Magnaporthe grisea</i> 70-15	EAA56258.1 XP_369714.1	
MG07631.4	<i>Magnaporthe grisea</i> 70-15	EAA53354.1 XP_367720.1	
MG06621.4 (fragment)	<i>Magnaporthe grisea</i> 70-15	XP_370106.1	
NCU07898.1	<i>Neurospora crassa</i> OR74A	EAA33178.1 XP_328604.1	
NCU05969.1	<i>Neurospora crassa</i> OR74A	EAA29347.1 XP_325824.1	
NCU02916.1	<i>Neurospora crassa</i> OR74A	EAA36362.1 XP_330104.1	
NCU03000.1 (B24P7.180)	<i>Neurospora crassa</i> OR74A	CAB97283.2 EAA36150.1 XP_330187.1	Q9P3R7
NCU07760.1	<i>Neurospora crassa</i> OR74A	EAA29018.1 XP_328466.1	
NCU07520.1	<i>Neurospora crassa</i> OR74A	EAA29132.1 XP_327806.1	
NCU01050.1 (G15G9.090)	<i>Neurospora crassa</i> OR74A	CAD21296.1 EAA32426.1 XP_326543.1	Q8WZQ2

NCU02240.1	<i>Neurospora crassa</i> OR74A	EAA30263.1 XP_331016.1	
NCU02344.1 (B23N11.050)	<i>Neurospora crassa</i> OR74A	CAF05857.1 EAA30230.1 XP_331120.1	
NCU00836.1	<i>Neurospora crassa</i> OR74A	EAA34466.1 XP_325016.1	
NCU08760.1	<i>Neurospora crassa</i> OR74A	EAA26873.1 XP_330877.1	
NCU07974.1	<i>Neurospora crassa</i> OR74A	EAA33408.1 XP_328680.1	
NCU03328.1 (B10C3.010)	<i>Neurospora crassa</i> OR74A	CAD70347.1 EAA26656.1 XP_322586.1	Q873G1
NCU01867.1 (B13N4.070)	<i>Neurospora crassa</i> OR74A	CAE81966.1 EAA36262.1 XP_329057.1	Q7SHD9
beta-1,3-1,4-glucanase (peptide fragment)	<i>Paecilomyces thermophila</i> J18		
Pc20g11100	<i>Penicillium chrysogenum</i> Wisconsin 54-1255	CAP86439.1	B6HG02
Pc12g13610	<i>Penicillium chrysogenum</i> Wisconsin 54-1255	CAP80988.1	B6H016
Pc13g07400	<i>Penicillium chrysogenum</i> Wisconsin 54-1255	CAP91809.1	B6H3U0
Pc13g13110	<i>Penicillium chrysogenum</i> Wisconsin 54-1255	CAP92380.1	B6H3A3
Cel61 (Cel61A)	<i>Phanerochaete chrysosporium</i> BKM-F-1767	AAM22493.1	Q8NJI9
Pa_4_1020	<i>Podospira anserina</i> S mat+	CAP61476.1	B2ADG1

unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP68173.1	B2AUV0
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP68309.1	B2AV86
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP61650.1	B2ADY5
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP68352.1	B2AVC8
Pa_7_3160	<i>Podospora anserina</i> S <i>mat+</i>	CAP68375.1	B2AVF1
unnamed protein product (fragment)	<i>Podospora anserina</i> S <i>mat+</i>	CAP71532.1	B2B346
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP71839.1	B2B403
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP72740.1	B2B4L5
Pa_5_8940	<i>Podospora anserina</i> S <i>mat+</i>	CAP64619.1	B2AKU6
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP73072.1	B2B5J7
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP64732.1	B2AL94
Pa_2_6530	<i>Podospora anserina</i> S <i>mat+</i>	CAP73254.1	B2B629
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP73311.1	B2B686
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP73320.1	B2B695
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP64865.1	B2ALM7
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP65111.1	B2AMI8
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP65855.1	B2APD8
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP65866.1	B2APE9

unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP65971.1	B2API9
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP66744.1	B2ARG6
Pa_1_500	<i>Podospora anserina</i> S <i>mat+</i>	CAP59702.1	B2A9F5
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP61048.1	B2AC83
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP67176.1	B2AS05
Pa_1_22040	<i>Podospora anserina</i> S <i>mat+</i>	CAP67190.1	B2AS19
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP67201.1	B2AS30
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP67466.1	B2ASU3
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP67481.1	B2ASV8
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP67493.1	B2ASX0
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP70156.1	B2AZV6
Pa_4_350	<i>Podospora anserina</i> S <i>mat+</i>	CAP61395.1	B2AD80
Pa_1_16300	<i>Podospora anserina</i> S <i>mat+</i>	CAP67740.1	B2ATL7
unnamed protein product	<i>Podospora anserina</i> S <i>mat+</i>	CAP70248.1	B2AZD4
SMU2916 (fragment)	<i>Sordaria macrospora</i> k- <i>hell</i>	CAQ58424.1	
cellulase-enhancing factor (GH61A)	<i>Thermoascus</i> <i>aurantiacus</i>	ABW56451.1 ACS05720.1	
unnamed protein product	<i>Thielavia terrestris</i>	CAG27576.1	
cellulase-enhancing factor (GH61B)	<i>Thielavia terrestris</i> NRRL 8126	ACE10231.1	

Sequence 4 from U.S. Patent No. 7,361,495 (GH61C)	<i>Thielavia terrestris</i> NRRL 8126	ACE10232.1	
Sequence 4 from U.S. Patent No. 7,361,495 (GH61C)	<i>Thielavia terrestris</i> NRRL 8126	ACE10232.1	
Sequence 6 from U.S. Patent No. 7,361,495 (GH61D)	<i>Thielavia terrestris</i> NRRL 8126	ACE10233.1	
Sequence 6 from U.S. Patent No. 7,361,495 (GH61D)	<i>Thielavia terrestris</i> NRRL 8126	ACE10233.1	
cellulase-enhancing factor (131562) (GH61E)	<i>Thielavia terrestris</i> NRRL 8126	ACE10234.1	
Sequence 10 from U.S. Patent No. 7,361,495 (GH61G)	<i>Thielavia terrestris</i> NRRL 8126	ACE10235.1	
Sequence 10 from U.S. Patent No. 7,361,495 (GH61G)	<i>Thielavia terrestris</i> NRRL 8126	ACE10235.1	
endoglucanase (EnGluIV;EndoGluIV)	<i>Trichoderma saturnisporum</i>	ADB89217.1	
Endoglucanase IV	<i>Trichoderma</i> sp. SSL	ACH92573.1	B5TYI4
endoglucanase IV (EgiV)	<i>Trichoderma viride</i> AS 3.3711	ACD36973.1	B4YEW3
endoglucanase VII (EgvII)	<i>Trichoderma viride</i> AS 3.3711	ACD36971.1	B4YEW1
Endoglucanase II (EgII)	<i>Volvariella volvacea</i>	AAT64005.1	Q6E5B4
unknown (ZM_BFc0036G02)	<i>Zea mays</i> B73	ACF78974.1 ACR36748.1	B4FA31

GH61 proteins from *Phanerochaete chrysosporium* are also preferred (see Vanden Wymelenberg *et al.*, 2009, *Appl Environ Microbiol.* 75(12): 4058-68; Hori *et al.*, 2011, *FEMS Microbiol Lett.* 321(1): 14-23).

- 5 Preferred GH61 proteins are from fungi, in particular from *Thielavia*, especially preferably from *Thielavia terrestris* or *Thielavia aurantiacus*. Especially preferably said GH61 protein is GH61A from *Thielavia aurantiacus* or GH61B, GH61C, GH61D, GH61E or GH61G

from *Thielavia terrestris* as described above. Other preferred GH61 proteins include GH61A and B from *Hypocrea jecorina* (SEQ ID NOs: 15 and 16).

The GH61 protein can thus be or correspond to or comprise a naturally occurring GH61 protein that is found in nature or a biologically active fragment thereof. In the alternative the GH61 protein may be a non-native variant as disclosed hereinafter.

In an alternative preferred feature the oxidohydrolytic enzyme is a class CBM33 family protein. The CBM33 family comprises a carbohydrate-binding module (CBM) which is defined as a contiguous amino acid sequence within a carbohydrate binding protein with a discreet fold having carbohydrate-binding activity. For example, chitinases are known which contain one or more chitin binding modules in addition to catalytic regions. ChiA of *Serratia marcescens* contains a fibronectin type III - type CBM, ChiB of *Serratia marcescens* contains a family 5 CBM and ChiC of *Serratia marcescens* contains a family 12 and a fibronectin type III - like CBM. See Bourne & Henrissat, 2001, *Curr. Opin. Struct. Biol.* 11: 593 for domain nomenclature. Likewise, many cellulases contain CBMs that bind to cellulose. Proteins binding to chitin and containing CBMs that stimulate such binding may for example be structural or signalling molecules or they can be enzymes and the overall function of the protein may be determined by domains that are present in addition to the carbohydrate binding module. The CBMs for use in methods of the invention must, however, have oxidohydrolytic activity as defined above. So far such oxidohydrolytic activity has been detected in only one CBM family, namely CBM family 33. This is exemplified by the function of the chitin-binding protein (CBP) CBP21.

Members of family 33 of Carbohydrate Binding Modules (CBM33) may be identified according to the CAZY classification system (cazy.org/CAZY/fam/acc_CBM.html), which is based on sequence similarities (Davies & Henrissat, 2002, *Biochem Soc T* 30: 291-297 and Bourne & Henrissat, 2001, *supra*). Proteins in this family are known to bind to chitin, but binding to other polysaccharides, including cellulose, has also been observed (Moser *et al.*, 2008, *Biotechnol. Bioeng.* 100(6):1066-77). For some of these proteins it has been shown that they act synergistically with chitinases and cellulases in the degradation of chitin and cellulose, respectively (Vaaje-Kolstad *et al.*, 2005, *J. Biol. Chem.* 280(31): 28492-7; Vaaje-Kolstad *et al.*, 2009, *FEBS J.* 276(8):2402-15; Moser *et al.*, 2008, *supra*), as described in the Examples.

Studies of the action of the chitin-binding protein CBP21 (and other CBM33 proteins) have now led to the identification of CBM33 proteins as oxidohydrolases.

As described herein, all members of CBM family 33 contain a family 33 carbohydrate binding module. In several cases, the CBM33 module makes up the whole protein, *i.e.*, the protein consists of or consists essentially of a single family 33 CBM, which is in nature synthesized and secreted as such. However some family 33 CBMs may be fused to one or more additional non-catalytic carbohydrate binding modules (*e.g.*, CBM family 2, CBM family 3

and CBM family 5 modules). These proteins are bi- or multi-domain proteins. There is also one known example of a family 33 carbohydrate binding module that is present as an individual module within a much larger catalytic protein. This is the beta-1,4-mannanase protein of *Caldibacillus cellulovorans* (Sunna *et al.*, 2000, *Appl. Environ. Micro.* 66(2): 664-670).

5 The family 33 CBMs are usually approximately 150-250 amino acids, e.g., 160-240, 170-230, 180-220, 190-210 amino acids in size and have a molecular weight of approximately 20 kDa, preferably 19-21 kDa, 18-21 kDa, 19-22 kDa or 18-20 kDa in size, though CBM33 domains as large as 300-400 amino acids with a molecular weight of approximately 30-40 kDa may also be used. The size of a protein can readily be determined by standard methods that
10 are known in the art.

Preferably, the oxidohydrolytic enzyme consists of a single family 33 CBM, or consists essentially of a family 33 CBM.

If said oxidohydrolytic enzyme "consists essentially of" a family 33 CBM, it is meant that additional amino acids may be present in the protein, in addition to those that make up the
15 family 33 CBM. Preferably there are 1-3, 1-5, 1-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90 or 90-100 or more additional amino acids present. These additional amino acids are in general present C terminal to the family 33 CBM.

Alternatively, the oxidohydrolytic enzyme can comprise a family 33 CBM. Additional modules or domains may thus be present in the protein. Examples of such modules are CBM
20 family 2, CBM family 3 and CBM family 5 modules. If additional domains or modules are present, they are in general found C-terminal to the family 33 CBM.

Thus in a preferred aspect, the oxidohydrolytic enzyme can contain, consist or consist essentially of a naturally occurring family 33 CBM (or CBM33 family protein) such as CBP21 (or to a homologue thereof from another species) or a biologically active fragment thereof. It can
25 alternatively contain, consist or consist essentially of a variant of a naturally occurring family 33 CBM (or CBM33 family protein) or a biologically active fragment thereof.

Oxidohydrolytic enzymes which comprise or consist of a family 33 CBM module or the full family 33 CBM protein (which comprises the family 33 CBM module) or its fragments or variants are referred to herein, collectively, as CBM33 proteins or CBM33 family members or
30 proteins.

Naturally occurring CBM33 proteins that can be used in the invention include microbial (e.g., bacterial), eukaryotic (e.g., *Dictyostelium*) or viral CBM33 proteins. Bacterial CBM33 proteins are, however, preferred.

Examples of known CBM33 proteins which may be used in methods of the invention
35 and relevant database accession numbers (which are hereby incorporated by reference) are set out in Table 1:

BACTERIA

PROTEIN	ORGANISM	GENBANK/GENPEPT	
Cbp1	<i>Alteromonas</i> sp. O-7	AB063629	BAB79619.1
chitin binding protein ChbA	<i>Bacillus amyloliquefaciens</i> 2718	ALKOAF181997	AAG09957.1
BA_3348	<i>Bacillus anthracis</i> str. A2012	NC_003995	NP_656708.1
BA2827	<i>Bacillus anthracis</i> str. Ames	AE017033	AAP26659.1
		NC_003997	NP_845173.1
BA2793	<i>Bacillus anthracis</i> str. Ames	AE017032	AAP26628.1
		NC_003997	NP_845142.1
GBAA2827	<i>Bacillus anthracis</i> str. Ames 0581	AE017334	AAT31944.1
GBAA2793	<i>Bacillus anthracis</i> str. Ames 0581	AE017334	AAT31910.1
BAS2636	<i>Bacillus anthracis</i> str. Sterne	AE017225	AAT54946.1
BAS2604	<i>Bacillus anthracis</i> str. Sterne	AE017225	AAT54914.1
BCE2855	<i>Bacillus cereus</i> ATCC 10987	AE017273	AAS41767.1
		NC_003909	NP_979159.1
BCE2824	<i>Bacillus cereus</i> ATCC 10987	AE017273	AAS41736.1
		NC_003909	NP_979128.1
BC2827	<i>Bacillus cereus</i> ATCC 14579	AE017007	AAP09778.1
		NC_004722	NP_832577.1
BC2798	<i>Bacillus cereus</i> ATCC 14579	AE017007	AAP09751.1
		NC_004722	NP_832550.1
pE33L466_0276 (ChbA)	<i>Bacillus cereus</i> E33L	CP000040	AAU60428.1
BTZK2523 (ChB)	<i>Bacillus cereus</i> ZK	CP000001	AAU17736.1
BTZK2552 (ChB)	<i>Bacillus cereus</i> ZK	CP000001	AAU17707.1
ABC1161	<i>Bacillus clausii</i> KSM-K16	AP006627	BAD63699.1
BH1303	<i>Bacillus halodurans</i> C-125	AP001511	BAB05022.1
		NC_002570	NP_242169.1
BLi00521 or BL00145	<i>Bacillus licheniformis</i> DSM 13 ATCC 14580	CP000002	AAU22121.1
		AE017333	AAU39477.1
BT9727_2586 (ChB)	<i>Bacillus thuringiensis</i> serovar <i>konkukian</i> str. 97-27	AE017355	AAT61310.1

BT9727_2556 (ChB)	<i>Bacillus thuringiensis</i> serovar <i>konkukian str. 97-27</i>	AE017355	AAT61323.1
BMAA1785	<i>Burkholderia mallei</i> ATCC 23344	CP000011	AAU45854.1
BMA2896	<i>Burkholderia mallei</i> ATCC 23344	CP000010	AAU48386.1
BURPS1710b_0114	<i>Burkholderia pseudomallei</i> 1710b	CP000124	ABA49030.1
BURPS1710b_A2047	<i>Burkholderia pseudomallei</i> 1710b	CP000125	ABA53645.1
BPSL3340	<i>Burkholderia pseudomallei</i> K96243	BX571965	CAH37353.1
BPSS0493	<i>Burkholderia pseudomallei</i> K96243	BX571966	CAH37950.1
Bcep18194_C6726	<i>Burkholderia</i> sp. 383	CP000150	ABB05775.1
BTH_II1925	<i>Burkholderia thailandensis</i> E264; ATCC 700388	CP000085	ABC34637.1
BTH_I3219	<i>Burkholderia thailandensis</i> E264; ATCC 700388	CP000086	ABC38514.1
Beta-1,4-mannanase (ManA)	<i>Caldibacillus cellulovorans</i>	AF163837	AAF22274.1
CV0554	<i>Chromobacterium violaceum</i> ATCC 12472	AE016911	AAQ58230.1
		NC_005085	NP_900224.1
CV0553	<i>Chromobacterium violaceum</i> ATCC 12472	AE016911	AAQ58229.1
		NC_005085	NP_900223.1
CV2592 (CpbD)	<i>Chromobacterium violaceum</i> ATCC 12472	AE016919	AAQ60262.1
		NC_005085	NP_902262.1
CV3489	<i>Chromobacterium violaceum</i> ATCC 12472	AE016922	AAQ61150.1
		NC_005085	NP_903159.1
CV3323 (CbpD1)	<i>Chromobacterium violaceum</i> ATCC 12472	AE016921	AAQ60987.1
		NC_005085	NP_902993.1
EF0362	<i>Enterococcus faecalis</i> V583	AE016948	AAO80225.1
		NC_004668	NP_814154.1
Sequence 4287 from U.S. Patent No. 6,583,275	<i>Enterococcus faecium</i>	-	AAQ43729.1

FTL_1408	<i>Francisella tularensis</i> subsp. AM233362	CAJ79847.1
	<i>holarctica</i> LVS	
FTT0816c	<i>Francisella tularensis</i> subsp. AJ749949	CAG45449.1
	<i>tularensis</i> Schu 4	
HCH_00807	<i>Hahella chejuensis</i> KCTC 2396	CP000155 ABC27701.1
HCH_03973	<i>Hahella chejuensis</i> KCTC 2396	CP000155 ABC30692.1
lp_1697	<i>Lactobacillus plantarum</i> WCFS1	AL935256 CAD64126.1
	NC_004567	NP_785278.1
LSA1008	<i>Lactobacillus sakei</i> subsp. <i>sakei</i> 23K	CR936503 CAI55310.1
YucG	<i>Lactococcus lactis</i> subsp. <i>lactis</i> AE006425	AAK06049.1
	IL1403	
	NC_002662	NP_268108.1
lpp0257	<i>Legionella pneumophila</i> Paris	CR628336 CAH11404.1
Lin2611	<i>Listeria innocua</i>	AL596173 CAC97838.1
	NC_003212	NP_471941.1
Lmo2467	<i>Listeria monocytogenes</i> EGD-e	AL591983 CAD00545.1
	NC_003210	NP_465990.1
LMOF2365_2440	<i>Listeria monocytogenes</i> str. 4b AE017330	AAT05205.1
	F2365	
OB0810	<i>Oceanobacillus iheyensis</i> HTE831	AP004595 BAC12766.1
	NC_004193	NP_691731.1
PBPRB0312	<i>Photobacterium profundum</i> SS9	CR378676 CAG22185.1
plu2352	<i>Photorhabdus luminescens</i> subsp. BX571866	CAE14645.1
	<i>laumondii</i> TTO1	
	NC_005126	NP_929598.1
Sequence 6555 from U.S. <i>Proteus mirabilis</i>	-	AAR43285.1
Patent No. 6,605,709		
chitin-binding protein ChiB	<i>Pseudoalteromonas</i> sp. S9	AF007895 AAC79666.1
chitin-binding protein	<i>Pseudomonas aeruginosa</i> PAO1	AE004520 AAG04241.1
(CbpD;PA0852)		
	NC_002516	NP_249543.1
chitin-binding protein	<i>Pseudomonas aeruginosa</i> PAO25	AF196565 AAF12807.1
(CbpD)		
PFL_2090	<i>Pseudomonas fluorescens</i> Pf-5	CP000076 AAY91365.1
Pfl_3569	<i>Pseudomonas fluorescens</i> PfO-1	CP000094 ABA75307.1

Psyr_2856	<i>Pseudomonas syringae</i> pv. <i>syringae</i> B728a	CP000075	AAY37892.1
PSPTO2978	<i>Pseudomonas syringae</i> pv. <i>tomato</i> str. DC3000	AE016866	AAO56470.1
		NC_004578	NP_792775.1
RF_0708	<i>Rickettsia felis</i> URRWXCal2	CP000053	AAY61559.1
chitin-binding (CbpA)	protein <i>Saccharophagus degradans</i> 2-40	BK001045	DAA01337.1
ORF	<i>Salinivibrio costicola</i> 5SM-1	AY207003	AAP42509.1
chitin-binding (Cbp21)	protein <i>Serratia marcescens</i> 2170	AB015998	BAA31569.1
chitin-binding (Cbp21)	protein <i>Serratia marcescens</i> BJL200	AY665558	AAU88202.1
ORF2	<i>Serratia marcescens</i> KCTC2172	L38484	AAC37123.1
SO1072	<i>Shewanella oneidensis</i> MR-1	AE015551	AAN54144.1
		NC_004347	NP_716699.1
SG1515 fragment)	(possible <i>Sodalis glossinidius</i> str. 'morsitans'	AP008232	BAE74790.1
SAV6560	<i>Streptomyces avermitilis</i> MA-4680	AP005047	BAC74271.1
		NC_003155	NP_827736.1
SAV2168	<i>Streptomyces avermitilis</i> MA-4680	AP005029	BAC69879.1
		NC_003155	NP_823344.1
SAV5223 (Chb)	<i>Streptomyces avermitilis</i> MA-4680	AP005042	BAC72935.1
		NC_003155	NP_826400.1
SAV2254 (CelS2)	<i>Streptomyces avermitilis</i> MA-4680	AP005030	BAC69965.1
		NC_003155	NP_823430.1
SCO7225 or SC2H12.24	<i>Streptomyces coelicolor</i> A3(2)	AL359215	CAB94648.1
		NC_003888	NP_631281.1
SCO6345 or SC3A7.13	<i>Streptomyces coelicolor</i> A3(2)	AL031155	CAA20076.1
		NC_003888	NP_630437.1
SCO2833 (Chb)	<i>Streptomyces coelicolor</i> A3(2)	AL136058	CAB65563.1
		NC_003888	NP_627062.1
SCO0643 or SCF91.03c	<i>Streptomyces coelicolor</i> A3(2)	AL132973	CAB61160.1
		NC_003888	NP_624952.1

SCO0481 or SCF80.02	<i>Streptomyces coelicolor</i> A3(2)	AB017013	BAA75647.1
		AL121719	CAB57190.1
		NC_003888	NP_624799.1
SCO1734 or SCI11.23	<i>Streptomyces coelicolor</i> A3(2)	AL096849	CAB50949.1
		NC_003888	NP_626007.1
CelS2 (SCO1188 or <i>Streptomyces coelicolor</i> A3(2) SCG11A.19)		AL133210	CAB61600.1
		NC_003888	NP_625478.1
chitin binding protein	<i>Streptomyces griseus</i>	AB023785	BAA86267.1
cellulose binding protein (ORF2)	<i>Streptomyces halstedii</i>	U51222	AAC45430.1
chitin-binding protein	<i>Streptomyces olivaceoviridis</i> ATCC X78535 11238		CAA55284.1
chitin binding protein (Chb2)	<i>Streptomyces reticuli</i>	Y14315	CAA74695.1
chitin-binding protein (Cbp1)	<i>Streptomyces thermoviolaceus</i> OPC-520	AB110078	BAD01591.1
chitin-binding protein celS2	<i>Streptomyces viridosporus</i>	AF126376	AAD27623.1
Tfu_1665 (E8)	<i>Thermobifida fusca</i> YX	CP000088	AAZ55700.1
Tfu_1268 (E7)	<i>Thermobifida fusca</i> YX	CP000088	AAZ55306.1
VCA0140	<i>Vibrio cholerae</i> N16961	AE004355	AAF96053.1
		NC_002506	NP_232540.1
VCA0811	<i>Vibrio cholerae</i> N16961	AE004409	AAF96709.1
		NC_002506	NP_233197.1
VFA0143	<i>Vibrio fischeri</i> ES114	CP000021	AAW87213.1
VFA0013	<i>Vibrio fischeri</i> ES114	CP000021	AAW87083.1
VPA0092	<i>Vibrio parahaemolyticus</i> RIMD AP005084 2210633		BAC61435.1
		NC_004605	NP_799602.1
VPA1598	<i>Vibrio parahaemolyticus</i> RIMD AP005089 2210633		BAC62941.1
		NC_004605	NP_801108.1
VV21258	<i>Vibrio vulnificus</i> CMCP6	AE016812	AAO08152.1
		NC_004460	NP_763162.1
VV20044	<i>Vibrio vulnificus</i> CMCP6	AE016808	AAO07021.1

		NC_004460	NP_762031.1
VVA0086	<i>Vibrio vulnificus</i> YJ016	AP005344	BAC96112.1
		NC_005140	NP_936142.1
VVA0551	<i>Vibrio vulnificus</i> YJ016	AP005346	BAC96577.1
		NC_005140	NP_936607.1
ChiY	<i>Yersinia enterocolitica</i> (type 0:8) WA-314	AJ344214	CAC83040.2
YP0706	<i>Yersinia pestis</i> biovar <i>Medievalis</i> str. 91001	AE017129	AAS60972.1
		NC_005810	NP_992095.1
YPO3227	<i>Yersinia pestis</i> CO92	AJ414156	CAC92462.1
		NC_003143	NP_406699.1
Y0962	<i>Yersinia pestis</i> KIM	AE013699	AAM84543.1
		NC_004088	NP_668292.1
YPTB3366	<i>Yersinia pseudotuberculosis</i> 32953	IPBX936398	CAH22604.1
YPTB0899	<i>Yersinia pseudotuberculosis</i> 32953	IPBX936398	CAH20139.1

EUKARYOTA

PROTEIN	ORGANISM	GENBANK/GENPEPT	
ORF-26	<i>Agrotis segetum</i> nucleopolyhedrovirus	DQ123841	AAZ38192.1
spheroidin-like protein (Gp 37)	<i>Autographa californica</i> nucleopolyhedrovirus	L22858	AAA66694.1
		D00583	BAA00461.1
		NC_001623	NP_054094.1
Fusolin	<i>Bombyx mori</i> nuclear polyhedrosis virus	U55071	AAB47606.1
		L33180	AAC63737.1
		NC_001962	NP_047468.1
Spheroidin	<i>Choristoneura biennis</i> entomopoxvirus	M34140	AAA42887.1

VIRUSES

PROTEIN	ORGANISM	GENBANK/GENPEPT
ORF-26	<i>Agrotis segetum</i> <i>nucleopolyhedrovirus</i>	DQ123841 AAZ38192.1
Spheroidin-like (Gp 37)	protein <i>Autographa californica</i> <i>nucleopolyhedrovirus</i>	D00583 BAA00461.1 L22858 AAA66694.1 NC_001623 NP_054094.1
Fusolin	<i>Bombyx mori</i> nuclear polyhedrosis virus	U55071 AAB47606.1 NC_001962 NP_047468.1 L33180 AAC63737.1
Spheroidin	<i>Choristoneur biennis</i> <i>entomopoxvirus</i>	M341140 AAA42887.1
ORF60	<i>Choristoneura fumiferana</i> <i>defective nucleopolyhedrovirus</i>	AY327402 AAQ91667.1 NC_005137 NP_932669.1
spindle-like protein	<i>Choristoneura fumiferana</i> nuclear <i>polyhedrosis virus</i>	U26734 AAC55636.1 NC_004778 NP_848371.1
GP37 (ORF-67 GP37)	<i>Chrysodeixis chalcites</i> <i>nucleopolyhedrovirus</i>	AY864330 AAY83998.1
ORF57	<i>Epiphyas postvittana</i> <i>nucleopolyhedrovirus</i>	AY043265 AAK85621.1 NC_003083 NP_203226.1
GP37	<i>Helicoverpa armigera</i> single <i>nucleocapsid polyhedrovirus</i>	AF266696 AAK57880.1 AF303045 AAK96305.1 NC_003094 NP_203613.1
ORF59	<i>Helicoverpa zea</i> <i>nucleopolyhedrovirus</i>	AF334030 AAL56204.1 NC_003349 NP_542682.1
gp37	<i>Heliocoverpa armigera</i> <i>nucleopolyhedrovirus G4</i>	AF271059 AAG53801.1 NC_002654 NP_075127.1
Fusolin	<i>Heliothis armigera</i> entomopoxvirus	L08077 AAA92858.1

HynVgp086 (slp)	<i>Hyphantria</i>	<i>cunea</i>	AP009046	BAE72375.1
	<i>nucleopolyhedrovirus</i>			
Gp37 protein	<i>Leucania separata</i>	<i>nuclear</i>	AB009614	BAA24259.1
	<i>polyhedrosis virus</i>			
fusolin-like protein	<i>Lymantria</i>	<i>dispar</i>	U38895	AAB07702.1
	<i>nucleopolyhedrovirus</i>			
			AF081810	AAC70254.1
			NC_001973	NP_047705.1
gp37 protein	<i>Mamestra</i>	<i>brassicae</i>	AF108960	AAD45231.1
	<i>nucleopolyhedrovirus</i>			
ORF 37 (Gp37)	<i>Mamestra</i>	<i>configurata</i>	U59461	AAM09145.1
	<i>nucleopolyhedrovirus A</i>			
			AF539999	AAQ11056.1
Gp37	<i>Mamestra</i>	<i>configurata</i>	AY126275	AAM95019.1
	<i>nucleopolyhedrovirus B</i>			
			NC_004117	NP_689207.1
spheroidin-like protein (Gp 37)	<i>Orgyia pseudotsugata</i>	<i>nuclear</i>	U75930	AAC59068.1
	<i>polyhedrosis virus</i>			
			D13306	BAA02566.1
			NC_001875	NP_046225.1
enhancing factor	<i>Pseudaletia</i>	<i>separata</i>	D50590	BAA09138.1
	<i>entomopoxvirus</i>			
ORF25	<i>Spodoptera</i>	<i>exigua</i>	AF169823	AAF33555.1
	<i>nucleopolyhedrovirus</i>			
			NC_002169	NP_037785.1
gp37 (fragment)	<i>Spodoptera frugiperda</i>	<i>MNPV</i>	AY250076	AAP79107.1
ubiquitin GP37 fusion protein	<i>Spodoptera</i>	<i>litura</i>	AF325155	AAL01718.1
	<i>nucleopolyhedrovirus G2</i>			
			NC_003102	NP_258300.1
gp37	<i>Trichoplusia</i>	<i>ni</i>	single DQ017380	AAZ67435.1
	<i>nucleopolyhedrovirus</i>			
Fusolin	<i>unidentified</i>	<i>entomopoxvirus</i>	X77616	CAA54706.1
ORF107	<i>Xestia c-nigrum</i>	<i>granulovirus</i>	AF162221	AAF05221.1
			NC_002331	NP_059255.1

Other preferred bacterial CBM33 proteins include:

PROTEIN	ORGANISM	GENBANK/GENPEPT
Cfla_0175	<i>Cellulomonas flavigena</i> DSM 20109	DSMADG73094.1 D5UGB1
Cfla_0172	<i>Cellulomonas flavigena</i> DSM 20109	DSMADG73091.1 D5UGA8
Cfla_0316	<i>Cellulomonas flavigena</i> DSM 20109	DSMADG73234.1 D5UH31
Cfla_0490	<i>Cellulomonas flavigena</i> DSM 20109	DSMADG73405.1 D5UHY1
CJA_2191 (Cbp33A)	<i>Cellvibrio japonicus</i> Ueda107	ACE83992.1 B3PJ79
CJA_3139 (cbp33/10B)	<i>Cellvibrio japonicus</i> Ueda107	ACE84760.1 B3PDT6

Bacterial CBM33 proteins can be from any appropriate source but are preferably from a genus selected from the group consisting of *Bacillus*, *Chromobacterium*, *Enterococcus*, *Francisella*, *Hahella*, *Lactobacillus*, *Lactococcus*, *Legionella*, *Listeria*, *Oceanobacillus*, *Photobacterium*, *Photothabodus*, *Proteus*, *Pseudoalteromonas*, *Pseudomonas*, *Rickettsia*, *Saccharophagus*, *Salinivibrio*, *Serratia*, *Shewanella*, *Sodalis*, *Streptomyces*, *Thermobifida*, *Vibrio* and *Yersini* and optionally *Cellulomonas* and *Cellvibrio*.

Preferably said CBM33 protein is a CBP21 as described in U.S. Patent Application No. 2007/0218046 which is incorporated herein by reference. For example the CBP21 of *Serratia marescens* (SEQ ID NO: 4) is preferred. Alternatively, the EfCBM33 of *Enterococcus faecalis* (SEQ ID NO: 5), E7 of *Thermobifida fusca* (SEQ ID NO: 6), CelS2 of *Streptomyces coelicolor* A3(2) (SEQ ID NO: 7), Cfla_0175 of *Cellulomonas flavigena* DSM 20109 (SEQ ID NO: 8), Cfla_0172 of *Cellulomonas flavigena* DSM 20109 (SEQ ID NO: 9), Cfla_0316 of *Cellulomonas flavigena* DSM 20109 (SEQ ID NO: 10), Cfla_0490 of *Cellulomonas flavigena* DSM 20109 (SEQ ID NO: 11), CJA_2191 (Cbp33A) of *Cellvibrio japonicus* Ueda107 (SEQ ID NO: 12), CJA_3139 (Cbp33/10B) of *Cellvibrio japonicus* Ueda107 (SEQ ID NO: 13) and SCO1734 of *Streptomyces coelicolor* A3(2) (SEQ ID NO: 14), may be used. ChbA of *B. amyloliquefaciens* (Chu *et al.*, 2001, *Microbiology* 147 (Pt 7):1793-803) CHB1, 2 & 3 of *Streptomyces* (Svergun *et al.*, 2000, *Biochemistry* 39(35):10677-83, Zeltins *et al.*, 1997, *Eur. J. Biochem.* 246(2):557-64, Zeltins *et al.*, 1995, *Anal. Biochem.* 231(2):287-94, Schnellmann *et al.*, 1994, *Mol. Microbiol.* 13(5):807-19; Kolbe *et al.*, 1998, *Microbiology* 144 (Pt 5):1291-7; Saito *et al.*, 2001, *Appl. Environ. Microbiol.* 67(3):1268-73) and CBP1 of *Alteramonas* (Tsujiibo *et al.*, 2002, *Appl. Environ. Microbiol.* 68:263-270) are also preferred CBM33 proteins for use in the invention. All of these references are incorporated herein by reference.

The oxidohydrolytic enzyme can thus be or correspond to or comprise a naturally occurring CBM33 family protein (such as CBP21, EfCBM33, ChbA, CHB1, 2 & 3 and CBP1 or E7, CelS2, Cfla_0175, Cfla_0172, Cfla_0316, Cfla_0490, CJA_2191 (Cbp33A), CJA_3139 (Cbp33/10B) and SCO1734) or GH61 family protein in that it is found in nature or a biologically active fragment thereof. In the alternative the oxidohydrolytic enzyme may be a non-native variant as disclosed hereinafter.

As mentioned above, the oxidohydrolytic enzymes may be native proteins or biologically active fragments thereof or molecules containing those enzymes. Furthermore, non-native proteins may be derived from a naturally occurring protein, *e.g.*, from a GH61 or CBM33 family protein.

Such fragments are preferably at least 200, 300 or 400 amino acids in length and preferably comprise simple, short deletions from the N or C terminal, *e.g.*, a C terminal deletion of 1, 2, 3, 4 or 5 amino acids.

All such variants or fragments must retain the functional property of the protein from which they are derived such that they are "biologically active". Thus they must retain oxidohydrolytic activity, *e.g.*, under the conditions described in the Examples (*e.g.*, exhibit enhanced activity when used in the presence of a reducing agent and one or more saccharolytic enzymes when compared to performing the method without the reducing agent, see, *e.g.*, Fig. 4). Furthermore, said biologically active fragments and variants must be able to enhance degradation as described herein, *i.e.*, enhance degradation of the polysaccharide substrate (*e.g.*, when said degradation is performed in the presence of one or more saccharolytic enzymes) when used in the presence of a reducing agent and a divalent metal ion, relative to degradation omitting the reducing agent and divalent metal ion. Some loss of activity is contemplated, *e.g.*, the biologically active fragment or variant may have at least 50, 60, 70, 80, 90 or 95% of the oxidohydrolytic activity of the native full length sequence wherein said activity may be assessed in terms of the extent or level of degradation, *e.g.*, hydrolysis, achieved over a set time period, *e.g.*, as assessed by the production of reaction products such as oligo and/or di-saccharides.

Variants include or comprise naturally occurring variants of the oxidohydrolytic enzymes described above such as comparable proteins or homologues found in other species or more particularly variants found within other microorganisms, which have the functional properties of the enzymes as described above.

Variants of the naturally occurring oxidohydrolytic enzymes as defined herein can also be generated synthetically, *e.g.*, by using standard molecular biology techniques that are known in the art, for example standard mutagenesis techniques such as site directed or random mutagenesis. Such variants further include or comprise proteins having at least 70, 80, 85, 90,

91, 92, 93, 94, 95, 96, 97, 98 or 99% sequence similarity or identity with a naturally occurring oxidohydrolytic enzyme at the amino acid level.

Thus in a preferred aspect the oxidohydrolytic enzyme for use in the methods described herein is a polypeptide which comprises an amino acid sequence as set forth in any one of
 5 SEQ ID NOs: 1 to 14 (e.g., SEQ ID NOs: 1-4 or 1-5) and/or 15 to 16 (optionally with or without the leader peptide, where present) or a sequence with at least 30, 40, 50, 60, 70, 80, 90, 95, 97, 98 or 99% sequence identity thereto or a biologically active fragment thereof comprising at least 100 amino acids (preferably at least 200 or 300 amino acids) of said sequence.

In the sequences below, the leader peptides, where present, are underlined.

10 SEQ ID NO: 1 - GH61E from *T. terrestris* (Acc. No. ACE10234)
MLANGAIVFLAAALGVSGHYTWPRVNDGADWQQVRKADNWQDNGYVGDVTSPQIRCFQATP
 SPAPSVLNTTAGSTVTYWANPDVYHPGPVQFYMARVPDGEDINSWNGDGAVWFKVYEDHPT
 FGAQLTWPSTGKSSFAVPIPPCIKSGYYLLRAEQIGLHVAQSVGGAQFYISCAQLSVTGGGSTE
 PPNKVAFFPGAYSATDPGILINIYYPVPTSYQNPGPAVFSCMLANGAIVFLAAALGVSGHYTWPR
 15 VNDGADWQQVRKADNWQDNGYVGDVTSPQIRCFQATPSPAPSVLNTTAGSTVTYWANPDV
 YHPGPVQFYMARVPDGEDINSWNGDGAVWFKVYEDHPTFGAQLTWPSTGKSSFAVPIPPCIK
 SGYYLLRAEQIGLHVAQSVGGAQFYISCAQLSVTGGGSTEPPNKVAFFPGAYSATDPGILINIY
 YPVPTSYQNPGPAVFSC

20 SEQ ID NO: 2 - GH61A from *T. aurantiacus* (Acc. No. ABW56451)
MSFSKIIATAGVLASASLVAGHGFVQNIVIDGKKYYGGYLVNQYPYMSNPPEVIAWSTTATDLG
 FVDGTGYQTPDIICHRGAKPGALTAPVSPGGTVELQWTPWPDSHHGPVINY LAPCNGDCSTV
 DKTQLEFFKIAESGLINDNPPGIWASDNLIAANNSWTVTIPTTIAPGNYVLRHEIIALHSAQNQD
 GAQNPQCINLQVTGGGSDNPAGTLGTALYHDTDPGILINIYQKLSSYIIPGPPLYTG

25 SEQ ID NO: 3 - GH61B from *T. terrestris* (Acc. No. ACE10231)
MKSFTIAALAALWAQEAAAHATFQDLWIDGVDYGSQCVRLPASNSPVTNVASDDIRCNVGTSR
 PTVKCPVKAGSTVTIEMHQQPGDRSCANEAGGDHYGPVMVYMSKVDDAVTADGSSGWFKV
 FQDSWAKNPSGSTGDDDYWGTDKLNCCGKMNVKIPEDIEPGDYLLRAEVIALHVAASSGGA
 30 QFYMSCYQLTGTGSGSATPSTVNFPGAYSASDPGILINIHAPMSTYVVPGPVYAGGSTKSAG
 SSCSGCEATCTVGSGPSATLTQPTSTATATSAPGGGGSGCTAAKYQQCGGTGYTGCTTCAS
 GSTCSAVSPPYYSQCL

SEQ ID NO: 4 - CBP21 of *Serratia marescens*
 35 MNKTSRTLLSLGLLSAAMFGVSQQANAHGYVESPASRAYQCKLQLNTQCGSVQYEPQSVEG
 LKGFPQAGPADGHASADKSTFFELDQQTPTRWNKLNLTGPNSFTWKLTARHSTTSWRYFIT

KPNWDASQPLTRASFDLTPFCQFNDGGAIPAAQVTHQCNIPADRSGSHVILAVWDIADTANAF
YQAIDVNLISK

In the above sequence (SEQ ID NO: 4), amino acid residues 1 to 27 correspond to the leader peptide that is necessary for secretion of the protein in a natural system and amino acids 28-196 correspond to the mature protein. Using Pfam for domain/module discovery ("The Pfam protein families database" by Finn *et al.*, 2010, *Nucleic Acids Research Database* Issue 38: D211-222), for SEQ ID NO: 4 residues 28-194, *i.e.*, essentially the complete mature protein, are classified as CBM33. Similarly, in relation to the sequence presented in SEQ ID NO: 5, below, the mature protein starts at position 29 (H).

SEQ ID NO: 5 – EfCBM33 from *Enterococcus faecalis* (Acc. No. Q838S1)

MKKSLLTIVLAFSFLGGAALAPTIVSEAHGYVASPGSRAFFGSSAGGNLNTNVGRAQWEPQSI
EAPKNTFITGKLASAGVSGFEPLDEQTATRWHKTNITTGPLDITWNLTAQHRTASWDYYITKNG
WNPNQPLDIKNFDKIASIDGKQEVNPKVVKQTINIPTDRKGYHVIYAVWGIGDTVNAFYQAIDVN
IQ

SEQ ID NO: 6 – E7 from *Thermobifida fusca* (Acc. No. Q47QG3)

MHRYSRTGKHRWTVRALAVLFTALLGLTQWTAPASAHGSVINPATRNYGCWLRWGHDLNLP
NMQYEDPMCWQAWQDNPANMWNWNGLYRDWVGGNHRAALPDGQLCSGGLTEGGYRS
MDAVGPWKTTDVNNTFTIHLTDQASHGADYFLVYVTKQGFDPTTQPLTWDSLELVHQTGSYP
PAQNIQFTVHAPNRSGRHVFTIWKASHMDQTYLCSDEVNFV

SEQ ID NO: 7 – CelS2 from *Streptomyces coelicolor* A3(2) (Acc. No. Q9RJY2)

MVRRTLLTLAAVLATLLGSLGVTLGLGQGRAEA
HGVAMMPGSRTYLCQLDAKTGTGALDPTNPACQAALDQSGATALYNWFAVLDSNAGGRGAG
YVPDGTLCAGDRSPYDFSAYNAARSDWPRTHLTSGATIPVEYSNWAAHPGDFRVYLTKEG
WSPTSELGWDDLELIQTVTNPPQQGSPGTDGGHYYWDLALPSGRSGDALIFMQWVRSDSQE
NFFSCSDVVFDDGGNGEVTGIRGSGSTPDPTPTPTPTPTPTHTGSCMAVYSVENSWSGG
FQGSVEVMNHGTEPLNGWAVQWQPGGGTTLGGVWNGSLTSGSDGTVTVRNVDHNRVPP
DGSVTFGFTATSTGNDFPVDSIGCVAP

The signal peptide for the proteins in SEQ ID NOs: 1, 2, 3, 4, 5, 6 and 7 is underlined. The two conserved histidines in the metal binding motif of these proteins are shown in bold formatting. The signal peptides and histidines are similarly shown for SEQ ID Nos: 8-14, below.

MPRHRSTRRALAGLAATAVVTTALVTVPTVAQA**H**GGLTNPPTRTYACYQDGLAGGAAAGEAG
NIRPRNAACVNAFDNEGNYSFYNWYGNLLGTIAGRHETIADGKVCGPDARFASYNTPSSAWP
TTKVTPGQTMTFQYAAVAR**H**PGWFTTWITKDGWNQNEPIGWDDLEPAPFDRVLDPPLREGG
PAGPEYWWNVKLPSNKGKHLFNIWERTDSPESFYNCVDVDFGGGGTVTPSPTPSVTPTRT
PTPSPTPSVTPSPTPSVTPTPTPTPTPTPSPTPTLTVTPTPTPTSVPGDSVCELEVDTS
SAWPG
GFQGTVTVFNATMEPVNGWQVSWKFTNGETIAQSWSGVTSQSGSTVTVKNADWNSTIAHHN
AVNFGFIGSGTPKAVTDATLNGKPCIVR

MFIPTRSRFGRLARLALAVPLALAATGIVATSASAHGSVTDPPSRNYGCWEREGGTHMDPAM
 AQRDPMCWQAFQANPTMWNWNGNFREGVGGRHEQVIPDDQLCSAGKTQNGLYASLDTP
 GPWIMKTVPHNFTLTLDGAMHGADYMRIYVSKAGYDPTTDPLGWDDIELIKETGRYGTGLY
 QADVSIPSNRTGRAVLFTIWQASHLDQPYYICSDININGTAPTQQPTQQPTQQPTQQPT
 QQPTQQPTQQPTQQPTQQPTQNP GTGACTATVKAASTWGNGWQGEVTVTAGSSAINGWKV
 TVGGASITQAWSGSYSGGTFSNAEWNGKLAAGASTTAGFIASGTPGTLTATCTAA

MSRISPLRRVAAACGALAIGAATVVGSI~~LAAPASA~~HGAVSDPPSRIYGCWERWASNFTDPAM
ATSDPQCWDAWQSEPQAMWNWNGMFKEGAAGQHEQSPDGKLCADNPLYAAADDPGPW
RTTPVDHDFRLTLHDPSNHGADYLKIYVTKQGYDARSEALTWADLELVKTTGRYATSSPYVTD
VSVPRDRTGHHVVFTIWQASHLDQPYQCS~~DVT~~FGGGGTPTTSPTTPAPTPTTPAPTTPAPT
TTPAPTTPAPTTPAPTTPAPTTPAPTQPADGACTAAIEVVS~~AWQ~~GGYQATVTVTAGSGGLDG
WTVTVPGATITQAWNGTATGSTITAAGWNGTVAAGGTAGVGFLGSGSPDGLTATCAA

MRSHALPR SARPTPGRLLLSVLAVIALAFAVLTVAPAPSAQA**H**GWISDPPSRQDLCYTGA VSN
CGPVMYEPWSVEAKKGSMQCSGGGRFTELDNESRSWPRQNLKTNQVFTWDIVAN**H**STSTW
EYFVDGRLHTTIDDKGALPPNRFTH TINLPEGNHKIFVRWNIADTVNAFYQCIDAYITPGGTPG
PTQQPTQQPTQQPTQQPTQQPTQQPTQQPTQQPGNGACTATFKTNNAWNGNGYQGEITVTA
GSSAIRGWKVTVNGATITQAWSSQLSGSTLSNASWNGSLNAGASTTLGFIANGTPSGVTATCA
AA

MFNTRHLLAGVSQLVKPASMMILAMASTLAIHEASA**H**GYVSSPKSRVIQCKENGNIENPHTPACI
AAKAAGNGGLYTPQEVAVGGVDRNDHDYYIPDGR LCSANRANLFGMDLARNDWPATSVTPGA
REFVWTNTAA**H**KTKYFRYYITPQGYDHSQPLRWSDLQLIHDSGPADQEWVSTHNVLPRYTG

RHIIYSIWQRDWRDAAEGFYQCIDVDFGNGTGTGSSSSSVASSVVSSVTSSSVASSVASSLSN
 DTCATLPSWDASTVYTNPQQVKHNSKRYQANYWTQNQNPSTNSGQYGPWLDLGNCVTSSG
 SSSVASSSVASSVASSVTSSVASSVVSGNCISPVYVDGSSYANNALVQNNGSEYRCLVGGWC
 TVGGPYAPGTGWAWANAWELVRSCQ

5

SEQ ID NO: 13 - CJA_3139 (Cbp33/10B) from *Cellvibrio japonicus* Ueda107

MNNKFVKMGGMGALLAFSALSFGHGFVDSPGARNYFCGAVTKPDHVMNGVARYPECAGAF
 ANDFNGGYSYMSVLTHHQGRKVLGPVARNVCGFDSETWNGGKTPWDNAINWPVNNINSGL
 TFSWDISNGPHFDDTSDFRYWITKPGFVYQVGREL TWADFEDQPFCDLAYNDDNPGAYPNVR
 10 ADKPNTHFHTTCTVPARTGRHVIYAEWGREPPTYERFHGCIDVQIGGGSNSSVPVSSSSSSRS
 SSSSSLAPSSSSSRSSSSSSSVSSSRSSSSSVSSSSSSSRPASSSSSSTGGSTEYCNWYGWQ
 VAICKNTTSGWSNENQQTCIGRDTCNAPR

SEQ ID NO: 14 - SCO1734 from *Streptomyces coelicolor* A3(2)

15

MPAPSASRRAAVAVAGLAPLALTTLAAAPASAHGSMGDPVSRVSQCHAEGPENPKSAACRA
 AVAAGGTQALYDWNIGIRIGNAAGKHQELIPDGR LCSANDPAFKGLDLARADWPATGVSSGSY
 TFKYRVTAPHKGTGFKVYLTKPGYDPSKPLGWGDLDSAPVATSTDPVASGGFYTFSGTLPERS
 GKHLLYAVWQSRSDSPEAFYSCSDVTFGGDGDGDGDGSGSGAATGDDTASGD AEAGAAPA
 PEASAPSEEQLAAAAEKSTIEHHGHGDQDAATTTDPTDPAAAP EEPGTAAEPHQVKAAGGG
 20 TENLAETGGDSTTPYIAVGGAALALGAAVL FASVRRRATTGGRHGH

SEQ ID NOs: 15 and 16 are shown without a leader peptide. The two conserved
 histidines in the metal binding motif of these proteins are shown in bold formatting.

25

SEQ ID NO: 15 - HjGH61A from *Hypocrea jecorina*

HGHINDIVINGVWYQAYDPTTFPYESNPPIVVGWTAADLDNGFVSPDAYQNPDIICHKNATNAK
 GHASVKAGDTILFQWVPVPWPHPGPIVDYLANCNGDCETVDKTTLEFFKIDGVLLSGGDPGT
 WASDVLISNNNTWVVKIPDNLAPGNYVLRHEIALH**S**AGQANGAQNPQCFNIAVSGSGSLQP
 SGVLGTDLYHATDPGVLINIYTSPLNYIIPGPTVVSGLP TSVAQGSSAATATASATVPGGGSGPT
 30 SRTTTTARTTQASSRPSSTPPATTSAPAGGPTQTLYGQCGSGSGYSGPTRCAPPATCSTLNPY
 YAQCLN

SEQ ID NO: 16 - HjGH61 from *Hypocrea jecorina*

35

HGQVQNFTINGQYNQGFILDYYYQKQNTGHFPNVAGWYAEDLDLGFISPDQYTTTPDIVCHKNA
 APGAISATAAAGSNIVFQWGPVWPHPYGPVITYVVECSGSCCTTVNKNLNRWVKIQEAGINYN
 TQVWAQQDLINQGNKWTVKIPSSLRPGNYVFRHELLAAH**G**ASSANGMQNPQCVNIAVTGSG
 TKALPAGTPATQLYKPTDPGILFNPYTTITSYTIPGPALW

When variants are generated, it should be noted that appropriate residues to modify depend on the properties that are being sought in such a variant. In the case that a variant having the same oxidohydrolytic activity as the native parent molecule is being sought, the residues are in general those residues that are not involved in the catalytic reaction or interaction of the enzyme with the chitin substrate. However, those residues may be targeted, in the alternative, to develop variants with improved reactivity. This could be achieved by standard protein engineering techniques or by techniques based on random mutagenesis followed by screening, all techniques that are well known in the art. Attempts to improve the function of oxidohydrolytic enzymes may include improving the binding and catalytic ability of the enzyme, e.g., to act on other substrates, e.g., carbohydrate containing copolymers, e.g., protein-carbohydrate co-polymers.

A person skilled in the art will recognize the potential of using the native proteins' framework to create variants that are optimised for other insoluble polymeric polysaccharide substrates (e.g., other forms of chitin or cellulose), or insoluble carbohydrate-containing co-polymers.

In the case of GH61 proteins as set forth in SEQ ID NOs: 1, 2 and 3 (and 15-16), preferably the residues at positions 19, 86, 169, 171 and 210 of SEQ ID NO: 1 are conserved (see Harris *et al.*, 2010, *Biochemistry* 49:3305-3316, in which His-1 of the mature protein appears at position 19) or the corresponding residues in other GH61 proteins. Such corresponding residues can easily be found by sequence alignment.

In the case of CBP21, several residues have been shown to be important in the binding of CBP21 to chitin and more specifically to the ability of CBP21 to enhance the degradation of chitin (Vaaje-Kolstad *et al.*, 2005, *J. Biol. Chem.* 280: 11313-11319 and 28492-28497). Several mutations have been shown not to affect binding, but to affect the ability of CBP21 to enhance the degradation of chitin. These results may be predicted for other CBM33 proteins such as EfCBM33, E7, CelS2, Cfla_0175, Cfla_0172, Cfla_0316, Cfla_0490, CJA_2191 (Cbp33A), CJA_3139 (Cbp33/10B) and SCO1734. These residues are preferably not modified relative to the wild type CBP21 sequence as set out in SEQ ID NO: 4 (or any one of SEQ ID NOs: 5 to 14, e.g., SEQ ID NO: 5), if the aim is to modify, e.g., the stability of the CBP (for example under process conditions), but these residues may be targeted if one's aim is to improve or change the CBP21's functional properties.

Preferred variants of CBP21 retain one or more and preferably all of: a tyrosine residue at position 54, a glutamic acid residue at position 55, a glutamic acid residue at position 60, a histidine residue at position 114, an aspartic acid residue at position 182 and an asparagine at position 185 (sequence numbering according to SEQ ID NO: 4).

In connection with amino acid sequences, "sequence similarity", preferably "sequence identity", refers to sequences which have the stated value when assessed using, e.g., using the SWISS-PROT protein sequence databank using FASTA pep-cmp with a variable pamfactor and gap creation penalty set at 12.0 and gap extension penalty set at 4.0 and a window of 2 amino acids). Sequence identity at a particular residue is intended to include identical residues which have simply been derivatized. Sequence identity assessments are made with reference to the full length sequence of the recited sequence used for comparison.

Preferred "variants" include those in which instead of the naturally occurring amino acid the amino acid which appears in the sequence is a structural, e.g., non-native analogue thereof. Amino acids used in the sequences may also be derivatized or modified, e.g., labelled, glycosylated or methylated, providing the function of the oxidohydrolytic enzyme is not significantly adversely affected.

Further preferred variants are those in which relative to the above described amino acid sequences, the amino acid sequence has been modified by single or multiple amino acid (e.g., at 1 to 10, e.g., 1 to 5, preferably 1 or 2 residues) substitution, addition and/or deletion or chemical modification, including deglycosylation or glycosylation, but which nonetheless retain functional activity, insofar as they bind to the polysaccharide substrate and enhance its degradation, particularly when used in conjunction with one or more saccharolytic enzymes.

Within the meaning of "addition" variants are included amino and/or carboxyl terminal fusion proteins or polypeptides, comprising an additional protein or polypeptide or other molecule fused to the enzyme sequence. Carboxyl terminal fusions are preferred. It must be ensured that any such fusion to the enzyme does not adversely affect the functional properties required for use in the methods of the invention as set out elsewhere herein.

"Substitution" variants preferably involve the replacement of one or more amino acids with the same number of amino acids and making conservative substitutions.

Such functionally-equivalent variants mentioned above include in particular naturally occurring biological variations (e.g., found in other microbial species) and derivatives prepared using known techniques. In particular functionally equivalent variants of the oxidohydrolytic enzymes described herein extend to enzymes which are functional in (or present in), or derived from different genera or species than the specific molecules mentioned herein.

Variants such as those described above can be generated in any appropriate manner using techniques which are known and described in the art, for example using standard recombinant DNA technology.

Preferably the variants or fragments described herein are derived from the native sequences set forth above, particularly those of any one of SEQ ID NOs: 1 to 14 (e.g., SEQ ID NOs: 1-4 or 1-5) and/or 15 to 16.

As referred to herein a "reducing agent" is an element or compound in a redox (reduction-oxidation) reaction that reduces another species and in so doing becomes oxidized and is therefore the electron donor in the redox reaction. Preferably the reducing agent is non-enzymatic. In this particular invention, the reduced compound is oxygen which by the reduction becomes activated, enhancing the oxidohydrolytic function of, *e.g.*, GH61 or CBM33 proteins. The reducing agent may function as an electron donor in the enzymatic process and it is possible that electron donation takes place via the generation of reactive oxygen species such as O_2^- . The reducing agent promotes electron donation and/or the generation of reactive oxygen. Preferably said reducing agent is ascorbic acid, reduced glutathione or $Fe(II)SO_4$. Further preferred reducing agents are $LiAlH_4$ and $NaBH_4$. Other preferred reducing agents include organic acids (such as succinic acid, gallic acid, coumaric acid, humic acid and ferulic acid) and reducing sugars (such as glucose, glucosamine and N-acetylglucosamine). Alternatively, lignin, which contains reducing groups, or fragments thereof, may be used as the reducing agent. As noted above, $Fe(II)SO_4$ may be used as a reducing agent and in so doing will also contribute the required divalent metal ion. Whilst a single compound may provide both the reducing agent and metal ion, it is preferred that these features are provided by different compounds, *i.e.*, that the reducing agent and metal ion are separate compounds.

More than one of such agents may be used in line with methods of the invention and may be selected according to the substrate and conditions used (*e.g.*, pH and temperature). It will be appreciated that the efficacy and stability of reducing agents varies between these agents and depends on pH. Thus the pH and reducing agent should be optimized for the oxidohydrolytic enzyme to be used.

Preferably said divalent metal ion is Ca, Co, Mg, Mn, Ni or Zn. In an alternative embodiment the divalent metal ion is Cu. Thus, for example salts such as $MgCl_2$, $ZnCl_2$ or $CoCl_2$ (or alternatively $CuCl_2$) may be used.

The following description sets out conditions that can be used for performance of the method of the invention, but it should be noted that any appropriate conditions can be used.

Prior to contacting the polysaccharide-containing material with the oxidohydrolytic enzyme, the polysaccharide-containing material may be pre-treated.

The polysaccharide-containing material may be pre-treated, *e.g.*, to disrupt plant cell wall components, using conventional methods known in the art. Prior to pre-treatment, where appropriate, the polysaccharide-containing material may be subjected to pre-soaking, wetting, or conditioning using methods known in the art. Physical pre-treatment techniques include, for example, various types of milling, irradiation, steaming/steam explosion and hydrothermolysis; chemical pre-treatment techniques can include dilute acid, alkaline (*e.g.*, lime pre-treatment), organic solvent (such as organosolv pre-treatments), ammonia treatments (*e.g.*, ammonia percolation (APR) and ammonia fibre/freeze explosion (AFEX)), sulfur dioxide, carbon dioxide,

wet oxidation and pH-controlled hydrothermolysis; and biological pre-treatment techniques can involve applying lignin-solubilizing microorganisms (see, for example, Hsu, 1996, Pre-treatment of biomass, in "Handbook on Bioethanol: Production and Utilization", Wyman, ed., Taylor & Francis, Washington, DC, 179-212; Ghosh & Singh, 1993, *Adv. Appl. Microbiol.* 39: 295-333; 5 McMillan, 1994, Pretreating lignocellulosic biomass: a review, in "Enzymatic Conversion of Biomass for Fuels Production", Himmel *et al.* eds., ACS Symposium Series 566, American Chemical Society, Washington, DC, Chapter 15; Gong *et al.*, 1999, Advances in Biochemical Engineering/Biotechnology, Scheper, ed., Springer-Verlag Berlin Heidelberg, Germany, 65: 207-241; Olsson & Hahn-Hagerdal, 1996, *Enz. Microb. Tech.* 18: 312-331; and Vallander & 10 Eriksson, 1990, *Adv. Biochem. Eng./Biotechnol.* 42: 63-95). Additional pre-treatments include ultrasound, electroporation, microwave, supercritical CO₂, supercritical H₂O and ammonia percolation.

Pre-treated corn stover is a cellulose-containing material derived from corn stover, *e.g.*, by treatment with heat and dilute acid.

15 Following optional pre-treatment, the polysaccharide-containing material (the substrate) may be exposed to the oxidohydrolytic enzyme *in vitro* in any appropriate vessel, *e.g.*, by mixing together the substrate and the enzyme in an appropriate medium (*e.g.*, a solution, such as an aqueous solution) or by applying the enzyme to the substrate (*e.g.*, by applying the enzyme in a solution to a substrate).

20 In a preferred embodiment the oxidohydrolytic enzyme is present in a buffer such as a phosphate buffer, *e.g.*, a sodium phosphate buffer, or Tris buffer. Suitable concentration ranges for such a buffer are 1-100 mM. The oxidohydrolytic enzyme may be provided as a purified preparation (as described hereinafter) or may be present in a composition, wherein it may be a major component, preferably comprising at least 20, 30, 40, 50, 60 or 70% w/w dry weight in 25 the composition, or it may be a minor component (*e.g.*, in a mixture with one or more saccharolytic enzymes), preferably comprising at least 1, 2, 5 or 10%, *e.g.*, 1-5%, w/w dry weight in the composition.

The enzyme can be present in the solution at any suitable concentration, such as a concentration of 0.001-1.0 mg/ml, *e.g.*, 0.01-0.1 mg/ml or 0.05-0.5 mg/ml.

30 The polysaccharide substrate is present in the reaction mix at any suitable concentration which will depend to some extent on the purity of the polysaccharide in the material containing it. Conveniently, however, the polysaccharide itself is present at a concentration of from 0.1 to 200 mg/ml, preferably 0.2 to 20 mg/ml or 0.5 to 50 mg/ml, or more preferably 25 to 150 mg/ml, especially preferably at least 25 mg/ml. Preferably the 35 polysaccharide is present in the material containing the polysaccharide to a level of >50%, *e.g.*, >60, 70, 80 or 90%, w/w dry weight in the material.

Preferably the polysaccharide substrate is exposed to the enzyme, *e.g.*, by incubation together, for a period of 4, 6, 12 or 24 hours or more, such as 4-24 or 6-24 hours, *e.g.*, 36 or 48 hours or more, or 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 days or more. In a preferred aspect the incubation is 6-24 hours. This incubation is in general carried out at or about 50°C, although
5 appropriate temperatures for optimizing the enhancement of polysaccharide degradation can readily be determined by the skilled person in the art. For example, the temperature can be in the range of 20-65°C, *e.g.*, 30-60°C, preferably 50-55°C.

It will be appreciated that the necessary incubation times, pH, temperature, substrate and enzyme concentrations are not independent of each other. Thus, a large range of
10 conditions can be envisaged, which can easily be evaluated. The oxidohydrolytic enzymes serve to enhance degradation by the saccharolytic enzymes and thus may allow the use of lower concentrations of the latter or shorter reaction times.

Preferably a pH in the range of 4 to 9 is used. Preferably the pH is in the range of 5 to 8. The preferred pH is about pH 5-6.

Where a reducing agent is used, the reducing agent is preferably added for the duration of the degradation reaction, though it may be added after that reaction has commenced and may be present only while the oxidohydrolytic enzyme is present or active. Reducing agents are preferably added to a final concentration range of 0.1 to 100 mM, preferably 0.5 to 20 mM, especially preferably 1-5 mM. Reducing agents may be present in the polysaccharide
20 substrate, *e.g.*, lignin present in a lignocellulosic biomass, but preferably said reducing agents are added to the reaction mix.

As with the reducing agent, the metal ion may be added at the start or during the degradation reaction. However, it may not always be necessary to add metal ions to the reaction mixture as some substrate-containing material may contain sufficient metal ions for the
25 reaction to proceed successfully. However, in a preferred aspect, metal ions are added to the reaction mix. In one embodiment, the metal ion may be added to the oxidohydrolytic enzyme during its production or isolation or to the enzyme prior to its addition to the reaction mix such that the enzyme is "pre-loaded" with the relevant metal ion. Metal ions are preferably added to a final concentration range of 0.001 to 50 mM, *e.g.*, 0.01 to 50 mM, preferably 0.1 to 5 mM. The
30 concentration to be used may be readily determined for the particular metal ion to be used in the reaction mix. For example, lower concentrations of Cu²⁺ (*e.g.*, 0.001 to 0.1 mM) may be appropriate relative to the concentration required for other metal ions. It will be appreciated that the optimal metal ion concentrations to some extent depend on the enzyme and substrate concentrations used in the enzymatic conversion reactions that are set up.

As noted herein, the oxidohydrolytic enzymes are believed to catalyze hydrolysis by oxidation by molecular oxygen. It is therefore imperative (as noted in the Examples) that
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molecular oxygen is available for use in the reaction. As such any conditions that result in an oxygen-free (anaerobic) environment must be avoided.

Thus, in a preferred aspect the method comprises contacting said polysaccharide with an oxidohydrolytic enzyme and adding at least one reducing agent and preferably at least one divalent metal ion to the reaction mixture.

Preferably the incubation is carried out with agitation, particularly when a cellulose-containing material is used.

In a preferred aspect, the oxidohydrolytic enzyme is used at a concentration of 0.01 to 0.5 mg/ml and the polysaccharide substrate at 25 to 150 mg/ml (when calculated according to the target substrate content and not taking into account the additional material that may be present with the substrate) and the reaction is conducted at pH 5-8 for 6 to 24 hours at 50 to 55°C.

In methods in which the degradation or hydrolysis is carried out with the oxidohydrolytic enzyme only, the result of said reaction is incomplete degradation of the polysaccharide to yield largely insoluble long oligosaccharides and minor fractions of soluble oligosaccharides, perhaps including very minor fractions of disaccharides. Preferably said degradation or hydrolysis is enhanced further or completed by the use of appropriate additional degradative glycoside hydrolases.

Thus in a further preferred aspect the present invention provides a method of degrading or hydrolyzing a polysaccharide comprising:

a) contacting said polysaccharide with one or more oxidohydrolytic enzymes, wherein said degradation or hydrolysis is carried out in the presence of at least one reducing agent and at least one divalent metal ion, and

b) contacting said polysaccharide (or the degradation or hydrolysis product thereof) with one or more saccharolytic enzymes selected from a cellulose hydrolase or chitin hydrolase.

Clearly in performing the method the oxidohydrolytic enzyme and the saccharolytic enzyme must be selected in accordance with the polysaccharide substrate, e.g., GH61 and a cellulose hydrolase for cellulose and CBP21 or another CBM33 family protein and a chitin hydrolase for chitin. Cross-reaction between different substrates may also be possible, e.g., CBM33 family proteins may be effective as oxidohydrolytic enzymes on cellulose, e.g., SEQ ID NO: 5 (EfCBM33) and other CBM33 proteins described herein may be used in methods of the invention performed on cellulose. Similarly, GH61 family members may be used on substrates other than cellulose, e.g., chitin or hemicellulose.

Until now enzyme activity of members of the CBM33 family has only been reported for chitin as a substrate (e.g., CBP21 from *Serratia marcescens*). However, the present invention demonstrates that members of the CBM33 family, e.g., E7 and CelS2, work on cellulose. The

enzymatic function entails hydrolysis (cleavage) and oxidation of cellulose chains in insoluble cellulose crystals, which enables a more rapid deconstruction of the cellulose by cellulases. CBM33 enzymes acting on cellulose work optimally in the presence of an external electron donor (e.g., a reducing agent) and divalent metal ions. These enzymatic traits are highly similar to those observed previously for CBM33 enzymes that act on chitin. In the experiments described herein a single domain CBM33 from *Thermobifida fusca* (Uniprot ID:Q47QG3; E7) and a multidomain CBM33 (Uniprot ID: Q9RJY2; a CBM33 with a CBM2 attached on the C-terminal side of the protein; CelS2) from *Streptomyces coelicolor* A3(2) were expressed and purified and their ability to potentate cellulose degrading enzyme activity was observed.

CBM33 proteins for reaction with cellulose are preferably obtained from cellulolytic bacteria, e.g., bacteria of the genera *Cellulomonas*, *Cellvibrio*, *Thermobifida* or *Streptomyces*, e.g., bacteria of the species *Cellulomonas flavigena*, *Cellvibrio japonicus*, *Thermobifida fusca* or *Streptomyces* spp. (preferably E7 and CelS2 as disclosed herein and Cfla_0175, Cfla_0172, Cfla_0316, Cfla_0490, CJA_2191 (Cbp33A), CJA_3139 (Cbp33/10B) and SCO1734) and/or have one or more cellulose binding modules (e.g., belonging to CBM family 2) attached to the C-terminal end.

Natural or engineered variants of these oxidohydrolytic enzymes with altered substrate specificity (e.g., from chitin to cellulose) may be combined with other substrates and saccharolytic enzymes.

It will be obvious to the expert in the field that polysaccharides such as chitin and, especially, cellulose may occur in complex co-polymeric matrices including for example hemicelluloses in the case of plant cell wall material. Since cellulose and hemicelluloses interact strongly, it is possible that loosening of the cellulose structure by an oxidohydrolase may make not only the cellulose but also the hemicellulose more accessible for attack by appropriate saccharolytic enzymes. Thus, oxidohydrolases such as GH61 and CBM33 family proteins may also be used concomitantly with, e.g., hemicellulases or other enzymes targeting the non-chitin and non-cellulose polymers in complex chitin- or cellulose- containing co-polymeric materials, in order to increase the saccharolytic efficiency of these enzymes.

As referred to herein a "saccharolytic enzyme" is an enzyme which is capable of cleaving glycosidic bonds between saccharide monomers or dimers in a polysaccharide, using a standard hydrolytic mechanism as employed by most enzymes classified in the glycoside hydrolase (GH) families in the CAZy database. These enzymes include cellulose hydrolases, chitin hydrolases and beta-glucosidases.

As referred to herein a "cellulose hydrolase" is an enzyme which hydrolyses cellulose or intermediate breakdown products. Preferably the hydrolase is a cellulase. Cellulases are classified as glycosyl hydrolases (GH) in families based on their degree of identity and fall within the GH families 1, 3, 5-9, 12, 44, 45, 48 and 74. Based on mechanism they can be

grouped into exo-1,4-beta-D-glucanases or cellobiohydrolases (CBHs, EC 3.2.1.91), endo-1,4-beta-D-glucanases (EGs, EC 3.2.1.4) and beta-glucosidases (BGs, EC 3.2.1.21). EGs cleave glycosidic bonds within cellulose microfibrils, acting preferentially at amorphous cellulose regions. EGs fragment cellulose chains to generate reactive ends for CBHs, which act "processively" to degrade cellulose, including crystalline cellulose, from either the reducing (CBH1) or non-reducing (CBHII) ends, to generate mainly cellobiose. Cellobiose is a water-soluble beta-1,4-linked dimer of glucose. Beta-glucosidases hydrolyze cellobiose to glucose. GH61 enzymes were previously classified as weak endoglucanases on the basis of activity of one member of the family though this is now not considered correct. As mentioned above, GH61 enzymes have been found to be oxido-hydrolytic by the present inventors.

The ability of cellulose hydrolases to hydrolyse cellulose may be assessed by using methods known in the art, including methods in which non-modified cellulose is used as substrate. Activity is then measured by measuring released products, using either HPLC-based methods or methods that determine the number of newly formed reducing ends (e.g., Zhang *et al.*, 2009, *Methods Mol. Biol.* 581:213-31; Zhang *et al.*, 2006, *Biotechnol. Adv.* 24(5): 452-81). In the alternative, the efficacy of the cellulose hydrolase may be assessed by using an appropriate substrate and determining whether the viscosity of the incubation mixture decreases during the reaction. The resulting reduction in viscosity may be determined by a vibration viscosimeter (e.g., MIVI 3000 from Sofraser, France). Determination of cellulase activity, measured in terms of Cellulase Viscosity Unit (CEVU), quantifies the amount of catalytic activity present in a sample by measuring the ability of the sample to reduce the viscosity of a solution of the substrate.

Cellulases may be obtained from commercial sources, i.e., companies such as Novozymes, Danisco and Biocatalysts. Examples of commercial cellulases include, for example, CELLIC[™] CTec (Novozymes A/S), CELLIC[™] CTec2 (Novozymes A/S), CELLUCLAST[™] (Novozymes A/S), NOVOZYME[™] 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.).

Alternatively cellulases may be produced using standard recombinant techniques for protein expression. The scientific literature contains numerous examples of the cloning, overexpression, purification and subsequent application of all types of cellulases, e.g., endoglucanases, cellobiohydrolases, and beta-glucosidases.

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); *Trichoderma reesei* endoglucanase II (Saloheimo, *et al.*, 1988, *Gene* 63:11-22; *Trichoderma reesei* Cel5A endoglucanase II; GENBANK™ accession no. M19373); *Trichoderma reesei* endoglucanase III (Okada *et al.*, 1988, *Appl. Environ. Microbiol.* 64: 555-563; GENBANK™ accession no. AB003694); *Trichoderma reesei* endoglucanase V (Saloheimo *et al.*, 1994, *Molecular Microbiology* 13: 219-228; GENBANK™ accession no. Z33381); *Aspergillus aculeatus* endoglucanase (Ooi *et al.*, 1990, *Nucleic Acids Research* 18: 5884); *Aspergillus kawachii* endoglucanase (Sakamoto *et al.*, 1995, *Current Genetics* 27: 435-439); *Erwinia carotovora* endoglucanase (Saarilahti *et al.*, 1990, *Gene* 90: 9-14); *Fusarium oxysporum* endoglucanase (GENBANK™ accession no. L29381); *Humicola grisea* var. *thermoidea* endoglucanase (GENBANK™ accession no. AB003107); *Melanocarpus albomyces* endoglucanase (GENBANK™ accession no. MAL515703); *Neurospora crassa* endoglucanase (GENBANK™ accession no. XM_324477); *Humicola insolens* endoglucanase V; *Myceliophthora thermophila* CBS 117.65 endoglucanase; basidiomycete CBS 495.95 endoglucanase; basidiomycete CBS 494.95 endoglucanase; *Thielavia terrestris* NRRL 8126 CEL6B endoglucanase; *Thielavia terrestris* NRRL 8126 CEL6C endoglucanase; *Thielavia terrestris* NRRL 8126 CEL7C endoglucanase; *Thielavia terrestris* NRRL 8126 CEL7E endoglucanase; *Thielavia terrestris* NRRL 8126 CEL7F endoglucanase; *Cladorrhinum foecundissimum* ATCC 62373 CEL7A endoglucanase; and *Trichoderma reesei* strain No. VTT-D-80133 endoglucanase (GENBANK™ accession no. M15665).

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

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

Cellulase mixtures may be used, e.g., a cellulase mixture which comprises at least one endoglucanase, e.g., belonging to GH family 5, 7 or 12, a cellobiohydrolase moving towards the reducing end, e.g., belonging to GH family 6, a cellobiohydrolase moving towards the non-

reducing end, e.g., belonging to GH family 7, and a beta-glucosidase. More preferably, more complex mixtures are used, in particular mixtures containing several endoglucanases with different substrate specificities (e.g., acting at different faces of the cellulose crystals). Appropriate cellulases may be readily identified taking into account the substrate to be degraded.

As referred to herein a "chitin hydrolase" is an enzyme which hydrolyses chitin or intermediate breakdown products. Preferably said chitin hydrolase is a chitinase, chitosanase or lysozyme. The degradation may be complete or partial. For example, the activity of some chitin hydrolase, e.g., chitinases on chitin substrates is not strong enough to result in complete degradation of the substrate. This is particularly the case for chitinases such as ChiG from *Streptomyces coelicolor* that do not have their own CBM, or chitinases such as ChiB from *S. marcescens*. In this case, the use of an oxidohydrolytic enzyme that acts on chitin in accordance with the present invention can result in enhanced chitin degradation and preferentially result in complete degradation that was not previously possible. Other chitinases, such as ChiC from *S. marcescens*, are capable of completely degrading chitin, but the speed of this process increases upon addition of an oxidohydrolytic enzyme such as CBP21.

Chitinase enzymes are found in plants, microorganisms and animals. Chitinases have been cloned from various species of microorganisms and have been categorised into two distinct families, designated family GH18 and family GH19 of the glycoside hydrolases, based on sequence similarities (Henrissat and Bairoch, 1993, *Biochem. J.* 293:781-788). These enzymes are referred to collectively herein as chitin hydrolases.

There are several ways to measure chitinase activity that are well known in the field, including methods in which non-modified chitin is used as substrate. Activity on non-modified chitin is measured by measuring released products, using either HPLC-based methods or methods that determine the number of newly formed reducing ends.

Chitinases may be obtained from commercial sources, i.e., companies such as Sigma. Alternatively chitinases may be produced using standard recombinant techniques for protein expression. The scientific literature contains numerous examples of the cloning, overexpression, purification and subsequent application of all types of chitinases (e.g., Horn *et al.*, 2006, *FEBS J.* 273(3):491-503 and references therein).

Other suitable hydrolytic enzymes for hydrolyzing additional non-cellulose (or non-chitin) polysaccharides include hemicellulases such as acetylxyylan esterases, arabinofurosidases, feruloyl esterases, glucuronidases, mannanases, xylanases, and xylosidases.

The cellulase mixtures may also be used in conjunction with hemicellulases. Hemicellulases may also be obtained from commercial sources. Examples of commercial hemicellulases 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 Limited, Wales, UK), DEPOL™ 740L (Biocatalysts Limited, Wales, UK), and DEPOL™ 762P (Biocatalysts Limited, 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 acetylxyylan esterases useful in the methods of the present invention include, but are not limited to, *Hypocrea jecorina* acetylxyylan esterase (WO 2005/001036), *Neurospora crassa* acetylxyylan esterase (UniProt accession number q7s259), *Thielavia terrestris* NRRL 8126 acetylxyylan esterase (WO 2009/042846), *Chaetomium globosum* acetylxyylan esterase (Uniprot accession number Q2GWX4), *Chaetomium gracile* acetylxyylan esterase (GeneSeqP accession number AAB82124), *Phaeosphaeria nodorum* acetylxyylan esterase (Uniprot accession number Q0UHH1), and *Humicola insolens* DSM 1800 acetylxyylan 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).

Whilst the use of native saccharolytic enzymes is preferred, variants defined in accordance with the properties described herein for the oxidohydrolytic enzyme's variants may also be used.

Preferably, when said polysaccharide is cellulose, said saccharolytic enzyme is an endo-1,4-beta-D-glucanase optionally used in combination with other 1,4-beta-D-glucanases such as cellobiohydrolases and/or beta-glucosidases.

Thus, the enzymes to be used in methods of the invention may be selected based on the polysaccharide substrate to be hydrolyzed. For example, CBP21 binds only to beta-chitin and would therefore be an appropriate oxidohydrolytic enzyme to use if the methods of the invention were to be applied to beta-chitin. Similarly ChbB from *B. amyloliquifaciens* as described in Chu *et al.* (*supra*) may be applied to beta-chitin.

CHB1, CHB2 and CHB3 have all been isolated from *S. olivaceovirides* (Svergun *et al.*, Zeltins *et al.*, Schnellman *et al.*, Kolbe *et al.*, Saito *et al.*, *supra*). The binding preferences of these three proteins have been determined and CHB1 and CHB2 bind preferably to alpha-chitin, whereas CHB3 binds to both alpha- and beta-chitin. CBP1 from *Alteromonas* s described by Tsujibo *et al.* binds to both alpha- and beta-chitin, with a preference for the alpha form.

Alternatively, GH61 family members, such as described herein, may be used to assist with chitin degradation in methods of the invention.

Similarly when the substrate is chitin, the saccharolytic enzyme can be selected accordingly. The properties of chitinases have been documented (*e.g.*, Hollis *et al.*, 1997, *Arch. Biochem. Biophys.* 344: 335-342 and Suzuki *et al.*, 1998, *Biosci. Biotech. Bioch.* 62: 128-135; Horn *et al.*, 2006).

Preferred combinations for beta-chitin hydrolysis are CBP21 (or variants or fragments thereof) with one or more of ChiA, ChiB, ChiC and ChiG. Preferred combinations for alpha-chitin hydrolysis are CHB1 or CHB2 (or variants or fragments thereof) with one or more of ChiA, ChiB, ChiC and ChiG. Alternatively GH61 family members as described herein may be used with appropriate chitinases.

When the substrate is cellulose, the oxidohydrolytic enzyme is preferably a GH61 family protein (as described herein), though in view of their ability to act on cellulose, CBM33 family proteins may also be used. Appropriate saccharolytic enzymes may be selected from known enzymes, *e.g.*, cellulases as described herein.

In a preferred aspect two or more oxidohydrolytic enzymes are employed in the methods of the invention. In view of their preferred substrate specificities, enhanced degradative effects may be expected when used together. Thus, for example, one may use two or more CBM33 family proteins and/or GH61 family proteins (as described herein), *e.g.*, two or more CBM33 family proteins, two or more GH61 family proteins or a combination of one or more of each of the CBM33 family proteins and GH61 family proteins in the methods (*e.g.*, at

least one CBM33 family protein and at least one GH61 family protein). Thus, for example chitin (or cellulose) may be contacted with one CBM33 family protein and one GH61 family protein, e.g., preferably selected from the proteins described herein (e.g., polypeptides which comprise an amino acid sequence as set forth in any one of SEQ ID NOs: 4 and/or 5 and/or 6 to 14 or related sequences or fragments described herein (CBM33 family proteins) and polypeptides which comprise an amino acid sequence as set forth in any one of SEQ ID NOs: 1 to 3 and/or 15 to 16 or related sequences or fragments described herein (GH61 family proteins)).

Appropriate enzymes for use in accordance with the invention can be determined by use of screening techniques to assess *in vitro* hydrolysis, e.g., as described in the Examples.

To identify oxido-hydrolytic enzymes which may be used in combination, the enzymes may be assessed to determine whether their activity will achieve enhanced effects on the substrate. For example, when degrading biomass one may combine members of the CBM33/GH61 families that are known from experiments, such as those described herein, to have different specificities for the various forms of chitin (e.g., alpha-chitin or beta-chitin) or cellulose (e.g., various types of cellulose fibers, cellulose pulps, filter paper, microcrystalline cellulose, AVICEL®, Carboxymethylcellulose) that occur in nature, biomass, and/or pretreated biomass, or can be obtained by chemical modification, many of these forms being easily accessible for experimentation. In biomass, chitin and cellulose often occur as heteropolymers, containing other polysaccharides often referred to as hemicelluloses or even proteins. The different members of the CBM33/GH61 families can be expected to have different activity on these different substrates. Biomass is often heterogeneous, either by nature, or because biomasses are mixed during process development in the factory. By mixing members of the CBM33/GH61 families with known differences in biomass preferences, more efficient processes may be obtained. Further synergies may be obtained using members of the CBM33/GH61 families that preferentially act on different polysaccharides in biomass, such as xylan.

Oxido-hydrolytic enzymes with different activities may also be identified by examining the periodicity of the reaction products (see the Examples herein). Thus, a combination may be made between oxido-hydrolytic enzymes (e.g., members of the CBM33/GH61 families) with different periodicities. The periodicity for CBP21 is shown in Figs. 2D, 6A and 7B; periodicity for EfCBM33 is shown in Fig. 15D; periodicity for CelS2 is shown in Figs. 17, 19, 23A, 27 and 31; and periodicity for E7 is shown in Fig. 29. One possible explanation for variation in periodicity is that crystalline cellulose has several forms and that crystals have different faces (*i.e.*, types of surface; see, e.g., Carrard *et al.*, 2000, *Proc Natl Acad Sci USA* 97(19):10342-7) and that different members of the CBM33 and GH61 families attack different faces. In view of the different activity of the enzymes, combinations of enzymes (which may achieve synergistic effects) may be made. Thus, for example, one could combine a GH61 with CelS2.

In the methods described above using both an oxidohydrolytic enzyme and a saccharolytic enzyme, the step with the oxidohydrolytic enzyme is carried out under conditions which allow the enzyme to interact or bind to the polysaccharide as described herein. The same conditions and considerations are applied to the additional step using additional saccharolytic enzymes (hydrolases), which step may be carried out simultaneously or subsequent to the first step. In total the incubation may be conducted for 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 days or more, but is typically performed for preferably about 8 to about 96 hours, more preferably about 8 to about 72 hours and most preferably about 8 to about 48 hours or 4 to 24 hours.

Preferably aqueous solutions of the enzymes are used and preferably the enzymatic hydrolysis is carried out in a suitable aqueous environment under conditions that can be readily determined by one skilled in the art.

Each enzyme used in the methods may be provided as a purified preparation (as described hereinafter) or may be present in a composition, (e.g., including the other enzymes for use in the methods) preferably at at least 1, 2, 5 or 10%, preferably 1-5% w/w dry weight in the composition.

For the methods described herein, the hydrolysis can be carried out as a fed batch or continuous process where the polysaccharide-containing material (substrate), which may be pre-treated, 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 as discussed herein. Suitable process time, temperature and pH conditions can readily be determined by one skilled in the art and are discussed herein and can depend on the substrate and enzymes used and their concentrations and whether the substrate has been pretreated and whether a fermenting organism is included, see hereinbelow.

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 15 to about 30 wt. %.

Each enzyme used in the reaction can be present in the solution at any suitable concentration, such as a concentration of 0.001-1.0 mg/ml, e.g., 0.01-0.1 mg/ml or 0.05-0.5 mg/ml. Alternatively expressed, the enzymes may be used at a concentration of 0.1-100 mg enzyme/g of polysaccharide substrate, e.g., 1-50 mg/g substrate. Suitable concentrations can be determined depending on the substrate and the material containing the substrate and the conditions of the reaction, e.g., temperature, pH and duration.

The steps in which the oxidohydrolytic enzyme and the saccharolytic enzyme(s) are contacted with the polysaccharide substrate may be performed separately or together or a combination thereof, e.g., the oxidohydrolytic enzyme may be added and after an initial incubation period the saccharolytic enzyme(s) may be added. In the alternative, the oxidohydrolytic enzyme may be removed before the saccharolytic enzyme is added. Any steps

in which the oxidohydrolytic enzyme is not present (e.g., a step in which only a saccharolytic enzyme is used) need not be conducted in the presence of a reducing agent and/or metal ion.

Other enzymes may also be added in addition to or as an alternative to the chitin or cellulose hydrolytic enzymes discussed above, depending on the nature of the substrate that is to be degraded. For example, if the polysaccharide to be degraded is a copolymer which contains protein, proteases may also be added. Suitable examples include Alcalase, Neutrase, Papain and other broad-specificity proteolytic enzymes. In each experimental set-up the suitability of proteases will need to be checked, especially if other enzymes (e.g., chitinases or cellulases), which may be destroyed by some of the available proteases, are present simultaneously. If the polysaccharide is a copolymer with hemicelluloses, hemicellulolytic enzymes may be added.

Furthermore, the product resulting from using the above described oxidohydrolytic enzyme and saccharolytic enzymes may include soluble short oligosaccharides (particularly disaccharides). Since dimeric products inhibit glycoside hydrolases and since monomers are the most desirable product resulting from the degradation/hydrolysis process for further processing (see hereinbelow), additional enzymes, namely beta-glucosidases are preferably also used in the methods of the invention.

Thus in a preferred aspect, said method of degrading or hydrolyzing a polysaccharide further comprising contacting said polysaccharide (or the degradation or hydrolysis product thereof) with one or more beta-glucosidases. Such enzymes may be identified and used as specified herein (e.g., in relation to their concentration) for other saccharolytic enzymes. For cellulose a beta-glucosidase(s) may be used and for chitin a beta-N-acetylglucosaminidase(s) may be used.

The steps in which the oxidohydrolytic enzyme, saccharolytic enzyme(s) and beta-glucosidase(s) are contacted with the polysaccharide substrate may be performed separately or together or a combination thereof, e.g., the oxidohydrolytic enzyme may be added and after an initial incubation period the saccharolytic enzyme(s) and beta-glucosidase(s) may be added, or the latter two enzymes may be added sequentially. In the alternative, the oxidohydrolytic enzyme may be removed before the other enzymes are added. Any steps in which the oxidohydrolytic enzyme is not present (e.g., a step in which only a saccharolytic enzyme(s) and/or beta-glucosidase(s) is used) need not be conducted in the presence of a reducing agent and/or metal ion.

The oxidohydrolytic enzymes and saccharolytic enzymes for use in the methods of the invention may be isolated, extracted or purified from various different sources or synthesised by various different means. As mentioned above the enzymes may be provided in purified preparations or in the presence of other components.

Chemical syntheses may be performed by methods well known in the art involving, in the case of peptides, cyclic sets of reactions of selection deprotection of the functional groups of a terminal amino acid and coupling of selectively protected amino acid residues, followed finally by complete deprotection of all functional groups. Synthesis may be performed in solution or on a solid support using suitable solid phases known in the art, such as the well known Merrifield solid phase synthesis procedure.

Preferably the enzymes for use in the invention are substantially purified, e.g., pyrogen-free, e.g., more than 70%, especially preferably more than 90% pure (as assessed for example, in the case of peptides or proteins, by an appropriate technique such as peptide mapping, sequencing or chromatography or gel electrophoresis). Purification may be performed for example by chromatography (e.g., HPLC, size-exclusion, ion-exchange, affinity, hydrophobic interaction, reverse-phase) or capillary electrophoresis.

Recombinant expression of proteins is also well known in the art and an appropriate nucleic acid sequence can be used to express the enzymes used herein for subsequent expression and optional purification using techniques that are well known in the art. For example, an appropriate nucleic acid sequence can be operably linked to a promoter for expression of the enzyme to be used in bacterial cells, e.g., *E. coli* which may then be isolated or if the enzyme is secreted, the culture medium or the host expressing the enzyme may be used as the source of the enzyme.

The methods described above have applications in a number of different fields in which hydrolysis of polysaccharides forms one of the method steps or in which the products of that hydrolysis are useful.

Thus in a further aspect the present invention provides a method of producing soluble saccharides, wherein said method comprises degrading or hydrolyzing a polysaccharide by contacting said polysaccharide with one or more oxido-hydrolytic enzymes, wherein said degradation or hydrolysis is carried out in the presence of at least one reducing agent and at least one divalent metal ion and said degradation or hydrolysis releases said soluble saccharides.

In an alternative preferred aspect the invention provides a method of producing soluble saccharides, wherein said method comprises degrading or hydrolyzing a polysaccharide by:

a) contacting said polysaccharide with one or more oxido-hydrolytic enzymes, wherein said degradation or hydrolysis is carried out in the presence of at least one reducing agent and at least one divalent metal ion,

b) contacting said polysaccharide (or the degradation or hydrolysis product thereof) with one or more saccharolytic enzymes selected from a cellulose hydrolase or chitin hydrolase, and optionally

c) contacting said polysaccharide (or the degradation or hydrolysis product thereof) with one or more beta-glucosidases;
wherein said degradation or hydrolysis releases said soluble saccharides.

The result of complete hydrolysis is soluble sugars. Usually, a mixture of monomeric sugars and higher order oligosaccharides (e.g., disaccharides) are generated. As discussed above, preferably beta-glucosidases are used to produce monomeric sugars. The partially or completed degraded polysaccharide-containing material is preferably recovered for further processing, e.g., fermentation. Soluble products of degradation of the polysaccharide-containing material can be separated from the insoluble material using technology well known in the art such as centrifugation, filtration and gravity settling.

Preferably said soluble saccharides are isolated or recovered after said degradation or hydrolysis process. Preferably the soluble saccharides which are isolated or recovered are chitobiose and/or N-acetylglucosamine (from chitin) or cellobiose and/or glucose (from cellulose) and/or oligosaccharides thereof.

N-acetylglucosamine and oligosaccharides of N-acetylglucosamine have a number of commercial uses including use as a food supplement. Chitin fragments have found utility in various applications including use as immune stimulants (Aam *et al.*, 2010, *Drugs* 8(5): 1482-517).

The soluble saccharides resulting from hydrolysis of cellulose have various applications, particularly for use as a source of energy in fermentation reactions.

Preferably the saccharide mixture released after hydrolysis containing monomeric sugars is fermented to generate an organic substance such as an alcohol, e.g., ethanol.

Thus the present invention further provides a method of producing an organic substance, preferably an alcohol, comprising the steps of:

i) degrading or hydrolyzing a polysaccharide by a method comprising:

a) contacting said polysaccharide with one or more oxido-hydrolytic enzymes, wherein said degradation or hydrolysis is carried out in the presence of at least one reducing agent and at least one divalent metal ion, and

b) contacting said polysaccharide (or the degradation or hydrolysis product thereof) with one or more saccharolytic enzymes selected from a cellulase or chitinase, and optionally

c) contacting said polysaccharide (or the degradation or hydrolysis product thereof) with one or more beta-glucosidases;

to produce a solution comprising soluble saccharides;

ii) fermenting said soluble saccharides, preferably with one or more fermenting microorganisms to produce said organic substance as the fermentation product; and optionally

iii) recovering said organic substance.

Optionally, said soluble saccharides produced in step (i) may be isolated or purified from said solution.

The organic substance thus produced forms a further aspect of the invention.

As referred to herein "soluble saccharides" include monosaccharides, disaccharides and oligonucleotides which are water soluble, preferably mono- and/or disaccharides. Preferably said soluble saccharides are fermentable, e.g., glucose, xylose, xylulose, arabinose, maltose, mannose, galactose and/or soluble oligosaccharides.

"Fermentation" refers to any fermentation process or any process comprising a fermentation step.

The above method may additionally comprise the use of one or more additional enzymes such as esterases (e.g., lipases, phospholipases and/or cutinases), proteases, laccases and peroxidases.

The steps of hydrolysis (saccharification) and fermentation may be performed separately and/or simultaneously and 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 cofermentation (HHCF) and direct microbial conversion (DMC). Conveniently, any method known in the art comprising pre-treatment, enzymatic hydrolysis (saccharification), fermentation, or a combination thereof, can be used in the practicing the above methods.

Conveniently, 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 (de Castilhos Corazza *et al.*, 2003, *Acta Scientiarum. Technology* 25: 33-38; Gusakov & Sinitsyn, 1985, *Enz. Microb. Technol.* 7: 346-352), an attrition reactor (Ryu & Lee, 1983, *Biotechnol. Bioeng.* 25: 53-65), or a reactor with intensive stirring induced by an electromagnetic field (Gusakov *et al.*, 1996, *Appl. Biochem. Biotechnol.* 56: 141-153). Additional reactor types include, for example, fluidized bed, upflow blanket, immobilized and extruder type reactors for hydrolysis and/or fermentation.

Pre-treatments that may be used were discussed herein and apply to all methods of the invention. The polysaccharide-containing material can be pre-treated before hydrolysis and/or fermentation. Pre-treatment is preferably performed prior to the hydrolysis step. Alternatively, the pretreatment can be carried out simultaneously with hydrolysis, such as simultaneously with treatment of the polysaccharide-containing material with the enzymes used in the methods (*i.e.*, oxidohydrolytic and saccharolytic enzymes) to release fermentable sugars, such as glucose and/or cellobiose. In most cases the pre-treatment step itself results in some conversion of biomass to fermentable sugars (even in the absence of enzymes).

The fermentable sugars obtained by the method of the invention can be fermented by one or more fermenting microorganisms capable of fermenting the sugars directly or indirectly into a desired fermentation product.

The fermentation conditions depend on the desired fermentation product and fermenting organism and can easily be determined by one skilled in the art.

In the fermentation step, sugars, released from the substrate are fermented to a product, e.g., ethanol, by a fermenting organism, such as yeast. The polysaccharide substrate to be used in the method may be selected based on the desired fermentation product.

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

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

Examples of fermenting microorganisms that can ferment C6 sugars include bacterial and fungal organisms, such as yeast. Preferred yeast includes strains of *Saccharomyces spp.*, preferably *Saccharomyces cerevisiae*.

Examples of fermenting organisms that can ferment C5 sugars include bacterial and fungal organisms, such as yeast. Preferred C5 fermenting yeast include strains of *Pichia*, preferably *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*; and *E. coli*, especially *E. coli* strains that have been genetically modified to improve the yield of ethanol.

In a preferred aspect, the yeast is a *Saccharomyces spp.* In a more preferred aspect, the yeast is *Saccharomyces cerevisiae*, *Saccharomyces distaticus*, *Saccharomyces uvarum*. In another preferred aspect, the yeast is a *Kluyveromyces*, e.g., *Kluyveromyces marxianus* or *Kluyveromyces fragilis*.

Other yeast that may be used include *Clavispora*, e.g., *Clavispora lusitaniae* or *Clavispora opuntiae*; *Pachysolen*, e.g., *Pachysolen tannophilus*; and *Bretanomyces*, e.g., *Bretanomyces clausenii*.

Bacteria that can efficiently ferment hexose and pentose to ethanol include, for example, *Zymomonas*, such as *Zymomonas mobilis* and *Clostridium*, such as *Clostridium thermocellum*.

Commercially available yeast suitable for ethanol production include, 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).

The fermenting microorganism(s) is typically added to the degraded polysaccharide-material 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 to 50°C and at about pH 3 to about pH 8, such as around pH 4-5, 6, or 7. The above conditions will of course depend on various factors including the fermenting microorganism that is used.

The fermenting microorganism(s) is preferably applied in amounts of approximately 10^5 to 10^{12} , preferably from approximately 10^7 to 10^{10} , especially approximately 2×10^8 viable cell count per ml of fermentation broth.

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

A fermentation stimulator can be used in combination with any of the enzymatic processes described herein to further improve the fermentation process, and in particular, the performance of the fermenting microorganism, such as, rate enhancement and ethanol yield. A "fermentation stimulator" refers to stimulators for growth of the fermenting microorganisms, in particular, yeast. Preferred fermentation stimulators for growth include vitamins and 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.

The organic substance which is the fermentation product can be any substance derived from the fermentation. The fermentation product can be, without limitation, an alcohol (e.g., arabinitol, butanol, ethanol, glycerol, methanol, 1,3-propanediol, sorbitol or xylitol); an organic acid (e.g., acetic acid, acetonetic acid, adipic acid, ascorbic acid, citric acid, 2,5-diketo-D-gluconic acid, formic acid, fumaric acid, glucaric acid, gluconic acid, glucuronic acid, glutaric acid, 3-hydroxypropionic acid, itaconic acid, lactic acid, malic acid, malonic acid, oxalic acid, propionic acid, succinic acid or xylonic acid); a ketone (e.g., acetone); an aldehyde (e.g., formaldehyde); an amino acid (e.g., aspartic acid, glutamic acid, glycine, lysine, serine or threonine); or a gas

(e.g., methane, hydrogen (H₂), carbon dioxide (CO₂) or carbon monoxide (CO)). The fermentation product may also be an alkane, a cycloalkane, an alkene, isoprene, or polyketide. The fermentation product can also be protein.

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. Preferably the alcohol is arabinitol, butanol, ethanol, glycerol, methanol, 1,3-propanediol, sorbitol or xylitol. Ethanol is the preferred product.

The fermentation product(s) may be recovered from the fermentation medium using any method 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), distillation or extraction. For example, ethanol is separated from the fermented cellulose-containing material and purified by conventional methods of distillation. Ethanol with a purity of up to about 96 vol. % can be obtained.

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

EXAMPLES

Example 1: Effects of oxidohydrolytic enzymes CBP21, EfCBM33, CelS2 and E7 on chitin or cellulose substrates.

Materials and Methods

Reagents

Pure beta-chitin powder (80# mesh) from squid pen was purchased from France Chitin (Marseille, France). H₂¹⁸O (containing 97% ¹⁸O) and ¹⁸O₂ (containing 99% ¹⁸O) was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA). 2,5-dihydroxy-benzoic acid (DHB) was purchased from Bruker Daltonics (Bremen, Germany). Dithionite, ascorbic acid, reduced glutathione, Fe(II)SO₄, Cu(I) acetate, MgCl₂, ZnCl₂, CoCl₂, LiCl, acetonitrile, Trisma-Base, HCl, EDTA and H₂O₂ (30% v/v) were all purchased from Sigma-Aldrich Inc. The Schlenk line was hand-made at the University of Oslo and used with an in-house N₂ supply (99.999% pure). The N₂ gas was purchased from YARA PRAXAIR (Oslo, Norway). Oligosaccharides of N-Acetyl-D-glucosamine ranging from dimer to hexamer were purchased from Seikagaku (Tokyo, Japan). Chitin beads for protein purification were purchased from New England Biolabs.

Nano-whiskers of beta-chitin were prepared according as described in (Fan *et al.*, 2008, *Biomacromolecules* 9: 1919) by sonication of 3.0 mg/mL beta-chitin particles suspended in 0.2

M acetic acid using a Vibracell Ultrasonic Processor equipped with a 3 mm sonication probe (Sonics, Newtown, CT) in four, one minute intervals with 30 s pauses between each interval. Before use, the buffer in the chitin-whisker suspension was changed to 20 mM Tris pH 8.0 by dialysis. These whiskers were used for the experiment displayed in Fig. 2A, where an increased surface area was needed to enable detection of CBP21 activity in the absence of reductants and additional enzymes. All other experiments were performed with non-treated beta-chitin.

Cloning, expression and purification of recombinant proteins

Chitin binding protein 21 (CBP21) from Serratia marcescens

CBP21 was cloned, produced and purified as previously described (Vaaje-Kolstad *et al.*, 2005, *J. Biol. Chem.* 280: 11313). Briefly, the *E. coli* BL21 DE3 strain harbouring the pRSET-B vector containing the *cbp21* gene was grown overnight and harvested. The periplasmic content of the cells containing CBP21 was extracted by cold osmotic shock, filtered through a 0.2 micron syringe filter and kept at 4°C. Further, CBP21 was purified from the periplasmic extract by chitin affinity chromatography, using chitin beads (NEB) as chromatographic medium. CBP21 was bound to the column using 20 mM Tris pH 8.0 and 1.0 M (NH₄)₂SO₄ as binding buffer. After non-bound protein had passed through the column, the binding buffer was run for one column volume before eluting CBP21 with 20 mM acetic acid. The fraction containing CBP21 was concentrated using an Amicon Ultra centrifugal filter unit with 10 kDa molecular weight cut off, dialysed into 20 mM Tris-HCl pH 8.0 and stored at 4°C until use. Protein purity was assessed by SDS-PAGE (always >99% pure) and protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., USA) according to the instructions supplied by the manufacturer. Single site mutants of CBP21 were made as previously described (Vaaje-Kolstad *et al.*, 2005, *J. Biol. Chem.* 280: 11313) using the QuickChange site-directed mutagenesis kit (Stratagene).

Chitinase C (ChiC) from Serratia marcescens

ChiC was cloned and produced as previously described (Synstad *et al.*, 2008, *Biosci. Biotechnol. Biochem.* 72: 715). Briefly, *E. coli* BL21 DE3 cells harbouring a pREST-B vector containing the *chic* gene under control of the T7 promoter were grown to OD 0.6 and induced by 0.4 mM IPTG. The enzyme was extracted from the cells by periplasmic extraction by cold osmotic shock. Further, ChiC was purified from the periplasmic extract by chitin affinity chromatography using chitin beads (NEB) as column material. Using 20 M Tris-HCl, pH 8.0 as running buffer, the periplasmic extract containing ChiC was passed through the column at 2.5 ml/min enabling binding of the chitinase to the chitin beads. After the non-bound proteins in the extract had passed through the column, one column volume of running buffer was run through the column before eluting the bound chitinase with 20 mM acetic acid. Following elution, the

purified protein was concentrated using an Amicon ultrafiltration device (Millipore) and finally dialysed into 20 mM Tris-HCl, pH 8.0 and stored at 4°C before use. Protein purity was routinely assayed by SDS-PAGE and protein concentration was determined by the Bio-Rad method.

5 *Chitin deacetylase (AnCDA) from Aspergillus nidulans*

Aspergillus nidulans FGSC A4 (obtained from FGSC) was grown at 37°C in solid YAG medium (5 g/L yeast extract, 20 g/L glucose, 20 g/L agar, 1 ml/L Cove's Trace Elements, 1.2 g/L MgSO₄·7H₂O) for 24-48h to provide an inoculum, and in liquid YG medium, typically for 16-24 hours with vigorous shaking (250-300 rpm). Genomic DNA was isolated with the SP Fungi
 10 DNA Mini Kit (Omega Bio-tek, USA). The gene (GeneBank accession number EAA66447.1) was amplified from *Aspergillus nidulans* FGSC A4 genomic DNA using overlap extension polymerase chain reactions, excluding introns as well as the gene part coding for the signal peptide. Primers for the reactions were P1f (*Bgl*II): cga aga tct acg cct ctg cct ttg gtt c (SEQ ID NO: 17), P2r: gag acg tgg tcg tat gta tgt gcg ccg act tga tg (SEQ ID NO: 18); P3f: caa gtc ggc
 15 gca cat aca tac gac cac gtc tcc ctc c (SEQ ID NO: 19); P4r: cca aca gtc gta gct atc aac cct cga gca tta ac (SEQ ID NO: 20); and P5r (*Hind*III): cag aag ctt tca atg ata cca cgc aat ctc tcc atc acc gag aca atc acc aac agt cgt agc tat caa c (SEQ ID NO: 21). A *Bgl*II and a *Hind*III site were incorporated at the start and the end of the *AnCDA* gene to produce an in-frame N-terminal His tag-fused construct in the pBAD/HisB(s) vector. This vector is a variant of the commercial
 20 vector pBAD/HisB (Invitrogen, USA) where the region between the N-terminal polyhistidine tail and the multiple cloning site has been shortened (Kallio *et al.*, 2006, *J. Mol. Biol.* 357: 210). The resulting plasmids were transformed into *Escherichia coli* TOP10 cells (Invitrogen) and the inserted gene was sequenced at the sequencing facility of the Department of Chemistry, Biotechnology and Food Science at the Norwegian University of Life Sciences.

25 For protein expression, the transformed *E. coli* strain was grown at 37°C in 2×TY medium (16 g/L Tryptone, 10 g/L yeast extract, 5 g/L NaCl) containing 100 mg of ampicillin per liter until OD₆₀₀=0.6 and subsequently induced with 0.02% (w/v; final concentration) arabinose before further incubation at 28°C overnight. Cells were harvested by centrifugation and the protein was purified to homogeneity by Ni²⁺ affinity chromatography. Protein concentrations
 30 were determined using the Bio-Rad protein assay, with bovine serum albumin as a standard.

E7 from Thermobifida fusca xy (Q47QG3)

The gene sequence encoding the mature variant of Q47QG3 from *T. fusca xy* (E7; residues 37-222) was cloned by amplifying the corresponding gene region from genomic DNA
 35 from *T. fusca xy* (purchased from ATCC) using primers designed according to the In-Fusion cloning protocol (Clontech). The resulting PCR product was inserted into a modified pRSETB vector (Invitrogen) using the In-Fusion™ technology (Clontech) in-frame with the signal

peptide for direction of the protein product to the periplasm upon expression in *E. coli*. The modified pRSETB vector has the His-tag containing region replaced by the signal sequence encoding region of the *cbp* gene from *Serratia marcescens* (Vaaje-Kolstad *et al.*, 2005, *J. Biol. Chem.* 280: 11313). By inserting genes of interest in frame with the signal sequence, the gene product will be transported to the periplasm upon expression in *E. coli* and the exported protein will have a native N-terminus, meaning that the protein sequence starts with a histidine. Successful constructs were sequenced for verification and transformed into *E. coli* BL21 DE(3) for protein expression. Cultures were grown overnight at 37°C. Cells were harvested by centrifugation and subjected to periplasmic extraction by cold osmotic shock. The mature E7 protein was purified by chitin affinity chromatography using the chitin-beads to capture the protein. The capture buffer contained 20 mM Tris-HCl pH 8.0 and 1.0 M ammonium sulphate. The protein was eluted from the column using 20 mM acetic acid. Peaks containing pure protein were pooled and concentrated using Sartorius Vivaspin devices with a 10 kDa cutoff. Using the same protein concentration device, the buffer was changed to 20 mM Tris pH 8.0.

CelS2 from Streptomyces coelicolor A3(2) (Q9RJY2)

A gene encoding the mature form of Q9RJY2 from *S. coelicolor* A3(2) (CelS2; residues 35-364) was cloned by amplifying the corresponding gene region from genomic DNA from *S. coelicolor* A3(2) (purchased from ATCC) using primers designed according to the LIC cloning protocol (Novagen) that places a hexa-histidine tag at the N-terminus of the protein that can be removed using the Factor Xa protease, leaving no non-native amino acids on the N-terminus of the protein. The PCR product was inserted into the pET-32 LIC vector according to the instructions supplied by the manufacturer (Novagen). Successful constructs were sequenced for verification and transformed into *E. coli* Rosetta DE(3) for protein expression. Expression of soluble target protein was obtained by growing a 5 ml pre-culture of the transformed Rosetta DE(3) cells overnight at 37°C, which was used the next day to inoculate a 300 ml volume of LB-medium and growth was continued with shaking at 250 rpm at 37°C. Gene expression was induced by adding IPTG to a final concentration of 0.1 mM when cell density reached an O.D. of 0.6, followed by immediate transfer of the culture to a shaking incubator having 20°C and for continuing the culturing overnight. The following day cells were harvested by centrifugation. Cell pellets were resuspended in sonication buffer (20 mM Tris-HCl pH 8.0, 100 M PMSF, lysozyme and DNase) followed by sonication using a Vibra Cell Ultrasonic processor equipped with a 3 mm sonication probe (Sonics) in order to release cytoplasmic proteins. Cell debris was removed by centrifugation and His-tagged Q9RJY2 was purified by standard IMAC (immobilized metal affinity chromatography) purification protocols using the Nickel-NTA IMAC resin (Qiagen). Purified protein was concentrated using Sartorius Vivaspin protein concentration devices with a 10 kDa cutoff, which also were used concomitantly to change to

buffer into a buffer suitable for Factor Xa removal of the His-tag (100 mM NaCl, 5.0 mM CaCl₂, 50 mM Tris pH 8.0). His-tags were cleaved off by adding Factor Xa and incubating overnight at room temperature, followed by His-tag removal using standard IMAC chromatography. The flow through protein fraction containing the processed Q9RJY2 protein and Factor Xa was collected and concentrated using Sartorius Vivaspin® protein concentration devices with a 10 kDa cutoff. Finally, Factor Xa was removed using Xarrest agarose beads according to the manufacturer's instructions (Novagen). The buffer of the pure protein was changed to 20 mM Tris pH 8.0 using Sartorius Vivaspin® protein concentration devices with a 10 kDa cutoff. Correct processing of the His-tag was verified by SDS-PAGE analysis.

Protein concentration was quantified using the Bio-Rad Bradford micro assay (Bio-Rad) and protein purity was validated by SDS-PAGE.

Site-directed mutagenesis of the gene encoding the CelS2 protein was done using the Quickchange® Mutagenesis Kit (Stratagene) and according to instructions provided by the manufacturer. The mutated protein was expressed and purified using methods identical to those used for the wild-type protein.

Purification of Cel7A from Trichoderma reesei / Hypocrea jecorina

The endo-acting family GH7 cellulase Cel7A from *Hypocrea jecorina* was purified from the commercially available *H. jecorina* extract CELLUCLAST™ (Novozymes) using purification protocols described by Jager *et al.*, 2010, *Biotechnology for Biofuels* 3(18). In short, the *H. jecorina* extract was adjusted to 10 mM AmAc pH 5.0 and the enzyme was purified using a DEAE-sepharose column attached to an Äcta Purifier running 10 mM AmAc pH 5.0 as a mobile phase. Relevant fractions were pooled and concentrated using Sartorius Vivaspin protein concentration devices with a 10 kDa cutoff. Purity was assessed using SDS-PAGE analysis.

Product analysis by mass spectrometry (MS)

Matrix-assisted laser desorption/ionization - time of flight (MALDI-TOF)

Two microliter of a 9 mg/mL mixture of 2,5-dihydroxybenzoic acid (DHB) in 30% acetonitrile was applied to a MTP 384 target plate ground steel TF (Bruker Daltonics). One microliter sample was then mixed into the DHB droplet and dried under a stream of air. The samples were analyzed with an Ultraflex MALDI-TOF/TOF instrument (Bruker Daltonics GmbH, Bremen, Germany) with a Nitrogen 337 nm laser beam. The instrument was operated in positive acquisition mode and controlled by the FlexControl 3.3 software package. All spectra were obtained using the reflectron mode with an acceleration voltage of 25kV, a reflector voltage of 26, and pulsed ion extraction of 40 ns in the positive ion mode. The acquisition range used was from *m/z* 0 to 7000. The data were collected from averaging 400 laser shots, with the lowest laser energy necessary to obtain sufficient signal to noise ratios. Peak lists were

generated from the MS spectra using Bruker FlexAnalysis software (Version 3.3). Post-source decay (PSD) spectra using the Bruker Daltonics LIFT system were recorded at 8 kV precursor ion acceleration voltage and fragment acceleration (LIFT voltage 19 kV). The reflector voltage 1 and 2 were set to 29 and 14.5 kV, respectively.

5

Product analysis by HPLC and UHPLC

High performance liquid chromatography (HPLC)

Isocratic HPLC was run on a Dionex Ultimate 3000 HPLC system set up with a 4.6x250 mm Amide-80 column (Tosoh Bioscience, Montgomeryville, PA, USA) with an Amide-80 guard column. The mobile phase consisted of 70% acetonitrile : 30% MilliQ H₂O and the flow rate was 0.7 ml/min. Eluted oligosaccharides were monitored by recording absorption at 190 nm. Chromatograms were recorded, integrated and analysed using the Chromeleon 6.8 chromatography software (Dionex). The major product of chitin degradation by ChiC is (GlcNAc)₂ (>95% of the total amount of degradation products on a molar basis), thus only (GlcNAc)₂ peaks were subject for data analysis and used for quantification of the extent of chitin degradation. A standard solution containing 0.10 mM (GlcNAc)₂ was analyzed at regular intervals during the sample series, and the resulting average values (displaying standard deviations of less than 3%) were used for calibration.

For the sake of experimental simplicity and throughput, the degradation of chitin in reactions with ChiC and CBP21 was quantitatively assessed by measuring concentration of the dominant product, (GlcNAc)₂, only. As a result of this simplification, product levels for maximally degraded chitin tend to be up to 25% lower than expected on the basis of the starting concentration of chitin. This "loss" is due to the lack of detection of the following products: (1) longer oligomers and monomers which may amount to an estimated 5 wt. % of the total product mixture, (2) partially deacetylated products; the chitin that we use contains a small fraction of deacetylated sugars (about 8%), (3) oxidized sugars. We have used conditions that boost CBP21 activity to a maximum. The amounts of undetected oxidized sugars may amount to as much as 10-15% of the starting material (Fig. 16).

Ultra high performance liquid chromatography (UHPLC)

UHPLC was run on an Agilent 1290 Infinity UHPLC system equipped with diode array detector, set up with a Waters Acquity UPLC BEH amide column (2.1x150 mm with a 2.1 x 30 mm pre column both having a column material particle size of 1.7 µm) using 5 µL sample injections. Separation of oxidized oligosaccharides was obtained at column temperature 30°C and a flow of 0.4 mL/min starting at 72% ACN (A):28% 15 mM Tris-HCl pH 8.0 (B) for 4 minutes, followed by an 11 minute gradient to 62% A: 38% B which was held for three minutes. Column reconditioning was obtained by a two minute gradient to initial conditions and

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subsequent running at initial conditions for 5 minutes. Eluted oligosaccharides were monitored by recording absorption at 205 nm. Chromatograms were recorded, integrated and analysed using the ChemStation rev. B.04.02 chromatography software (Agilent Technologies). The identity of the eluted oligosaccharides was verified by MALDI-TOF MS analysis according to the protocol described above.

Degradation reactions and sampling

General reaction conditions

Typical reactions were initiated by mixing beta-chitin (0.5 to 2 mg/mL) with CBP21 (0.1 to 1 μ M), ChiC (0.5 μ M) or AnCDA (1 μ M) or combinations of these enzymes at a total volume of 0.5 ml in 1.5 ml plastic reaction tubes (Axygen Scientific Inc, CA) or in 1.8 ml borosilicate glass vials with screw cap tops and TEFLON® lined rubber septa. All reactions were carried out in 20 mM Tris-HCl, pH 8.0 and incubated at 37°C with shaking at 1000 rpm in an Eppendorf Thermo mixer unless stated otherwise. All reactions used for quantification were run in triplicates. All reactions used for qualitative purposes were repeated at least three times.

Reactions with CBP21

Chitin solubilization by CBP21 was investigated by adding 1.0 μ M CBP21 to a reaction solution containing 2.0 mg/mL beta-chitin and 5.0, 1.0 or 0.2 mM ascorbic acid in 20 mM Tris-HCl pH 8.0. Reactions were incubated at 37°C and samples were taken at regular time intervals for analysis by MALDI-TOF MS and UHPLC. In order to investigate the effects of reducing agents on the function of CBP21, ascorbic acid was exchanged with either 1 mM reduced glutathione or 1 mM Fe(II)SO₄ in some reactions.

The effect of CBP21 on chitinase activity was studied by adding 0.5 μ M ChiC and 1.0 μ M CBP21 to a reaction solution containing 2.0 mg/mL beta-chitin and 1.0 mM ascorbic acid in 20 mM Tris-HCl pH 8.0. The reaction was incubated at 37°C and sampled at regular time intervals. Chitin degradation was measured by determining the concentration of (GlcNAc)₂ by HPLC. Control experiments where CBP21 and/or ascorbic acid were excluded from the reaction solution were performed in the same manner.

To investigate whether CBP21 was capable of cleaving and/or oxidizing soluble substrates, a 500 μ L reaction solution containing 1.0 μ M CBP21, 100 μ M (GlcNAc)₆ (0.12 mg/mL) and 1.0 mM ascorbic acid all dissolved in 20 mM Tris pH 8.0 was incubated for 16 hours at 37°C before product analysis by MALDI-TOF MS. The same was performed for control reactions where either CBP21 or ascorbic acid or both were excluded from the reaction solution.

An experiment designed to visualize the range of polymeric products generated by CBP21 was performed by combining 1.0 μ M CBP21 and 1.0 μ M AnCDA in a reaction solution

containing 2.0 mg/mL beta-chitin, 1.0 mM ascorbic acid and 10 μ l CoCl_2 (necessary for full AnCDA activity) in 20 mM Tris-HCl pH 8.0. Control reactions were performed where CBP21 was excluded from the reaction solution and/or replaced by 0.5 μ M ChiC. The reactions were incubated for 16 hours at 37°C, followed by product analysis with MALDI-TOF MS.

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Molecular oxygen free reaction and related control reactions

In order to obtain a di-oxygen free reaction solution, all reaction components except the enzyme or enzymes were mixed in a glass vial closed with a screw cap containing a rubber septum and degassed using a Schlenk line. The enzyme was added to a separate vial which was treated identically to the vial containing the reaction mixture. Before starting the degassing procedure, a freshly made 1.0 M dithionite solution was added the reaction solution to yield a final concentration of 10 mM to ensure total removal of molecular oxygen in the solution. The degassing procedure was performed by penetrating the rubber septum of the sealed vial with a needle connected to the Schlenk line, followed by five cycles of 5 minute degassing (vacuum) and 1 minute of N_2 saturation. The final cycle left the vials slightly pressurized by N_2 . After degassing both the reaction solution and the enzyme solution, a syringe was used to withdraw an appropriate amount of the enzyme solution which then was promptly injected into the vial containing the reaction solution in order to initiate the reaction, while injecting bubbles of air was avoided. The effect of a molecular oxygen free environment was assessed by analyzing the activity of 1.0 μ M CBP21 on 2.0 mg/mL beta-chitin in the presence of 1.0 mM ascorbic acid in 20 mM Tris-HCl pH 8.0 by MALDI-TOF MS. Additionally, the degradation of 0.1 mg/ml beta-chitin by 0.5 μ M ChiC in the presence of 1.0 μ M CBP21 and 1.0 mM ascorbic acid in 20 mM Tris-HCl pH 8.0 was analyzed in the same di-oxygen free environment. Samples were analysed by HPLC after 16 hours incubation at 37°C.

This latter experiment was also conducted in the absence of sodium dithionite and at a higher chitin concentration (0.45 mg/mL), but with otherwise identical reaction conditions. It should be noted that even though every precaution was taken to avoid oxygen entering the reaction solution, the dioxygen removal is not 100% efficient. This can be seen by studying the result from the $^{18}\text{O}_2$ experiment (see Fig. 7C), where products resulting from oxidation by $^{16}\text{O}_2$ are detected.

Additional control experiments were performed by running experiments where 0.45 mg/mL beta-chitin, 1.0 μ M CBP21, 0.5 μ M ChiC and 1.0 mM reduced glutathione in 20 mM Tris-HCl pH 8.0, were run in the presence of either 2.0 mM sodium azide or 2.0 mM potassium cyanide. The reactions were incubated at 37°C, sampled at 30, 60 and 90 minutes, and products were analyzed by UHPLC.

Reactions under metal chelating conditions

Divalent cations were removed by chelation through dialysis of a 10 mg/mL CBP21 solution in a buffer containing 20 mM Tris-HCl and 5 mM EDTA. The protein solution was present in a Slide-A-Lyzer cassette (Pierce) with 10 kDa MW cut-off dialysis membrane. Dialysis was performed for 16 hours at 4°C with a protein to buffer volume ratio of 1:1000 with moderate magnetic stirring. Reactions with metal-free CBP21 were performed as described above, except that EDTA was added to the reaction buffer to a final concentration of 5 mM. Re-activation of metal-free CBP21 was achieved by adding either ZnCl₂ or MgCl₂ to the reaction mixture to a final concentration of 25 mM. Reactions were run for 180 minutes and sampled at 30 minute intervals. For the re-activation experiment, the divalent cations were added to the appropriate reaction solutions immediately after the third sampling (90 minutes). Chitin degradation was measured by determining the concentration of (GlcNAc)₂ by HPLC.

Reactions in buffered H₂¹⁸O

Beta-chitin and ascorbic acid were each suspended/dissolved in H₂¹⁸O to yield concentrations of 2 mg/mL and 1.0 M, respectively. In order to achieve the correct pH in the H₂¹⁸O reaction solution 10 µL 1.0 M Tris-HCl pH 8.0 was transferred to a glass vial, which was heated with dry air at 60°C until all liquid had evaporated. 498 µL of the beta-chitin suspension was transferred to the same glass vial achieving the intended pH for the reaction. Concomitantly 0.5 µL ascorbic acid (dissolved in H₂¹⁸O) and 0.75 µL of a 660 µM solution of CBP21 (dissolved in H₂¹⁶O) were added to the solution to start the reaction, yielding final concentrations of 1 mM for ascorbic acid and 1 µM for CBP21. The glass vial was sealed with a screw cap with a TEFLON® coated rubber liner to ensure as little as possible contamination of H₂¹⁶O from the air phase into the reaction solution. After incubation for 16 hours at 37°C reaction products were analyzed by MALDI-TOF MS.

Reactions in an ¹⁸O₂ gas saturation solution

In a glass vial containing a reaction mixture of 2.0 mg/mL beta-chitin and 1.0 mM ascorbic acid in 20 mM Tris pH 8.0, CBP21 was added to yield a final concentration of 1.0 µM. Immediately after reaction initiation a screw cap containing a TEFLON® lined rubber septum was used to close the vial and the Schlenk line was used to remove dissolved molecular oxygen and fill the head space with N₂ (according to the procedure described under the heading "molecular oxygen free reaction"). After the five cycles of degassing and N₂ filling were completed, a gas cylinder containing compressed ¹⁸O₂ gas was connected to the vial by pushing a needle through the septum of the vial. The vial was then placed under vacuum, removing atmospheric gas residing in the tubing and the head space of the vial. After isolating the vial and the ¹⁸O₂ gas cylinder from the rest of the Schlenk line by closing appropriate in-line

valves, the head space of the vial was filled with $^{18}\text{O}_2$ gas by slowly opening the gas cylinder regulator. The vial was then removed from the needle connections and after incubation at 37°C for 16 hours reaction products were analyzed by MALDI-TOF MS.

5 *Reaction of beta-chitin with Fenton chemistry*

In order to determine whether Fenton chemistry (Fe^{2+} and H_2O_2 combined in an oxygen saturated solution to yield reactive hydroxyl radicals; Sawyer *et al.*, 1996, *Acc. Chem. Res.* 29: 409) would yield soluble products from chitin, 2 mg/mL beta-chitin suspended in 20 mM Tris-HCl pH 8.0 was incubated for 16 hours with 10 mM Fe(II)SO_4 and 0.3, 0.03 or 0.003% (v/v) H_2O_2 in plastic sample tubes having perforated lids (for release of gas generated during the reaction). Samples were analyzed by MALDI-TOF MS.

Control experiment with another CBM33 protein identified by genome mining - CBM33 (EF0362) from Enterococcus faecalis

15 The gene encoding the mature family 33 CBM from *Enterococcus faecalis*, EfCBM33 (Uniprot ID:Q838S1; EF0362; uniprot.org/uniprot/Q838S1), without its native leader peptide, was cloned into the pRSET-B-CBP21 vector in frame with the CBP21 leader peptide, replacing the gene encoding CBP21. The protein was expressed in *E. coli* BL21 DE3 cells, harvested from the periplasmic fraction by cold osmotic shock and purified to homogeneity by chitin affinity chromatography. Thus, this protein was expressed and purified in exactly the same way as CBP21, using CBP21's leader peptide to drive secretion. The fractions containing pure protein (assessed by SDS-PAGE) were pooled and concentrated using an Amicon centrifugal concentrator with 10 kDa cutoff to yield a 20 mg/ml solution. The protein was crystallized by hanging drop vapor diffusion experiments using a crystallization liquor containing 1.0 M Na Tartrate, 0.1 M imidazol pH 8.0 and 0.2 M NaCl. Crystals pyramidal in shape and measuring approximately 0.2 mm in width (see Fig. 15A) were obtained after 48 hours incubation at room temperature. A 0.95 Å dataset has been collected from a single crystal and the structure has been solved by molecular replacement using CBP21 (PDB ID 2BEM) as template. Refinement is complete, but not published. The quality of the data is illustrated by panel B of Fig. 15. Panel C of this Fig. 15 shows a structural superposition of CBP21 and EfCBM33.

25 For use in chitin degradation experiments, eight crystals were harvested from a 2 μL drop using a nylon loop and transferred to a 4 μL drop containing the crystallization liquor from the buffer reservoir. The crystals were mixed around in order to "rinse off" potential contaminants. After the first rinse, the crystals were transferred to a new 4 μL drop containing the crystallization liquor for a second rinsing cycle. Finally, all crystals were transferred to and dissolved in a 4 μL drop containing 20 mM Tris-HCl, pH 8.0. The resulting solution was diluted by adding it to a test tube containing 46 μL 20 mM Tris-HCl, pH 8.0. For reactions with beta-

chitin, 5 μ L of the EfCBM33 solution was mixed with a 95 μ L solution containing 2 mg/ml beta-chitin and 2 mM ascorbic acid in 20 mM Tris-HCl pH 8.0; the reaction mixture was then incubated for 90 minutes at 37°C in a test tube incubator rotating at 1400 rpm. Soluble products in the supernatant of the reaction were analyzed by MALDI-TOF, using the same methods as those used for testing the activity of CBP21. Further, the ability of EfCBM33 to boost degradation of alpha-chitin was probed by conducting an experiment where 2.0 mg/ml alpha-chitin (shrimp shells) was incubated with 0.3 μ M of the chitinase from *Entreococcus feacalis* (protein name (EF0361)) in the presence or absence of 0.3 μ M EfCBM33 and 1.0 mM reductant (R: reduced glutathione) incubated at 37°C with agitation at 900 rpm. A boost of the chitinase activity is clearly observed in the presence of EfCBM33 and reductant.

Determination of CBP21 reaction speed and degree of substrate oxidation

Using the UHPLC method for separating oxidized chitooligosaccharides, pure GlcNAc3GlcNAcA and GlcNAc4GlcNAcA samples were obtained by fractionation of beta-chitin samples treated by CBP21 in the presence of ascorbic acid. Fractions were dried under vacuum (SpeedyVac), and resuspended in 50 μ L MilliQ water. Purity was verified by MALDI-TOF MS. Isolated GlcNAc3GlcNAcA or GlcNAc4GlcNAcA were each incubated for 2 hours at 37°C with 7.0 μ M of a pure recombinant family 19 chitinase (ChiG from *Streptomyces coelicolor* (Hoell *et al.*, 2006, *FEBS J.* 273: 4889)) resulting in production of equimolar amounts of GlcNAc2 and GlcNAcGlcNAcA or GlcNAc2GlcNAcA, respectively. The amount of GlcNAc2 resulting from the hydrolysis was estimated using a predetermined standard curve. Response factors for the GlcNAcA containing oligosaccharides were obtained by determining GlcNAcGlcNAcA/ GlcNAc2 and GlcNAc2GlcNAcA/ GlcNAc2 peak area ratios and found to be 0.71 and 0.81, respectively. A response factor for GlcNAc3GlcNAcA was approximated to be 0.88 by extrapolation of the two experimentally determined response factors. Using the response factors determined, GlcNAcGlcNAcA and GlcNAc2GlcNAcA peaks could be quantified using GlcNAc2 for calibration. In experiments for the simultaneous detection and quantification of GlcNAc2 and oxidized oligomers 1.0 mM of reduced glutathione was used as reductant instead of ascorbic acid because the latter interferes with the chromatographic analysis. Additionally, the reactions contained 0.45 mg/mL beta-chitin, 1.0 μ M CBP21 and 0.5 ChiC μ M in 20 mM Tris-HCl pH 8.0.

The reactions were incubated at 37°C in an Eppendorf Thermo mixer with shaking at 1000 rpm, and sampled at 30, 60, 120 and 300 minutes. All samples were mixed 1:1 with 100% acetonitrile in order to stop the reaction and soluble products were analyzed by UHPLC. Separation of the oxidized oligosaccharides and GlcNAc2 was achieved using a column temperature of 30°C and a flow of 0.4 mL/min, with a gradient starting at 80% ACN (A):20% 15 mM Tris-HCl pH 8.0 (B) for 4.5 minutes, followed by an 11 minute gradient to 63% A: 37% B

which was held for 3.5 minutes. Column reconditioning was achieved by a two minute gradient to initial conditions and subsequent running at initial conditions for 5 minutes. Eluted oligosaccharides were monitored by recording absorption at 205 nm. Chromatograms were recorded, integrated and analysed using the ChemStation rev. B.04.02 chromatography software (Agilent Technologies).

In order to approximate the rate of the CBP21 oxidohydrolytic activity, reactions containing 0.45 mg/mL beta-chitin, 1.0 μ M CBP21 and 1.0 mM reduced glutathione in 20 mM Tris-HCl pH 8.0 were incubated at 37°C and sampled at 10, 15, 30, 45, 60 and 300 minutes. Instead of stopping the reaction with acetonitrile, a cocktail of purified recombinant chitinases containing 28 μ M ChiC (see above), 71 μ M ChiG (Hoell *et al.*, 2006, *supra*), 63 μ M ChiB (Brurberg *et al.*, 1995, *Microbiology* 141: 123, Brurberg *et al.*, 1996, *Microbiology* 142: 1581) and 15 μ M ChiA (Brurberg *et al.*, 1996, *supra*, Brurberg *et al.*, 1994, *FEMS Microbiol. Lett.* 124: 399) was added to the sample (0.1 volume) in order to obtain rapid complete degradation of the chitin. Under these conditions, insoluble chitin completely disappeared within 30 minutes. The quantities of the oxidized products (exclusively GlcNAcGlcNAcA and GlcNAc₂GlcNAcA) were determined using the UHPLC method outlined above.

Analysis and quantization of glucose and cellobiose by HPLC (E7 and CelS2)

Samples containing glucose and cellobiose were analysed by isocratic HPLC run on a Dionex Ultimate 3000 HPLC system set up with a 7.8x100 mm Rezex RFQ-Fast Fruit H+ column (Phenomenex) heated to 80°C. The mobile phase consisted of 5 mM sulfuric acid and the flow rate used was 1.0 ml/min. Eluted glucose and cellobiose were monitored by recording refractive index. Quantification was obtained by running glucose and cellobiose standards. Chromatograms were recorded, integrated and analysed using the Chromeleon 6.8 chromatography software (Dionex).

Analysis of native and oxidized cellooligosaccharides using HPAEC (E7 and CelS2)

Separation of native and oxidized cellooligosaccharides was achieved using a Dionex Bio-LC equipped with a CarboPack PA1, a column temperature of 30°C and a flow of 0.25 ml/min, with starting conditions, *i.e.*, 0.1 M NaOH. A stepwise linear gradient with increasing amounts of sodium acetate was applied, going from 0.1 M NaOH and 0.1 M sodium acetate in 10 minutes, then to 0.1 M NaOH and 0.3 M sodium acetate for 25 minutes then increasing to 0.1 M NaOH and 1.0 M sodium acetate at 30 minutes which was kept for 10 minutes. Column reconditioning was achieved by a one minute gradient to initial conditions and subsequent running at initial conditions for 14 minutes. Eluted oligosaccharides were monitored by PAD detection. Chromatograms were recorded and analysed using Chromeleon 7.0 Peak identification was achieved by a procedure including the following steps: oxidized

cellooligosaccharides were separated using a Dionex Ultimate 3000 UHPLC system carrying a Hypercarb 150x2.1 mm column (Thermo Scientific) running a gradient of water/ 0.1% TFA and acetonitrile/ 0.1% TFA (from 20 to 80% acetonitrile) according to the method developed by Westphal *et al.*, 2010, *Journal of Chromatography A* 1217: 689-695. Eluted peaks were manually fractioned, freeze-dried, re-dissolved in MilliQ water and identified using MALDI-TOF MS. Pure oxidized cellooligosaccharides with known identity were then analyzed using the HPEAC method described above in order to establish the identity of the oxidized cellooligosaccharides generated by CelS2 and E7.

Cellulose degradation experiments (E7 and CelS2)

Assays performed to evaluate the function of E7 and CelS2 were set up using a variety of cellulosic substrates (AVICEL®, filter paper and steam exploded wood chips from poplar), at either pH 5.5 (20 mM sodium acetate buffer), 6.5 (20 mM Bis-Tris buffer) or 8.0 (20 mM Tris buffer) in reaction mixtures containing 1.0 mM MgCl₂. The effect of the presence of an external electro donor was probed by adding 1.0 mM or 0.5 mM reduced glutathione or ascorbic acid to the reaction mixture (see figure legends for details).

Results

We show here that CBP21, a single-domain protein comprising one CBM33 domain, in fact is an enzyme that catalyzes an oxidohydrolytic cleavage of glycosidic bonds in crystalline chitin, thus opening up the inaccessible polysaccharide material for hydrolysis by normal glycoside hydrolases. This enzymatic activity was first discovered when we detected traces of non-native chito-oligosaccharides upon incubation of beta-chitin nano-whiskers with CBP21 (Fig. 2A). The products were identified as chitin oligosaccharides with a 2-(acetylamino)-2-deoxy-D-gluconic acid (GlcNAcA) at the reducing end (Figs. 2B and 3). We then found that addition of reductants dramatically increased the efficiency of the reaction (Figs. 4 and 6), enabling the destructuring of large crystalline beta-chitin particles by CBP21 alone (Fig. 2C), releasing a range of oxidized products (Fig. 5 and below) and boosting chitinase efficiency to much higher levels than previously observed (compare Fig. 1C with Fig. 4).

If CBP21 acted randomly on crystalline surfaces, one would expect generation of longer oligosaccharides, which are difficult to detect due to their low solubility. The majority of soluble products generated by CBP21 in the presence of a reductant had a DP below 10 (Fig. 2A; Fig. 5). To visualize longer products, we exploited a newly cloned chitin deacetylase from *Aspergillus nidulans* (AnCDA) to increase the solubility of longer chitin fragments by deacetylation. This approach indeed revealed the formation of chitin fragments with high DP, either with CBP21 (Fig. 2D) or with an endochitinase (ChiC; Fig. 2E). Both CBP21 and ChiC generated long products, indicative of an "endo-" type of activity.

Two important features stand out. Firstly, when using CBP21 all detected products are oxidized (*i.e.*, they contain a GlcNAcA moiety), confirming the observation that CBP21 catalyzes oxidative hydrolysis of glycosidic bonds. Secondly, whereas the products released by ChiC represent a continuum of lengths, the products released by CBP21 are dominated by even-numbered oligosaccharides (Figs. 2D and 6). This shows that ChiC tends to cleave any glycosidic bond, whereas CBP21 shows a strong preference for cleaving every second glycosidic bond. Keeping in mind the disaccharide periodicity in the substrate (Fig. 1A), this crucial observation implies that ChiC approaches single polymer chains from “any side”, whereas CBP21 must approach the substrate from one fixed side. The latter situation would be obtained if the polysaccharide chain cleaved by CBP21 is part of an intact crystalline structure. Clearly, the endochitinase ChiC and CBP21 have different roles in chitin hydrolysis.

The CBP21 mediated cleavage mechanism was probed in more detail by isotope-labelling. Experiments in H₂¹⁸O showed that one of the oxygen atoms introduced at the oxidized new chain end comes from water (Fig. 7A). The only plausible source for the second oxygen was molecular oxygen and this was confirmed by experiments performed in ¹⁸O₂ saturating conditions (Figs. 7B and C). Removal of dissolved molecular oxygen or in the reaction solution inhibited CBP21 activity (Figs. 8A, B and C), confirming the requirement for molecular oxygen for catalysis. The strong inhibition by cyanide, a common mimic of molecular oxygen, supports the crucial role of the oxidative step (Figs. 8C and D). Thus, the reaction catalyzed by CBP21 comprises a hydrolytic step and an oxidation step, as summarized in Fig. 7D. Enzymatic oxidohydrolysis of polysaccharides has so far not been described. CBP21 is referred to herein as a “chitin oxidohydrolase”. Likewise, GH61 proteins are referred to as “cellulose oxidohydrolases”.

CBP21 catalysis was found to be dependent on the presence of a divalent cation (Fig. 9), which may bind to the conserved histidine motif (Fig. 1F). Interestingly, the activity of GH61 proteins also depends on divalent cations (Harris *et al.*, 2010, *Biochemistry* 49: 3305). Structural studies of both CBP21 (Vaaje-Kolstad *et al.*, 2005, *J. Biol. Chem.* 280: 11313) and GH61 proteins (Harris *et al.*, 2010, *Biochemistry* 49: 3305; Karkehabadi *et al.*, 2008, *J. Mol. Biol.* 383: 144) show considerable structural plasticity in the metal-binding site, explaining why the metal binding site is promiscuous and why the need for divalent cations is rather unspecific (as shown in Fig. 9 and in Harris *et al.*, 2010, *Biochemistry* 49: 3305). Mutation of the second histidine (His114 in CBP21 and His89 in GH61E from *Thielavia terrestris*) in the metal binding motif knocked out activity of both proteins (Fig. 10 and Harris *et al.*, 2010, *Biochemistry* 49, 3305, respectively). It is conceivable that the metal ion binding aids directly in the binding/stabilization/activation of the reactive oxygen species and/or the hydrolytic water during catalysis. Alternatively, the metal ion may be essential for stabilizing the structure of the active site region of the protein without interacting directly with the oxygen or the water.

Reductants boosted the oxidohydrolytic activity of CBP21 to extreme levels (Figs. 2C, 4, 6, 11), which very well may be beyond what is ever achieved in nature. The reductants are likely to function as electron donor in the oxidative enzymatic process, but the exact flow of electrons from the reductant to molecular oxygen is not yet clear. One option is that electron donation takes place via the generation of reactive oxygen species such as O_2^- . Another option is that electrons are somehow channelled to a complex involving CBP21, the substrate and O_2 , implying that reactive oxygen species such as O_2^- only emerge on the substrate and will not or hardly be present in solution. Most interestingly, and of biotechnological importance, our data show that activity of these new enzymes can be boosted considerably by simply adjusting the reaction conditions.

We conducted numerous control experiments that all confirmed the conclusion that formation of oxidized products only occurs in the presence of CBP21 and crystalline substrates. The presence of reductants alone did not yield oxidized products (Fig. 12) and did not potentiate or inhibit chitinase action (Figs. 4 and 10B). When incubated under optimal conditions with hexameric *N*-acetylglucosamine, neither degradation products nor oxidized oligosaccharides were observed (Fig. 13). We also studied the possibility that CBP21 would work without directly interacting with the cleaved bond itself, since one could envisage some sort of CBP21-induced "destructuring" mechanism making the substrate more accessible for the action of reactive oxygen species generated in a CBP21-independent manner, e.g., by Fenton chemistry. We could however not detect any soluble products upon subjecting beta-chitin to Fenton chemistry (Fig. 14). All these experiments confirm that CBP21 actively participates in the oxidohydrolytic cleavage reaction.

As shown in Fig. 8, CBP21 activity is strongly inhibited by cyanide, a known O_2 mimic, but not by azide, a known inhibitor of haem proteins. Several reductants capable to function as electron donors boosted the activity of CBP21 (Figs. 2C and 11). The experimental data indicate that the oxidation step catalyzed by CBP21 is co-factor independent and depends on an external electron donor. Co-factor independent oxygenases have been described before, but these enzymes are normally thought to use conjugated carbanions in the substrates as electron donors (Fetzner and Steiner, 2010, *Appl. Microbiol. Biotechnol.* 86:791), a mechanism that is not likely in the case of a polysaccharide substrate. If the oxidation step was to happen first, this would imply that CBP21 catalyzes co-factor independent oxygenation of a saturated carbon, which is unprecedented and perhaps not very likely. On the other hand, such a mechanism could yield an intermediate product, for example an ester bond, that may be more prone to hydrolysis than the original glycosidic bond. Alternatively, the hydrolytic step could occur first, which would imply that CBP21 is capable of hydrolyzing glycosidic bonds in a crystalline environment using a hitherto unknown mechanism. Such a hydrolytic step would require some degree of substrate distortion (Davies *et al.*, 2003, Mapping the conformational itinerary of beta-

glycosidases by X-ray crystallography. *Biochem. Soc. Trans.* 31: 523 and Vocadlo and Davies, 2008, Mechanistic insights into glycosidase chemistry. *Curr. Opin. Chem. Biol.* 12: 539), which seems challenging in a crystalline packing. However, in favour of this mechanism, the subsequent oxidation of the resulting sugar aldehyde ("reducing end") is more straightforward than oxidation of a saturated carbon.

CBP21 introduces chain breaks in what probably are the most inaccessible and rigid parts of crystalline polysaccharides substrates and its mode of action differs fundamentally from the mode of action of glycoside hydrolases. The key difference is that the glycoside hydrolases are designed to host a single "soluble" polysaccharide chain in their catalytic clefts or pockets and that their affinity and proximity to the crystalline substrate tends to be mediated by non-hydrolytic binding domains. In contrast, CBP21 and GH61 enzymes have flat surfaces (Figs. 1D and E) that bind to the flat, solid, well-ordered surfaces of crystalline material and catalyze chain breaks by an oxidohydrolytic mechanism. The chain break will result in disruption of crystalline packing and increased substrate accessibility, an effect that may be augmented by the modification of one of the new chain ends. At the cleavage point one of the new ends is a normal non-reducing end (indicated by R-OH in Fig. 7D). The other new end would have been a new reducing end if the cleavage had been performed by a normal glycoside hydrolase. However, in this case the product is different and the last sugar is oxidized to become 2-(acetylamino)-2-deoxy-D-gluconic acid (Figs. 2B and 7D; GlcNAcA). This new "acidic chain end" will interfere with normal crystal packing because it will not have the normal chair conformation of the sugar ring and because it carries a charge.

The enzyme activity demonstrated in this study is difficult to discover, because one depends on detecting products with low solubility and potentially a high tendency to remain attached to the crystalline material. In this sense, working with chitin is easier than working with cellulose because product solubilities are slightly higher and because crystalline packing is less compact (Eijsink *et al.*, 2008, *Trends Biotechnol.* 26: 228). The experiment with the chitin deacetylase (Figs. 2D and E) provided essential insights, but this approach can simply not be used for cellulose. Looking for chain-breaking activities of GH61 proteins with commonly used reducing end assays obviously will not work due to the oxidative mechanism.

Using methods for quantification of oxidized products, we were able to estimate the speed and degree of oxidation under various conditions (Fig. 16). When CBP21 acts alone on beta-chitin under optimal conditions the oxidation rate is in the order of 1 per minute and the maximum extent of oxidation is about 7.6% of the sugars. Simultaneous degradation of beta-chitin with CBP21 and ChiC under optimal conditions led to oxidation of approximately 4.9% of the sugars. It must be noted that in nature enzymes such as CBP21 normally act simultaneously with at least one, and in the case of *S. marcescens*, three chitinases (Suzuki *et al.*, 1998, *Biosci. Biotechnol. Biochem.* 62: 128).

The experiments conducted on CBM33 family protein EfCBM33 revealed that this protein is functionally similar to CBP21. The results of the MS analysis, depicted in panel D of Fig. 15 resemble those obtained with CBP21, clearly showing the formation of oxidized products and the oxidohydrolytic properties of this protein. It is interesting to note that EfCBM33 not only works on beta-chitin (Fig. 15D) but also on alpha-chitin (Fig. 15E).

The efficacy of CBM33 proteins on cellulose as the substrate were examined. Mature wild type E7, CelS2 and a cellulase classified as a belonging to GH family 7 and originating from *Trichoderma reesei* called Cel7A (Harjunpaa *et al.*, 1999, *FEBS Letters* 443: 149-153) were purified to ~95% purity using the cloning, expression and purification strategies described in the Material & Methods section. Finally, a cellulase mixture called CELLUCLAST™, which is an easily available and well known commercial product from Novozymes, was used.

To determine whether CelS2 and/or E7 had the ability to release soluble sugars from crystalline cellulose, CelS2 or E7 was incubated with AVICEL® in the presence or absence of an external electron donor (reduced glutathione or ascorbic acid). Putative reaction products were analyzed using MALDI-TOF MS for qualitative detection of product types and HPAEC (high pressure anion exchange chromatography), a chromatographic method enabling product identification and, in principle, quantification. Indeed, soluble oligomeric products were observed with both proteins (only shown for CelS2; Figs. 17-19) and with both detection methods. Native cellooligosaccharides were observed in both conditions with and without external electron donor, but in substantial larger amounts in the former condition. Oxidized cellooligosaccharides were only observed in experiments where an external electron donor was present.

More specifically, MALDI-TOF MS analysis revealed the presence of cellooligosaccharides with an oxidized reducing end (*i.e.*, cellooligosaccharides with the reducing glucose moiety replaced with a gluconic acid moiety; Figs. 17 and 18). The degree of polymerization (DP) of the released soluble oxidized oligosaccharides visible in Fig. 17 ranged from DP4 to DP7 and the product signals showed alternating intensity/ quantity depending on the cellooligosaccharide being even or odd numbered in terms of DP (even numbered cellooligosaccharides dominated). HPAEC analysis of the same samples reflected the results provided by the MALDI-TOF MS analysis, showing the presence of peaks with alternating intensities representing oxidized cellooligosaccharides (Fig. 19).

The dominance of even numbered oligosaccharides is a logical consequence of the fact that CBM33 enzymes attack the polysaccharide chains in their crystalline environment. As the repeating unit of cellulose and chitin is a dimer, only every second sugar/ glycosidic bond on the crystalline polysaccharide chain is prone for cleavage by the CBM33 enzyme, meaning that released products would tend to have an even-numbered DP. (NB. acid hydrolysis of either

crystalline cellulose or chitin gives an even distribution of even and odd numbered oligosaccharides).

Fig. 19 shows that incubation with CelS2 in the presence of a reducing agent, leads to cleavage of the cellulose chains in the crystal in an oxidohydrolytic manner, releasing soluble oxidized cellooligosaccharides (light grey, upper line). This also leads to additional release of non-oxidized cellooligosaccharides, which is likely to result in part from the disruption the AVICEL® crystal surface that facilitates the release of shorter oligomers that are captured in the crystal. Additionally, chain cleavage by CelS2 near the reducing end of a cellulose chain will give rise to such products.

Reactions containing crystalline cellulose (filter paper) and cellulases (either a cellulase mixture called CELLUCLAST™, or a single component cellulase, Cel7A) were monitored for cellobiose and glucose release in the presence and absence of CelS2/E7 and/or an external electron donor.

When CELLUCLAST™ was incubated with filter paper in the presence of CelS2 or E7, the glucose yield was indeed higher than when the filter paper was incubated with only CELLUCLAST™ (Fig. 20; ~3.0 fold increase for CelS2 and E7 after 24 hours incubation and ~9 fold increase for CelS2 and ~3.5 fold increase for E7 after 120 hours incubation). When both CelS2 and an external electron donor were added, the glucose yields were even higher (Fig. 20; ~5 fold increase for CelS2 and 3.5-fold increase for E7 after 24 hours incubation and ~11 fold increase for CelS2 and ~4.5 fold increase for E7 after 120 hours incubation), indicating that the presence of an external electron donor indeed is of importance for the boosting effect of CelS2 and E7.

Since the CELLUCLAST™ product contains a complex mixture of hydrolytic activities that may complicate the interpretation of the results, a monocomponent cellulase (Cel7A) was purified from CELLUCLAST™ (Novozymes) and used to probe the boosting efficiency of CelS2. Effects similar to those observed when combining CELLUCLAST™ and CelS2 in the presence and absence of an external electron donor were also observed when incubating Cel7A with CelS2 (Fig. 21). In the absence of an external electron donor, CelS2 improved Cel7A activity (in terms of cellobiose yield) 5.5- and 7.3-fold after 18 and 40 hours incubation, respectively. In the presence of an external electron donor, CelS2 improved Cel7A activity 6.7- and 9.8-fold after 18 and 40 hours incubation, respectively.

Figs. 20 and 21 show that in addition to boosting cellulose activity the presence of CelS2 in both cases also aids in maintaining the cellulase activity over time. Whereas the activity of the cellulases incubated in the presence or absence of an external electron donor comes to an almost complete stop within the first 18 – 24 hours, cellulase activity continues in the samples incubated with CelS2. This effect may be a partial explanation for the synergistic

effects that CBM33 proteins have on the degradation of chitin or cellulose by hydrolytic enzymes (chitinase and cellulases, respectively).

The oxidized products generated by CelS2 in the presence of an external electron donor exists in an equilibrium of two forms, the glucono delta-lactone which gains in population at mildly acidic pH and the gluconic acid form that dominates at mildly alkaline pH. At mildly alkaline pH (e.g., pH 8.0) it is likely that the charge developing on the cellulose crystal surface due to CelS2 activity may aid the distortion and disruption/solubilization of the cellulose crystal and thus increase the accessibility of the substrate for the cellulases. However, it is conceivable that oxidized oligosaccharides and or oxidized chain ends in cellulose crystals may also inhibit certain cellulases (e.g., exo-acting enzymes), making it likely that the degree of CelS2/ CBM33 activity should be carefully adjusted for optimal boosting efficiency.

When probing the cellulose boosting properties of CelS2 at various pHs using monocomponent Cel7A as the cellulose hydrolytic component, pH 5.5 came out as the optimal pH for activity. Less activity was seen at pH 6.5 and no activity could be detected at pH 8.0 (data not shown). The most obvious reason for the decrease in cellulose hydrolysis at increasing pH is the pH stability and efficiency of Cel7A. The cellulase is close to inactive at pH 8.0, which is a common trait for fungal cellulases in general (Garg & Neelakantan, 1981, *Biotechnology and Bioengineering* 23: 1653-1659 and Wood, 1985, *Biochemical Society Transactions* 13: 407-410.). CelS2 is a bacterial enzyme that originates from *Streptomyces* species that are known to grow optimally on cellulosic substrates in approximately neutral pH conditions (Kontro et al., 2005, *Letters in Applied Microbiology* 41: 32-38.). The data presented in Fig. 19 show that CelS2 is active at pH 8.0.

Because of the pH-dependent properties of the cellulases, the synergy experiments reported here were performed at slightly acidic pH, which may be suboptimal for the particular CBM33 used (primarily CelS2 from *Streptomyces*). It is thus possible that the observed effects of CelS2 are smaller than they could be under conditions optimized for CelS2 activity.

More generally, an expert in the field will know that natural enzymes vary in terms of their optimum pH and temperatures for activity. The expert will know that this will also apply to hydrolytic enzymes, such as cellulases and chitinases and to oxidohydrolytic enzymes, such as CBP21 and CelS2. It is obvious that for obtaining optimal overall reaction efficiency and/or for maximizing the boosting effect of a CBM33, one needs to take into account the pH optima and temperature optima of both the CBM33 and the hydrolytic enzymes. One may obtain enzymes with varying pH and temperature optima by selecting appropriate enzymes from nature or by modification of properties such as pH optimum of natural enzymes using protein engineering type of technologies.

It is conceivable that pH affects the performance of a CBM33 + hydrolase(s) type of enzymatic system because pH affects the equilibrium between lactone and the acid form of the

oxidized products, which again may affect the efficiency of the hydrolytic enzymes. The pH may also affect the reductant.

These experiments show that some family 33 CBMs, like CelS2 and E7, are active on crystalline cellulose, boost cellulase activity in both the absence and presence of an external electron donor, but show substantially higher activity/boosting effect with the electron donor present. These observations, as well as the results previously obtained for CBP21 acting on chitin, indicate that the oxidation of one of the newly generated chain ends is important for the function of these CBM33 enzymes. That the oxidation indeed also is part of the mechanism for CBM33s that act on cellulose is further supported by data showing that cyanide, a well known oxygen mimic, inhibits the generation of oxidized cellooligosaccharides when CelS2 is incubated with AVICEL® in the presence of an external electron donor (Fig. 22).

Additionally, a variant of CelS2 was generated containing a mutation of a putatively essential conserved residue, histidine 144, to alanine. This histidine is one of the strongly conserved residues in the metal binding motif characteristic for family 33 CBMs and corresponds to His114 in CBP21 (Figs. 1D and F; Fig. 10; Vaaje-Kolstad *et al.*, 2010, *Science* 330: 219-222). Fig. 23 shows that CelS2-H144A was unable to generate oxidized cellooligosaccharides in the presence of an external electron donor (Fig. 23).

It is important to emphasize once more that experiments have been performed at only one pH, which is likely to be not optimal for one of the two enzyme components (CBM33 or hydrolytic enzyme), as discussed above. The cellulases used work optimally at the acidic range of the pH scale, while the cellulose oxidating CBM33s are more active at the basic range of the pH scale. An expert in the field will understand that one may get different and better results when adapting the pH of the reaction and/or by selecting enzyme variants that are better suited for the pH used here and to work together at this particular pH. The full potential of the oxidative boosting effect may thus not be seen in these experiments.

Finally, other cellulosic variants (filter paper and steam exploded sawdust from poplar) were also probed as substrates for CelS2. Soluble oxidized cellooligosaccharides were not observed by MALDI-TOF MS analysis (results not shown). This is most likely due to the high DP of the cellulose in these substrates; cleavages on the crystalline surfaces will not easily lead to release of soluble (=short) cellooligosaccharides when the overall DP is very high. AVICEL® is special in that it is a form of microcrystalline cellulose that has a very low DP (~60-100; Wallis *et al.*, 1992, *Carbohydrate Polymers* 17: 103-110 and Mormann and U, 2002, *Carbohydrate Polymers* 50: 349-353) compared to that of the other substrates tested.

However, when the cellulase boosting effect of the CBM33s, in this case CelS2, was probed with a more "natural" substrate (represented by steam exploded sawdust from poplar), the effect of CelS2 was indeed present as shown by a ~2-fold increase; Fig. 24). The data shown for the boosted CELLUCLAST™ effect on steam exploded sawdust from poplar, which

represents a substrate more like what is used in industrial biomass degrading applications, also show that CelS2 activity does not dependent on the substrate being of high purity. Clearly, CelS2 also acts on cellulose present in a more natural environment (*i.e.*, embedded in a plant/wood cell wall matrix).

5 Apart from showing a completely novel enzyme activity for modifying solid polysaccharide surfaces, our results point into new directions for enzymatic conversion of recalcitrant polysaccharides. Clearly, CBM33 family proteins (such as CBP21, EfCBM33 (Fig. 15), E7 and CelS2 (Figs. 17-24)) and GH61 family proteins (such as the GH61 proteins found in *T. terrestris*, *Phanerochaete chrysosporium* or *Hypocrea jecorina*) can dramatically increase the efficiency of hydrolytic enzyme mixtures for chitin and cellulose and a first glimpse of the potential of GH61 proteins for cellulose conversion has indeed been presented very recently (Harris *et al.*, 2010, *Biochemistry* 49: 3305). The present invention shows how the beneficial effects of CBM33 and GH61 family proteins may be boosted by adjusting the reaction conditions, *i.e.*, the combined presence of an appropriate metal ion and agents that are reducing and/or generate reactive oxygen species. Clearly, the dependency of these oxidohydrolases on the presence of molecular oxygen and reductants acting as external electron donors provides guidelines for process design.

20 **Example 2: Effects of CelS2 on cellulose degradation by cellulases, in the presence of reduced glutathione.**

 Figs. 20 and 21 show that one of the effects of CelS2 and E7 is to prolong cellulase activity. While reactions with CELLUCLAST™ alone or Cel7A alone hardly produce any additional soluble glucose after the first time point (24 hours in Fig. 20, 18 hours in Fig. 21), release of soluble glucose continues in most cases where a CBM33 protein is present and this effect is increased in the presence of a reductant. To further analyze this, we conducted a time course experiment to examine the effect of CelS2 on CELLUCLAST™ activity over time.

Materials and Methods

30 As in Example 1. Further experimental details, including minor deviations from the standard protocols described in Example 1 are provided in the figure legends, where necessary, for this Example and the Examples which follow.

Results

35 The results (Fig. 25) clearly show that the production of soluble glucose by CELLUCLAST™ levels off, while production of soluble glucose continues if CelS2 is present. This effect is increased if a reducing agent is also present. It is conceivable that CelS2 somehow prevents the cellulases from becoming irreversibly and non-productively bound to the

substrate by modifying the surface of the cellulosic substrate, a phenomenon that is generally considered to reduce cellulase efficiency (Jalak and Väljamäe, 2010, *Biotechnology and Bioengineering* 106:871-883).

5 **Example 3: Effects of CelS2 and Cel7A on cellulose degradation by cellulases in the presence of reduced glutathione.**

To examine the functionality of CelS2, including its effect on prolonging cellulase activity, in more detail, the effect of CelS2 on the efficiency of a monocomponent cellulase was studied. The monocomponent enzyme was HjCel7A, obtained by purification from a mixture of
10 cellulases from *Hypocrea jecorina* (*Trichoderma reesei*).

Materials and Methods

As in Example 1.

15 **Results**

The results, depicted in Fig. 26 clearly show that CelS2 boosts the activity of the cellulase, Cel7A, and that this boosting effect is larger in the presence of a reductant. The results also show that the presence of CelS2 prolongs the activity of Cel7A.

20 **Example 4: Effects of different reductants on CBM33 efficiency – studies with recombinantly produced N-terminal CBM33 domain of CelS2.**

To check for the functionality of different reductants the recombinantly produced N-terminal CBM33 domain of CelS2 was incubated with 0.8 mM of reduced glutathione, gallic acid or ascorbic acid and the release of oxidized oligosaccharides from AVICEL® was monitored,
25

Materials and Methods

As in Example 1.

Results

30 Fig. 27 shows that all of the tested reductants boosted the activity of the CBM33 protein, although to a different extent. The choice of optimal reductant will, among other things, depend on pH, the substrate and the type of GH61/CBM33 protein.

Example 5: Effects of reductant on the boosting effect of E7 on cellulase activity.

35 According to the Pfam bioinformatic analysis (pfam.org), E7 is a single domain CBM33 protein (Uniprot ID:Q47QG3; E7) whereas CelS2 (Uniprot ID: Q9RJY2) comprises a CBM33 domain with a CBM2 (Carbohydrate-Binding Module 2; see cazy.org) attached on the C-

terminal side of the protein. It is important to note that single domain proteins such as E7 are active by themselves, as illustrated in Fig. 20. To further illustrate this, Fig. 28 was prepared which shows the degradation of cellulose by CELLUCLAST™ or a combination of CELLUCLAST™ and E7 in the presence or absence of reductant.

5

Materials and Methods

As in Example 1.

Results

10

It is clearly seen in Fig. 28 that E7 acts in synergy with CELLUCLAST™, and that the presence of reductants has a boosting effect on E7. Note also that Fig. 28 shows that one of the effects of E7 is that it prolongs cellulase activity over time, similar to what was observed for CelS2, as described above. Fig. 29 shows a MALDI spectrum of the oxidized products produced by E7 upon incubation with AVICEL® and a reductant.

15

Example 6: Effects of additional CBMs (the N-terminal CBM33 domain of CelS2 and the C-terminal CBM2 domain of CelS2).

As noted in Example 6, E7 is a single domain CBM33 protein, whereas CelS2 comprises a CBM33 domain with a CBM2 attached on the C-terminal side of the protein. It is important to note that single domain proteins such as E7 are active by themselves, as illustrated in Fig. 28. To further illustrate this, Fig. 30 was prepared which shows the products released from cellulose by full length CelS2, the recombinantly expressed N-terminal CBM33 domain of CelS2, and the recombinantly expressed C-terminal CBM2 domain of CelS2.

25

Materials and Methods

As in Example 1. The sequence of the recombinantly produced N-terminal CBM33 domain is:

HGVAMMPGSRTYLCQLDAKTGTGALDPTNPACQAALDQSGATALYNWFAVLDSNAGGRGAG
YVPDGTLC SAGDRSPYDFSAYNAARSDWPRTHLTSGATIPVEYSNWAHPGDFRVYLTKPG
30 WSPTSELGWDDLELIQTVTNPPQQGSPGTDGGHYYWDLALPSGRSGDALIFMQWVRSDSQE
NFFSCSDVVFDDGG (SEQ ID NO: 22).

Results

As expected the CBM2 domain alone did not show any activity on cellulose (Fig. 30). However, the CBM33 domain shows activity on AVICEL® without its CBM2, although lower than the activity of the whole protein.

35

These data clearly show that CBM33 domains alone can act synergistically with cellulases and that the activity of these single domains is stimulated by the presence of reductants (see also Fig. 27). The data also show that the presence of additional CBM domains, such as the CBM2 in CelS2, may be beneficial for CBM33 activity. By analogy to what is known in the scientific literature about the effects of adding various types of CBMs to various types of carbohydrate-active enzymes, it will be understood that the activity and efficiencies of naturally occurring CBM33 and GH61 proteins may be manipulated by removing, adding or changing additional CBMs that are fused to the CBM33 or GH61 domain.

Example 7: Metal activation of CelS2.

To identify which is the preferred metal for CelS2, the activity of the N-terminal CBM33 domain of CelS2 was inhibited by EDTA and different metals were tested to reactivate the protein.

Materials and Methods

As in Example 1.

Results

Fig. 31 clearly shows that under the conditions used in this experiment, including very low metal concentrations (compared to, e.g., the concentrations used in Fig. 9) only copper of the tested metals was able to reactivate CelS2 (10 μ M metal concentration in the presence of 20 μ M EDTA). Thus, copper ions may be the preferred metal ion for CelS2. It should be noted though that at slightly higher (but still low) concentrations, many other metal ions will also work, at least for practical purposes (Vaaje-Kolstad *et al.*, 2010, *supra*; Harris *et al.*, 2010, *supra*; Fig. 9).

Exactly which metal ion is employed by CBM33s and GH61s remains somewhat uncertain, but for practical purposes, several metal ions will work at low (1 mM range) concentrations. If CBM33s and GH61 prefer copper, the fact that other bivalent metal ions have been observed to activate both CBM33 and GH61 enzymes (e.g., Fig. 9 and Harris *et al.*, 2010, *supra*) may be due to the following: Cu^{2+} ions naturally present in very low amounts in the reaction mixtures (e.g., as part of the substrate) may be inaccessible to the enzyme because they are bound to, e.g., the substrate. By adding other bivalent metal ions that tend to bind to the same "binding sites", bound copper ions may be released and thus become available for the enzymes (they are "outcompeted" from the binding sites by the added bivalent metals). At concentrations in the 1 mM range, that work well for other metals, Cu^{2+} inhibits CBM33 action which may be due to unspecific binding (not shown).

Example 9: Sequence and activity comparisons.

GH61 enzymes that are known to be active on cellulose (TtGH61E, Harris *et al.*, 2010, *supra*) or that are likely to be active on cellulose by analogy, such as the two GH61 proteins encoded on the genome of *Hypocrea jecorina* (or *Trichoderma reesei*; HjGH61A & HjGH61B) share several conserved residues in addition to the two histidines making up the metal binding site (Fig. 32). Interestingly, these same residues are conserved in cellulose-active E7, whereas several of them are absent in the chitin-active CBP21 (Fig. 33).

Example 10: Cleavage of cellulose by GH61 proteins in the presence of reductants.

GH61 proteins TtGH61E (SEQ ID NO: 1) and TaGH61A (SEQ ID NO: 2) were incubated with cellulose and ascorbic acid to demonstrate that these proteins can cleave cellulose and yield oxidized products in the presence of ascorbic acid. The two proteins were cloned and produced as described in Harris *et al.*, 2010, *supra*. Fig. 34 clearly shows that TtGH61E cuts cellulose producing oxidized and native celluoligosaccharides in a similar way as CBM33 proteins. While the product pattern is similar to that obtained with CBM33s, it is not identical. The patterns typically obtained with CBM33s acting on cellulose in the presence of reductants (Figs. 19, 22, 27, 30, and 31) show different peridiodicities (*i.e.*, relative abundances of the various products, both oxidized and native oligosaccharides). This may indicate that TtGH61E acts on the substrate in a slightly different way, *e.g.*, by attacking another face of the crystal. Clearly, however, the overall outcome of the reaction of TtGH61E in the presence of the reductant is similar to the outcome of reactions of CBM33s on cellulose in the presence of reductant. Fig. 34 also shows that TaGH61A is much less active than TtGH61E, at least on this substrate, the production of oxidized sugars being very low. Notably, this enzyme produces relatively more native cello-oligosaccharides, *i.e.*, a product pattern that is clearly different from the product patterns obtained with TtGH61E and the CBM33s, which again may indicate slight variations in substrate binding specificity.

Example 11: Effect of reductants on Cellic™ CTec2, a cellulase preparation containing GH61.

Since the commercially available cellulase preparation Cellic™ CTec2 contains GH61 proteins, the effect of ascorbic acid on the efficiency of this enzyme preparation was tested. In this experiment the effect of ascorbic acid on glucose release from cellulose by Cellic™ CTec2 was investigated. Fig. 35 shows that the presence of ascorbic acid increased the release of glucose from both Filter Paper and AVICEL® by about 30%, demonstrating the beneficial effects of ascorbic acid.

Claims

What is claimed is:

- 5 1. A method of degrading or hydrolyzing a polysaccharide comprising contacting said polysaccharide with one or more oxidohydrolytic enzymes, wherein said degradation or hydrolysis is carried out in the presence of at least one reducing agent and at least one divalent metal ion.
- 10 2. A method as claimed in claim 1, wherein said polysaccharide is chitin or cellulose.
3. A method as claimed in claim 1 or 2, wherein said oxidohydrolytic enzyme is a CBM33 family protein (preferably CBP21) or a GH61 family protein.
- 15 4. A method as claimed in any one of claims 1 to 3, wherein two or more oxidohydrolytic enzymes are used in said method.
5. A method as claimed in claim 4, wherein one of said oxidohydrolytic enzymes is a CBM33 family protein and another of said oxidohydrolytic enzymes is a GH61 family protein.
- 20 6. A method as claimed in any one of claims 1 to 5, wherein said oxidohydrolytic enzyme is a polypeptide which comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 1 to 16 or a sequence with at least 30% sequence identity thereto or a biologically active fragment thereof comprising at least 100 amino of said sequence.
- 25 7. A method as claimed in any one of claims 1 to 6, wherein said polysaccharide is cellulose.
8. A method as claimed in claim 7, wherein said oxidohydrolytic enzyme is a GH61 protein, E7, CelS2, Cfla_0175, Cfla_0172, Cfla_0316, Cfla_0490, CJA_2191, CJA_3139 or SCO1734.
- 30 9. A method as claimed in claim 7 or 8, wherein said oxidohydrolytic enzyme is a polypeptide which comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 1 to 3 or 6 to 16 or a sequence with at least 30% sequence identity thereto or a biologically active fragment thereof comprising at least 100 amino acids of said sequence.
- 35

10. A method as claimed in any one of claims 1 to 9, wherein said reducing agent is ascorbic acid, reduced glutathione, Fe(II)SO_4 , LiAlH_4 , NaBH_4 or lignin or a fragment thereof.

11. A method as claimed in any one of claims 1 to 10, wherein said divalent metal ion is Ca^{2+} , Co^{2+} , Mg^{2+} , Mn^{2+} , Ni^{2+} or Zn^{2+} .

12. A method as claimed in any one of claims 1 to 10, wherein said divalent metal ion is Cu^{2+} .

13. A method as claimed in any one of claims 1 to 12, further comprising:

(a) contacting said polysaccharide (or the degradation or hydrolysis product thereof) with one or more saccharolytic enzymes selected from a cellulose hydrolase or chitin hydrolase, and

(b) optionally contacting said polysaccharide (or the degradation or hydrolysis product thereof) with one or more beta-glucosidases, wherein preferably said enzymes are contacted with said polysaccharide simultaneously with said oxidohydrolytic enzyme.

14. A method as claimed in claim 13, wherein said saccharolytic enzyme is chitinase or cellulase.

15. A method of producing soluble saccharides, wherein said method comprises degrading or hydrolyzing a polysaccharide by a method as defined in any one of claims 1 to 14 wherein said degradation or hydrolysis releases said soluble saccharides and optionally isolating said soluble saccharides.

16. A method as claimed in claim 15, wherein said soluble saccharides are chitobiose and/or N-acetylglucosamine and/or oligosaccharides thereof or cellobiose and/or glucose and/or oligosaccharides thereof.

17. A method of producing an organic substance, comprising the steps of:

(i) degrading or hydrolyzing a polysaccharide by a method as claimed in any one of claims 1 to 16 to produce a solution comprising soluble saccharides;

(ii) fermenting said soluble saccharides, to produce said organic substance as the fermentation product; and optionally

(iii) recovering said organic substance.

18. A method as claimed in claim 17, wherein said organic substance is an alcohol.

19. A method as claimed in claim 18, wherein said alcohol is ethanol.

20. A process for producing a fermentation product, comprising:

(a) degrading or hydrolyzing a cellulosic material with an enzyme composition which comprises one or more enzymes selected from the group consisting of a cellulase, a GH61 polypeptide having cellulolytic enhancing activity, a hemicellulase, an esterase, an expansin, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin, wherein said degradation or hydrolysis is carried out in the presence of at least one reducing agent and at least one divalent metal ion;

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

(c) recovering the fermentation product from the fermentation.

21. The process as claimed in claim 20, wherein the cellulosic material is pretreated.

22. The process as claimed in claim 20 or 21, wherein the cellulase is one or more enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

23. The process as claimed in any one of claims 20 to 22, wherein the hemicellulase is one or more enzymes selected from the group consisting of a xylanase, an acetylxytan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase.

24. The process as claimed in any one of claims 20 to 23, wherein steps (a) and (b) are performed simultaneously in a simultaneous saccharification and fermentation.

25. The process as claimed in any one of claims 20 to 24, 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.

26. A process for producing a fermentation product, comprising:

(a) saccharifying a cellulosic material with an enzyme composition comprising an endoglucanase, a cellobiohydrolase, a beta-glucosidase, a GH61 polypeptide having cellulolytic enhancing activity, and a CBM33;

(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.

27. The process as claimed in claim 26, wherein the cellulosic material is pretreated.

28. The process as claimed in claim 26 or 27, wherein the enzyme composition further comprises one or more enzymes selected from the group consisting of an esterase, an expansin, a hemicellulase, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.

29. The process as claimed in claim 28, wherein the hemicellulase is one or more enzymes selected from the group consisting of an acetylxylan esterase, an arabinofuranosidase, a feruloyl esterase, a glucuronidase, a mannanase, a xylanase, and a xylosidase.

30. The process as claimed in any one of claims 26-29, wherein steps (a) and (b) are performed simultaneously in a simultaneous saccharification and fermentation.

31. The process as claimed in any of claims 26-30, 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.

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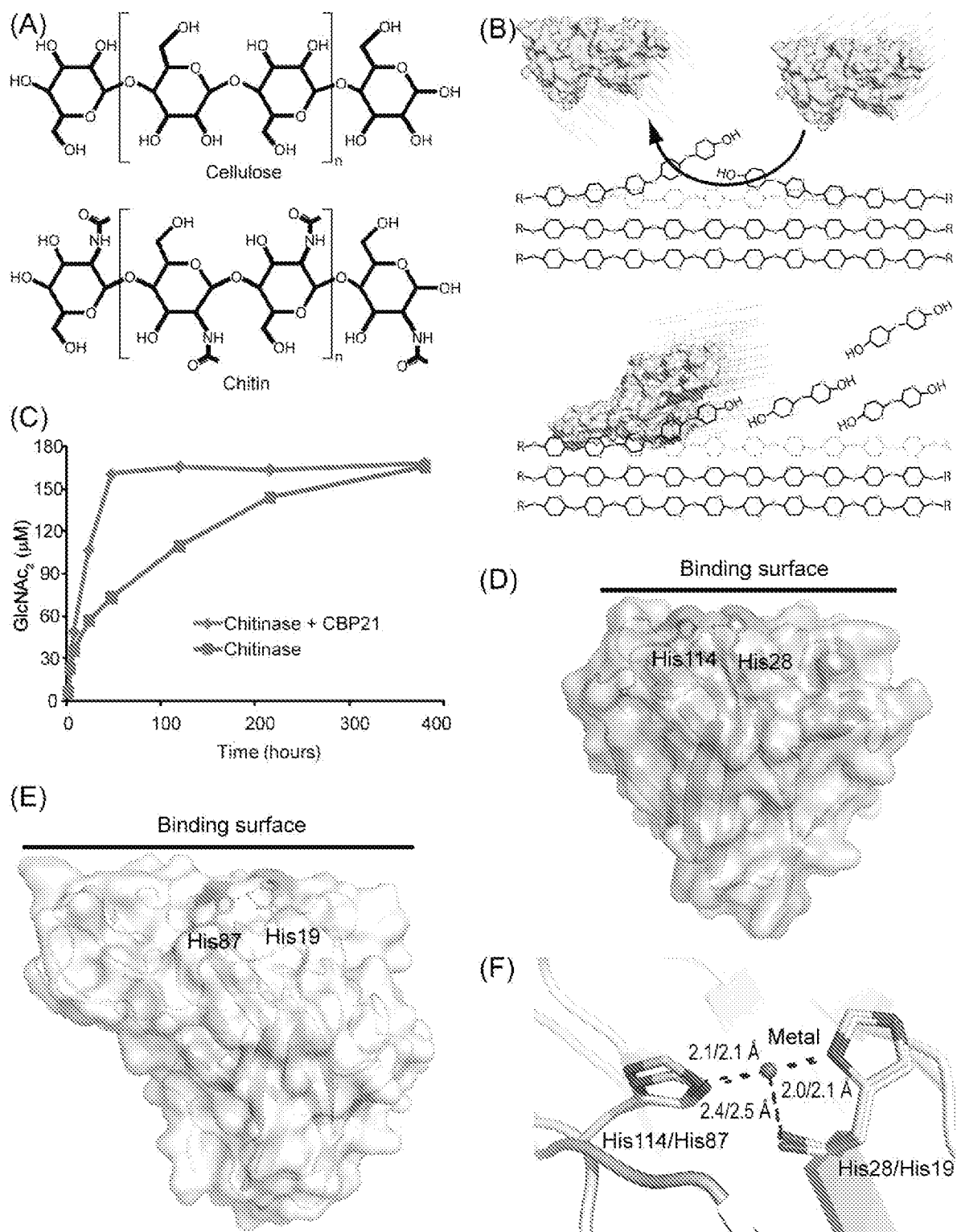


Figure 1

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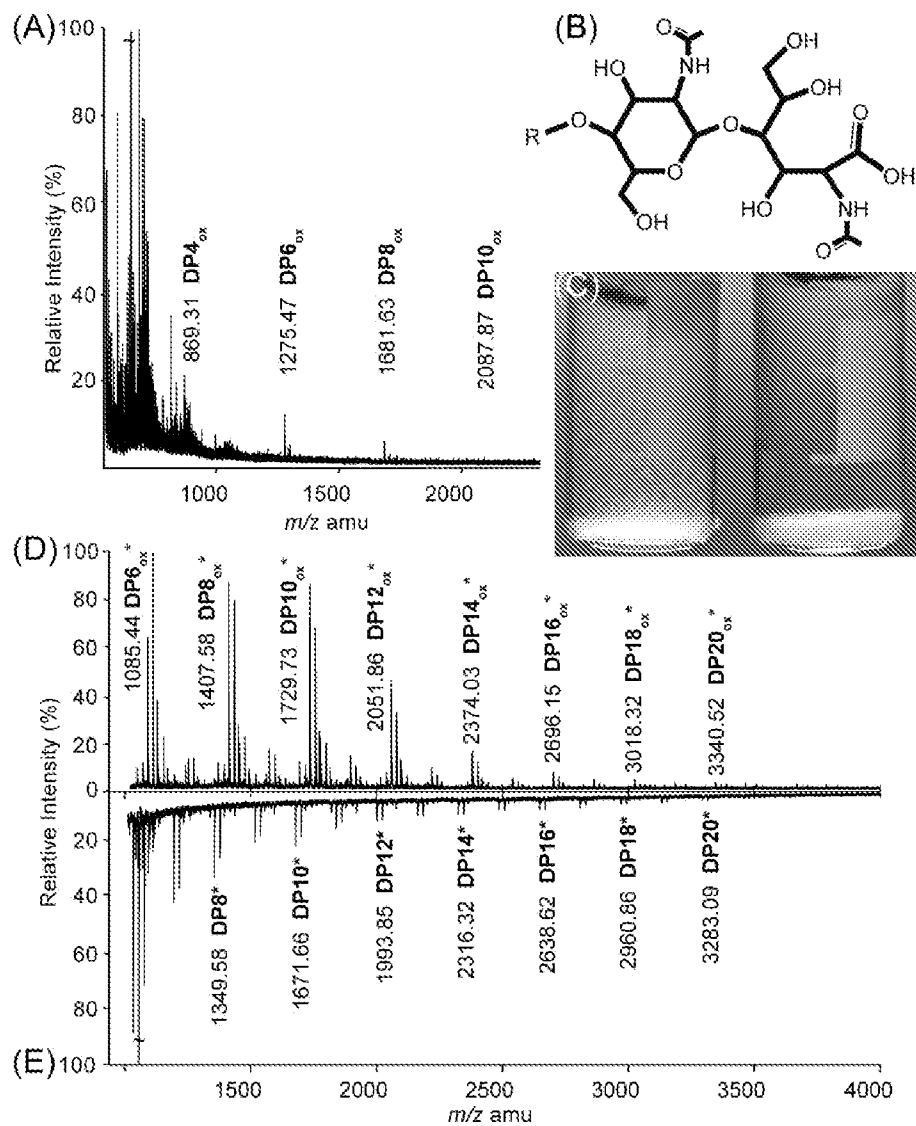


Figure 2

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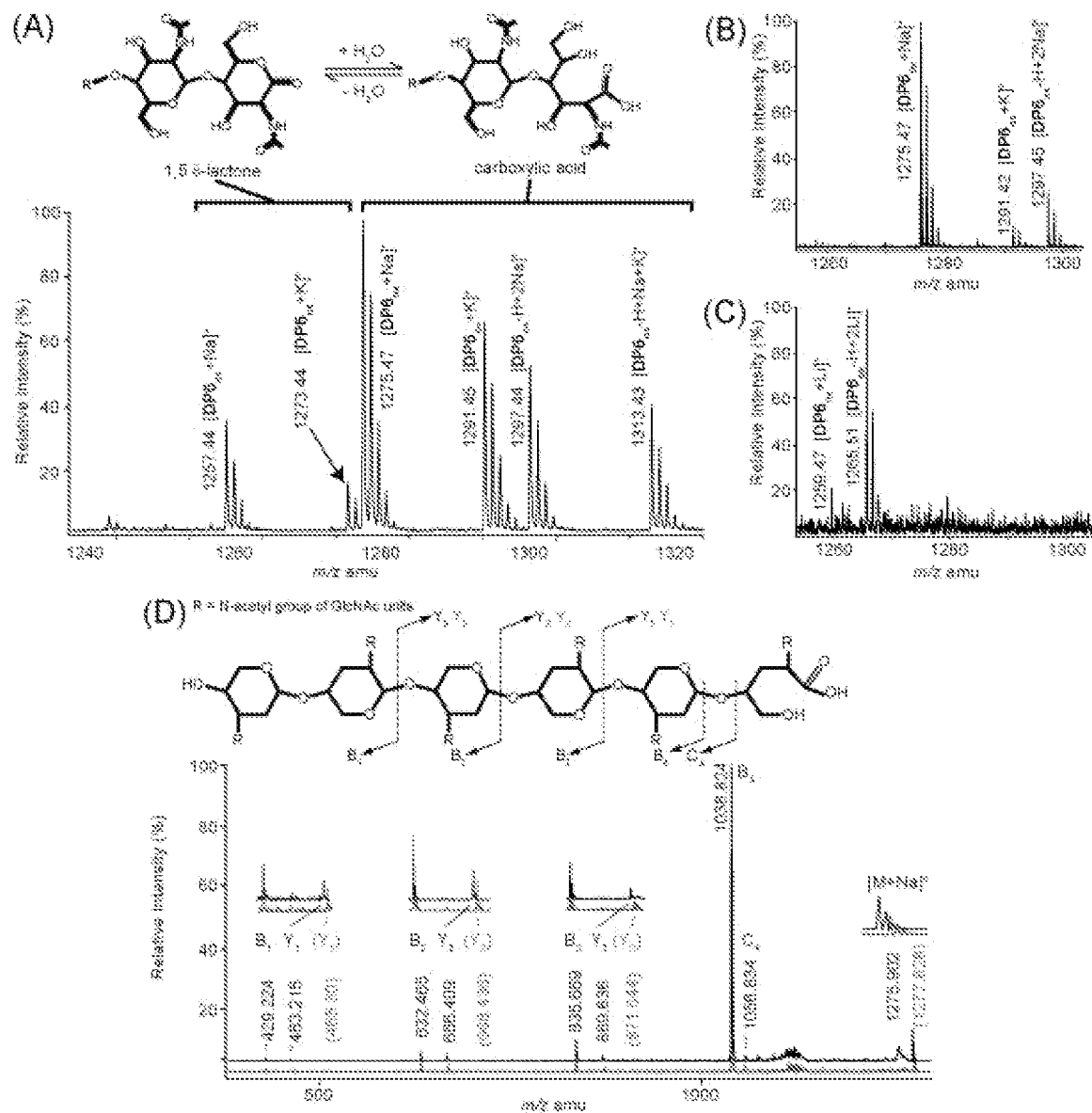


Figure 3

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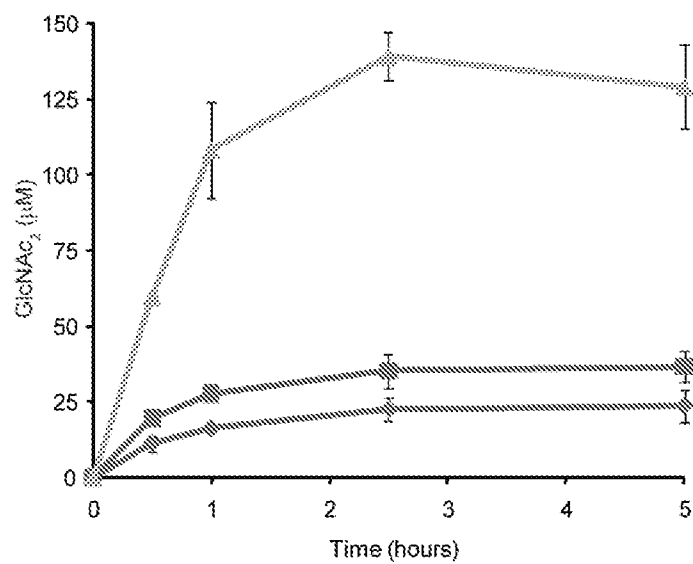


Figure 4

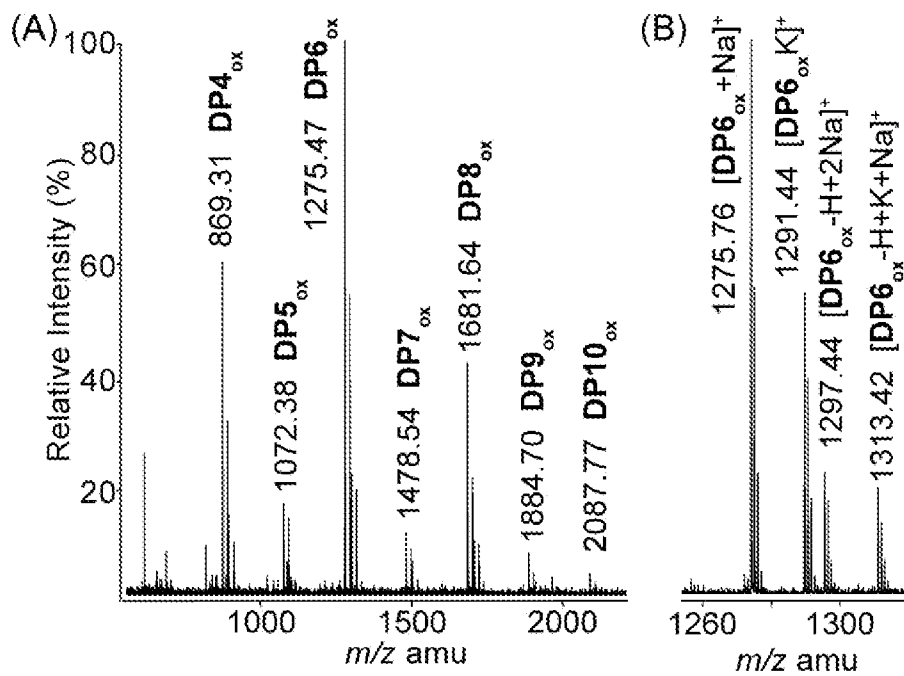


Figure 5

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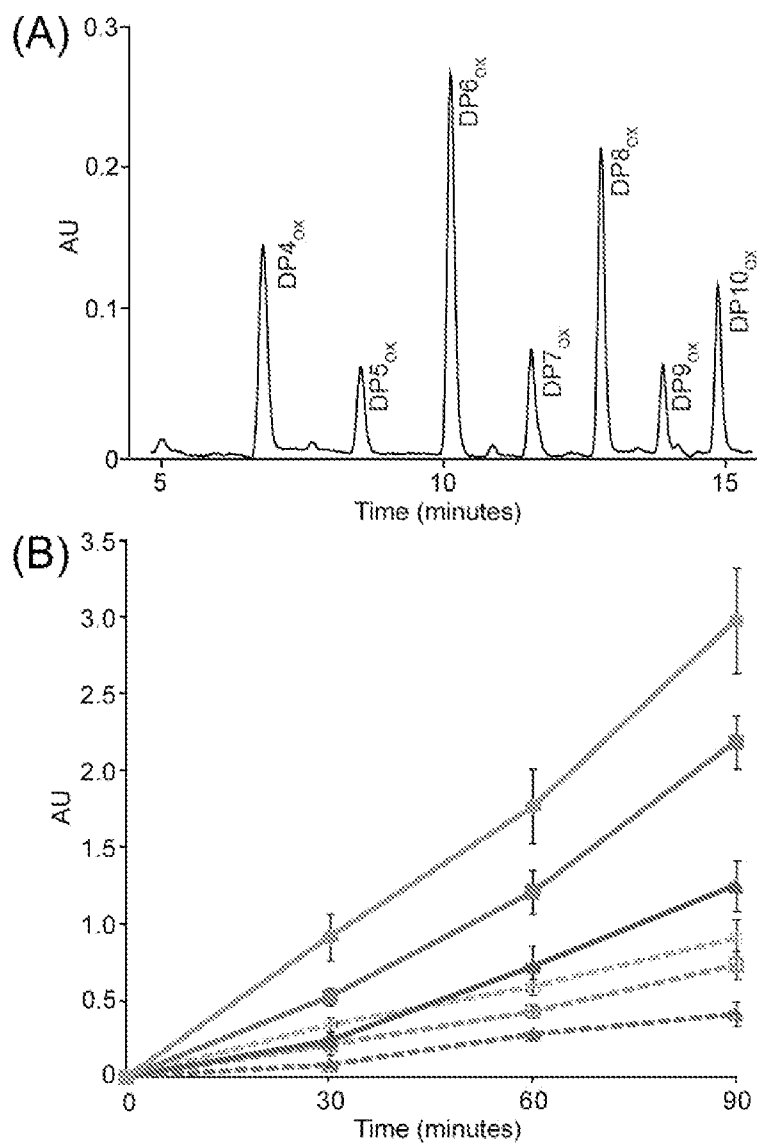


Figure 6

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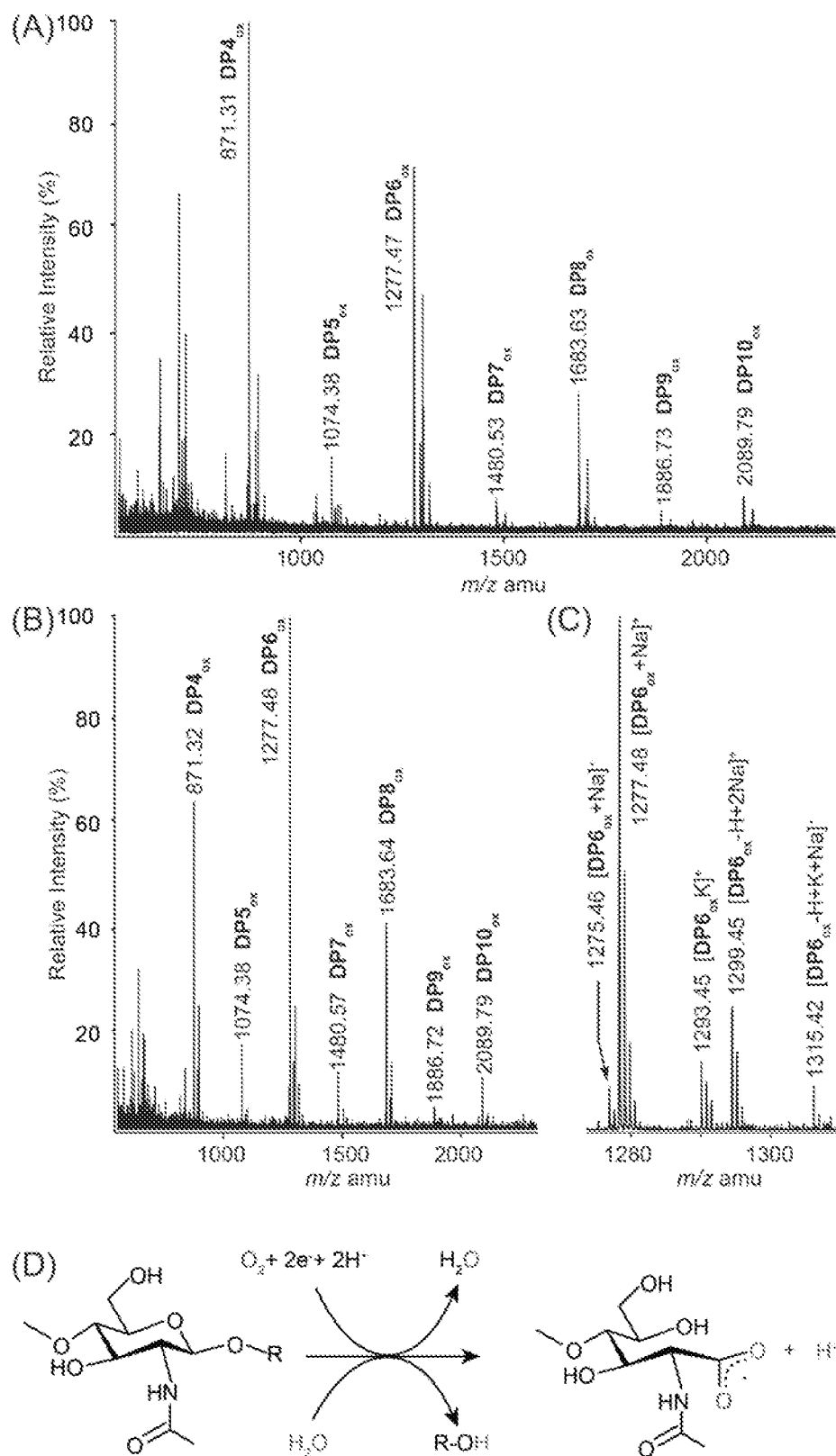


Figure 7

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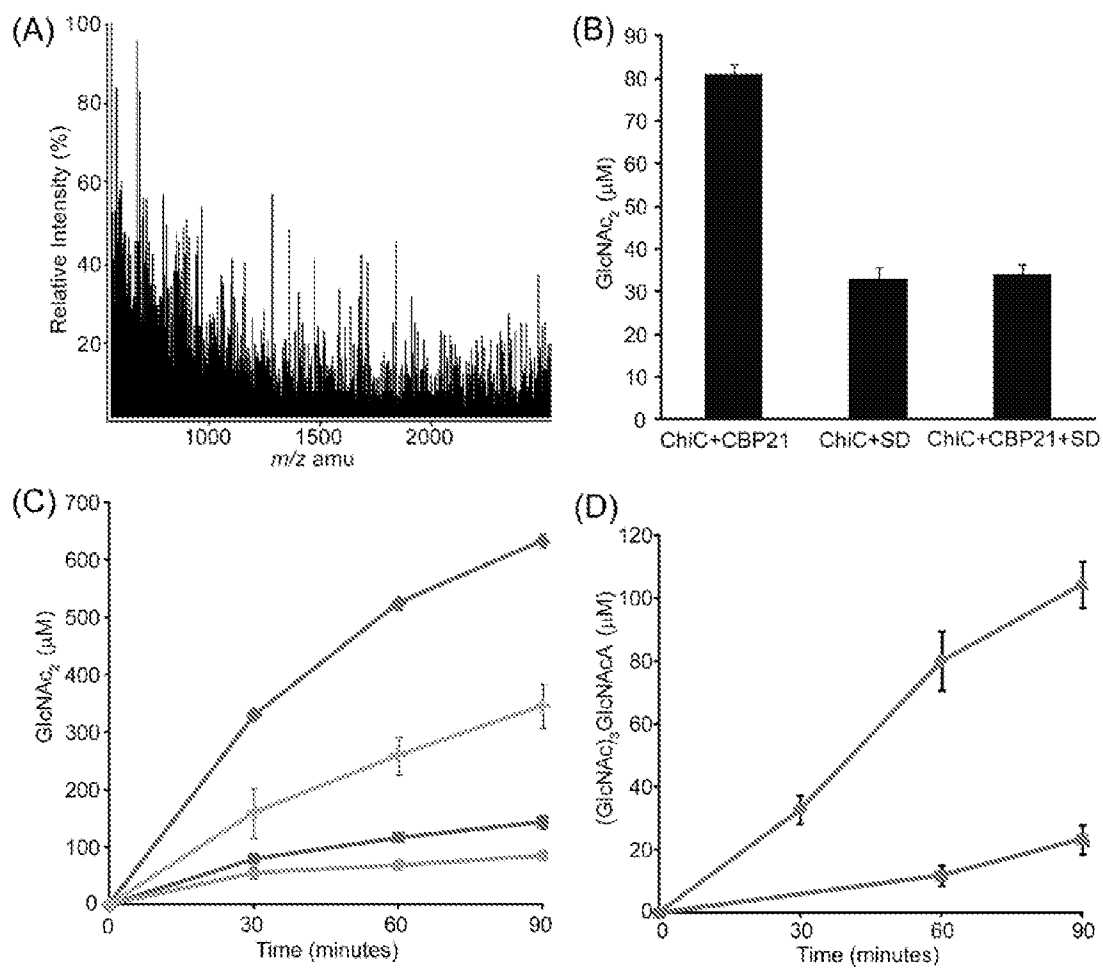


Figure 8

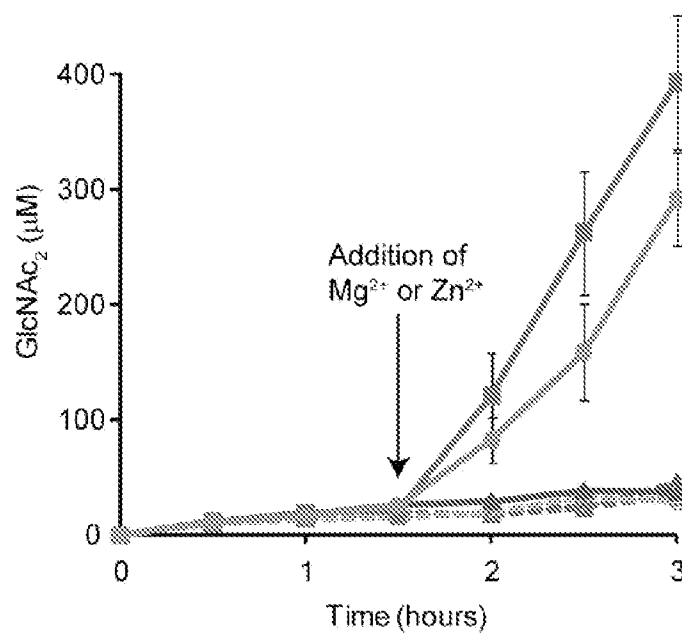


Figure 9

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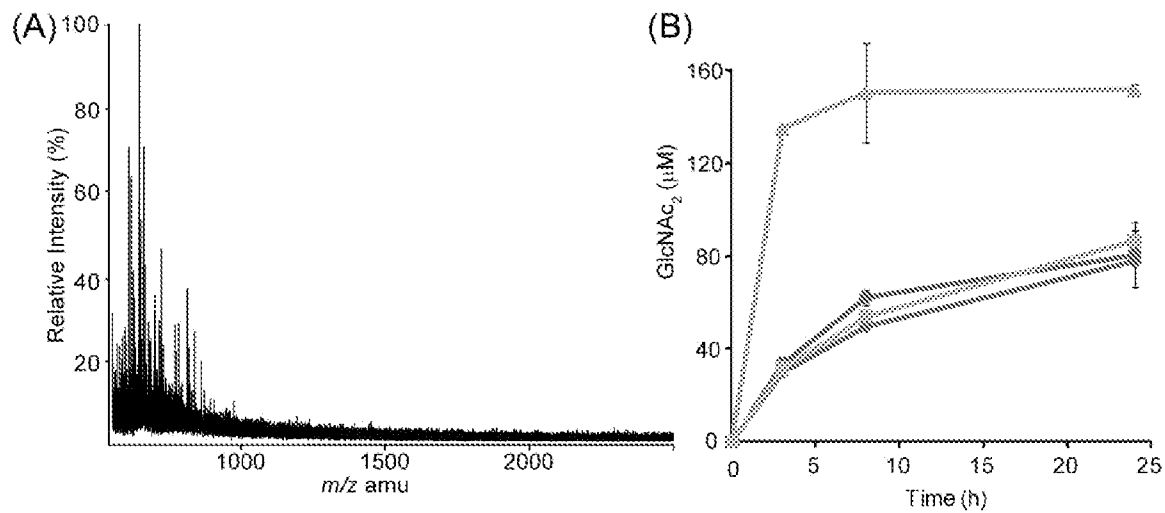


Figure 10

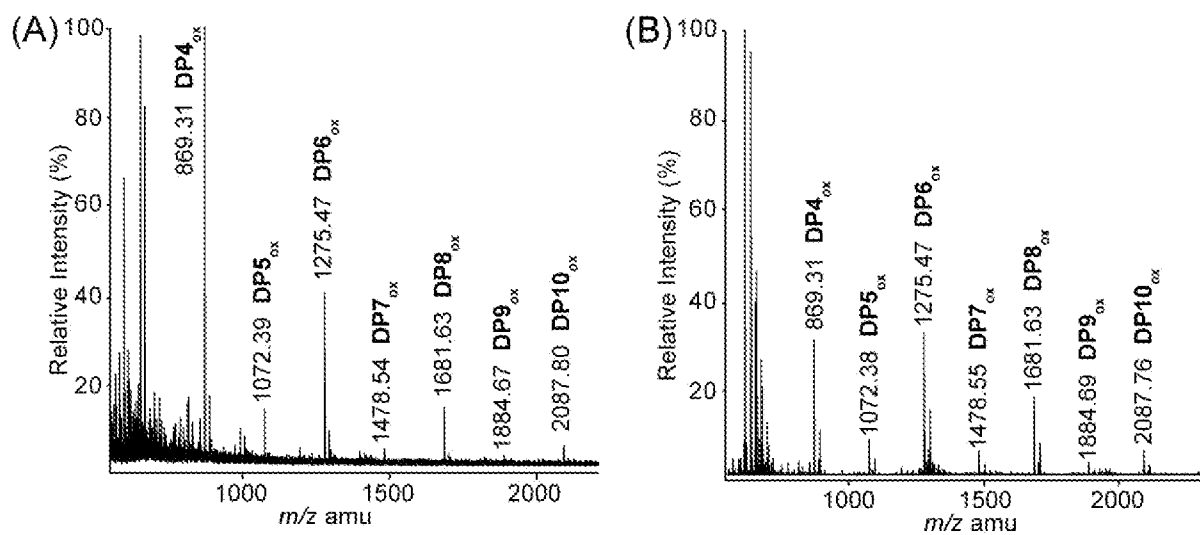


Figure 11

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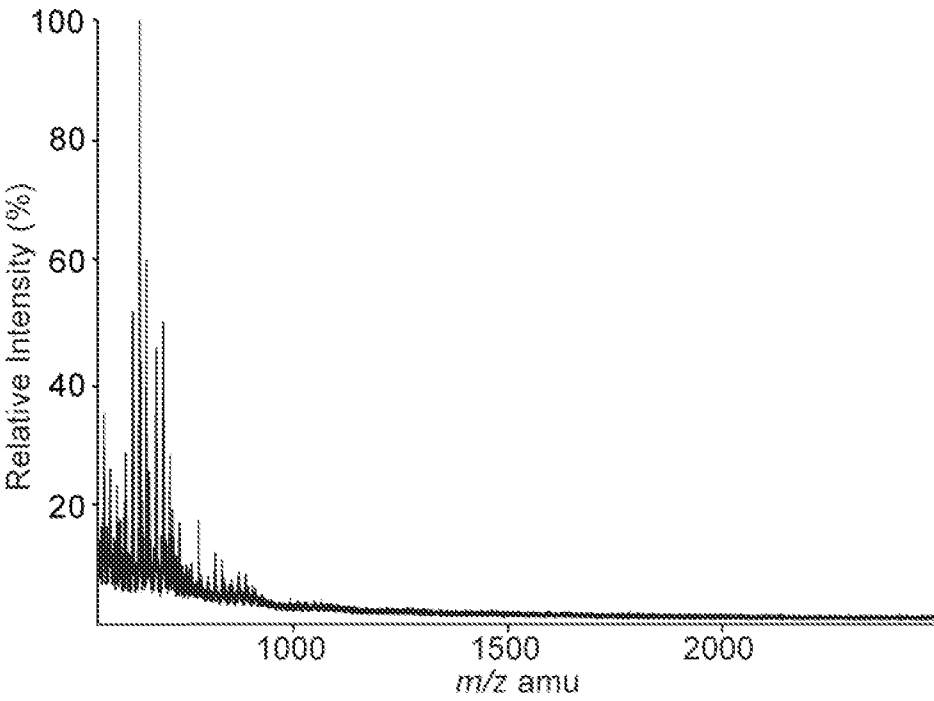


Figure 12

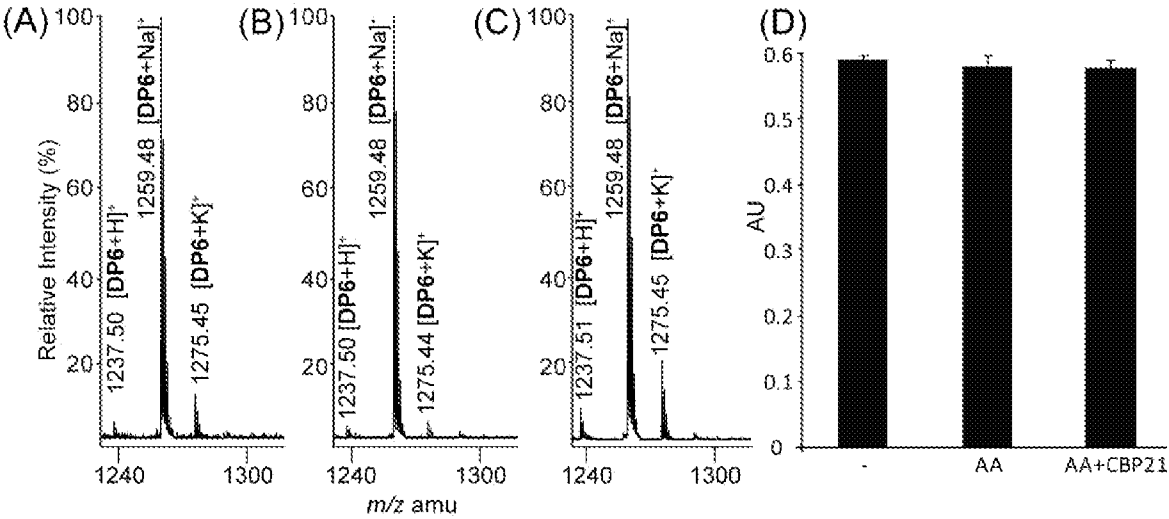
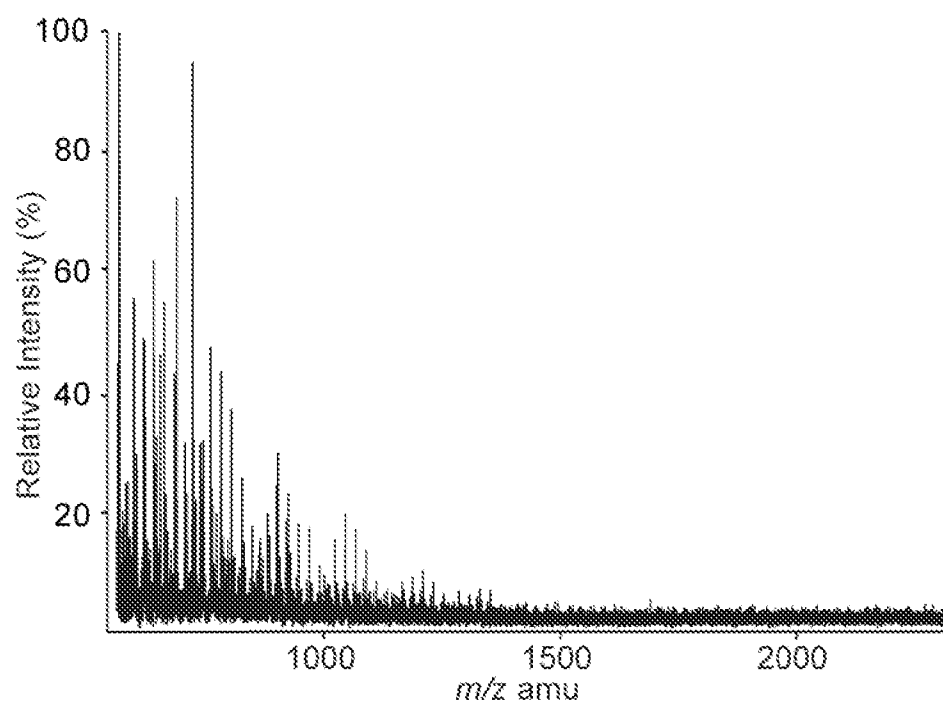


Figure 13

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**Figure 14**

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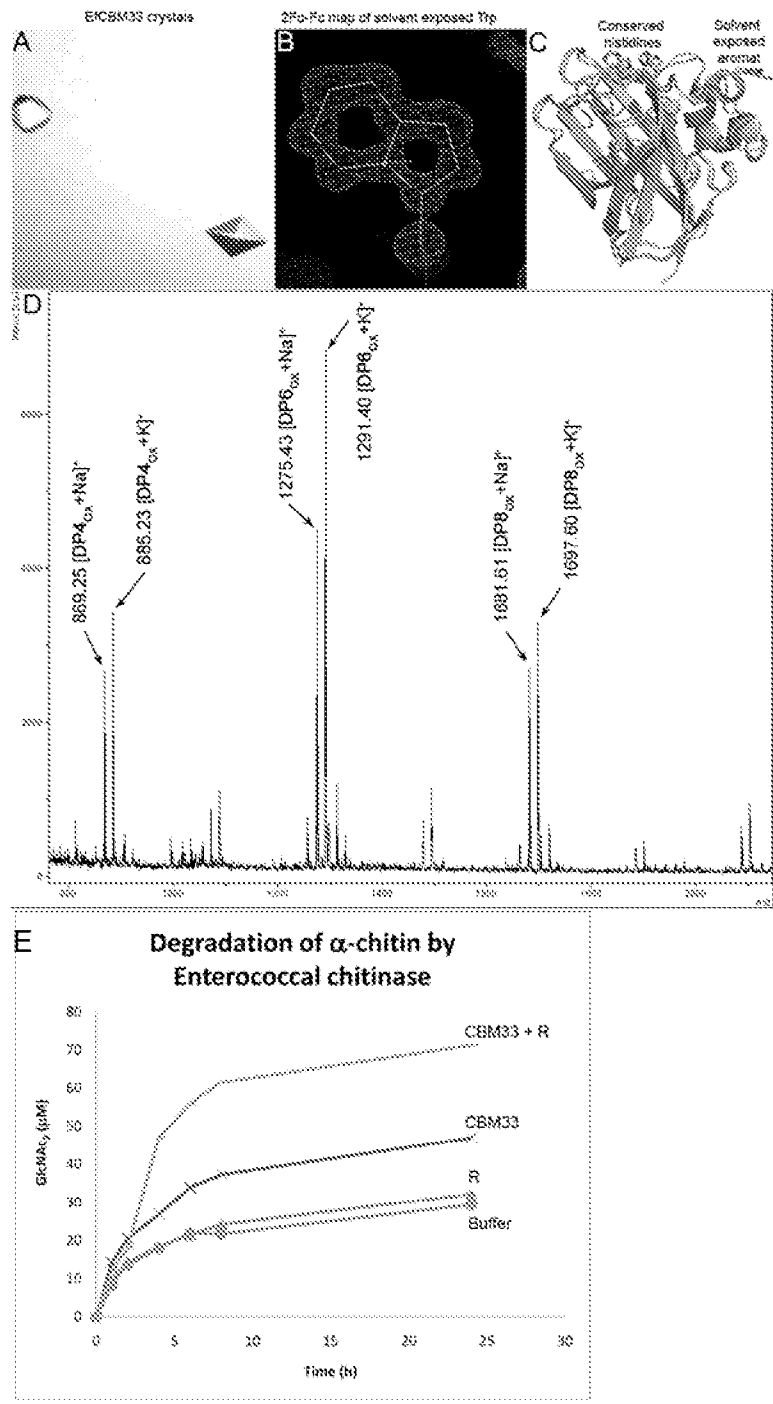


Figure 15

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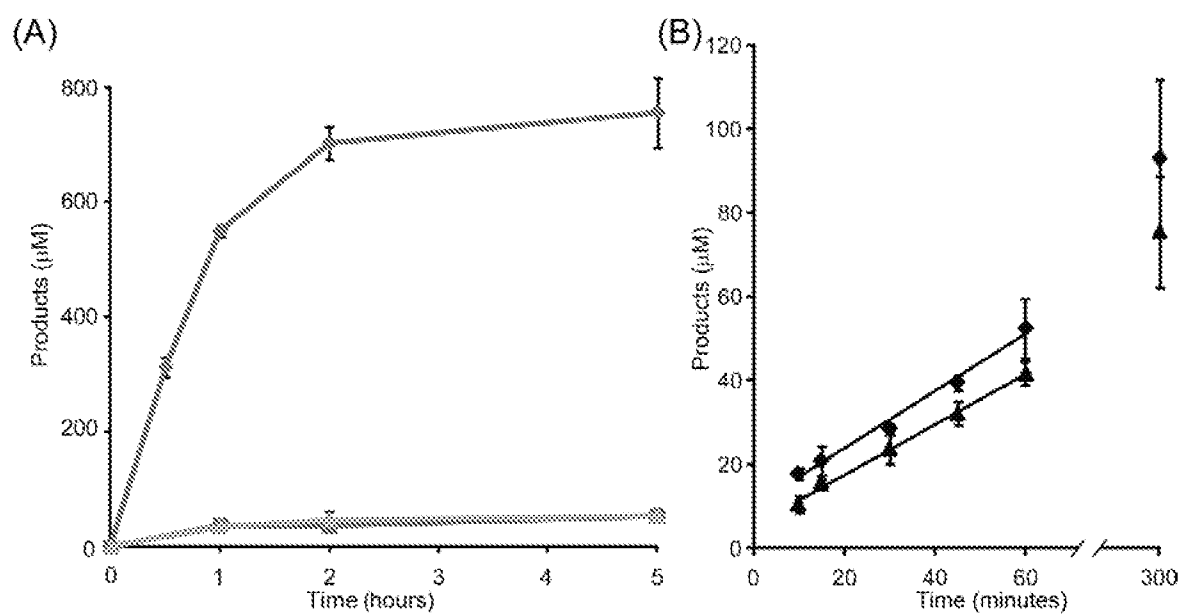


Figure 16

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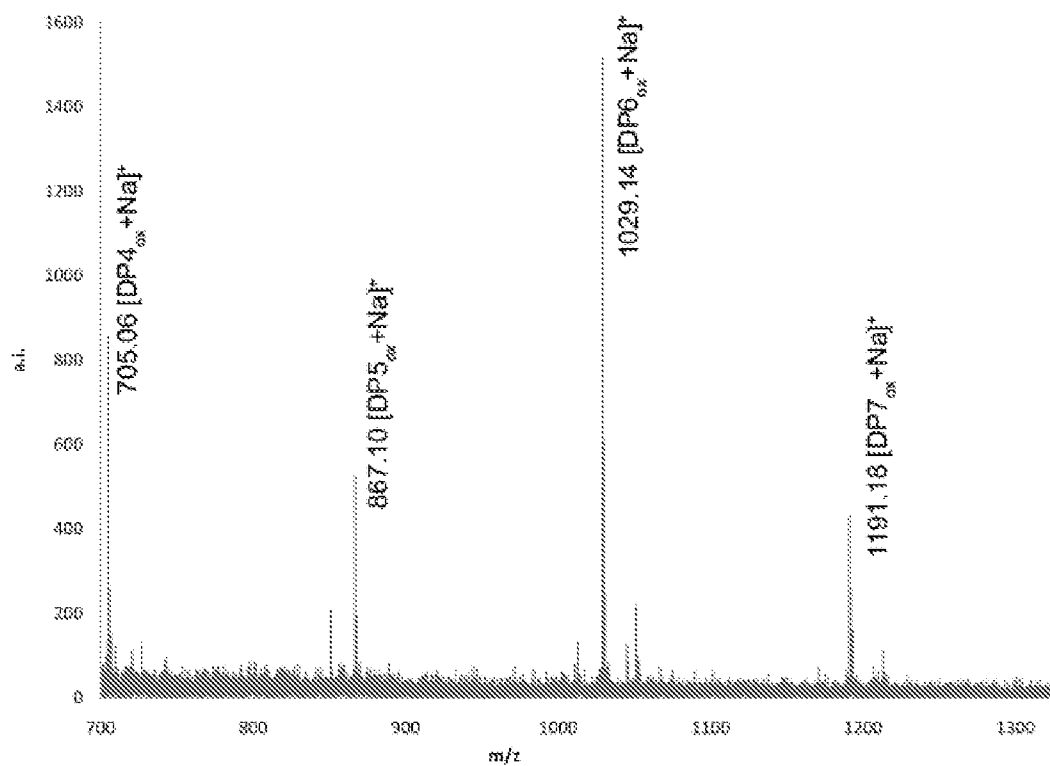


Figure 17

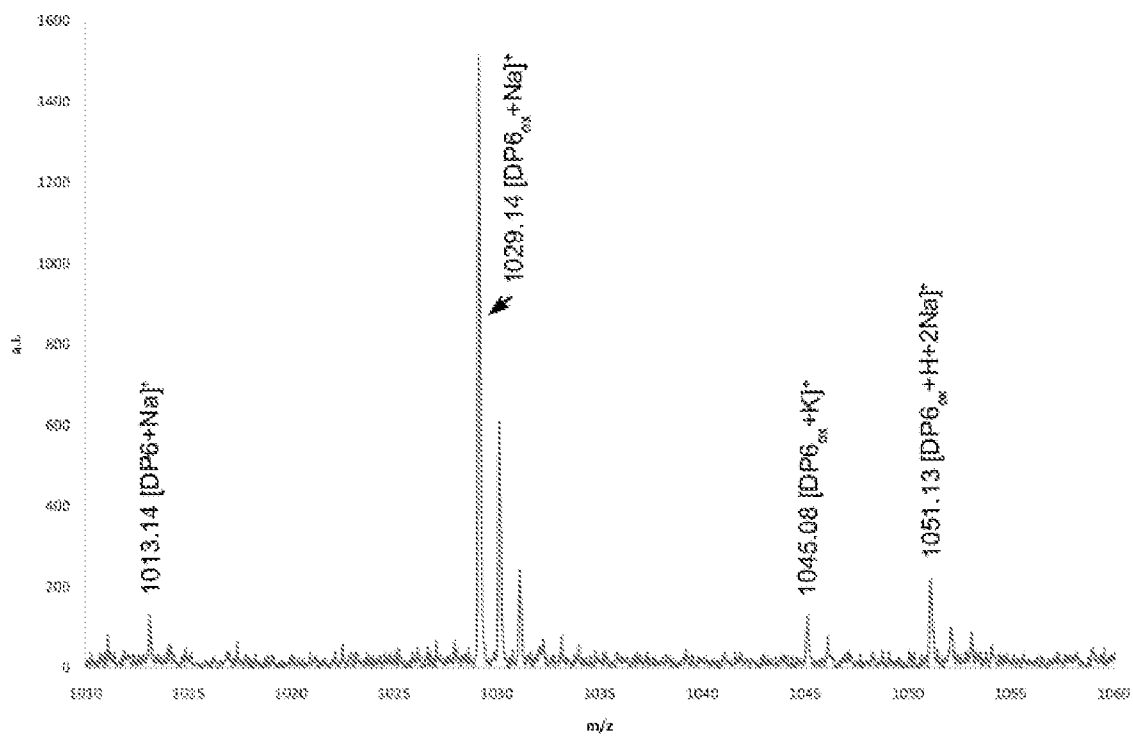


Figure 18

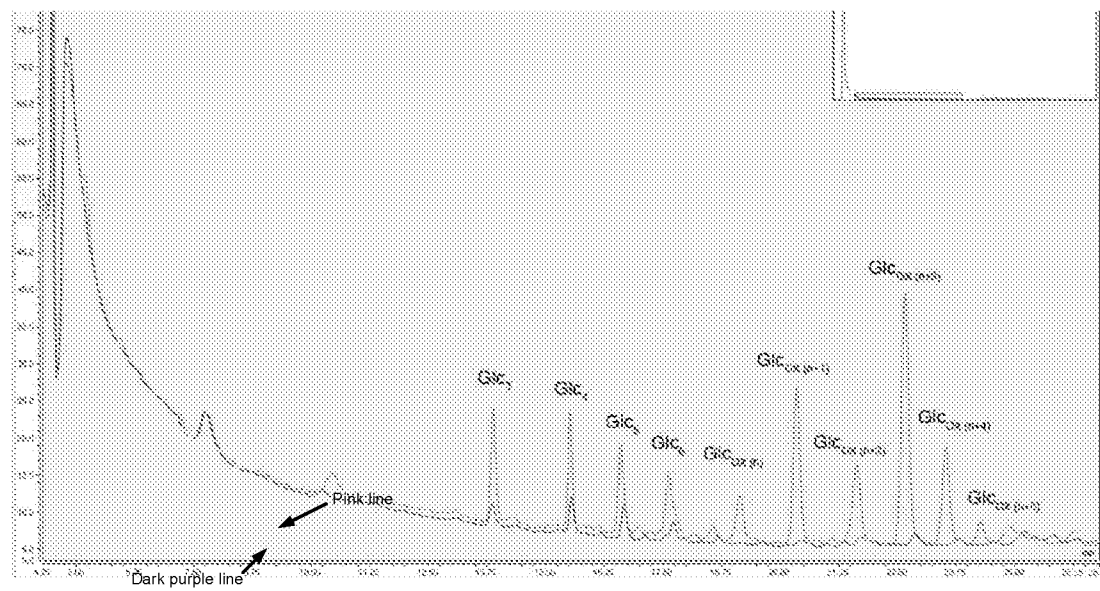


Figure 19

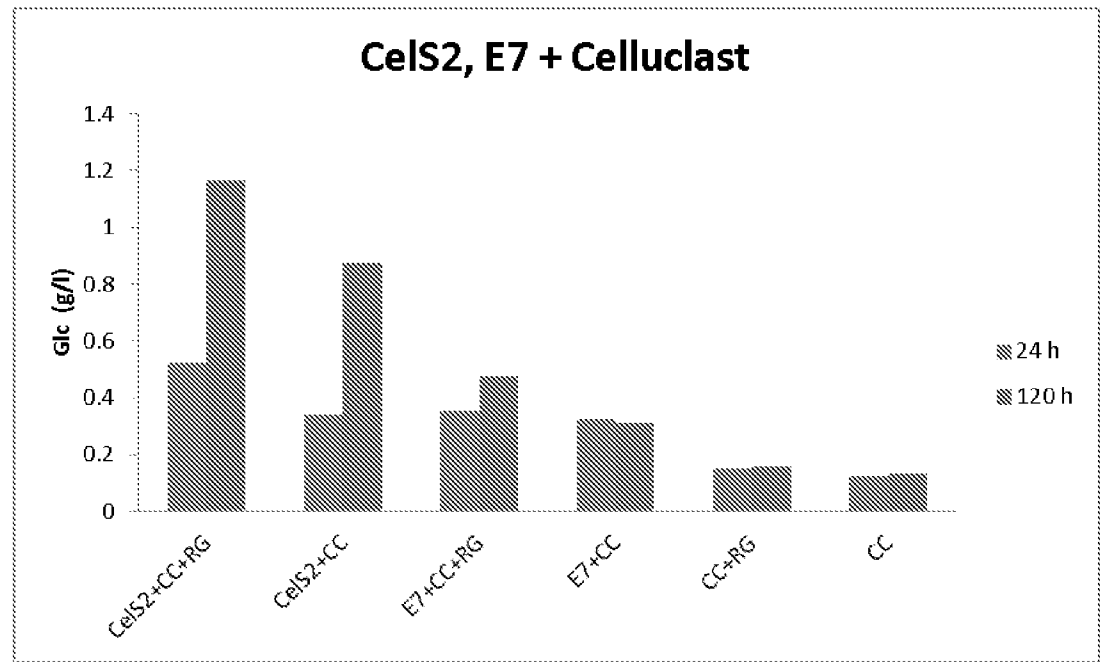


Figure 20

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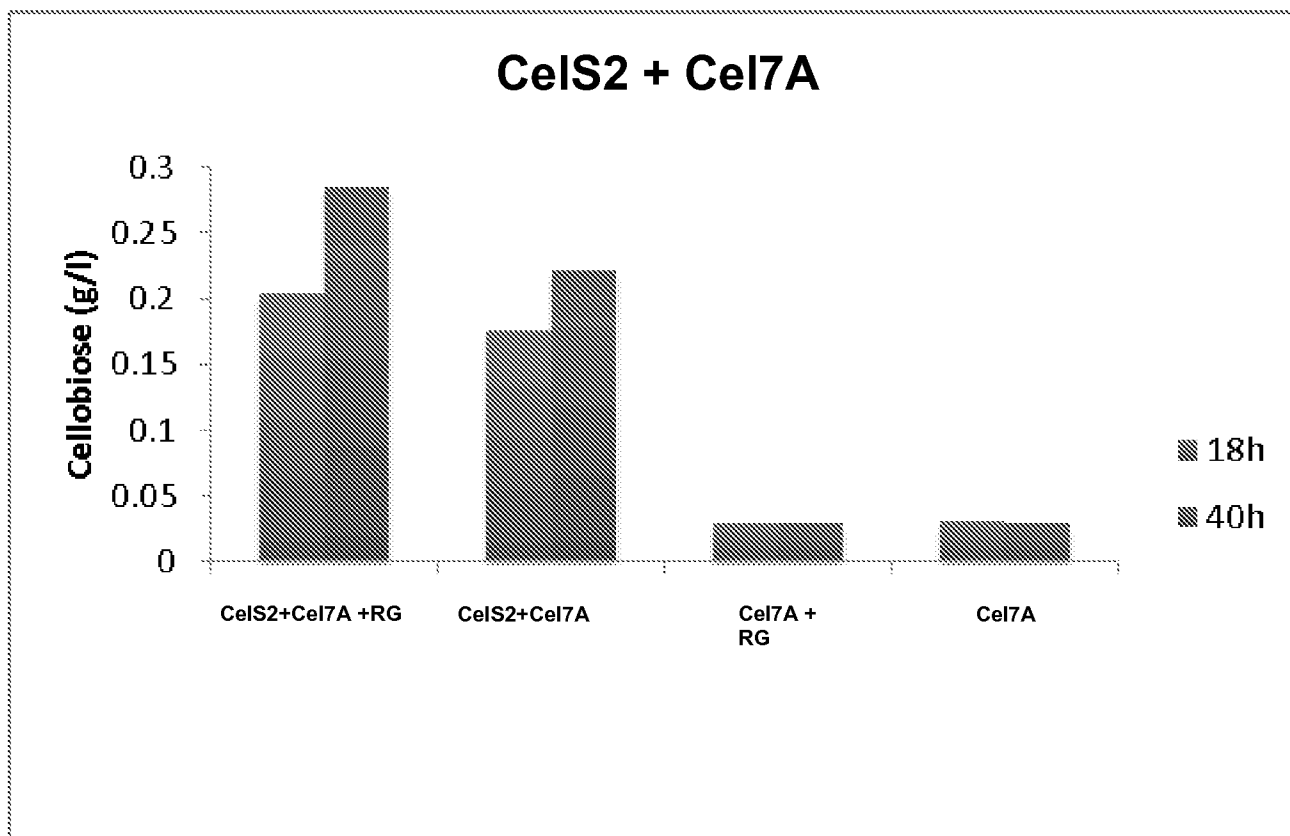


Figure 21

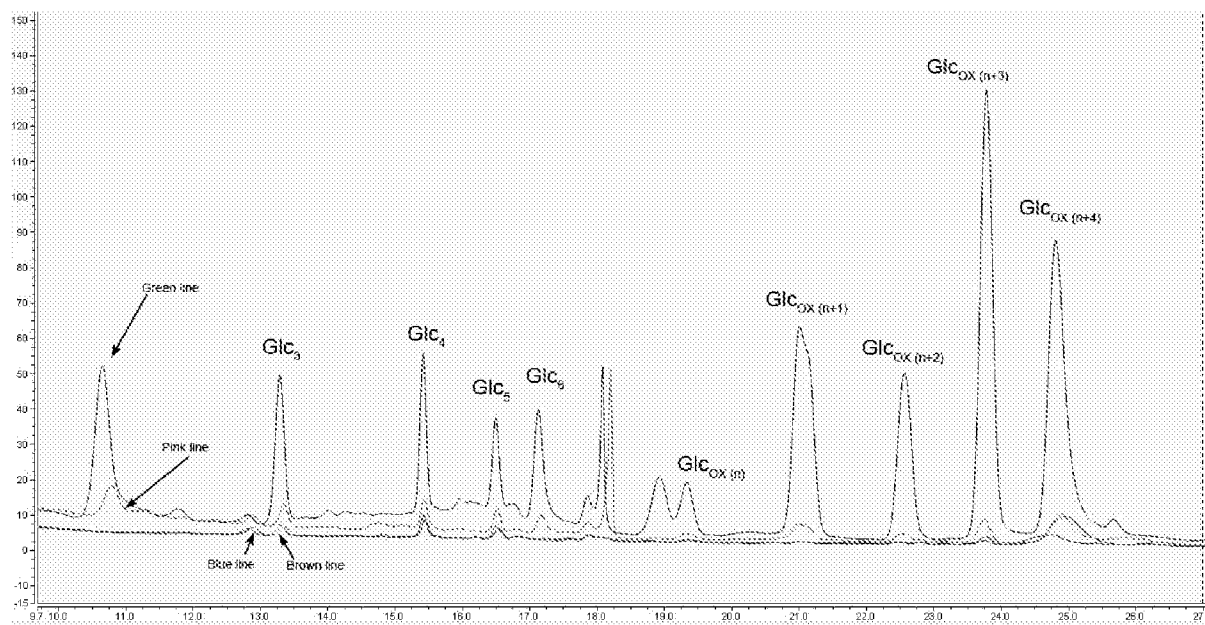


Figure 22

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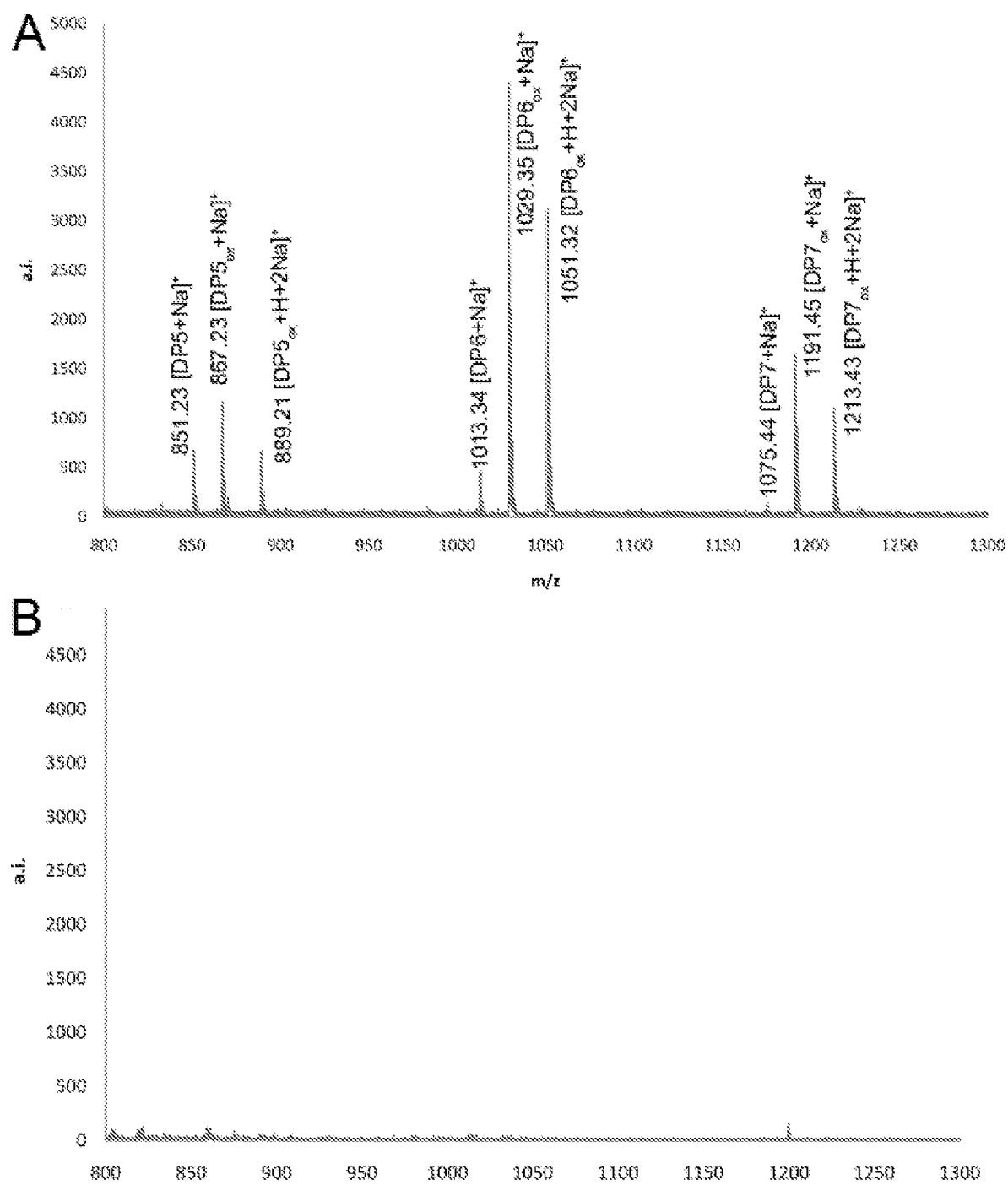


Figure 23

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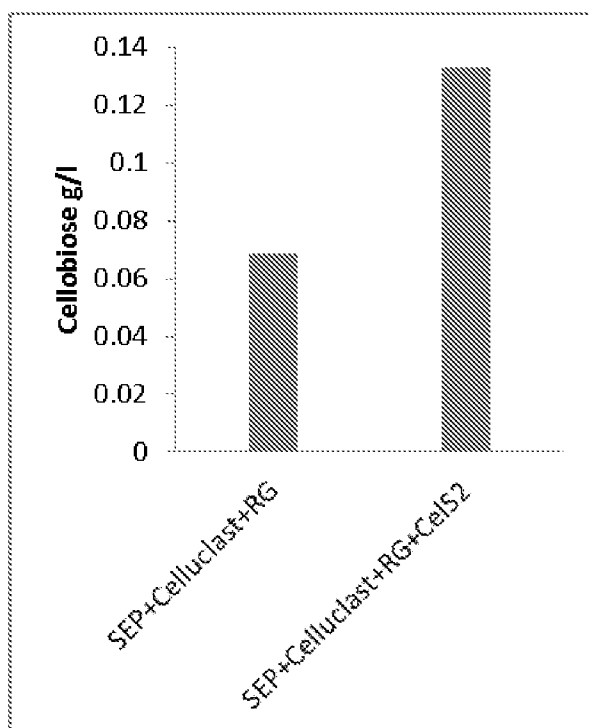


Figure 24

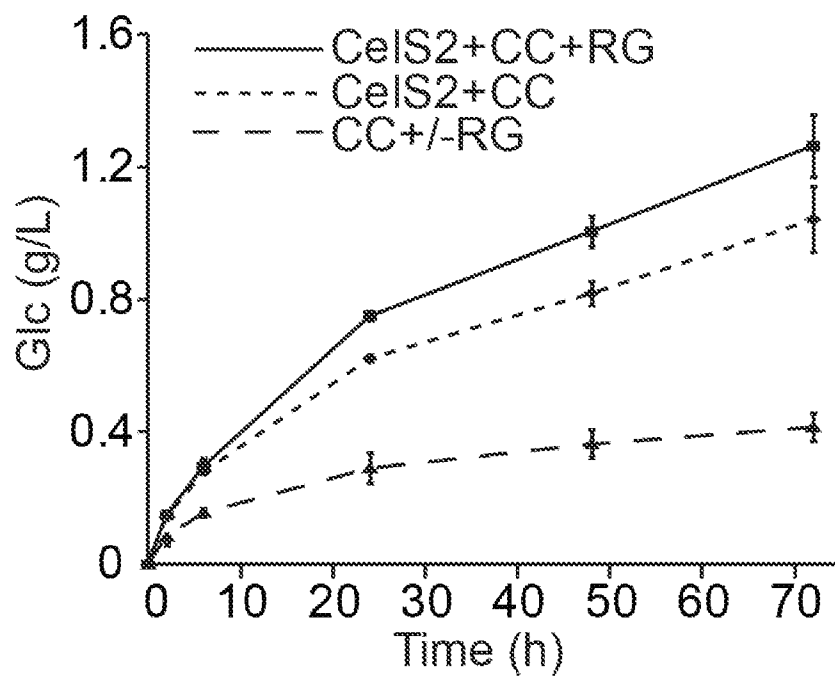
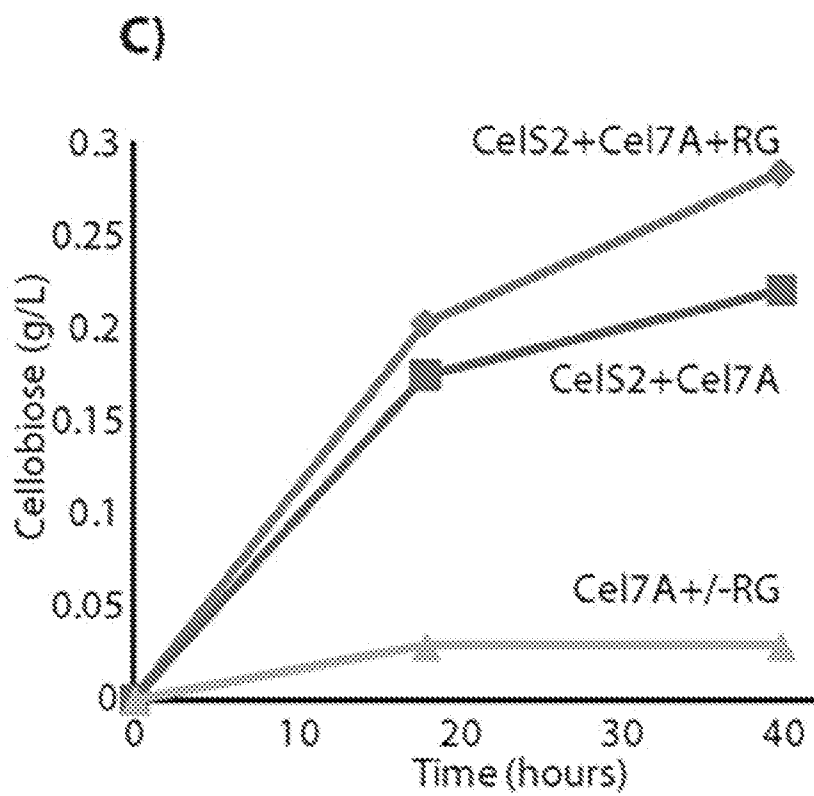


Figure 25

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**Figure 26**

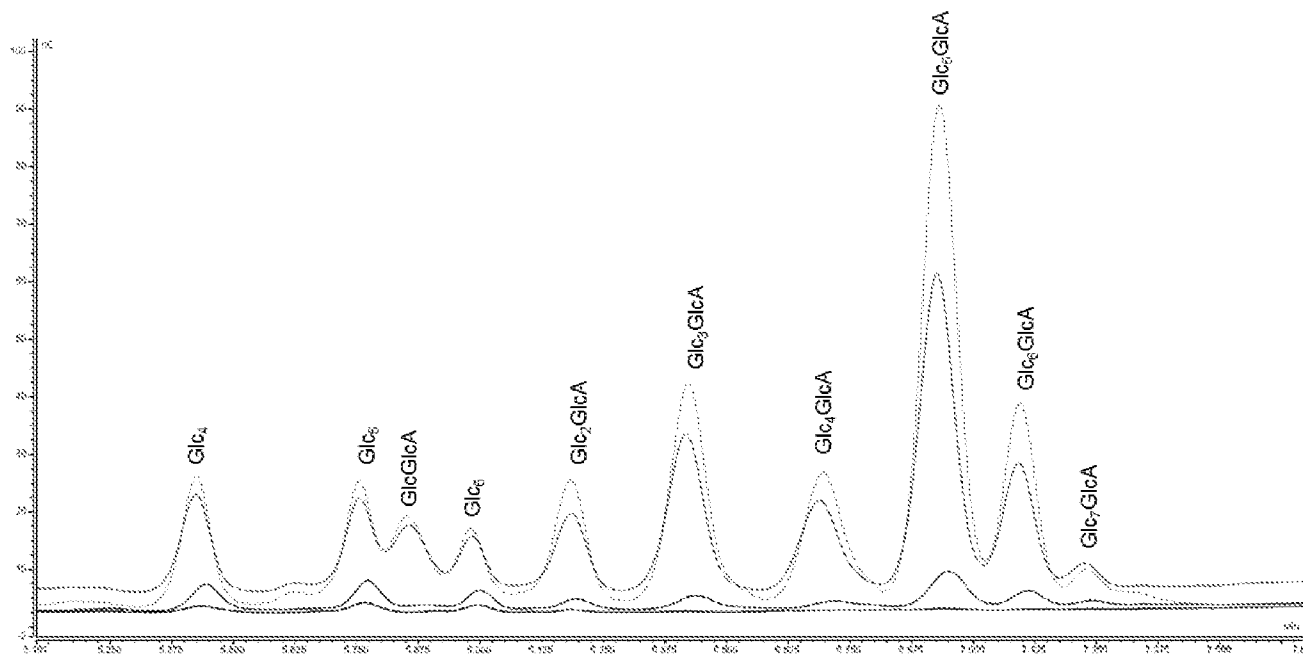


Figure 27

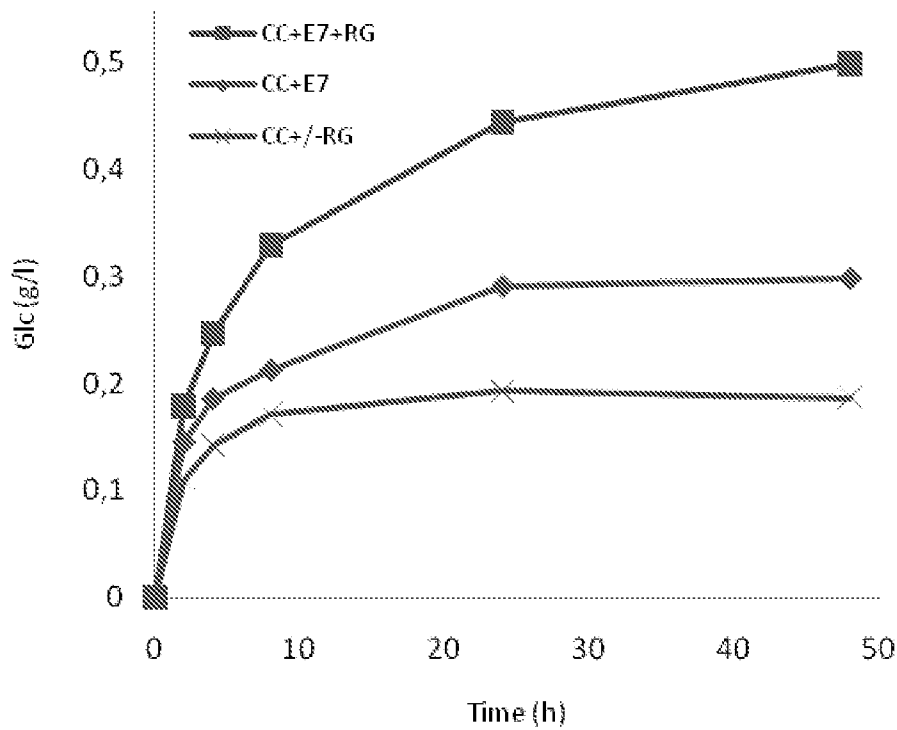


Figure 28

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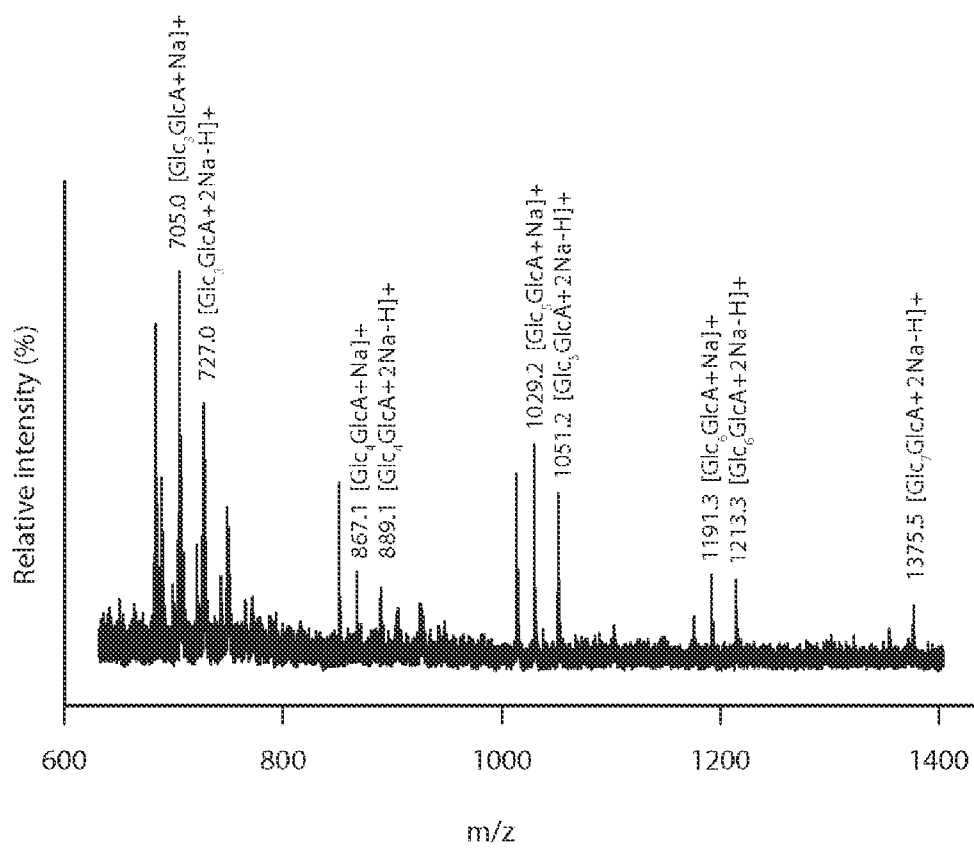


Figure 29

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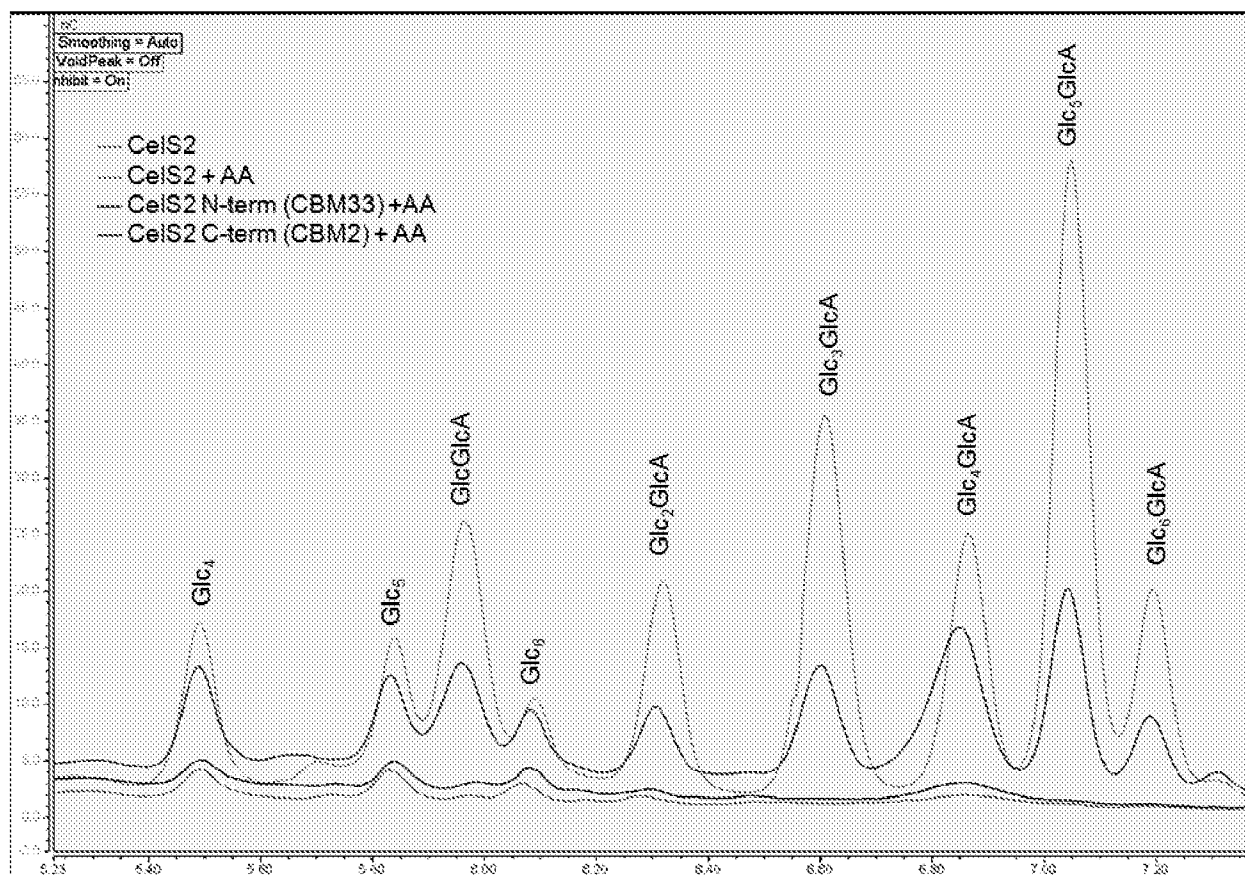


Figure 30

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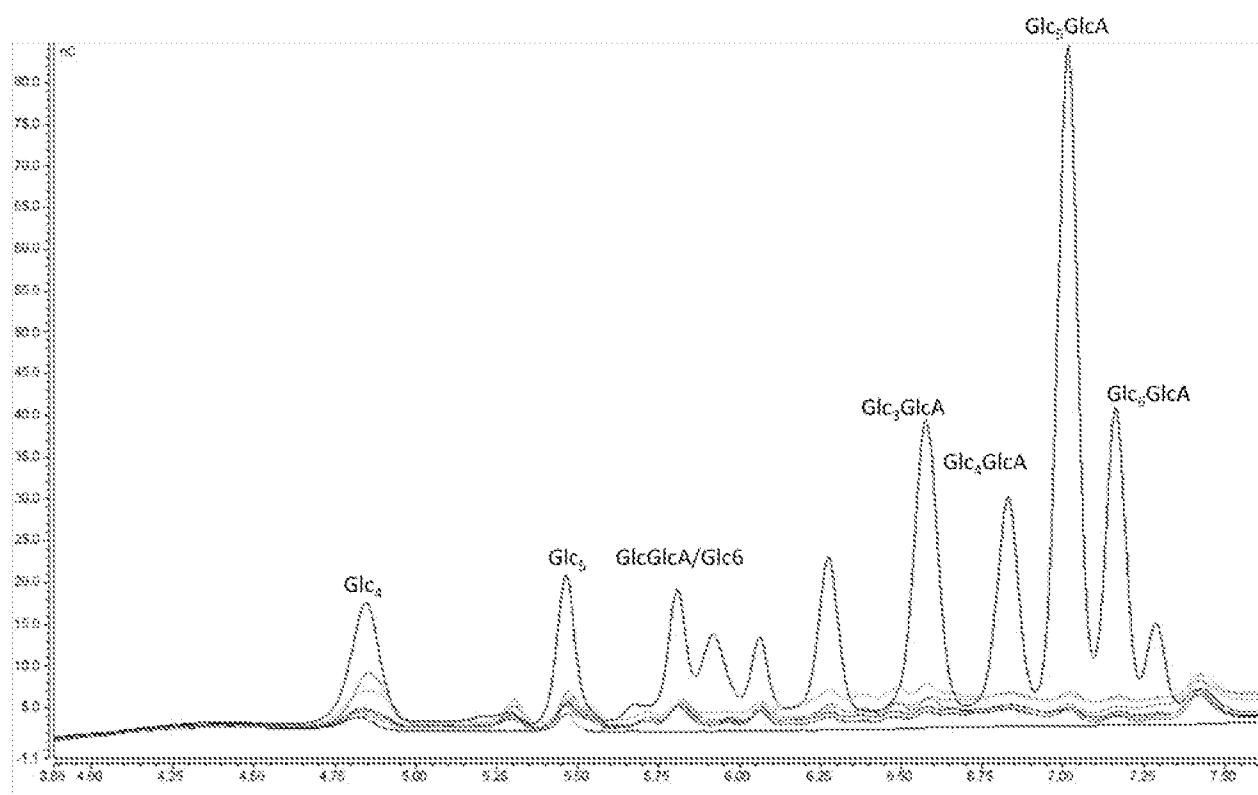


Figure 31

TtGH61E	YTWPRVN-----DGADWQQVRKA-----DNWQDNGYVG--DVTS
HjGH61B	GQVQNFT-----INGQYNQGFILDYYYQKNTGHFPNVAGWYAEDL-DLGFISPDQYTT
HjGH61A	GHINDIV-----INGVWYQAYDPTTFPYESN---PPIVVGW-TAADLDNGFVSPDAYQN
	* . . : . * :
TtGH61E	PQIRCFQATPS---PAPSVLNTTAGSTVTYWANPDVY-P-GPVQFYMARVPDGEDINSW
HjGH61B	PDIVCHKNAAP---GAISATAAA-GSNIVFQWGPVWP-PYGPVITYVVECS--GSCTTV
HjGH61A	PDIICHKNATN---AKGHASVKAGDTILFQWVPVPWP-P-GPIVDYLANCN--GDCETV
	*:: *.: . *.: : . : ** * : :
TtGH61E	NGDGAVWFKVYEDHPTFG-AQLTWPSTGK---SSFVPIPPCIKSGYLLRAEQIGLV
HjGH61B	NKNNLRWVKIQEAGINYN-TQV-WAQDDLINQGNKWTVKIPSSLRPGNYVFRHELLAAG
HjGH61A	DKTTLEFFKIDGVGLLSGGDPGTWASDVLISSNNTWVVKIPDNLAPGNYVLRHEIIALS
	:.*: : .. ** : .* *::* * .. *
TtGH61E	AQSVGGAISCAQLSVTGGGSTEPNKAFAFGAYSATDPGILINIYPVP--TSYQNP
HjGH61B	ASSANGMNPQCVNIAVTGSGTKALPAGTPATQLYKPTDPGILFNPTTI---TSYTIP
HjGH61A	AGQANGANPQCFNIAVSGSGSLQ-PSGVLGTDLYHATDPGVLINITSP---LNYIIP
	* . .* * * * : : * ..* . . * :*:*: * * . * *
TtGH61E	GPAVFSC-----
HjGH61B	GPALW-----
HjGH61A	GPTVVSGLPTSVAQGSSAATATASATVPGGGSGPSTRTTTTARTTQASSRPSSTPPATTS
	**::
TtGH61E	-----
HjGH61B	-----
HjGH61A	APAGGPTQTLYGQCGSGYSGPTRCAPPATCSTLNPYYAQCLN

Figure 32A

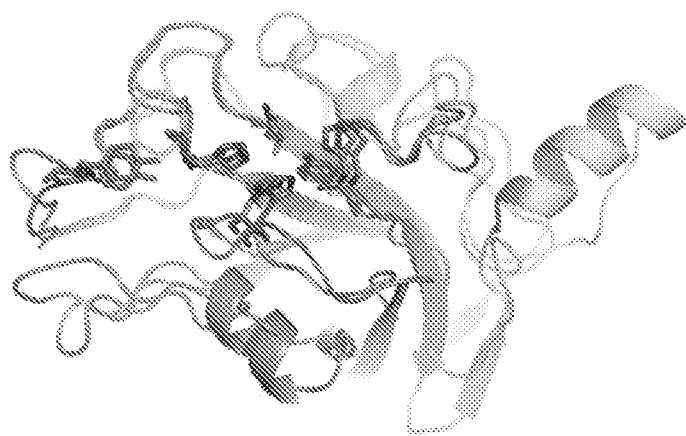
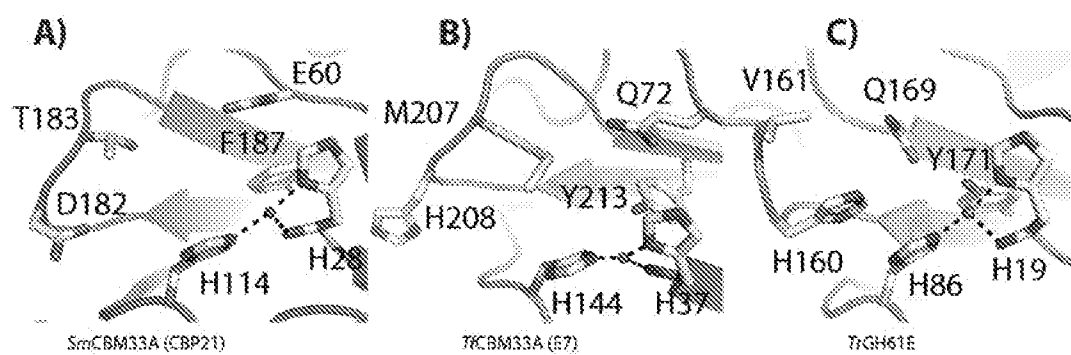


Figure 32B

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**Figure 33**

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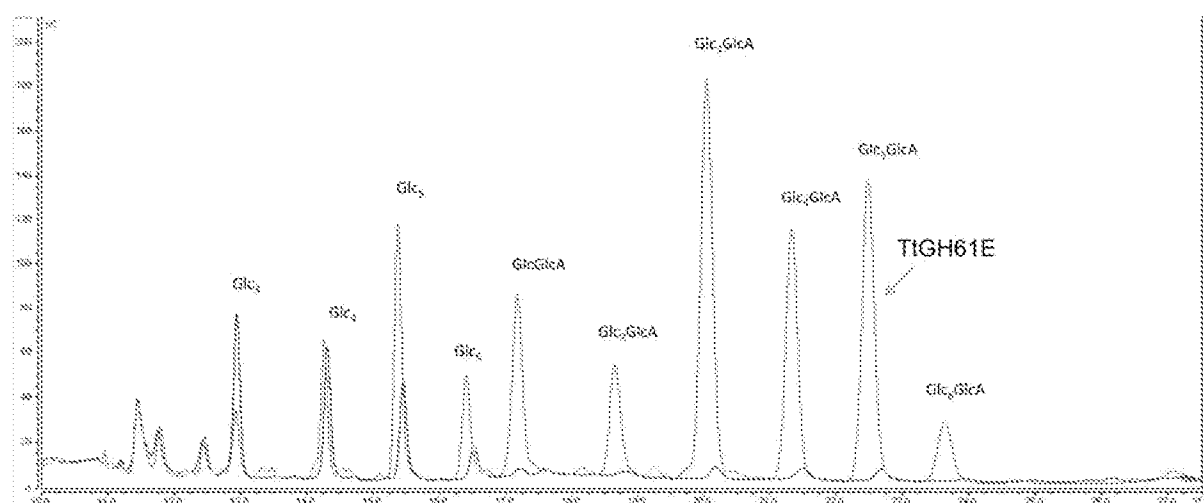


FIGURE 34

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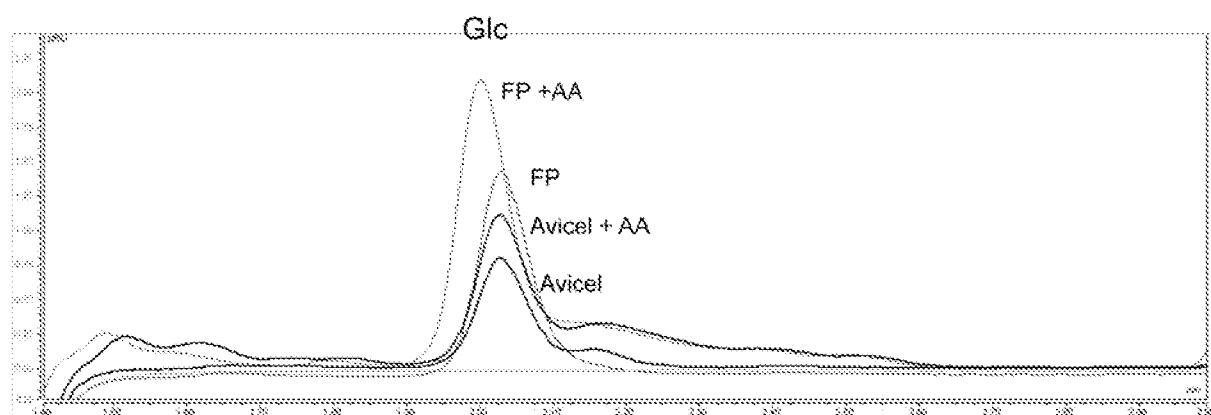


FIGURE 35

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/046838

A. CLASSIFICATION OF SUBJECT MATTER INV. C12P7/10 C12P19/02 C12P19/12 C12P19/14 C12P19/26 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		
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	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] cited in the application abstract; figures 2, 3, 8 page 3309, left-hand column, last paragraph - right-hand column, paragraph 1 page 3313, left-hand column, paragraph 2-3 ----- -/--	1-3,6-9, 11,13-19
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "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 9 November 2011		Date of mailing of the international search report 29/12/2011
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Schröder, Gunnar

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2011/046838

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	H. J. GILBERT: "The Biochemistry and Structural Biology of Plant Cell Wall Deconstruction", PLANT PHYSIOLOGY, vol. 153, no. 2, 1 June 2010 (2010-06-01), pages 444-455, XP055011488, ISSN: 0032-0889, DOI: 10.1104/pp.110.156646 page 449, left-hand column, paragraph 3 - page 450, left-hand column, paragraph 1 -----	1-19
A	ARANTES VALDEIR ET AL: "Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis", BIOTECHNOLOGY FOR BIOFUELS, BIOMED CENTRAL LTD, LONDON UK, vol. 3, no. 1, 4, 23 February 2010 (2010-02-23), pages 1-11, XP021070031, ISSN: 1754-6834 abstract page 4, left-hand column, paragraph 2 - page 5, left-hand column, paragraph 1 page 7, right-hand column, last paragraph - page 8, right-hand column, paragraph 1 -----	1-19
A	US 4 540 664 A (JOHNSON ERIC A [US] ET AL) 10 September 1985 (1985-09-10) abstract; figures 1, 2 column 3, lines 58-68 -----	1-19
A	US 2008/251374 A1 (MCMANIGAL BRENT ALAN [US]) 16 October 2008 (2008-10-16) paragraphs [0015], [0016] -----	1-19
X,P	VAAJE-KOLSTAD GUSTAV ET AL: "An Oxidative Enzyme Boosting the Enzymatic Conversion of Recalcitrant Polysaccharides", SCIENCE (WASHINGTON D C), vol. 330, no. 6001, October 2010 (2010-10), pages 219-222, XP002663130, ISSN: 0036-8075 abstract; figures 1-3 page 219, column 2, paragraph 2 - page 220, column 2 -----	1-3,6, 10,11, 13-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2011/046838

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.:

1-19

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

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

1. claims: 1-19

Hydrolysis of a polysaccharide with an oxidohydrolytic enzyme in the presence of a reducing agent and a divalent metal ion

2. claims: 20-25

Hydrolysis of a cellulosic material with a hydrolytic enzyme in the presence of a reducing agent and a divalent metal ion.

3. claims: 26-31

Hydrolysis of a cellulosic material with a cellulase, a GH61 protein and a CBM33 protein

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2011/046838

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 4540664	A	10-09-1985	NONE	

US 2008251374	A1	16-10-2008	US 2008251374 A1	16-10-2008
			WO 2008128253 A1	23-10-2008
