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The effect of a lytic polysaccharide monooxygenase and a xylanase from *Gloeophyllum trabeum* on the enzymatic hydrolysis of lignocellulosic residues using a commercial cellulase

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ABSTRACT

Hydrolysis of lignocellulosic biomass depends on the concerted actions of cellulases and accessory proteins. In this work we examined the combined action of two auxiliary proteins from the brown rot fungus *Gloeophyllum trabeum*: a family AA9 lytic polysaccharide monooxygenase (GtLPMO) and a GH10 xylanase (GtXyn10A). The enzymes were produced in the heterologous host *Pichia pastoris*. In the presence of an electron source, GtLPMO increased the activity of a commercial cellulase on filter paper, and the xylanase activity of GtXyn10A on beechwood xylan. Mixtures of GtLPMO, GtXyn10A and Celluclast 1.5 L were used for hydrolysis of pretreated wheat straw. Results showed that a mixture of 60% Celluclast 1.5 L, 20% GtXyn10A and 20% GtLPMO increased total reducing sugar production by 54%, while the conversions of glucan to glucose and xylan to xylose were increased by 40 and 57%, respectively. This suggests that GtLPMO can contribute to lignocellulose hydrolysis, not only by oxidative activity on glycosidic bonds, but also to hemicellulose through the oxidation of xylosyl bonds in xylan. The concerted action of these auxiliary enzymes may significantly improve large-scale recovery of sugars from lignocellulose.

1. Introduction

Utilization of lignocellulosic residues provides an opportunity to advance sustainable human development. The complex structure and recalcitrance of lignocellulose makes the derivation of bio-products from these residues technically challenging. Despite important manufacturing advances [1], it is still necessary to identify efficient enzyme complexes that will increase the speed of biomass degradation.

Wheat straw is one of the most abundant agricultural residues available, Chile alone generates 1.5 million tons every year [2]. Currently, various groups are investigating alternative uses of this raw material which is composed of 33–40% cellulose, 20–25% hemicellulose and 15–20% lignin [3]. The relatively low level of lignin in wheat straw, compared to that in woody biomasses, suggests that its degradation should be a simpler process. Furthermore, the composition of the hemicellulosic fraction also differs from that of woody biomasses, primarily consisting of xylans in hardwoods and straw as opposed to galactomannans in softwoods [4]. Xylans from different sources differ in composition as well. Hardwood xylans contain primarily xylose and glucuronic acid, whereas grass xylans contain up to 33.5% (w/w) arabinose [5]. The nature of the acetylated polymer and the extent of acetylation also differs between plant species. About half of the β-D-xylopyranosyl units in hardwood hemicellulose are acetylated moieties [6], while only 13% and 1.7% (w/w) in cottonwood and wheat straw, respectively [7]. These differences are relevant because acetyl-substituents inhibit the enzymatic degradation of wall polymers and the released acetate can be a potent inhibitor in microbial fermentation.

The ascomycete *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) is a widely used industrial fungi due to its capacity to produce cellulolytic enzymes [8]. The *T. reesei* cellulolytic system has an abundance of *endo*-acting cellulase (EC 3.2.1.4, endoglucanase) and *exo*-acting cellulase (EC 3.2.1.91 and EC 3.2.1.176, cellobiohydrolase). Despite this, the *T. reesei* genome has a relatively low number and diversity of carbohydrate-degrading enzymes [9]. For example, *T. reesei* must be supplemented with β glucosidase in order to yield glucose [10,11]. Accessory enzymes help remove barriers to cellulose hydrolysis, assisting in plant biomass degradation [12,13]. This group includes hemicellulases, lytic polysaccharide monooxigenases, expansin-like proteins, ligninases and oxidases, among others. Endoxylanases from glycoside hydrolase family 10 (GH10) [14] are the most used due to their ability to hydrolyze

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highly substituted xylose linkages [15]. Several reports highlight the synergy between cellulases and xylanases [16,17] as an essential tool for complete hemicellulose removal and efficient carbohydrate conversion.

Lytic polysaccharide monooxygenases (LPMOs, former GH61s) contribute to cellulose deconstruction by completely different mechanisms than glycoside hydrolases [18–20]. LPMOs are metal ion dependent monooxygenases that use the ion metal copper (II) for enzyme activation [21–23]. The discovery that GH61s are copper-dependent polysaccharide monooxygenases ultimately led to the reclassification of this family to an Auxiliary Activity family number 9 (AA9) in the Carbohydrate-Active Enzymes Database (CAZy) [24]. AA9 enzymes depend on reducing cofactors for activity [22], accomplished by ascorbic acid and other reductants for *in vitro* reactions and by cellobiose dehydrogenase (CDH) for *in vivo* reactions [25]. LPMOs are interesting from both fundamental and practical points of view as they increase the activity of cellulases on pure crystalline cellulosic substrates [22,25–29] as well as on real lignocellulosic substrates [21,30–32].

Unlike *T. reesei*, brown rot fungi such as *Gloeophyllum trabeum* have many genes encoding proteins with accessory activity [33]. They metabolize the hemicellulose and cellulose in wood by a two-step mechanism comprising non-enzymatic and enzymatic reactions, finally producing a brown lignin residue [34]. The non-enzymatic reactions involve wood cell wall decomposition by oxidative mechanisms, in which hydroxyl radicals selectively depolymerize cellulose [35]. Subsequently, cellulases and hemicellulases complete the breakdown of the carbohydrates [36]. For this purpose, *G. trabeum* secretes a battery of enzymes, several of which have been produced by recombinant methods. For example, the endoglucanase Cel5A is unusually active on microcrystalline cellulose (Avicel) [37], which probably compensates for the absence of cellobiohydrolases in this fungus. Endoxylanases GtXyn10A and GtXyn10B are thermostable enzymes [38], a property that makes them very useful in the hemicellulose processing industry. Furthermore, β-Glucosidase [39], alcohol oxidase [40,41] and two polysaccharide monooxygenases, one of which is active on xyloglucan [31,42], have been also identified. Despite their potential, *G. trabeum* enzymes are not widely used as cellulase cocktail additives to increase saccharification efficiency. In our laboratory, we have identified protein fractions from *G. trabeum* cultures that improve the hydrolysis of wheat straw by cellulases. The most abundant proteins in these extracts were the xylanase GtXyn10A and the polysaccharide monooxygenase GtLPMO (unpublished results). This suggests that these enzymes are important contributors towards the efficiency of *G. trabeum*'s cellulolytic system.

The objective of this study was to examine the auxiliary effects of GtXyn10A and GtLPMO, mixed with commercial cellulase, in the degradation of wheat straw. The effect of different proportions of these enzymes was examined, finding that the efficiency of the hydrolysis can significantly increase at optimal concentrations. Additionally, these experiments suggest that *G. trabeum* GtLPMO not only cleaves cellulose as previously reported, but also degrades xylan by a mechanism that is dependent on the presence of a reducing factor. GtLPMO activity on xylan can therefore be used to increase the efficiency of sugar recovery from lignocellulosic biomasses.

2. Material and methods

2.1. Fungus cultivation and maintenance

The fungus *Gloeophyllum trabeum* isolate CTB (925-B) was isolated from a native Chilean forest by members of the Laboratory of Biodegradation and Wood Preservation at the Faculty of Forest Sciences of the University of Chile. The fungus was maintained in Potato Dextrose Agar (PDA) plates at 4 °C and transferred onto fresh plates every month. For cellulolytic enzyme production, the fungus was cultured at 28 °C in liquid medium as reported by Chouiter et al. [43], except that 0.005 g/L $MgSO_4$ was used. The medium contained 1% (w/v) milled wheat straw as the only carbon source.

2.2. Wheat straw pretreatment

Wheat (*Triticum aestivum)* straw was obtained from a local farm in the Maule district of Chile. The biomass was physically processed using a SM2000 cutting mill. Pretreatment was conducted in 10% (w/v) NaOH for two hours at 80 °C [44]. After extensive rinsing with distilled water, the biomass was dried over-night at 50 °C and sieved through a 0.5 mm mesh. The composition of the pretreated wheat straw was determined following the National Renewable Energy Laboratory (NREL) protocols [45]. Glucose and xylose quantification was carried out by high performance liquid chromatography in a Shimadzu Prominence chromatograph, model 20A, equipped with a Refraction Index Detector and a Biorad Aminex HPX-87P column.

2.3. G. trabeum RNA isolation and cDNA synthesis

The fungus was grown on 1.5% (w/v) agar plates containing the medium previously described [43] except that 3% (w/v) milled wheat straw was used as the only carbon source. The plates with the solid medium were covered by a sterile nitrocellulose membrane and inoculated with one square centimeter agar of fresh fungal mycelia previously grown in a PDA medium [46]. After 8 days at 28 °C, the mycelium covering the entire plate was scraped with a scalpel and used as the starting material for RNA extraction. The RNA extraction was carried out using the Ambion® TRIzol® Reagent following the manufacturer's instructions. cDNA synthesis was prepared using Superscript III Reverse Transcriptase (Invitrogen) following the manufacturer's instructions.

2.4. Isolation and cloning of the GtLPMO and GtXyn10A genes

The sequences encoding for GtLPMO and GtXyn10A were identified from the *G. trabeum* genome sequence available in the JGI database (http://genome.jgi.doe.gov/Glotr1_1/Glotr1_1.home.html) [33,47]. For cloning the GtLPMO gene, the encoding sequence was isolated from the *G. trabeum* cDNA by a two-step PCR. In the first step, specific

Table 1
Primers used in this work.

Primer name	Sequence (5′®3′)	Purpose
GAXyl1For	GAACATGATTTCTAAGACCTTCACCG	Cloning GtXyn10A in pGemTeasy
GAXyl1Rev	TTTCCACTGCGCTCCACC	Cloning GtXyn10A in pGEM Teasy
XGT1FwEcoRI	GAATTCGCACCTCCTACTCCCGCC	Cloning GtXyn10A in pPIC9k
XGT1Rev AvrII	CCTAGGTTACTGCCAGCCAGCAAC	Cloning GtXyn10A in pPIC9k
GH61F1	ATGACGACAGACACGGGTACGTTG	Cloning GtPMO in pPIC9k
GH61R1	TGATGATGATGATTGCCGGTCCAG	Cloning GtPMO in pPIC9k
GH61F2	GAATTCCCTCTCGAGAAAAGAGATGATGACGACAGA	Cloning GtPMO in pPIC9k
GH61R2	CGCGGCCGTCAATGATGATGATGATGATG	Cloning GtPMO in pPIC9k

primers GH61F1 and GH61R1 (Table 1) were used to amplify the part of the gene corresponding to the predicted mature enzyme (i.e. without secretion signal). The amplified fragment was ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into *E. coli* strain Top10. This construct was used as a template in a second PCR with primers GH61F2 and GH61R2 (Table 1), which contained restriction sites for *Eco*RI and *Not*I, respectively. The presence of the expected sequence elements was confirmed by sequencing by Macrogen (Korea). The recombinant fragment was recovered from the pGEM-T Easy vector by *Eco*RI and *Not*I restriction and cloned into pPIC9 K using standard DNA cloning procedures. All the PCRs were performed using Phusion High-Fidelity DNA polymerase (New Englands Biolabs), according to the provider's guidelines.

For cloning the GTXyn10A gene, primers GAXyl1For and GAXyl1Rev were used in the amplification of the entire gene from the cDNA (Table 1). The fragment was ligated to the pGEM-T Easy vector and transformed into *E. coli* Top10. The recombinant plasmid produced was used as template for a PCR with primers XGT1FwEcoRI and XGT1RevAvrII in order to amplify the sequence encoding for the mature enzyme. The PCR product was restricted with *Eco*RI and *Avr*II and ligated to pPIC9K vector digested with the same restriction enzymes. DNA sequencing (Macrogen, Korea) confirmed the integrity of all the recombinant constructs.

2.5. Recombinant expression and purification of GtLPMO and GtXyn10A

Production of recombinants was carried out using the Multicopy Pichia Expression Kit (Life Technologies), according to the supplier's directions. *Sal*I-digested recombinant pPIC9 K plasmids were introduced into *Pichia pastoris* KM71 by electroporation. Transformants were selected in RDB agar plates and then in YPD plates supplemented with 0.25–2.0 mg/mL geneticin. Clones exhibiting the highest resistance to geneticin were preserved at −80 °C as 20% glycerol stocks. For protein production, the stocks were spread on YPD agar plates and isolated colonies were cultured in BMGY (Buffered Complex Medium containing Glycerol) until OD_{600} 6. For the induction phase, cells were transferred to BMMY medium (Buffered complex Medium with 0.5% (v/v) methanol) and cultured for 4 days at 25 °C at 300 rpm. Proteins in the supernatant were concentrated by ammonium sulfate precipitation. GtLPMO and GtXyn10A were purified by anion exchange chromatography in a 20 mL Q-Sepharose column (GE) equilibrated with 20 mM Tris HCl pH 7.6 using an Äkta Protein Purifier System (GE). Proteins were eluted with a linear gradient of 0–50% 1 M NaCl in 10 column volumes of 20 mM Tris HCl pH 7.6. Fractions containing the pure proteins, as determined by SDS PAGE, were pooled, concentrated and desalted in a 10 kDa Vivaspin 20 centrifugal ultrafiltration cassette (Sartorius). Concentrated proteins were stored at 4 °C for short periods (days) and frozen at −80 °C for longer periods.

2.6. Enzyme activity assays

The accessory effect of GtLPMO on the activity of Celluclast 1.5 L was measured in 50 mM sodium acetate pH 5 with one disc of 0.5 cm diameter Whatman N°1, 1 mM freshly prepared ascorbic acid, with or without 0.01–0.1 mg/mL Celluclast 1.5 L. Reactions were incubated for 2 h at 50 °C with 200 rpm agitation. The reducing sugars produced were quantified after reacting with dinitrosalicylic acid (DNS) [48] by measuring the absorbance at 550 nm in a microplate reader (Asys UVM 340) and compared to a glucose calibration curve. The percentage increase in activity was calculated as the amount reducing sugars produced by Celluclast 1.5 L mixed with GtLPMO compared to those produced with Celluclast 1.5 L alone.

The accessory effect of GtLPMO on the activity of GtXyn10A 1.5 L was evaluated on 1% (w/v) beechwood xylan (Megazyme) in a 50 mM sodium acetate buffer pH 5, 0.05–0.2 μg/mL GtXyn10A, with or without 1 mM ascorbic acid. Reducing sugars were measured with the DNS method described previously. The values of absorbance at 550 nm were converted to xylose reducing equivalents using a calibration curve made with xylose standard.

All the activity assays were carried out using purified GtLPMO and GtXyn10A. All the experiments were conducted at least three times.

2.7. Wheat straw hydrolysis

GtXyn10A (10–40 μg/mL) and GtLPMO (4–20 μg/mL) were combined with Celluclast 1.5 L (Sigma; 0.454 FPU per mg protein) in hydrolysis reactions containing 2% (w/v) dry matter (DM) of pretreated wheat straw. The hydrolysis reactions were carried out in 50 mM sodium acetate buffer pH 5.0, 5 g/L Tween 20, with orbital agitation (300 rpm) at 50 °C for up to 48 h. Celluclast 1.5 L and the auxiliary enzymes were included in concentrations that varied depending on the particular experiment. β-Glucosidase from *Aspergillus niger* (Sigma) was maintained at 30% of the Celluclast 1.5 L concentration to ensure rapid and complete conversion of cellobiose to glucose. All of the mixtures were supplemented with bovine serum albumin (BSA) to complete 2 mg protein per gram of DM. Reducing sugars were measured using the DNS method. Glucose and xylose were determined enzymatically with kits obtained from Randox Laboratories and Megazyme, respectively, following the providers directions. Relative increase in the production of each sugar was calculated as the experimental value, divided by the value obtained in an experiment with Celluclast 1.5 L alone, at the same concentration and performed in parallel.

2.8. Statistical analysis

The results were subjected to Student's *t*-test analyzes using Minitab 17, and values of $P \ll 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Recombinant expression of GtLPMO and GtXyn10A in Pichia pastoris

The gene sequences of *Gloeophyllum trabeum* GtLPMO and GtXyn10A were isolated from the *G. trabeum* cDNA. In each case, the gene sequence encoding for the mature part of the enzyme was fused to the alpha factor signal peptide present on the expression vector pPIC9K. The recombinant plasmid was transferred into *Pichia pastoris* and the protein was expressed in the culture's extracellular fraction after methanol induction. The separation process produced highly purified proteins (Fig. 1). GtXyn10A was produced at 22 mg per liter of culture, while GtLPMO was produced at 1 mg per liter. GtXyn10A migrated close to 38 kDa in SDS PAGE as was predicted from the sequence. On the other hand, GtLPMO produced a 50 kDa protein, almost twice the size predicted from the sequence; however, after deglycosylation with Rapid™ PNGase F, the protein migrated close to the expected 27 kDa (see lanes 2 and 3 in Fig. 1). This result shows that GTLPMO produced in *P. pastoris* is glycosylated, mainly modified with *N*-linked oligosaccharides.

3.2. GtLPMO auxiliary activity in model substrates

The auxiliary activity of the recombinant GtLPMO was evaluated by measuring its effect on the hydrolysis of filter paper (FP) by cellulase. Celluclast 1.5 L, a LPMO-free commercial mixture, was used as source of cellulase activity. Results in Fig. 2 indicate that GtLPMO improves the FP hydrolysis in a dose-dependent manner, reaching 60% improvement with 3.2 μg/mL, which corresponds to 20% of the total protein load. The improvement was observed only in presence of 1 mM ascorbic acid. In the absence of Celluclast 1.5 L, virtually no production of reducing sugars was observed. Similar experiments were carried out on carboxymethyl cellulose, resulting in no effect of GtLPMO on the Celluclast 1.5 L activity, whether in presence or absence of ascorbic

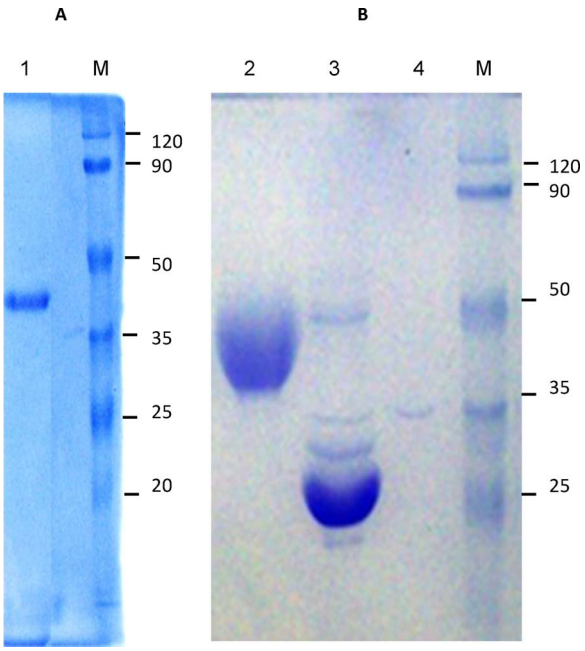

Fig. 1. SDS-PAGE of purified GtXyn10A (A) and GtLPMO (B) A) Lane 1, GtXyn10A; B) Lane 2, GtLPMO; Lane 3, GtLPMO after treatment with Rapid PNGase, lane 4, Rapid PNGase F. M: Molecular mass ladder.

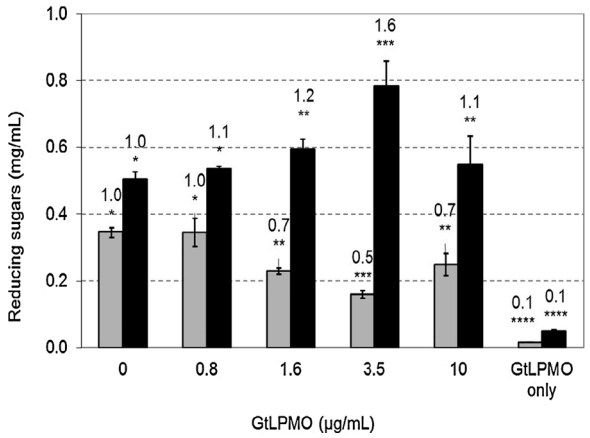

Fig. 2. Effect of GtLPMO on cellulose hydrolysis by Celluclast 1.5 L. Filter paper hydrolysis was carried out with 15 µg/mL Celluclast 1.5 L and supplemented with GtLPMO, without ascorbic acid (gray bars) or with 1 mM ascorbic acid (black bars). In "GtLPMO only" the GtLPMO concentration was 3.5 µg/mL. Error bars represent standard deviation of experiments ran in triplicate. For each condition, different numbers of asterisks (*) indicate a significant difference between GtLPMO concentrations by one-way ANOVA followed by Student's t – test ($P \ll 0.05$).

acid (data not shown). These results are consistent with previous data, that demonstrated the preference of LPMOs by crystalline substrates [22].

When a similar experiment was performed using beechwood xylan as substrate and GtXyn10A as the main hydrolytic enzyme, GtLPMO significantly boosted the GtXyn10A xylanolytic activity (Fig. 3A). After two hours at 50 °C, reducing sugar production increased 2.3 times with GtPMO and xylanase compared with xylanase alone, but after 18 h of incubation the maximum increase was only 20 percent with 8 ng/mL GtLPMO. The smaller boosting effect at 18 h incubation compared to 2 h may be due to thermo-inactivation of the monooxygenase. Inactivation of the xylanase is unlikely, based on literature [38] and our own results (data not shown). After 20 h at 50 °C the enzyme retained full hydrolytic activity on beechwood xylan. Results of control experiments carried out with the monooxygenase in the absence of xylanase demonstrated that when ascorbic acid is included in the reaction, a small but significant amount of reducing sugar is produced (Fig. 3B). The magnitude of reducing sugars production depends on the GtLPMO concentration in the reaction. In the absence of ascorbic acid (Fig. 3C), there was no production of reducing sugars, even after 18 h incubation. This suggests that the reducing sugars in Fig. 3A and B are not the result of residual hydrolytic contamination with an endoxylanase activity in the GtLPMO preparation.

Based on the sequence similarity and the product specificity, LPMOs can be classified into at least three types [49]. In a similar comparison we included GtLPMO in the amino acid sequence alignment and found that it shares several structural features with type 3 LPMOs (Supplementary Material S1). Type 1 and type 2 LPMOs hydroxylate C1 or C4 respectively, while type 3 LPMOs hydroxylate both C1 and C4. Based on the proposed cleavage mechanism of *gluco*-oligosaccharides in C4 [23,50,51], oxidation at the C4 position of a β-(1 → 4)-xylosyl bond in beechwood xylan could lead to a 4-ketoaldose. Oxidation of carbon 4 occurs simultaneously with the production of a reducing end at C1 [52,53]. This may explain the dose-dependent production of reducing sugars with GtLPMO exclusively in presence of ascorbic acid, as seen in Fig. 3B and C. Detailed characterization of the xylan cleavage reaction products is needed in order to complete our understanding of current results.

3.3. GtLPMO auxiliary activity on wheat straw

Firstly, the sugar composition of the wheat straw was determined. The alkali-pretreated biomass was completely hydrolyzed with sulfuric acid and the main monomeric sugars and lignin were quantified. Results showed that the after pretreatment with 10% NaOH the wheat straw was 83.3% (w/w) glucose, 6.5% (w/w) xylose, 0.57% (w/w) arabinose and 10% (w/w) total lignin (Table S1 Supplementary Material). This substrate was then used to investigate the effect of GtLPMO on wheat straw hydrolysis. Fig. 4A shows that when 50%, 25% and 10% Celluclast 1.5 L (in protein base) is substituted with GtLPMO, reducing sugar production is enhanced by 10%, 32% and 35% respectively, compared to hydrolysis with Celluclast 1.5 L with the same total protein loading. Results showed that substituting 10% of Celluclast 1.5 L with GtLPMO returns the highest reducing sugar production, c.a. 17% higher than by Celluclast 1.5 L alone. This effect was also observed with higher protein loads (Fig. 4B), although the degree of enhancement diminished as the protein loading increased. These results show that GtLPMO produced in *P. pastoris* in this work is active as an accessory protein on pretreated wheat straw degradation. The increments in hydrolysis are similar to those reported by Jung et al. [31], who studied GtLPMO combined with *Gloeophyllum trabeum* Xyl10G (a different xylanase from *G. trabeum*) and the endoglucanase GtCel5B on pretreated oak and kenaf.

The synergy observed between Celluclast 1.5 L and GtLPMO on wheat straw probably results from the complementary functions of the monooxygenase and endoglucanase activity present in the commercial mixture. Endoglucanases in cellulolytic mixtures create a synergistic interaction with LPMO, because the endoglucanases prefer the amorphous regions in cellulose and show very low activity on crystalline zones.

3.4. GtXyn10A activity on wheat straw hydrolysis

To study the ability of GtXyn10A to complement a cellulase mixture in wheat straw degradation, experiments were carried out where the total enzyme loading was maintained constant while different amounts of Celluclast 1.5 L were substituted with GtXyn10A. Results showed that when replacing 25% of Celluclast 1.5 L with GtXyn10A, production of reducing sugars was maintained without significant loss in glucose production and a 17% increase in xylose production (Fig. 5). An increase in xylose was observed even after decreasing Celluclast 1.5 L

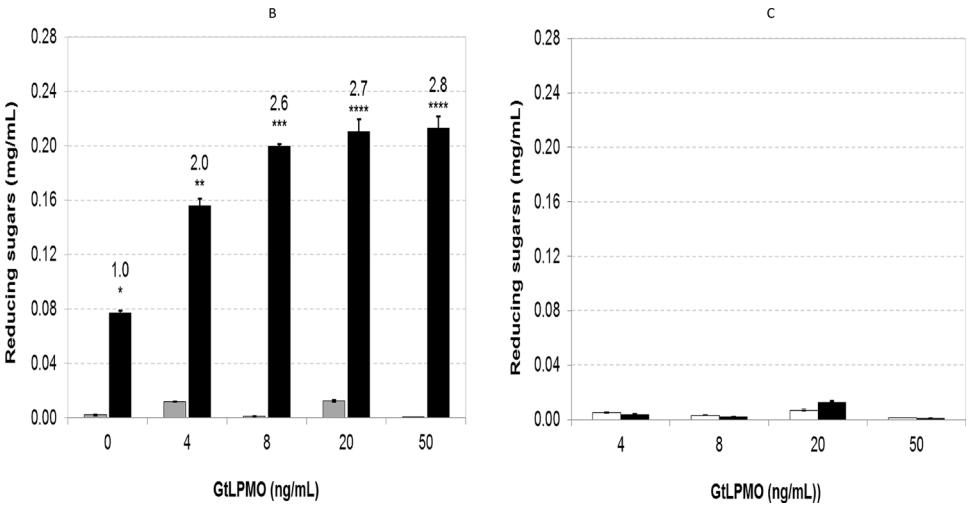

Fig. 3. Effect of GtLPMO on beechwood xylan hydrolysis by GtXyn10A. In A) 1% beechwood xylan was incubated with 0, 4, 8, 20 or 50 ng/mL GtLPMO, 75 ng/mL GtXyn10A and 1 mM ascorbic acid during 2 h (gray bars) or 18 h (black bars). "GtLPMO only" reactions contained 20 ng/mL GtLPMO with or without 1 mM ascorbic acid. In B) reactions were set up as in part A, but omitting GtXyn10A. In C) reactions were set up as in A, but omitting GtXyn10A and ascorbic acid. For each incubation time, the number of asterisks (*) indicates a significant difference between GtLPMO concentrations, determined by one-way ANOVA followed by Student's *t* – test ($P \ll 0.05$).

concentration by 50%, although that also resulted in a 20% reduction in glucose produced.

Removal of the hemicellulose covering the cellulose microfibrils by xylanase probably facilitated access of Celluclast 1.5L to the cellulose by increasing fiber porosity [13]. Processive cellobiohydrolases seemed to be the most affected by the blocking effect of the hemicellulose. Selig et al. showed that the addition of xylanolytic enzymes enhances the performance of cellobiohydrolases on pretreated corn stover [54], an effect attributed to the removal of non-cellulosic polysaccharides that coat cellulose fibers. The synergy between Celluclast 1.5 L and GtXyn10A in glucan conversion is probably a consequence of the co-operation between the xylanase and cellobiohydrolase components in Celluclast 1.5 L. GtXyn10A may also affect wheat straw hydrolysis by removing xylooligosaccharides that are accumulated due to insufficient xylanase activity in Celluclast 1.5 L. Qing et al. demonstrated that xylooligosaccharides, xylan, and xylose to a lesser degree, are strong inhibitors of cellulose hydrolysis [55].

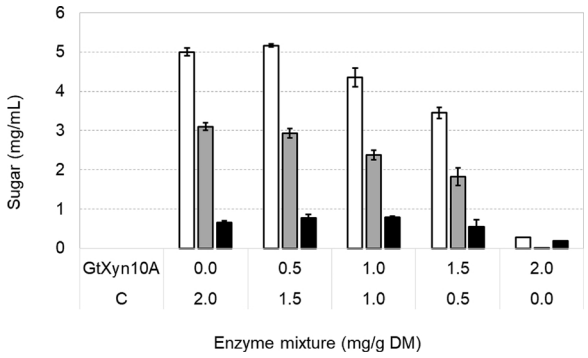

Fig. 5. Effect of Celluclast 1.5 L substitution by GtXyn10A on wheat straw hydrolysis. Pretreated wheat straw was incubated with a mixture of Celluclast 1.5 L and GtXyn10A. In all cases the total protein loading was maintained at 2 mg enzyme per g dry matter (DM). Total reducing sugars (white bars), glucose (gray bars) and xylose (black bars) were evaluated. "C" stands for Celluclast 1.5 L.

3.5. Effect of combinations of GtXyn10A and GtLPMO on wheat straw hydrolysis

The effect of double and triple combinations of Celluclast 1.5 L, GtLPMO and GtXyn10A on wheat straw hydrolysis were examined. The wheat straw hydrolysis was carried out with 1.5 mg Celluclast 1.5 L per g DM. GtLPMO was added during loadings at 0.2 or 0.5 mg per gDM, corresponding to approximately 10% and 25% of the total protein loading respectively. Additional reactions were carried out that included 0.5 mg GtXyn10A per g DM (equivalent to 25% of total protein loading). Hydrolysis was evaluated as the production of reducing sugars, i.e. the glucan to glucose conversion and the xylan to xylose conversion (Fig. 6). In the absence of GtXyn10A, 0.2 mg GtLPMO per g DM enhanced production of reducing sugars by 20%; however when 0.5 mg/g DM GtLPMO was used, production of reducing sugars was

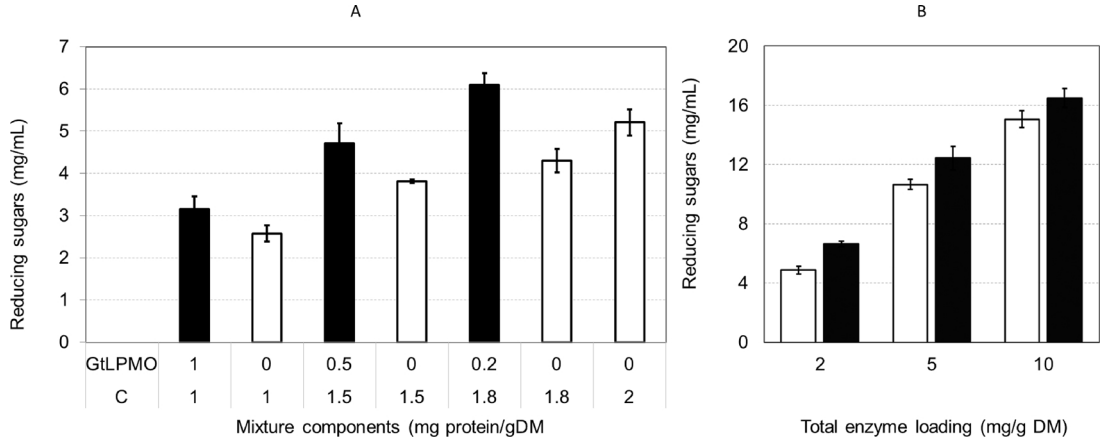

Fig. 4. Auxiliary role of GtLPMO on wheat straw hydrolysis by Celluclast 1.5 L. (A) Wheat straw hydrolysis by Celluclast 1.5 L supplemented with GtLPMO (black bars) or bovine serum albumin (white bars) at different proportions. (B) Wheat straw hydrolysis by Celluclast 1.5 L with 10% (w/w) protein loading substituted with GtLPMO (black bars) or bovine serum albumin (white bars). (C) Celluclast 1.5 L. Differences between Celluclast 1.5 L with or without GtLPMO were all statistically significant as evaluated by a Student's *t*- test ($P \ll 0.05$).

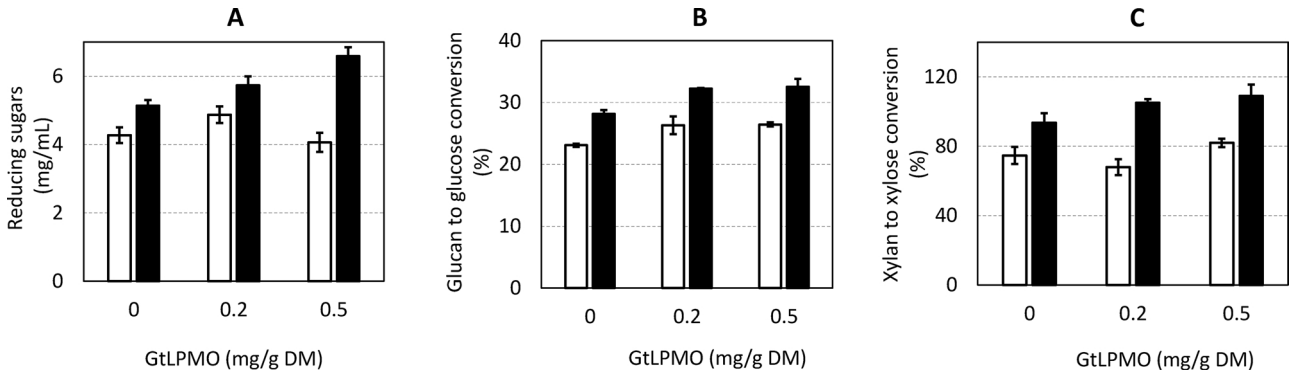

Fig. 6. Wheat straw hydrolysis by Celluclast 1.5 L, GtXyn10A and GtLPMO in double and triple combinations. Hydrolysis was carried out with 1.5 mg Celluclast 1.5 L/g dry matter in mixture with GtLPMO (white columns) or with GtLPMO plus 0.5 mg GtXyn10A/g dry matter (black columns). (A) Reducing sugars. (B) Glucan to glucose conversion and (C) Xylan to xylose conversion. Error bars represent standard deviation of experiments run by triplicate. Differences between binary (Celluclast 1.5 L plus GtLPMO) and tertiary mixtures (Celluclast 1.5 L plus GtLPMO and GtXyn10A) were all statistically significant as evaluated by a Student's *t*-student test ($P \ll 0.05$).

reduced by 10%. When Celluclast 1.5 L was combined with GtXyn10A, there was no hydrolysis inhibition due to increased GtLPMO concentrations, producing a synergistic increase of 54% in reducing sugars production, an additive increase of 40% in glucose production and a synergistic increase of 57% in the of xylan to xylose conversion.

The inhibition observed in hydrolysis reactions supplemented with 0.5 mg/g DM GtLPMO may result from oxidative inactivation of Celluclast 1.5 L caused by hydrogen peroxide [56,57]. Under limiting or non-available substrate concentrations, the LPMO's catalytic cycle produces superoxide ions, which spontaneously transform into H_2O_2 and react with transition metals to form highly toxic hydroxyl radicals and other reactive oxygen species [56]. Also, Celluclast 1.5 L could be inhibited by products of the LPMO reaction, such as oxidized cellodextrins, cellobionic acid and gluconic acid. It has been reported that cellobionic acid is a poor substrate for β-glucosidase, therefore it accumulates and competitively inhibits cellobiohydrolases and β-glucosidases [30].

As shown in Fig. 6, the negative impact of GtLPMO on the Celluclast 1.5 L activity was not observed when GtXyn10A was present. The presence of GtXyn10A and GtLPMO during hydrolysis produced additive increments in the glucan to glucose conversion, but synergistic enhancement of the xylan to xylose conversion. These results, together with the finding of GtLPMO being active in xylan (Fig. 3), allow us to infer that GtLPMO and GtXyn10A functionally cooperate to degrade xylan. The mechanism and the molecular bases for this cooperation are uncertain and must still be investigated.

Our results suggest that GtLPMO is able to act on xylose polysaccharides. Beechwood xylan is a polysaccharide with a low substitution degree, composed of *O*-acetyl-(4-*O*-methylglucurono) xylans and containing approximately one 4-*O*-methylglucuronic acid substituent for every 15 D-xylose residues [58]. To date, most of LPMOs that are active on hemicellulose use xyloglucan as a substrate [52,53] including GtLPMO9A-2, which is a second *G. trabeum* LPMO that shares 75% if its identity with GtLPMO [59]. Recently, Frommhagen et al. [60] described a *Myceliophthora thermophila* LPMO (MtLPMO9A) that cleaves β-(1 → 4)-xylosyl bonds in xylan, forming oxidized *xylo*-oligosaccharides, while simultaneously cleaving β-(1 → 4)-glucosyl bonds in cellulose, forming oxidized *gluco*-oligosaccharides. However, although MtLPMO9A was shown to be active on several commercial xylans, this effect occurs only if xylan is mixed with cellulose. Recently, Kim et al. [32] reported that a AA9 LPMO from *Chaetomium globosum* is active on cellulose and beechwood xylan. To our knowledge, this enzyme and GtLPMO are the only LPMOs that are active on both cellulose and linear xylan described to date. We have focused our efforts on optimizing reaction conditions, in order to take better advantage of the bi-functional characters of these enzymes in hydrolysis. They represent a valuable tool for improving high-scale recovery of simple sugars from lignocellulose.

4. Concluding remarks

Experimental results have confirmed the auxiliary role of GtLPMO and GtXyn10A on the hydrolytic activity of a commercial cellulase on pretreated wheat straw. GtLPMO improves the hydrolysis of model cellulose substrates by cellulase and xylan substrate by xylanase. At low concentrations GtLPMO boosts the hydrolysis of pretreated wheat straw by Celluclast 1.5 L and at high concentrations GtLPMO exerts an inhibitory effect on the hydrolytic activity. However, this inhibition is counteracted when GtXyn10A is included in the mixture. We show that, the combination of 20% GtLPMO, 20% GtXyn10A and 60% Celluclast 1.5 L resulted in significant improvements in total wheat straw hydrolysis, glucan conversion and xylan conversion, at 54%, 40% and 57% respectively. Our results suggest that, not only does GtLPMO plays an auxiliary role in lignocellulose hydrolysis by oxidative activity on glycosidic bonds, but also in hemicellulose hydrolysis by the oxidation of xylosyl bonds in xylan.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmictec.2017.11.007.

References

[1] V.K. Gupta, C.P. Kubicek, J.-G. Berrin, D.W. Wilson, M. Couturier, A. Berlin, E.X.F. Filho, T. Ezeji, Fungal enzymes for bio-products from sustainable and waste biomass, Trends Biochem. Sci. 41 (2016) 633–645, http://dx.doi.org/10.1016/j.tibs.2016.04.006.
[2] A.E.E. García, R.J.J. Carmona, M.E.E. Lienqueo, O. Salazar, The current status of liquid biofuels in Chile, Energy 36 (2011) 2077–2084, http://dx.doi.org/10.1016/j.energy.2010.06.005.
[3] S. Prasad, A. Singh, H.C. Joshi, Ethanol as an alternative fuel from agricultural, industrial and urban residues, Resour. Conserv. Recycl. 50 (2007) 1–39, http://dx.doi.org/10.1016/j.resconrec.2006.05.007.
[4] J. Puls, Chemistry and biochemistry of hemicelluloses: relationship between hemicellulose structure and enzymes required for hydrolysis, Macromol. Symp. 120 (1997) 183–196, http://dx.doi.org/10.1002/masy.19971200119.
[5] W.B. Betts, R.K. Dart, A.S. Ball, S.L. Pedlar, 10.1007/978-1-4471-3470-1, Biosynthesis and Structure of Lignocellulose, (1991), pp. 139–155.
[6] D.V. Evtuguin, J.L. Tomás, A.M. Silva, C.P. Neto, Characterization of an acetylated heteroxylan from Eucalyptus globulus Labill, Carbohydr. Res. 338 (2003) 597–604,

http://dx.doi.org/10.1016/S0008-6215(02)00529-3.
[7] J.C. Garcia, M. Diaz, M. Garcia, M. Feria, D. Gomez, F. Lopez, Search for optimum conditions of wheat straw hemicelluloses cold alkaline extraction process, Biochem. Eng. J. 71 (2013) 127–133, http://dx.doi.org/10.1016/J.BEJ.2012.12.008.
[8] R.H. Bischof, J. Ramoni, B. Seiboth, Cellulases and beyond: the first 70 years of the enzyme producer Trichoderma reesei, Microb. Cell Fact. 15 (2016) 106, http://dx.doi.org/10.1186/s12934-016-0507-6.
[9] D. Martinez, R.M. Berka, B. Henrissat, M. Saloheimo, M. Arvas, S.E. Baker, J. Chapman, O. Chertkov, P.M. Coutinho, D. Cullen, E.G.J. Danchin, I.V. Grigoriev, P. Harris, M. Jackson, C.P. Kubicek, C.S. Han, I. Ho, L.F. Larrondo, A.L. de Leon, J.K. Magnuson, S. Merino, M. Misra, B. Nelson, N. Putnam, B. Robbertse, A.A. Salamov, M. Schmoll, A. Terry, N. Thayer, A. Westerholm-parvinen, C.L. Schoch, J. Yao, R. Barabote, M.A. Nelson, C. Detter, D. Bruce, C.R. Kuske, G. Xie, P. Richardson, D.S. Rokhsar, S.M. Lucas, E.M. Rubin, N. Dunn-coleman, M. Ward, T.S. Brettin, A.L. De Leon, J.K. Magnuson, S. Merino, M. Misra, B. Nelson, N. Putnam, B. Robbertse, A.A. Salamov, M. Schmoll, A. Terry, N. Thayer, A. Westerholm-parvinen, C.L. Schoch, J. Yao, R. Barabote, M.A. Nelson, C. Detter, D. Bruce, C.R. Kuske, G. Xie, P. Richardson, D.S. Rokhsar, S.M. Lucas, E.M. Rubin, N. Dunn-coleman, M. Ward, T.S. Brettin, Genome sequencing and analysis of the biomass-degrading fungus Trichoderma reesei (syn Hypocrea jecorina), Nat. Biotechnol. 26 (2008) 553–560, http://dx.doi.org/10.1038/nbt1403.
[10] A. Berlin, V. Maximenko, N. Gilkes, J. Saddler, Optimization of enzyme complexes for lignocellulose hydrolysis, Biotechnol. Bioeng. 97 (2007) 287–296, http://dx.doi.org/10.1002/bit.21238.
[11] J. Hu, V. Arantes, J.N. Saddler, The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition o, Biotechnology 36 (2011).
[12] J.S. Van Dyk, B.I. Pletschke, A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-factors affecting enzymes, conversion and synergy, Biotechnol. Adv. (2012), http://dx.doi.org/10.1016/j.biotechadv.2012.03.002.
[13] K. Ulaganathan, B. Goud, M. Reddy, V. Kumar, J. Balsingh, S. Radhakrishna, Proteins for breaking barriers in lignocellulosic bioethanol production, Curr. Protein Pept. Sci. 16 (2015) 100–134, http://dx.doi.org/10.2174/1389203716021502151657l8.
[14] V. Lombard, H.G. Ramulu, E. Drula, P. Coutinho, B. Henrissat, The carbohydrate-active enzymes database (CAZy) in 2013, Nucleic Acids Res. 42 (2014) D490–D495, http://dx.doi.org/10.1093/nar/gkt1178.
[15] A. Pollet, J.A. Delcour, C.M. Courtin, Structural determinants of the substrate specificities of xylanases from different glycoside hydrolase families, Crit. Rev. Biotechnol. 30 (2010) 176–191, http://dx.doi.org/10.3109/07388551003645599.
[16] J. Hu, R. Chandra, V. Arantes, K. Gourlay, J.S. van Dyk, J.N. Saddler, The addition of accessory enzymes enhances the hydrolytic performance of cellulase enzymes at high solid loadings, Bioresour. Technol. 186 (2015) 149–153, http://dx.doi.org/10.1016/j.biortech.2015.03.055.
[17] X.-Y. Song, Y. Gao, Y.-M. Yang, W.-J. Xiao, S.-H. Liu, W.-C. Xia, Z.-L. Liu, L. Yi, Z.-B. Jiang, Synergistic effect of cellulase and xylanase during hydrolysis of natural lignocellulosic substrates, Bioresour. Technol. 219 (2016) 710–715, http://dx.doi.org/10.1016/j.biortech.2016.08.035.
[18] Z. Forsberg, A.K. Mackenzie, M. Sorlie, A.K. Rohr, R. Helland, A.S. Arvai, G. Vaaje-Kolstad, V.G.H. Eijsink, Structural and functional characterization of a conserved pair of bacterial cellulose-oxidizing lytic polysaccharide monooxygenases, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 8446–8451, http://dx.doi.org/10.1073/pnas.1402771111.
[19] I. Morgenstern, J. Powlowski, A. Tsang, Fungal cellulose degradation by oxidative enzymes: fromdysfunctional GH61 family to powerful lytic polysaccharide monooxygenase family, Brief. Funct. Genomics 13 (2014) 471–481, http://dx.doi.org/10.1093/bfgp/elu032.
[20] T.L.R. Corrêa, L.V. dos Santos, G.A.G. Pereira, AA9 and AA10: from enigmatic to essential enzymes, Appl. Microbiol. Biotechnol. 100 (2016) 9–16, http://dx.doi.org/10.1007/s00253-015-7040-0.
[21] P. Harris V, D. Welner, K.C. McFarland, E. Re, J.-C. Navarro Poulsen, K. Brown, R. Salbo, H. Ding, E. Vlasenko, S. Merino, F. Xu, J. Cherry, S. Larsen, L. Lo Leggio, Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family, Biochemistry 49 (2010) 3305–3316, http://dx.doi.org/10.1021/bi100009p.
[22] R.J. Quinlan, M.D. Sweeney, L. Lo, H. Otten, J.-C.N. Poulsen, T. Tryfona, C.P. Walter, P. Dupree, F. Xu, G.J. Davies, P.H. Walton, L. Lo Leggio, H. Otten, J.-C.N. Poulsen, K.S. Johansen, B.R.M. Krogh Kristian, C.I. Jorgensen, M. Tovborg, A. Anthonsen, T. Tryfona, C.P. Walter, P. Dupree, F. Xu, G.J. Davies, P.H. Walton, Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 15079–15084, http://dx.doi.org/10.1073/pnas.1105776108.
[23] W.T. Beeson, V. van Vu, E.A. Span, C.M. Phillips, M.A. Marletta, Cellulose degradation by polysaccharide monooxygenases, Annu. Rev. Biochem. 84 (2015) 923–946, http://dx.doi.org/10.1146/annurev-biochem-060614-034439.
[24] A. Levasseur, E. Drula, V. Lombard, P.M. Coutinho, B. Henrissat, Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes, Biotechnol. Biofuels 6 (2013) 41, http://dx.doi.org/10.1186/1754-6834-6-41.
[25] J.A. Langston, T. Shaghasi, E. Abbate, F. Xu, E. Vlasenko, M.D. Sweeney, Oxidoreductive cellulose depolymerization by the enzymes cellobiose dehydrogenase and glycoside hydrolase 61, Appl. Environ. Microbiol. 77 (2011) 7007–7015, http://dx.doi.org/10.1128/AEM. 05815-11.
[26] G. Vaaje-Kolstad, B. Westereng, S.J. Horn, Z. Liu, H. Zhai, An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides, Science 330 (2010) 219–222, http://dx.doi.org/10.1126/science.1192231.
[27] B.B. Westereng, T. Ishida, G. Vaaje-kolstad, M. Wu, V.G.H. Eijsink, K. Igarashi, M. Samejima, J. Stahlberg, S.J. Horn, M. Sandgren, The putative endoglucanase PcGH61D from Phanerochaete chrysosporium is a metal-dependent oxidative enzyme that cleaves cellulose, PLoS One 6 (2011) e27807, http://dx.doi.org/10.1371/journal.pone.0027807.
[28] I.J. Kim, K.H. Nam, E.J. Yun, S. Kim, H.J. Youn, H.J. Lee, I.-G. Choi, K.H. Kim, Optimization of synergism of a recombinant auxiliary activity 9 from Chaetomium globosum with cellulase in cellulose hydrolysis, Appl. Microbiol. Biotechnol. 99 (2015) 8537–8547, http://dx.doi.org/10.1007/s00253-015-6592-3.
[29] A.G. Bulakhov, A.V. Gusakov, A.V. Chekushina, A.D. Satrutdinov, A.V. Koshelev, V.Y. Matys, A.P. Sinitsyn, Isolation of homogeneous polysaccharide monooxygenases from fungal sources and investigation of their synergism with cellulases when acting on cellulose, Biochemistry 81 (2016) 530–537, http://dx.doi.org/10.1134/S0006297916050102.
[30] D. Cannella, C.-W.C. Hsieh, C. Felby, H. Jorgensen, Production and effect of aldonic acids during enzymatic hydrolysis of lignocellulose at high dry matter content, Biotechnol. Biofuels 5 (2012) 26, http://dx.doi.org/10.1186/1754-6834-5-26.
[31] S. Jung, Y. Song, H.M. Kim, H. Bae, Enhanced lignocellulosic biomass hydrolysis by oxidative lytic polysaccharide monooxygenases (LPMOs) GH61 from Gloeophyllum trabeum, Enzyme Microb. Technol. 77 (2015) 38–45, http://dx.doi.org/10.1016/j.enzmictec.2015.05.006.
[32] I.J. Kim, H.J. Youn, K.H. Kim, Synergism of an auxiliary activity 9 (AA9) from Chaetomium globosum with xylanase on the hydrolysis of xylan and lignocellulose, Process Biochem. 51 (2016) 1445–1451, http://dx.doi.org/10.1016/j.procbio.2016.06.017.
[33] D, M. Floudas, R. Binder, K. Riley, R.A. Barry, B. Blanchette, Henrissat, R. Martínez, J.W. Otillar, J.S. Spatafora, A. Yadav, I. Aerts, A. Benoit, A. Boyd, A. Carlson, P.M. Copeland, R.P. Coutinho, P. de Vries, K. Ferreira, B. Findley, J. Foster, D. Gaskell, P. Glotzer, J. Górecki, C. Heitman, C. Hesse, K. Hori, J.A. Igarashi, N. Jurgens, P. Kallen, A. Kersten, U. Kohler, T.K.A. Kües, A. Kumar, K. Kuo, L.F. LaButti, E. Larrondo, A. Lindquist, V. Ling, S. Lombard, T. Lucas, R. Lundell, D.J. Martin, I. McLaughlin, E. Morgenstern, C. Morin, L.G. Murat, M. Nagy, R.A. Nolan, A. Ohm, A. Patyshakuliyeva, G. Ruiz-Dueñas, A. Sabat, M. Salamov, J. Samejima, J.C. Schmutz, J. John, H. Stenlid, S. Sun, K. Sun, A. Syed, A. Tsang, D. Wiebenga, A. Young, D.C. Pisabarro, F. Eastwood, D. Martin, I.V. Cullen, D.S. Grigoriev, The paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes, Science 336 (2012) 1715, http://dx.doi.org/10.1126/science.1221748.
[34] B. Goodell, Brown-rot fungal degradation of wood: our evolving view, Wood Deterior Preserv. 845 (2003) 97–118, http://dx.doi.org/10.1021/bk-2003-0845(ch006).
[35] Z. Kerem, K.A. Jensen, K.E. Hammel, Biodegradative mechanism of the brown rot basidiomycete Gloeophyllum trabeum: evidence for an extracellular hydroquinone-driven fenton reaction, FEBS Lett. 446 (1999) 49–54, http://dx.doi.org/10.1016/S0014-5793(99)00180-5.
[36] E. Varela, T. Mester, M. Tien, Culture conditions affecting biodegradation components of the brown-rot fungus Gloeophyllum trabeum, Arch. Microbiol. 180 (2003) 251–256, http://dx.doi.org/10.1007/s00203-003-0583-y.
[37] R. Cohen, M.R. Suzuki, K.E. Hammel, Processive endoglucanase active in crystalline cellulose hydrolysis by the brown rot basidiomycete Gloeophyllum trabeum, Appl. Environ. Microbiol. 71 (2005) 2412–2417, http://dx.doi.org/10.1128/AEM.71.5.
[38] R. Sydenham, Y. Zheng, A. Riemens, A. Tsang, J. Powlowski, R. Storms, Cloning and enzymatic characterization of four thermostable fungal endo-1, 4-beta-xylanases, Appl. Microbiol. Biotechnol. 98 (2014) 3613–3628, http://dx.doi.org/10.1007/s00253-013-5244-8.
[39] H. Herr, D. Baumer, F. Dellweg, Purification and properties of an extracellular b-glucosidase from Lenzites trabea, Eur. J. Appl. Microbiol. Biotechnol. 5 (1978) 29–39, http://dx.doi.org/10.1007/BF00515684.
[40] G. Daniel, J.J. Volc, L. Filonova, O. Plíhal, E. Kubátová, P. Halada, O. Plihal, E. Kubatova, P. Halada, O. Plíhal, E. Kubátová, P. Halada, Characteristics of Gloeophyllum trabeum alcohol oxidase, an extracellular source of H2O2 in brown rot decay of wood, Appl. Environ. Microbiol. 73 (2007) 6241–6253, http://dx.doi.org/10.1128/AEM.00977-07.
[41] J. Tewalt, J. Schilling, Assessment of saccharification efficacy in the cellulase system of the brown rot fungus Gloeophyllum trabeum, Appl. Microbiol. Biotechnol. 86 (2010) 1785–1793, http://dx.doi.org/10.1007/s00253-010-2462-1.
[42] Y. Kojima, A. Várnai, T. Ishida, N. Sunagawa, D.M. Petrovic, K. Igarashi, J. Jellison, B. Goodell, G. Alfredsen, B. Westereng, V.G.H. Eijsink, M. Yoshida, Characterization of an LPMO from the brown-rot fungus Gloephyllum trabeum with broad xyloglucan specificity, and its action on cellulose-xyloglucan complexes, Appl. Environ. Microbiol. 82 (2016) 6557–6572, http://dx.doi.org/10.1128/AEM.01768-16.
[43] R. Chouiter, I. Roy, C. Bucke, Optimisation of \textgreek{b}-glucuronidase production from a newly isolated Ganoderma applanatum, J. Mol. Catal. B Enzym. 50 (2008) 114–120, http://dx.doi.org/10.1016/j.molcatb.2007.09.015.
[44] S. Suwannarangsee, B. Bunterngsook, J. Arnthong, A. Paemanee, A. Thamchaipenet, L. Eurwilaichitr, N. Laosiripojana, V. Champreda, Optimisation of synergistic biomass-degrading enzyme systems for efficient rice straw hydrolysis using an experimental mixture design, Bioresour. Technol. 119 (2012) 252–261, http://dx.doi.org/10.1016/j.biortech.2012.05.098.
[45] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, D. Crocker, Determination of Structural Carbohydrates and Lignin in Biomass: Determination of Structural Carbohydrates and Lignin in Biomass; NREL/TP-510-42618, US National Renewable Energy Determination of Sugars, Byproducts and degradation Products in Liquid Fract, 2008.

[46] U. Schumann, N.A. Smith, M.-B. Wang, A fast and efficient method for preparation of high-quality RNA from fungal mycelia, BMC Res. Notes 6 (2013) 71, http://dx.doi.org/10.1186/1756-0500-6-71.
[47] H. Nordberg, M. Cantor, S. Dusheyko, S. Hua, A. Poliakov, I. Shabalov, T. Smirnova, I.V. Grigoriev, I. Dubchak, The genome portal of the department of energy joint genome institute: 2014 updates, Nucleic Acids Res. 42 (2014) D26–D31, http://dx.doi.org/10.1093/nar/gkt1069.
[48] G.L. Miller, Determination of reducing sugar by DNS method, Anal. Chem. 31 (1959) 426–428.
[49] V.V. Vu, W.T. Beeson, C.M. Phillips, J.H.D. Cate, M.A. Marletta, Determinants of regioselective hydroxylation in the fungal polysaccharide monooxygenases, J. Am. Chem. Soc. 136 (2014) 562–565, http://dx.doi.org/10.1021/ja409384b.
[50] K.E.H. Frandsen, T.J. Simmons, P. Dupree, J.-C.N. Poulsen, G.R. Hemsworth, L. Ciano, E.M. Johnston, M. Tovborg, K.S. Johansen, P. von Freiesleben, L. Marmuse, S.S. Fort, S. Cottaz, H. Driguez, B. Henrissat, N. Lenfant, F. Tuna, A. Baldansuren, G.J. Davies, L. Lo Leggio, P.H. Walton, The molecular basis of polysaccharide cleavage by lytic polysaccharide monooxygenases, Nat. Chem. Biol. 12 (2016) 298–303, http://dx.doi.org/10.1038/nchembio.2029.
[51] P.H. Walton, G.J. Davies, On the catalytic mechanisms of lytic polysaccharide monooxygenases, Curr. Opin. Chem. Biol. 31 (2016) 195–207, http://dx.doi.org/10.1016/j.cbpa.2016.04.001.
[52] J.W. Agger, T. Isaksen, A. Várnai, S. Vidal-Melgosa, W.G.T. Willats, R. Ludwig, S.J. Horn, V.G.H. Eijsink, B. Westereng, Discovery of LPMO activity on hemicelluloses shows the importance of oxidative processes in plant cell wall degradation, Proc. Natl. Acad. Sci. 111 (2014) 6287–6292, http://dx.doi.org/10.1073/pnas.1323629111.
[53] A.S. Borisova, T. Isaksen, M. Dimarogona, A.A. Kognole, G. Mathiesen, A. Varnai, A.K. Rohr, C.M. Payne, M. Sorlie, M. Sandgren, V.G.H. Eijsink, A. Várnai, Å.K. Røhr, C.M. Payne, M. Sørlie, M. Sandgren, V.G.H. Eijsink, Structural and functional characterization of a lytic polysaccharide monooxygenase with broad substrate specificity, J. Biol. Chem. 290 (2015) 22955–22969, http://dx.doi.org/10.1074/jbc.M115.660183.
[54] M.J. Selig, T.B. Vinzant, M.E. Himmel, S.R. Decker, The effect of lignin removal by alkaline peroxide pretreatment on the susceptibility of corn stover to purified cellulolytic and xylanolytic enzymes, Appl. Biochem. Biotechnol. 155 (2009) 94–103, http://dx.doi.org/10.1007/s12010-008-8511-x.
[55] Q. Qing, B. Yang, C.E. Wyman, Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes, Bioresour. Technol. 101 (2010) 9624–9630, http://dx.doi.org/10.1016/j.biortech.2010.06.137.
[56] C.H. Kjaergaard, M.F. Qayyum, S.D. Wong, F. Xu, G.R. Hemsworth, D.J. Walton, N.A. Young, G.J. Davies, P.H. Walton, K.S. Johansen, K.O. Hodgson, B. Hedman, E.I. Solomon, Spectroscopic and computational insight into the activation of O2 by the mononuclear Cu center in polysaccharide monooxygenases, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 8797–8802, http://dx.doi.org/10.1073/pnas.1408115111.
[57] B.R. Scott, H.Z. Huang, J. Frickman, R. Halvorsen, K.S. Johansen, Catalase improves saccharification of lignocellulose by reducing lytic polysaccharide monooxygenase-associated enzyme inactivation, Biotechnol. Lett. 38 (2016) 425–434, http://dx.doi.org/10.1007/s10529-015-1989-8.
[58] A. Teleman, M. Tenkanen, A. Jacobs, O. Dahlman, Characterization of O-acetyl-(4-O-methylglucurono)xylan isolated from birch and beech, Carbohydr. Res. 337 (2002) 373–377, http://dx.doi.org/10.1016/S0008-6215(01)00327-5.
[59] Y. Kojima, A. Varnai, T. Ishida, N. Sunagawa, D.M. Petrovic, K. Igarashi, J. Jellison, B. Goodell, G. Alfredsen, B.B. Westereng, V.G.H. Eijsink, M. Yoshida, A. Várnai, T. Ishida, N. Sunagawa, D.M. Petrovic, K. Igarashi, J. Jellison, B. Goodell, G. Alfredsen, B.B. Westereng, V.G.H. Eijsink, M. Yoshida, A lytic polysaccharide monooxygenase with broad xyloglucan specificity from the brown-rot fungus Gloeophyllum trabeum and its action on cellulose-xyloglucan complexes, Appl. Environ. Microbiol. 82 (2016) 6557–6572, http://dx.doi.org/10.1128/AEM.01768-16.
[60] M. Frommhagen, S. Sforza, A.H. Westphal, J. Visser, S.W.A. Hinz, M.J. Koetsier, Willem J.H. van Berkel, H. Gruppen, M.A. Kabel, Discovery of the combined oxidative cleavage of plant xylan and cellulose by a new fungal polysaccharide monooxygenase, Biotechnol. Biofuels 8 (2015) 101, http://dx.doi.org/10.1186/s13068-015-0284-1.