Podospora anserina Hemicellulases Potentiate the *Trichoderma reesei* Secretome for Saccharification of Lignocellulosic Biomass[▽]

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To improve the enzymatic hydrolysis (saccharification) of lignocellulosic biomass by *Trichoderma reesei*, a set of genes encoding putative polysaccharide-degrading enzymes were selected from the coprophilic fungus *Podospora anserina* using comparative genomics. Five hemicellulase-encoding genes were successfully cloned and expressed as secreted functional proteins in the yeast *Pichia pastoris*. These novel fungal CAZymes belonging to different glycoside hydrolase families (*Pa*Man5A and *Pa*Man26A mannanases, *Pa*Xyn11A xylanase, and *Pa*Abf51A and *Pa*Abf62A arabinofuranosidases) were able to break down their predicted cognate substrates. Although *Pa*Man5A and *Pa*Man26A displayed similar specificities toward a range of mannan substrates, they differed in their end products, suggesting differences in substrate binding. The N-terminal CBM35 module of *Pa*Man26A displayed dual binding specificity toward xylan and mannan. *Pa*Xyn11A harboring a C-terminal CBM1 module efficiently degraded wheat arabinoxylan, releasing mainly xylobiose as end product. *Pa*Abf51A and *Pa*Abf62A arabinose-debranching enzymes exhibited differences in activity toward arabinose-containing substrates. Further investigation of the contribution made by each *P. anserina* auxiliary enzyme to the saccharification of wheat straw and spruce demonstrated that the endo-acting hemicellulases (*Pa*Xyn11A, *Pa*Man5A, and *Pa*Man26A) individually supplemented the secretome of the industrial *T. reesei* CL847 strain. The most striking effect was obtained with *Pa*Man5A that improved the release of total sugars by 28% and of glucose by 18%, using spruce as lignocellulosic substrate.

Lignocellulosic biomass is a desirable feedstock for the supply of second-generation bioethanol, being the largest renewable source of carbohydrates, but the recalcitrance of raw materials makes biotechnological conversion complex and costly (34).

The main components of plant cell walls are cellulose, lignin, and hemicellulose, which form a tight complex with varying proportions between plants (for a review, see reference 45). Cellulose microfibrils are formed by crystalline-like combination of linear and highly ordered polymer chains of β-1,4-linked D-glucose residues, and lignin consists of an amorphous aromatic heteropolymer with hydrophobic properties. Hemicellulose is a combined designation of a diverse set of noncrystalline carbohydrate polymers from plant tissues that form closely associated networks with cellulose microfibrils and lignin (6, 24). Typically, the most abundant component of hemicellulose in the cell walls of monocots (e.g., cereals) is β-1,4-xylan, which consists of β-1,4-linked D-xylose residues substituted with L-arabinosyl, 4-*O*-methyl-glucuronosyl and acetyl side chains (46, 53). Arabinofuranosyl residues of arabinoxylan may also be esterified with hydroxycinnamic acid residues, e.g., ferulic and *p*-coumaric acids (53). In softwoods (gymnosperms), the main hemicellulose component is β-1,4-mannan, which consists of a backbone formed by β-1,4-linked

D-mannose and D-glucose residues substituted with α-1,6-linked D-galactose residues (39). Linear mannan and glucomannan chains containing more than 5% (wt/wt) D-galactose are called galactomannans and galactoglucomannans, respectively (39).

The proportion of monosaccharide residues in plant biomass degradation products depends on the raw material and the hydrolysis procedure, but significantly, pentose sugars such as D-xylose, constitute the second most abundant carbohydrate in plant raw materials after glucose (26). Thus, it is considered now that the use of all sugar monomers is one of the possible strategies to provide cost-efficient biorefinery processes (28), which is partly due to the recent progress made with pentose-fermenting yeast strains (36). Therefore, bioconversion of hemicelluloses into fermentable sugars is essential to enable the commercial production of lignocellulosic ethanol (24).

One of the crucial steps in the bioconversion of LCB to fuel-grade ethanol is the enzymatic degradation of this recalcitrant biomass (28). The ascomycete *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) is one of the most widely used industrial fungi, able to produce cellulase-rich enzyme cocktails with up to 100 g of extracellular protein per liter of culture (13). Such high yields enable a wide application of *T. reesei* enzyme preparations in different fields of white biotechnology, in the bioconversion of biomass to fuel and other useful products (34). Recent genetic and biochemical technologies have improved our knowledge of the full set of *T. reesei* enzymes. Although analysis of the secretome of *T. reesei* CL847 industrial strain revealed only 22 secreted enzymes, most of them involved in biomass degradation (27), complete genome se-

quencing of the QM6a strain (35) identified about 200 genes encoding glycoside hydrolases as defined by the CAZy classification (10). Despite this high number, the *T. reesei* genome is surprisingly poor in terms of number and diversity of carbohydrate-degrading enzymes, including GHs, polysaccharide lyases, and carbohydrate esterases potentially able to participate in LCB degradation, compared to other fungi known for their carbohydrate-degrading capacity (35). In addition, the *T. reesei* genome has the smallest number of carbohydrate-binding modules of all of the *Sordariomycetes* sequenced to date (35). The greatest current challenge facing the development of second generation biofuels is enzyme cost reduction, the main strategy to meet this goal being the improvement of the efficiency of *T. reesei* enzymatic cocktail for the saccharification of LCB (34).

Possible strategies to circumvent *T. reesei* weaknesses in LCB hydrolysis include the search for enzymatic activities able to supplement those of *T. reesei* industrial enzyme preparations. The identification of novel glycosidases that are either distantly related to or absent from the *T. reesei* genome is a promising option for enhancing enzymatic hydrolysis of lignocellulosic materials. The increasing number of sequenced genomes annotated for their content of genes encoding for CAZymes (i.e., CAZomes) is a potential source of novel enzymes that warrants exploration. Among the fungal genomes already sequenced, that of the coprophilic ascomycete *Podospora anserina*, a late grower in the droppings of grass herbivores, encodes for a large, highly specialized set of genes involved in the utilization of carbon sources commonly found in its natural biotope, together with the highest number of CBMs of all of the fungal genomes sequenced to date (19). Interestingly, its CAZome has also revealed an increased potential for the digestion of resistant components of hemicellulose, presenting abundant enzyme sets dedicated to the hydrolysis of cross-linked xylan (19), from which no CAZymes have yet been characterized experimentally.

Owing to the heterogeneity of the hemicellulose fraction of LCB, complete hydrolysis requires a broad variety of cooperatively acting enzymes, such as xylanases, xylosidases, arabinofuranosidases, glucuronidases, acetylxylan esterases, ferulic acid esterases, and *p*-coumaric acid esterases for the xylan fraction and mannanases, galactosidases, glucosidases, and acetylmannan esterases for the mannan fraction. Until now, most of the characterization and applied studies on hemicellulases have focused on xylanases found in CAZy families GH10 and GH11. Accessory enzymes, such as arabinose-debranching enzymes, are found in six families (GH3, GH43, GH51, GH54, GH62, and GH93), whose members display different specificities. Although mannan is the principal hemicellulose component in softwood, studies of mannan-hydrolyzing enzymes found in families GH5 and GH26 have been largely neglected compared to xylanases.

The objective of the present study was to investigate the potential of single enzyme candidates from the hemicellulolytic enzymatic arsenal of *P. anserina* for improving the degradation of LCB by an industrial strain of *T. reesei*. By means of genome analysis using CAZy database tools, we identified genes encoding enzymes that degrade carbohydrates distantly related to or absent from *T. reesei*. We thus selected and cloned a set of genes from *P. anserina* encoding putative hemicellulases, which

were heterologously expressed and purified to give insight into their biochemical characteristics and enzymatic specificities. The *T. reesei* enzyme cocktail was further used as a reference to investigate the contribution made by each specific *P. anserina* auxiliary enzyme on wheat straw and/or spruce, taking advantage of the recently developed automated saccharification method (40).

MATERIALS AND METHODS

Abbreviations. Abf, α-L-arabinofuranosidase; AFMB, autoclaved fraction of maize bran; AX, arabinoxylan; CAZy, carbohydrate active enzyme; CBM, carbohydrate binding module; CE, carbohydrate esterase; DNS, dinitrosalycilic acid; d.m., dry matter; GH, glycoside hydrolase; HPAEC-PAD, high-performance anion exchange chromatography coupled with pulsed amperometric detection; IEF, isoelectric focusing; LBG, locust bean gum; LCB, lignocellulosic biomass; Man, endo-β-1,4-mannanase; PL, polysaccharide lyase; PX, mixture of pectin, birchwood, and larchwood xylans; Xyn, endo-β-1,4-xylanase.

Bioinformatics-guided sequence selection. Previous analysis of the *T. reesei* (anamorph of *Hypocrea jecorina*) strain QM6a genome had revealed weaknesses in hemicellulolytic capacity (35). Candidate hemicellulolytic CAZymes from the *P. anserina* genome were subjected to BLASTp analysis against all sequences present in CAZy (10) supplemented by *T. reesei* CAZyme sequences (35). A number of *P. anserina* candidates that were (i) absent or distant from those found in the *T. reesei* genome, (ii) distant from known characterized enzymes from other sources, and/or (iii) displayed an unusual combination of catalytic and carbohydrate-binding modules, were selected for cloning and characterization. For each individual target, the enzyme activity was predicted from the sequence comparisons with BLASTp against a specific library of characterized enzyme modules derived from CAZy. As a complement, the signal peptide cleavage sites were predicted using the signalP 3.0 server (http://www.cbs.dtu.dk/services /SignalP/).

Cloning of genes encoding hemicellulases. The homokaryotic *P. anserina* strain S mat+ used in the present study was kindly provided by P. Silar (CNRS, Paris, France). Currently used methods and culture media can be accessed at the *P. anserina* Genome Project web site (http://podospora.igmors.u-psud.fr). For the induction of enzymes, *P. anserina* was grown on M2 medium supplemented with AFMB (autoclaved fraction of maize bran at 15 g liter^{-1}) or PX (pectin, birchwood xylan, and larchwood xylan from Sigma-Aldrich (Lyon, France), each at 5 g liter^{-1}. AFMB was prepared as described previously (7). Growth was carried out in baffled flasks at 120 rpm and 27°C. The mycelium was recovered by sterile filtration using miracloth after 3, 4, and 5 days of culture and directly frozen at −80°C. Total RNA was extracted by using RNeasy plant kit (Qiagen, Courtaboeuf, France), and first-strand cDNA synthesis was performed with SuperScript reverse transcriptase (Invitrogen, Cergy-Pontoise, France) and oligo(dT$_{18}$) primer according to the manufacturer's instructions. DNA was amplified by PCR using *Pwo* Super yield DNA polymerase (GE Healthcare, Buc, France) and the specific primers (including restriction sites) designated in Table 1. The amplified fragments were subcloned into pCRII-TOPO vector (Invitrogen) and subjected to sequencing, and the corresponding genes were deposited in GenBank (for accession numbers, see Table 2 and the legend to Fig. 1). Amplified fragments were inserted at the corresponding sites into vector pPICZαA or pPICZαC (Table 1) in frame with both the yeast α-secretion factor and the C-term-His$_6$ tag encoding sequences. The resulting recombinant expression plasmids were linearized with PmeI and transformed into *P. pastoris* X-33 (Invitrogen) by electroporation. *P. pastoris* transformants were isolated on plates containing increasing concentrations of zeocin ranging from 100 to 1,000 μg ml^{-1}. Zeocin-resistant *P. pastoris* transformants were then screened for protein expression in 10 ml of BMGY (in 50-ml tubes) at 30°C in an orbital shaker (200 rpm) for 16 h to an optical density at 600 nm of 2 to 6, and expression was induced by transferring cells into 2 ml of BMMY, followed by growth for another 3 days. Each day the medium was supplemented with 3% (vol/vol) methanol. The supernatant was then analyzed by SDS-PAGE to determine the transformant with the best secretion yield for each enzyme.

Recombinant enzymes production and purification. For each enzyme, the best-producing transformant was grown in 200 ml of BMGY in a 1-liter baffled flask. The cells were then transferred to 40 ml of BMMY in 200-ml flasks stirred at 200 rpm and 30°C. The suspension was recovered after 3 days induction by centrifugation (10 min, 4,000 × g), and the supernatant was concentrated 10 times by using Amicon centrifugal units (10-kDa cutoff, 4,000 × g; Millipore, Molsheim, France). A nickel chelate His-Bind Resin (GE Healthcare) column

TABLE 1. Oligonucleotide sequences used in this study

Orientation and primer	Sequence (5′–3′)[a]	Destination vector
Forward		
PaMan5AEcoF	TTTGAATTCCTCCCCCAAGCACAAGGTG	pPiCZαA
PaXyn11AEcoF	TTTGAATTCCGTCCTTTCGACTTTCTCG	pPiCZαA
PaMan26AClaF	TTTATCGATAAAGCCTTGTAAGCCTCGTG	pPiCZαC
PaAbf51AEcoF	TTTTTTGAATTCATTGATTTGTTTGTCAAGTC	pPiCZαA
PaAbf62AEcoF	TTGAATTCTCTTCCCTGCCAGCTGGGC	pPiCZαA
Reverse		
PaMan5AXbaR	TTTTCTAGACCCGCCGGGAGAGCATTGATAG	pPiCZαA
PaXyn11AXbaR	TTTTCTAGACGTGTTCTGGACATAAATGTC	pPiCZαA
PaMan26AXbaR	TTTTCTAGACCACTCCTCCACCCCTGAATCTC	pPiCZαC
PaAbf51AXbaR	TTTTCTAGACCGTTACCCTTTCCCTTGCCCTTG	pPiCZαA
PaAbf62AXbaR	TTTTCTAGACCACAAGCAGGGTTGTTGTGTGCG	pPiCZαA

[a] The restriction sites added to insert genes into pPICZαA or pPICZαC are underlined.

(0.7 by 5 cm) was connected to a fast-performance liquid chromatography Äkta apparatus (GE Healthcare) and equilibrated with 50 mM Tris-HCl (pH 7.8), 150 mM NaCl, and 10 mM imidazole. The concentrated supernatant was diluted into the equilibration buffer and loaded onto the column at 4°C. The column was washed with equilibration buffer, and the enzyme was eluted with the same buffer containing 150 mM imidazole. The eluate was concentrated in the centrifugal units and repeatedly washed with sodium acetate buffer (50 mM, pH 5).

Biochemical characterization. Protein concentration was determined by using the Bio-Rad protein assay kit with bovine serum albumin as the standard (Bio-Rad, Marnes-la-Coquette, France). SDS-PAGE was performed in 12% (wt/vol) polyacrylamide gel (Bio-Rad) using a Pharmacia LMW electrophoresis calibration kit (GE Healthcare). Native IEF was carried out at 4°C in the Bio-Rad gel system, using pI standards ranging from 4.45 to 8.2 (Bio-Rad). Proteins were visualized with either Coomassie staining or IEF gel staining solution (0.04% [wt/vol] Coomassie blue R250, 0.05% [wt/vol] Cocrein Scarlet, 10% [vol/vol] acetic acid, 27% [vol/vol] isopropanol).

Enzyme assays. Unless otherwise indicated, assay mixtures contained substrate and suitably diluted enzyme in McIlvaine's buffer (pH 5). Reactions were carried out at 37°C for 5 to 30 min, and the enzyme activity was determined spectrophotometrically. The activity of enzymes toward xylan, mannan, or arabinan was measured by using the 3,5-dinitrosalicylic acid method described earlier (2). Briefly, 10 μl of enzyme was mixed with 190 μl of substrate (xylan or mannan at 10 mg ml^{-1}) in McIlvaine's buffer (pH 5) at 37°C. The reaction was terminated by the addition of 300 μl of dinitrosalicylic acid reagent at 1% (wt/vol) and boiled for 5 min. The reaction mixtures were cooled and centrifuged for 5 min at 13,000 rpm, and 200 μl was transferred to a microtiter plate. The absorbance at 550 nm was measured relative to a xylose or mannose standard curve (0 to 180 μg ml^{-1}). One unit of endo-glycosidase activity was defined as the amount of protein that released 1 μmol of sugar monomer per min. Low-viscosity wheat arabinoxylan, konjac glucomannan, carob galactomannan, ivory nut mannan, sugar beet arabinan, and debranched arabinan were purchased from Megazyme International (Wicklow, Ireland). Birchwood xylan, carboxymethyl cellulose, and locust bean gum galactomannan were purchased from Sigma-Aldrich. Activity toward pNP-α-ʟ-arabinofuranoside (Sigma-Aldrich) was determined by measuring the release of 4-nitrophenol from *p*-nitrophenyl-α-ʟ-arabinofuranoside (pNP-Ara; 10 mM; Sigma-Aldrich) in 50 mM sodium phosphate buffer (pH 5) at 37°C in a reaction volume of 100 μl. The reaction was

stopped by adding 900 μl of 1 M sodium carbonate (pH 9), and the release of 4-nitrophenol was quantified at 410 nm with a molar extinction coefficient for 4NP of 18,300 M^{-1} cm^{-1}. One unit of enzyme activity was defined as the amount of protein that released 1 μmol of arabinose per min.

The apparent optimal pH was estimated by using wheat arabinoxylan or LBG galactomannan at 10 mg ml^{-1} in McIlvaine's buffer in the pH range from 2.6 to 7.5 (11 pHs tested). The apparent optimal temperature was estimated in the range 30 to 70°C (nine temperatures tested) under the conditions used. For determination of apparent Michaelis-Menten constants, the initial velocities of the enzymes were measured at 37°C in McIlvaine's buffer (pH 5.0), with substrate concentrations ranging from 2 to 20 mg ml^{-1} for polymeric substrates and from 0.1 to 20 mM for pNP substrates. The molar concentrations of polymeric substrates could not be calculated because of their heterogeneous nature. Consequently, only an apparent value for the Michaelis constant, $K_{m(\text{app})}$, was determined. The kinetic parameters were estimated by using weighted nonlinear squares regression analysis with Grafit software (Erithacus Software, Horley, United Kingdom).

Determination of the *Pa*Man26A N-terminal CBM35 dissociation constant and the carbohydrate binding capacity. For the binding assay, the reaction mixture contained 1 mg of insoluble wheat arabinoxylan or insoluble ivory nut mannan (Megazyme) or Avicel PH101 (Sigma-Aldrich) and 10 to 50 μg of *Pa*Man26A ml^{-1} in a final volume of 225 μl of sodium citrate buffer (pH 5). For each assay, the mixture was incubated at 4°C for 1 h with vertical rotation (20 rpm). Polysaccharide was removed by centrifugation, and the concentration of unbound protein ([UB], μM) in the supernatant was measured by the Bradford method. The bound protein concentration ([B], μmol per g of polysaccharide) was determined by subtracting [UB] from the total protein concentration. All of the assays were carried out in triplicate. Adsorption parameters were determined as described previously (41) based on typical double-reciprocal plots using the equation [B] = [UB] × [B]$_{\text{max}}$/(K_d + [UB]), where K_d (μM) and [B]$_{\text{max}}$ (μmol per g of polysaccharide) are the equilibrium dissociation constant and the maximum amount of protein bound, respectively.

Supplementation of *T. reesei*. The lignocellulosic raw materials, wheat straw (*Triticum aestivum* cv. Apache; Aveyron, France) and spruce (*Picea abies*, Holmen plant; Braviken, Sweden) were kindly prepared by X. Rouau (INRA Montpellier, France). They were reduced to particles of roughly 100 μm by a multistep micronization procedure as previously described (40). Suspensions were made by

TABLE 2. Characteristics of the *P. anserina* hemicellulase-encoding genes selected

P. anserina gene	Enzyme family	CBM family	GenBank accession no. (locus)	Putative activity	% Identity to *T. reesei* characterized GH (amino acid sequence)	Condition of induction[a]
PaMan5A	GH5		HM357135 (Pa_6_490)	Endo-β-1,4-mannanase	62% with *TrMan5A*	AFMB/PX
PaMan26A	GH26	CBM35 (N-term)	HM357136 (Pa_5_1950)	Endo-β-1,4-mannanase	NA[b]	AFMB/PX
PaXyn11A	GH11	CBM1 (C-term)	HM357137 (Pa_2_13730)	Endo-β-1,4-xylanase	47% with *TrXyn1*, 64% with *TrXyn2*	AFMB/PX
PaAbf51A	GH51		HM357138 (Pa_5_11670)	α-ʟ-Arabinofuranosidase	NA	AFMB/PX
PaAbf62A	GH62		HM357139 (Pa_0_1370)	α-ʟ-Arabinofuranosidase	35% with *TrAbf2*	AFMB

[a] mRNA expression was induced using AFMB or pectin and xylan (PX) (see Materials and Methods).
[b] NA, not applicable. That is, no member of this family was present in *T. reesei* based on genomic data.
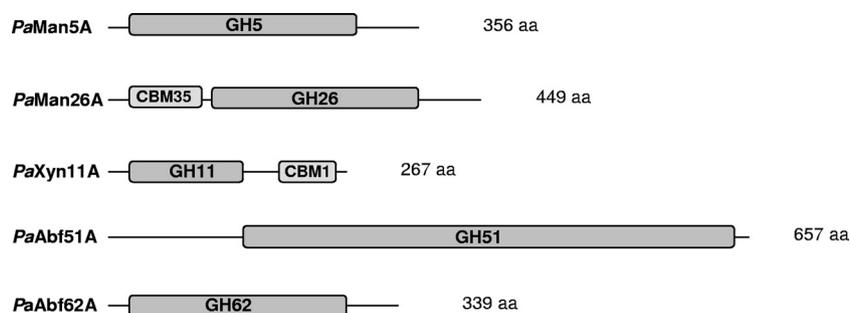
FIG. 1. Modular representation of hemicellulases used in the present study. GH, glycoside hydrolases; CBM, carbohydrate-binding modules. The corresponding GenBank accession numbers are HM357135 (*Pa*Man5A), HM357136 (*Pa*Man26A), HM357137 (*Pa*Xyn11A), HM357138 (*Pa*Abf51A), and HM357139 (*Pa*Abf62A).

adding 1 g of wheat straw or 2 g of spruce to 100 ml of 50 mM sodium acetate (pH 5) in the presence of 30 μg of cycloheximide ml^{-1} and 40 μg of tetracycline ml^{-1}. Using the handling robot (Tecan, Männedorf, Switzerland), 96-well microplates were filled with micronized wheat straw or spruce suspensions, heat sealed, and stored frozen at −20°C until use; then, 30 μg per well of *T. reesei* industrial xylanase-rich enzyme cocktail (E508) produced from *T. reesei* CL847 strain (49) was used as a reference in the assay as described previously (40). *T. reesei* enzyme cocktail contained 0.12 U of filter paper activity, 0.33 U of CMCase, 0.2 U of β-glucosidase, 1.6 U of xylanase, 0.02 U of mannanase, and 0.02 U of arabinofuranosidase per mg of total protein at 37°C (pH 5.0). To investigate the contribution of each specific *P. anserina* auxiliary enzyme to the saccharification of micronized wheat straw and/or spruce, 30 μg of *Pa*Man5A, 30 μg of *Pa*Man26A, 10 μg of *Pa*Xyn11A, 30 μg of *Pa*Abf51A, and 30 μg of *Pa*Abf62A were individually added to the 30 μg of *T. reesei* enzymatic cocktail. A total of 30 μg of equal amounts of *Pa*Man5A and *Pa*Man26A was also assayed in combination on spruce. The plate was heat sealed and transferred to a shaking incubator set at 40°C. The enzymatic reaction was left to proceed for 24 h, and the plate was filtered. The total reducing sugars were quantified by using the DNS assay as described previously (40). Monosaccharides (glucose, xylose, and mannose) were quantified by HPAEC-PAD (see below). Saccharification results were expressed in μmol of sugar released per mg of d.m.

Analysis of sugar release by HPAEC-PAD. Monosaccharides generated after hydrolysis of micronized LCB were analyzed by HPAEC coupled with PAD (ICS 3000; Dionex, Sunnyvale, CA) equipped with a Carbo-Pac PA-1 analytical column (250 by 4 mm). Enzymatic reactions were stopped by the addition of 50 mM NaOH before injection (10 μl) into the HPAEC system. Elution (1 ml min^{-1}) was carried out in 18 mM NaOH. Calibration curves were plotted using arabinose, glucose, xylose, mannose, and cellobiose standards (Sigma-Aldrich), from which response factors were calculated (Chromeleon program; Dionex) and used to estimate the amount of product released in test incubations. All of the assays were carried out in triplicate. The results are expressed in μmol of sugar released per mg of d.m. End products generated after hydrolysis of polymeric substrates (ivory nut mannan and wheat arabinoxylan) were analyzed as described earlier (4). The reference β-1,4-xylo-oligosaccharides and β-1,4-manno-oligosaccharides were obtained from Megazyme International.

RESULTS

Selection of genes encoding fungal hemicellulases and sequence analysis. Genes encoding polysaccharide-degrading enzymes were selected based on (i) structural features of lignocellulosic raw materials selected for the present study, i.e., wheat straw and spruce; (ii) BLASTp searches using CAZy database by comparison of *T. reesei* and *P. anserina* genomes; (iii) the presence and diversity of CBMs; and (iv) the predicted specificity of putative exo- and endo-acting CAZymes. The search yielded the sequences corresponding to the loci Pa_6_490 (designated *PaMan5A* following characterization), Pa_5_1950 (*PaMan26A*), Pa_2_13730 (*PaXyn11A*), Pa_5_11670 (*PaAbf51A*), and Pa_0_1370 (*PaAbf62A*).

One of our main goals was to select targets that would contribute to β-mannan degradation. Sequence comparison showed that *Pa*Man5A exhibited 62% identity to the catalytic module of *T. reesei* β-mannosidase *Tr*Man5A, a well-characterized enzyme (48). Unlike *Tr*Man5A, which bears a CBM1 module at C-term, *Pa*Man5A bears no additional module. Since genome analysis had revealed that likely orthologs from different *Trichoderma* bear the C-terminal CBM, while a variety of other fungi bear none (data not shown), *Pa*Man5A was selected as a target. The second selected enzyme degrading β-mannan was *Pa*Man26A, which has no equivalent in the *T. reesei* genome. Interestingly, only a very reduced number of fungal genomes present closely related catalytic modules (unpublished data). The closest characterized enzyme is the β-mannanase from *Humicola insolens* (30), with 79% identity at the catalytic module, followed by a number of bacterial β-mannanases. All closely related enzymes have CBM35 modules adjacent to the N-terminal side of the GH26 catalytic module, but the substrate affinity of the corresponding binding modules has never been studied.

The next hemicellulolytic target *Pa*Xyn11A was selected, although its catalytic module presented, respectively, 47 and 64% sequence identity to *T. reesei* xylanases TrXyn1 and TrXyn2, two long-established enzymes. (51). The main singularity of this sequence is the fact that it carries a C-term CBM1 module. The number of characterized GH11 enzymes bearing a C-term CBM1 module is relatively small. They include xylanases from *Penicillium funiculosum* XynB (9, 25), *Neocallimastix patriciarum* XynS20 (33), *Lentinula edodes* Xyn11A (32), and *Phanerochaete chrysosporium* XynB/XynB-1 (17). Although the substrate specificities of these fungal CBM1-containing enzymes have been described, none was chosen for that feature.

Finally, two enzyme targets for arabinofuranose-containing substrates were considered, *Pa*Abf51A and *Pa*Abf62A. The first has no equivalent in the *T. reesei* genome, and the closest characterized sequences are those of the α-L-arabinofuranosidases Afq1 from *Penicillium chrysogenum* (43) and Abf2 from *Penicillium purpurogenum* (21), both at ca. 50% identity. The second target had 35% identity to a still (to our knowledge) uncharacterized *Tr*Abf2 from *T. reesei*, although a number of transcription studies have identified its presence upon induction by a number of saccharides (1, 20). The closest characterized α-L-arabinofuranosidase to *Pa*Abf62A is *Cj*Abf62A from *Cellvibrio japonicus* at 43% identity (31).

TABLE 3. Expression yield and biochemical characterization of *P. anserina* hemicellulolytic CAZymes

CAZyme	Expression yield (mg/liter)[b]	Molecular mass (kDa)		pI		Apparent optimum pH and temp[a]	
		Theoretical	Experimental[c]	Theoretical	Experimental[d]	pH	Temp (°C)
*Pa*Man5A	440	39.4	46	7.8	7.5	3–6 (4)	50–65 (60)
*Pa*Man26A	100	49.8	62	4.7	4.3	4–6 (4.5)	40 (40)
*Pa*Xyn11A	30	28.5	41	8.3	6.2–6.3	5–7 (6)	50–60 (50)
*Pa*Abf51A	90	71.7	86	6.4	6.1	4–7 (4.5)	5–75 (70)
*Pa*Abf62A	300	37.7	48	4.8	4.6	4–6 (4)	50–60 (55)

[a] Values indicate that the relative enzyme activity remaining was above 50%. Values in parentheses indicate the apparent optimum pH and temperature under the conditions used.
[b] Expression yield is expressed in mg of recombinant protein purified from 1 liter of culture.
[c] Estimated from SDS-PAGE.
[d] Estimated from an IEF gel.

Cloning and expression of novel hemicellulases. Five genes (*PaMan5A*, *PaMan26A*, *PaXyn11A*, *PaAbf51A*, and *PaAbf62A*) encoding putative CAZymes were cloned by reverse transcription-PCR using mRNA purified from *P. anserina* grown on different natural inducers (i.e., AFMB or PX). Although both inducers used were found to induce the expression of *PaMan5A*, *PaMan26A*, *PaXyn11A*, and *PaAbf51A* genes, only AFMB was able to induce the expression of *PaAbf62A* gene (Table 2). After sequencing, all of the coding sequences were in agreement with those predicted from genomic data except for the *PaAbf51A* coding sequence (HM357138), which presented an insertion of 54 nucleotides, leading to 18 extra amino acids with no change in the open reading frame. The coding sequence of each gene was inserted into the *P. pastoris* expression vector in frame with sequences encoding the yeast α-factor secretion peptide and a His$_6$ tag located at the C terminus. Recombinant genes were then introduced into the *Pichia* genome under the control of the methanol-inducible promoter. Multicopy transformants were screened on small cultures to select transformants that exhibited satisfactory levels of production by the analysis of each culture supernatant by means of SDS-PAGE. All targeted hemicellulases were successfully detected in the supernatant after induction, indicating correct processing of the α-factor signal sequence. Secretion levels were between 0.1 and 1 g liter^{-1}, facilitating further purification of recombinant enzymes taking advantage of His$_6$ tags. In addition, only trace amounts of endogenous proteins were present in the culture supernatant of transformants (data not shown). Purification yields ranged from 1.2 to 17.6 mg of recombinant protein from 40 ml of supernatant after 3 days induction (Table 3).

Characterization and kinetic properties of individual enzymes. First, *P. anserina* hemicellulolytic CAZymes purified to homogeneity were biochemically characterized in terms of apparent molecular mass on SDS-PAGE and pI on IEF (Fig. 2 and Table 3). The recombinant proteins displayed apparent molecular masses slightly higher that the theoretical ones (Fig. 2A), probably as a result of posttranslational modification (e.g., glycosylation). Experimental pIs were in close agreement with pIs deduced from the amino acid sequences (Fig. 2B; Table 3). Apparent optimum pH and temperature were first determined on each enzyme, together with temperature stability (Table 3). All of the enzymes were active in the pH 4 to 6 range at temperatures up to 60°C and were stable at 40°C for

1 h with >90% residual activity. *P. anserina* glycosidases were characterized in depth in terms of activity toward various polysaccharides. Kinetic parameters were further determined at pH 5 and 40°C on the best substrates of each enzyme.

*Pa*Man5A and *Pa*Man26A enzymes efficiently hydrolyzed structurally different mannan polysaccharides, such as galactomannans, glucomannans, and β-1,4-mannans from different sources with specific activities ranging from 35 to 86 U mg^{-1} (Table 4). Neither of the mannanases was active on carboxymethyl cellulose or Avicel. Determination of kinetic parameters showed that *Pa*Man5A and *Pa*Man26A displayed the highest specificity toward konjac glucomannan, with K_m values of 0.7 and 0.8 mg ml^{-1} and catalytic efficiencies (k_{cat}/K_m) of 3,142 and 5,750 mg^{-1} min^{-1} ml, respectively. *Pa*Man5A and *Pa*Man26A enzymes also acted efficiently on unsubstituted mannan from ivory nut with K_m values of 1.6 and 1.0 mg.ml^{-1} and catalytic efficiencies (k_{cat}/K_m) of 1,044 and 1,928 mg^{-1} min^{-1} ml, respectively. The *Pa*Xyn11A enzyme efficiently hydrolyzed xylans from birchwood and wheat with a preference for wheat arabinoxylan (K_m = 5.8 mg ml^{-1} and k_{cat}/K_m = 2,443 mg^{-1} min^{-1} ml, Table 4). To evaluate the mode of action of these *P. anserina* glycosidases, the products generated by the action of *Pa*Man5A and *Pa*Man26A on unsubstituted mannan and *Pa*Xyn11A on arabinoxylan were analyzed by HPAEC-PAD. All three enzymes tested displayed typical endo-activity

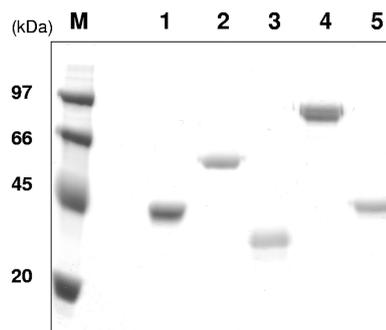
FIG. 2. SDS-PAGE analysis of *P. anserina* hemicellulases: 3 μg of purified recombinant was loaded onto a 12% Tris glycine SDS-PAGE, and the proteins were stained with Coomassie blue. *Pa*Man5A (lane 1), *Pa*Man26A (lane 2), *Pa*Xyn11A (lane 3), *Pa*Abf51A (lane 4), and *Pa*Abf62A (lane 5) are shown. The molecular masses of marker proteins (M) are indicated on the left.

TABLE 4. Enzymatic properties of *P. anserina* CAZymes acting on hemicellulases from different sources

CAZyme	Activity	Substrate	Enzymatic property			
			SA[a] ± SEM (U mg^{-1})	K_m[b] ± SEM (mg ml^{-1} or mM)	V_{max} ± SEM (U mg^{-1})	k_{cat}/K_m[c] (mg^{-1} min^{-1} ml or M^{-1} min^{-1})
*Pa*Man5A	Endo-β-1,4-mannanase	Konjac glucomannan	45 ± 0.9	0.7 ± 0.1	56.4 ± 1.7	3,142
		Carob galactomannan	65 ± 1.4	1.7 ± 0.1	75.5 ± 1.3	1,732
		LBG galactomannan	68 ± 3.2	4.7 ± 1.4	107 ± 14	899
		Ivory nut mannan	35 ± 1.8	1.6 ± 0.4	42.3 ± 2.4	1,044
*Pa*Man26A	Endo-β-1,4-mannanase	Konjac glucomannan	56 ± 1.9	0.8 ± 0.0	74.2 ± 1.8	5,750
		Carob galactomannan	60 ± 3.4	2.6 ± 0.6	79.5 ± 5.8	1,895
		LBG galactomannan	86 ± 4.1	5.5 ± 1.2	126 ± 13	904
		Ivory nut mannan	46 ± 2.7	1.0 ± 0.1	48.8 ± 1.7	1,928
*Pa*Xyn11A	Endo-β-1,4-xylanase	Wheat arabinoxylan	249 ± 7.4	5.8 ± 0.4	405 ± 12	2,443
		Birchwood xylan	95 ± 4.2	3.7 ± 0.5	124 ± 5	1,172
*Pa*Abf51A	α-L-Arabinofuranosidase	pNP-Ara	22.8 ± 0.6	1.2 ± 0.1	26.3 ± 0.5	1,571
		Wheat arabinoxylan	6.9 ± 0.4	ND[d]	ND	ND
		Sugar beet arabinan	3.1 ± 0.3	ND	ND	ND
		Debranched arabinan	0.9 ± 0.0	ND	ND	ND
*Pa*Abf62A	α-L-Arabinofuranosidase	pNP-ara	0.38 ± 0.01	6.1 ± 0.3	0.61 ± 0.02	3.7
		Wheat arabinoxylan	1.0 ± 0.1	ND	ND	ND
		Sugar beet arabinan	0.11 ± 0.02	ND	ND	ND
		Debranched arabinan	0.22 ± 0.03	ND	ND	ND

[a] SA was determined at 10 mg ml^{-1} for polysaccharides and at 10 mM for pNP sugars at 40°C and pH 5 in McIlvaine's buffer.
[b] Units are in mg ml^{-1} for polysaccharides and in mM for pNP substrates.
[c] Units are in mg^{-1} min^{-1} ml for polysaccharides and in mM^{-1} min^{-1} for pNP substrates.
[d] ND, not detected. Although enzyme activity was present, it was too low to measure the individual kinetic constant.

against polymeric substrates, i.e., a mixture of oligosaccharides was generated during the initial stages of hydrolysis (not shown). As the reaction continued, the oligosaccharides were progressively hydrolyzed, yielding principally mannobiose and xylobiose toward the end of the reaction for *Pa*Man5A and *Pa*Xyn11A, respectively, and a mixture of mannose, mannobiose, and mannotriose for *Pa*Man26A (Fig. 3).

To investigate the specificity of *Pa*Abf51A and *Pa*Abf62A enzymes, their capacity to hydrolyze pNP-α-L-arabinofuranoside, as well as arabinose-containing polysaccharides, was evaluated. Although *Pa*Abf51A and *Pa*Abf62A displayed similar K_m values in the micromolar range (1.2 and 6.1 mM, respectively) toward pNP-α-L-arabinofuranoside, *Pa*Abf51A was approximately 60 times more active than *Pa*Abf62A in terms of specific activity (Table 4). These arabinose-releasing enzymes exhibited low activity against polymeric substrates, preventing accurate determination of the individual kinetic parameters. HPAEC analysis of the reaction products generated by *Pa*Abf51A and *Pa*Abf62A revealed that they released only arabinose from polymeric substrates (wheat arabinoxylan, sugar beet arabinan, and debranched arabinan), thus demonstrating that they cleaved terminal units only (not shown).

Substrate-binding capacities of *Pa*Man26A. The ability of CBM35 harbored by *Pa*Man26A to bind various insoluble polysaccharides was tested. Although under our experimental conditions *Pa*Man26A did not bind to Avicel (crystalline cellulose), binding to mannan and xylan was observed. Based on typical double-reciprocal plots, binding parameters of *Pa*Man26A were determined for mannan and xylan, yielding K_d values of 6.25 and 1.85 μM and B_{max} values of 19 and 12 mg of *Pa*Man26A per g of mannan and xylan, respectively (Table 5).

Supplementation of *T. reesei* enzyme cocktail with *P. anserina* hemicellulases. Each enzyme was added to the *T. reesei* enzymatic cocktail, E508, for the saccharification of micronized wheat straw and spruce. The release of both reducing
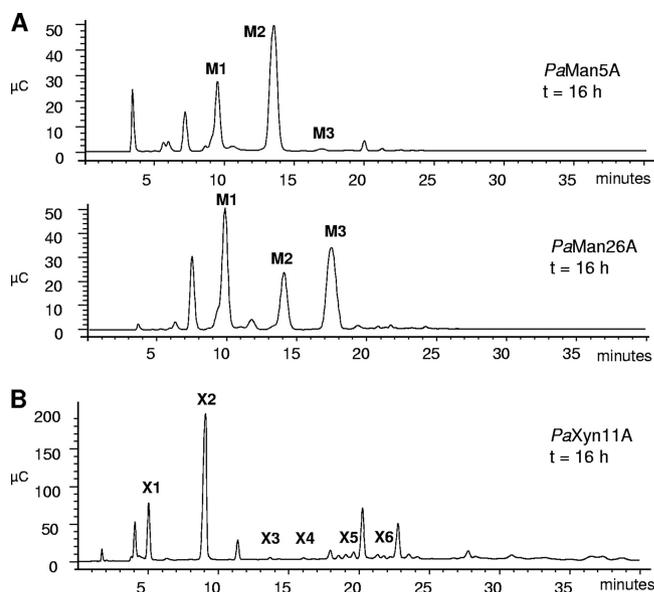

FIG. 3. HPAEC analysis of end products released by endo-acting *P. anserina* hemicellulases. (A) Ivory nut mannan hydrolysis by *Pa*Man5A and *Pa*Man26A: mannose (M1), mannobiose (M2), and mannotriose (M3). (B) Wheat arabinoxylan hydrolysis by *Pa*Xyn11A: xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6).

TABLE 5. Binding parameters of *P. anserina* *Pa*Man26A

Condition	Binding parameter[a]	
	K_d (μM)	B_{max} (mg bound per g of substrate)
Insoluble wheat xylan	1.85	12
Ivory nut mannan	6.25	19
Avicel PH101	NB	NB

[a] The binding parameters were determined in triplicate, and the K_d and B_{max} values were calculated using double-reciprocal plots. NB, no binding.

sugars and individual sugar monomers was quantified by using the DNS assay and HPAEC-PAD, respectively, and compared to E508 (Fig. 4). The addition of *Pa*Xyn11A resulted in an increase in reducing sugars, glucose, and xylose of ca. 10% from micronized wheat straw. However, the addition of exo-acting enzymes did not affect the release of sugar monomers compared to *T. reesei* alone. The combination of *Pa*Abf51A and *Pa*Abf62A exo-acting enzymes with *Pa*Xyn11A did not improve the results obtained with the xylanase alone on micronized wheat straw (data not shown). Using spruce as lignocellulosic substrate, a 15% increase in total sugars was observed when *Pa*Xyn11A was added, with no effect on the glucose release. Supplementation of E508 with either *Pa*Man5A or *Pa*Man26A significantly increased the release of total sugars by 28 and 17%, respectively, and the release of glucose by 18 and 10%, respectively. Supplementation of E508 with a combination of *Pa*Man5A and *Pa*Man26A improved the release of total sugars by 34% and the release of glucose by 23%. Also, the addition of mannanase alone or in combination improved the release of xylose and mannose by 40 to 50% (Fig. 4).

DISCUSSION

In the present study, we characterized five novel hemicellulases from different glycoside hydrolase families from *P. anserina* and assessed their individual contributions to the saccharification of LCB. *P. pastoris* was used as a heterologous expression system to express the set of *P. anserina* hemicellulases, since it is capable of secreting large amounts of correctly folded fungal proteins into the medium, with very few additional proteins (16), making it ideal for the production of recombinant fungal enzymes. It was recently used successfully to express a suite of polysaccharide-degrading enzymes from *Aspergillus* (3). Using this expression host, we efficiently produced and easily purified five fungal hemicellulases in one step, thus allowing their in-depth characterization.

*Pa*Man5A and *Pa*Man26A enzymes were both active on structurally different mannans. Determination of the kinetic parameters enabled us to demonstrate that their best substrate was the unsubstituted glucomannan in terms of affinity and catalytic efficiency, suggesting a negative impact of D-galactosyl side groups with hydrolysis for the galactomannan substrates as described for *T. reesei* mannanase (50). Although *Pa*Man5A and *Pa*Man26A displayed similar specificities toward a range of mannan substrates, they differed in terms of end product formation. *Pa*Man26A released mannobiose and mannotriose from mannan polymer (ivory nut mannan), as reported for the

mannanase from *T. reesei* (47). *Pa*Man5A released mainly mannobiose without accumulation of mannotriose, as described for the recently characterized *Aspergillus niger* GH5 mannanase (18). These results indicate differences in the mode of action of substrate binding within the active sites of the two characterized *P. anserina* mannanases.

Our data strongly suggest that the N-terminal CBM35 module harbored by *Pa*Man26A displays specificity toward hemicellulose polymers, mannan and xylan, with a similar constant K_d in the μM range. A CBM35 module is conserved in three
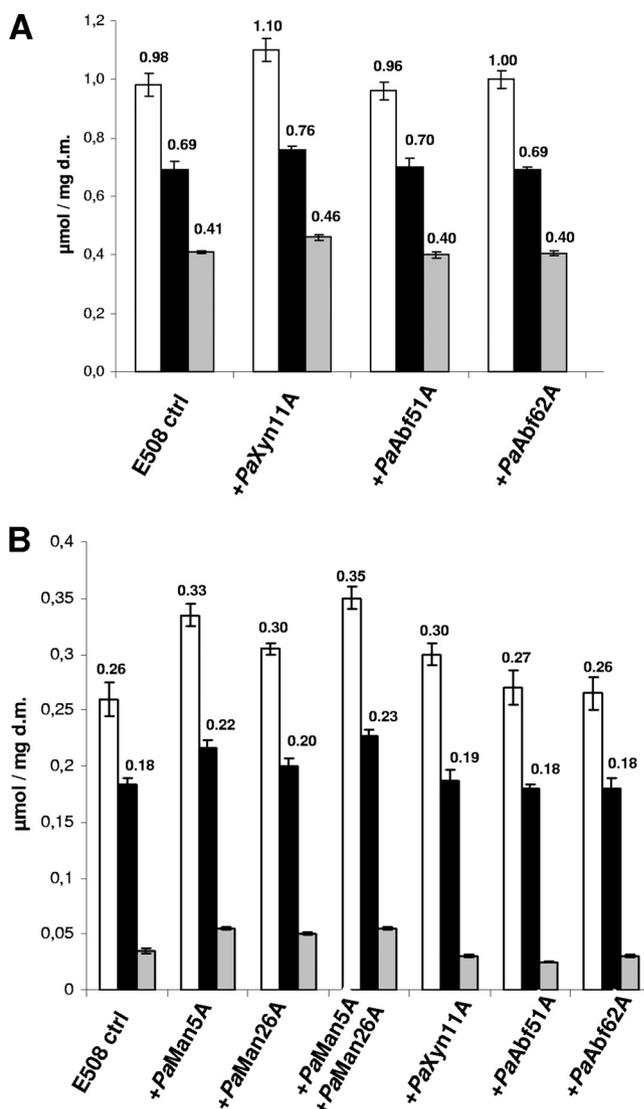

FIG. 4. Saccharification of lignocellulosic micronized substrates (wheat straw and spruce) by *T. reesei* enzymatic cocktail (E508) supplemented with *P. anserina* hemicellulases. Saccharification was carried out on wheat straw (A) and spruce (B) for 24 h at 40°C as described in Materials and Methods. The release of sugars was expressed in μmol per mg of dry matter (d.m.). White bars indicate the release of reducing sugars determined by using the DNS assay. Black bars indicate the release of glucose measured using HPAEC. Gray bars indicate the release of xylose from wheat straw (A) and the release of xylose plus mannose from spruce (B) measured using HPAEC. Values above each bar are means of sextuplicate measures, and error bars represent standard errors of the mean.

Cellvibrio xylan-degrading enzymes and has been shown to bind uronic acid of xylan with a calcium-dependent interaction (38), while a module from a *Cellvibrio* mannanase binds to decorated soluble mannans and manno-oligosaccharides (52). Subtle changes in amino acid chemistry and conformation within the binding site of these bacterial CBM35s have radical impacts on their specificity (14). In fungi, only one CBM35 module binding to β-galactan has been characterized to date in a *Phanerochaete chrysosporium* galactan β-1,3-galactosidase (29).

Although family GH11 glycoside hydrolases are one of the best-characterized CAZy families, containing exclusively endo-xylanases, *Pa*Xyn11A was selected because it harbors a CBM1 module. This family module, formerly designated cellulose-binding domain family 1 (CBD1), is a well-characterized module specific to cellulose and exclusively in fungi (12). *Pa*Xyn11A xylanase exhibited an "endo" mode of action on polymeric substrates, releasing mainly xylobiose as major end product with a high catalytic efficiency, a common characteristic of fungal GH11 xylanases (4, 11).

Fungal species have been found to produce several arabinofuranosidase isoenzymes with different modes of action and substrate specificity (21). The *Pa*Abf51A and *Pa*Abf62A arabinofuranosidases characterized in the present study followed Michaelis-Menten kinetics toward pNP-Ara. Since the activity of these enzymes is of the "exo" type, very low activity was observed toward natural substrates. The kinetic properties of *Pa*Abf51A toward pNP-Ara were comparable to the GH51 fungal enzymes characterized to date with a K_m value in the mM range, an optimum pH around 5, and an optimum temperature above 50°C (21). *P. anserina Pa*Abf62A displayed a 400-fold decrease in catalytic efficiency compared to its *Pa*Abf51A arabinofuranosidase counterpart, mainly due to a low k_{cat} value. This observed difference suggests that these two enzymes may act on different substitutions.

Genome sequencing of *T. reesei* has revealed the relatively small number of genes encoding enzymes involved in the breakdown of LCB in terms of diversity compared to other fungi. With only 16 genes encoding putative hemicellulases, *T. reesei* has the smallest set of hemicellulases of all of the saprophytic fungi analyzed (35). Among ascomycetes, the genome of *P. anserina* revealed the potential of this coprophilic fungus to hydrolyze recalcitrant lignocellulosic residues (19). It contains a significantly higher number of putative cellulases and xylanases compared to *A. niger*, *A. nidulans* and *A. oryzae*, reflected by its growth profile, which is better than *Aspergillus* species on cellulose and xylan (15). These observations fit well with the natural biotope of *P. anserina*, which is a late colonizer of herbivore dung, in which lignocellulose is the main remaining organic component. To our knowledge, none of the numerous putative glycoside hydrolases from different CAZy families encoded by the *P. anserina* genome have been characterized to date.

Since the *T. reesei* cocktail is xylanase-rich, the positive effect observed by adding *Pa*Xyn11A enzyme on the increase of both reducing sugars and glucose from wheat straw could be due to the presence of a CBM1 module linked to the enzyme that may contribute to the overall xylanase efficiency. To our knowledge, the *T. reesei* genome contains no gene encoding a GH11 xylanase harboring a CBM1. A possible explanation for the capacity of CBMs to increase the activity of glycoside hydrolases against insoluble substrates is that they reduce the "accessibility problem" by bringing the appended catalytic modules into intimate, prolonged association with their target substrate, thereby enhancing catalytic efficiency (23). Another hypothesis is that xylanases drastically change the structure and physicochemical properties of the AX population. Xylanases in the same GH family, i.e., with similar specificities, differ substantially in substrate selectivity, and the classification of GH family alone is not sufficient to predict the breakdown of insoluble AXs by xylanases (8). Although mechanistic information on the catalytic and substrate specificity of xylanases is helpful for comparing xylanase efficiencies, other factors, such as their degree of selectivity toward soluble versus insoluble substrate, may play a role in determining the functionality of these enzymes in the degradation of hemicellulose from raw materials (5). Although *T. reesei* has the smallest set of arabinofuranosidases (two GH43, no GH51, one GH62, and no GH93) of all plant cell wall-degrading ascomycetes (35), *Pa*Abf51A and *Pa*Abf62A did not potentiate the action of *T. reesei* enzymes in our experimental conditions.

In the present study, a positive effect on the release of reducing sugars and glucose from spruce was observed when *T. reesei* was supplemented with individual and combined addition of *Pa*Man5A and *Pa*Man26A mannanases. Galactoglucomannans are the main hemicellulose components in softwood (10 to 15% dry mass), with concentrations varying with the type of softwood and the location in the plant cell wall (39). Glucomannans can be found incorporated into aggregates of cellulose, as well as parallel to the cellulose fibrils, to which they are tightly connected (44). Our hypothesis is that *Pa*Man5A and *Pa*Man26A mannanases can facilitate the action of the *T. reesei* enzymatic cocktail by removing mannans that are found around cellulose fibrils. Also, *T. reesei* mannanase (*Tr*Man5a) is expressed at low levels of secretion with only ~0.02 U mg^{-1} detected in the E508 enzymatic cocktail. Although *Pa*Man5A does not carry a CBM, the supplementation effect was higher than that found for *Pa*Man26A. Further fusion of *Pa*Man5A to a CBM specific for cellulose (CBM1) could be way to further enhance the positive effect observed in the present study. We recently demonstrated that the fusion of the *Aspergillus niger* mannanase (*An*Man5) to a CBM1 module potentiates the action of the enzyme toward mannan-containing lignocellulosic substrates (42).

Helper enzymes can loosen up the lignocellulosic structure via degradation of hemicellulosic substrates (mannan/xylan), thus increasing the surface area of cellulose attacked by *T. reesei* cellulases. Because of the heterogeneity of hemicellulose between lignocellulosic substrates, enzymes with different characteristics are required in industrial applications, but the choice of the enzyme is still largely empirical (5). In the present study, hemicellulose-acting enzymes selected using comparative genomics brought a clearly marked improvement in both the liquefaction and the solubilization of LCB and the release of glucose and xylose/mannose when supplemented to *T. reesei*. The fact that this effect was more striking with the addition of mannanase might be due to the close association of softwood glucomannan with cellulose microfibrils, whereas xylan is interconnected to lignin. Since pretreatment of lignocellulose is also responsible for the high process cost of bioethanol pro-

duction from LCB, helper enzymes (e.g., hemicellulases) could be an alternative to chemical and/or physical pretreatments. With addition of xylan- or mannan-acting enzymes to the actual cocktail, we could consider lowering the dosage of the *T. reesei* cocktail to achieve the same glucose yield (22, 37), since cellulose would be more accessible to cellulases. Tailor-made enzymatic cocktails need to be developed according to the lignocellulosic structure of each biomass for optimal hydrolysis.

The analysis of *P. anserina* genome has recently revealed a large and highly specialized set of genes involved in utilization of natural carbon sources (e.g., lignocellulose) commonly found in its natural biotope (19). The present findings pave the way for further studies to give more insights into *P. anserina*'s enzymatic machinery. Among this unexpected unique enzymatic equipment, its genome presents a number of other interesting targets potentially acting on the recalcitrant components of plant cell wall. Finding complementary auxiliary enzymes displaying novel specificities will be required to deconstruct efficiently LCB for the production of biofuels and high-value products.

ACKNOWLEDGMENTS

REFERENCES

1. **Akel, E., B. Metz, B. Seiboth, and C. P. Kubicek.** 2009. Molecular regulation of arabinan and L-arabinose metabolism in *Hypocrea jecorina* (*Trichoderma reesei*). Eukaryot. Cell **8:**1837–1844.
2. **Bailey, M. J., P. Biely, and K. Poutanen.** 1992. Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. **23:**257–270.
3. **Bauer, S., P. Vasu, S. Persson, A. J. Mort, and C. R. Somerville.** 2006. Development and application of a suite of polysaccharide-degrading enzymes for analyzing plant cell walls. Proc. Natl. Acad. Sci. U. S. A. **103:** 11417–11422.
4. **Berrin, J. G., H. el Ajandouz, J. Georis, F. Arnaut, and N. Juge.** 2007. Substrate and product hydrolysis specificity in family 11 glycoside hydrolases: an analysis of *Penicillium funiculosum* and *Penicillium griseofulvum* xylanases. Appl. Microbiol. Biotechnol. **74:**1001–1010.
5. **Berrin, J. G., and N. Juge.** 2008. Factors affecting xylanase functionality in the degradation of arabinoxylans. Biotechnol. Lett. **30:**1139–1150.
6. **Biely, P.** 1993. Enzymological aspects of the production of microbial hemicellulases, p. 29–51. *In* M. P. Coughlan and G. P. Hazelwood (ed.), Hemicellulose and hemicellulases. Portland Press, London, England.
7. **Bonnin, E., M. Brunel, Y. Gouy, L. Lesage-Meessen, M. Asther, and J.-F. Thibault.** 2001. *Aspergillus niger* I-1472 and *Pycnoporus cinnabarinus* MUCL39533, selected for the biotransformation of ferulic acid to vanillin, are also able to produce cell wall polysaccharide-enzymes and feruloyl esterases. Enzyme Microb. Technol. **28:**70–80.
8. **Bonnin, E., S. Daviet, J. F. Sorenson, O. Sibbesen, A. Goldson, N. Juge, and L. Saulnier.** 2006. Behaviour of family 10 and 11 xylanases toward arabinoxylans with varying structure. J. Sci. Food Agric. **86:**1618–1622.
9. **Brutus, A., C. Villard, A. Durand, T. Tahir, C. Furniss, A. Puigserver, N. Juge, and T. Giardina.** 2004. The inhibition specificity of recombinant *Penicillium funiculosum* xylanase B toward wheat proteinaceous inhibitors. Biochim. Biophys. Acta **1701:**121–128.
10. **Cantarel, B. L., P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, and B. Henrissat.** 2009. The carbohydrate-active enzymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res. **37:**D233–D238.
11. **Cervera-Tison, M., G. Andre-Leroux, M. Lafond, J. Georis, N. Juge, and J. G. Berrin.** 2009. Molecular determinants of substrate and inhibitor specificities of the *Penicillium griseofulvum* family 11 xylanases. Biochim. Biophys. Acta **1794:**438–445.
12. **Chen, X. A., N. Ishida, N. Todaka, R. Nakamura, J. Maruyama, H. Takahashi, and K. Kitamoto.** 2010. Promotion of efficient saccharification of crystalline cellulose by *Aspergillus fumigatus* swo1. Appl. Environ. Microbiol. **76:**2556–2561.
13. **Cherry, J. R., and A. L. Fidantsef.** 2003. Directed evolution of industrial enzymes: an update. Curr. Opin. Biotechnol. **14:**438–443.
14. **Correia, M. A., D. W. Abbott, T. M. Gloster, V. O. Fernandes, J. A. Prates, C. Montanier, C. Dumon, M. P. Williamson, R. B. Tunnicliffe, Z. Liu, J. E. Flint, G. J. Davies, B. Henrissat, P. M. Coutinho, C. M. Fontes, and H. J. Gilbert.** 2010. Signature active site architectures illuminate the molecular basis for ligand specificity in family 35 carbohydrate binding module. Biochemistry **49:**6193–6205.
15. **Coutinho, P. M., M. R. Andersen, K. Kolenova, P. A. vanKuyk, I. Benoit, B. S. Gruben, B. Trejo-Aguilar, H. Visser, P. van Solingen, T. Pakula, B. Seiboth, E. Battaglia, G. Aguilar-Osorio, J. F. de Jong, R. A. Ohm, M. Aguilar, B. Henrissat, J. Nielsen, H. Stalbrand, and R. P. de Vries.** 2009. Post-genomic insights into the plant polysaccharide degradation potential of *Aspergillus nidulans* and comparison to *Aspergillus niger* and *Aspergillus oryzae*. Fungal Genet. Biol. **46:**S161–S169.
16. **Daly, R., and M. T. Hearn.** 2005. Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. J. Mol. Recognit. **18:**119–138.
17. **Decelle, B., A. Tsang, and R. K. Storms.** 2004. Cloning, functional expression and characterization of three *Phanerochaete chrysosporium* endo-1,4-β-xylanases. Curr. Genet. **46:**166–175.
18. **Do, B. C., T. T. Dang, J. G. Berrin, D. Haltrich, K. A. To, J. C. Sigoillot, and M. Yamabhai.** 2009. Cloning, expression in *Pichia pastoris*, and characterization of a thermostable GH5 mannan endo-1,4-β-mannosidase from *Aspergillus niger* BK01. Microb. Cell Fact. **8:**59.
19. **Espagne, E., O. Lespinet, F. Malagnac, C. Da Silva, O. Jaillon, B. M. Porcel, A. Couloux, J. M. Aury, B. Segurens, J. Poulain, V. Anthouard, S. Grossetete, H. Khalili, E. Coppin, M. Dequard-Chablat, M. Picard, V. Contamine, S. Arnaise, A. Bourdais, V. Berteaux-Lecellier, D. Gautheret, R. P. de Vries, E. Battaglia, P. M. Coutinho, E. G. Danchin, B. Henrissat, R. E. Khoury, A. Sainsard-Chanet, A. Boivin, B. Pinan-Lucarre, J. H. Sellem, P. Debuchy, P. Wincker, J. Weissenbach, and P. Silar.** 2008. The genome sequence of the model ascomycete fungus *Podospora anserina*. Genome Biol. **9:**R77.
20. **Foreman, P. K., D. Brown, L. Dankmeyer, R. Dean, S. Diener, N. S. Dunn-Coleman, F. Goedegebuur, T. D. Houfek, G. J. England, A. S. Kelley, H. J. Meerman, T. Mitchell, C. Mitchinson, H. A. Olivares, P. J. Teunissen, J. Yao, and M. Ward.** 2003. Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus *Trichoderma reesei*. J. Biol. Chem. **278:** 31988–31997.
21. **Fritz, M., M. C. Ravanal, C. Braet, and J. Eyzaguirre.** 2008. A family 51 α-L-arabinofuranosidase from *Penicillium purpurogenum*: purification, properties and amino acid sequence. Mycol. Res. **112:**933–942.
22. **Gao, D., S. P. Chundawat, C. Krishnan, V. Balan, and B. E. Dale.** 2009. Mixture optimization of six core glycosyl hydrolases for maximizing saccharification of ammonia fiber expansion (AFEX) pretreated corn stover. Bioresour. Technol. **101:**2770–2781.
23. **Gilbert, H. J.** 2010. The biochemistry and structural biology of plant cell wall deconstruction. Plant Physiol. **153:**444–455.
24. **Girio, F. M., C. Fonseca, F. Carvalheiro, L. C. Duarte, S. Marques, and R. Bogel-Lukasik.** 2010. Hemicelluloses for fuel ethanol: a review. Bioresour. Technol. **101:**4775–4800.
25. **Guais, O., G. Borderies, C. Pichereaux, M. Maestracci, V. Neugnot, M. Rossignol, and J. M. François.** 2008. Proteomics analysis of "Rovabio Excel", a secreted protein cocktail from the filamentous fungus *Penicillium funiculosum* grown under industrial process fermentation. J. Ind. Microbiol. Biotechnol. **35:**1659–1668.
26. **Hendriks, A. T., and G. Zeeman.** 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol. **100:**10–18.
27. **Herpoel-Gimbert, I., A. Margeot, A. Dolla, G. Jan, D. Molle, S. Lignon, H. Mathis, J. C. Sigoillot, F. Monot, and M. Asther.** 2008. Comparative secretome analyses of two *Trichoderma reesei* Rut-C30 and CL847 hypersecretory strains. Biotechnol. Biofuels **1:**18.
28. **Himmel, M. E., S. Y. Ding, D. K. Johnson, W. S. Adney, M. R. Nimlos, J. W. Brady, and T. D. Foust.** 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science **315:**804–807.
29. **Ichinose, H., M. Yoshida, T. Kotake, A. Kuno, K. Igarashi, Y. Tsumuraya, M. Samejima, J. Hirabayashi, H. Kobayashi, and S. Kaneko.** 2005. An exo-β-1,3-galactanase having a novel β-1,3-galactan-binding module from *Phanerochaete chrysosporium*. J. Biol. Chem. **280:**25820–25829.
30. **Kauppinen, M. S., M. Schulein, K. Schnorr, L. N. Andersen, and M. E. Bjørnvad.** 2003. Novel mannanases. U.S. patent 6566114.
31. **Kellett, L. E., D. M. Poole, L. M. Ferreira, A. J. Durrant, G. P. Hazlewood, and H. J. Gilbert.** 1990. Xylanase B and an arabinofuranosidase from *Pseudomonas fluorescens* subsp. *cellulosa* contain identical cellulose-binding domains and are encoded by adjacent genes. Biochem. J. **272:**369–376.
32. **Lee, C. C., D. W. Wong, and G. H. Robertson.** 2005. Cloning and characterization of the xyn11A gene from *Lentinula edodes*. Protein J. **24:**21–26.
33. **Liu, J. R., H. Duan, X. Zhao, J. T. Tzen, K. J. Cheng, and C. K. Pai.** 2008. Cloning of a rumen fungal xylanase gene and purification of the recombinant enzyme via artificial oil bodies. Appl. Microbiol. Biotechnol. **79:**225–233.
34. **Margeot, A., B. Hahn-Hagerdal, M. Edlund, R. Slade, and F. Monot.** 2009. New improvements for lignocellulosic ethanol. Curr. Opin. Biotechnol. **20:** 372–380.

35. **Martinez, D., R. M. Berka, B. Henrissat, M. Saloheimo, M. Arvas, S. E. Baker, J. Chapman, O. Chertkov, P. M. Coutinho, D. Cullen, E. G. 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, and T. S. Brettin.** 2008. Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). Nat. Biotechnol. **26:**553–560.

36. **Matsushika, A., H. Inoue, T. Kodaki, and S. Sawayama.** 2009. Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives. Appl. Microbiol. Biotechnol. **84:**37–53.

37. **Meyer, A. S., L. Rosgaard, and H. R. Sørensen.** 2009. The minimal enzyme cocktail concept for biomass processing. J. Cereal Sci. **50:**337–344.

38. **Montanier, C., A. L. van Bueren, C. Dumon, J. E. Flint, M. A. Correia, J. A. Prates, S. J. Firbank, R. J. Lewis, G. G. Grondin, M. G. Ghinet, T. M. Gloster, C. Herve, J. P. Knox, B. G. Talbot, J. P. Turkenburg, J. Kerovuo, R. Brzezinski, C. M. Fontes, G. J. Davies, A. B. Boraston, and H. J. Gilbert.** 2009. Evidence that family 35 carbohydrate binding modules display conserved specificity but divergent function. Proc. Natl. Acad. Sci. U. S. A. **106:**3065–3070.

39. **Moreira, L. R., and E. X. Filho.** 2008. An overview of mannan structure and mannan-degrading enzyme systems. Appl. Microbiol. Biotechnol. **79:**165–178.

40. **Navarro, D., M. Couturier, G. Ghizzi Damasceno da Silva, J.-G. Berrin, X. Rouau, M. Asther, and C. Bignon.** 2010. Automated assay for screening the enzymatic release of reducing sugars from micronized biomass. Microb. Cell Fact. **9:**58.

41. **Okazaki, F., Y. Tamaru, S. Hashikawa, Y. T. Li, and T. Araki.** 2002. Novel carbohydrate-binding module of β-1,3-xylanase from a marine bacterium, *Alcaligenes* sp. strain XY-234. J. Bacteriol. **184:**2399–2403.

42. **Pham, T. A., J. G. Berrin, E. Record, K. A. To, and J. C. Sigoillot.** 2010. Hydrolysis of softwood by *Aspergillus* mannanase: role of a carbohydrate-binding module. J. Biotechnol. **148:**163–170.

43. **Sakamoto, T., and H. Kawasaki.** 2003. Purification and properties of two type-B α-L-arabinofuranosidases produced by *Penicillium chrysogenum*. Biochim. Biophys. Acta **1621:**204–210.

44. **Salmen, L.** 2004. Micromechanical understanding of the cell-wall structure. C. R. Biol. **327:**873–880.

45. **Sanchez, C.** 2009. Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol. Adv. **27:**185–194.

46. **Saulnier, L., P.-E. Sado, G. Branlard, G. Charmet, and F. Guillon.** 2007. Wheat arabinoxylans: exploiting variation in amount and composition to develop enhanced varieties. J. Cereal Sci. **46:**261–281.

47. **Stålbrand, H., MattiSika-aho, M. Tenkanen, and L. Viikari.** 1993. Purification and characterization of two β-mannanases from *Trichoderma reesei*. J. Biotechnol. **29:**229–242.

48. **Stålbrand, H., A. Saloheimo, J. Vehmaanperä, B. Henrissat, and M. Penttilä.** 2005. Cloning and expression in *Saccharomyces cerevisiae* of a *Trichoderma reesei* beta-mannanase gene containing a cellulose binding domain. Appl. Environ. Microbiol. **61:**1090–1097.

49. **Tabka, M. G., I. Herpoel-Gimbert, F. Monod, M. Asther, and J. C. Sigoillot.** 2006. Enzymatic saccharification of wheat straw for bioethanol production by a combined cellulase xylanase and feruloyl esterase treatment. Enzyme Microb. Technol. **39:**897–902.

50. **Tenkanen, M., M. Makkonen, M. Perttula, L. Viikari, and A. Teleman.** 1997. Action of *Trichoderma reesei* mannanase on galactoglucomannan in pine kraft pulp. J. Biotechnol. **57:**191–204.

51. **Törrönen, A., R. L. Mach, R. Messner, R. Gonzalez, N. Kalkkinen, A. Harkki, and C. P. Kubicek.** 1992. The two major xylanases from *Trichoderma reesei*: characterization of both enzymes and genes. Biotechnology **10:**1461–1465.

52. **Tunnicliffe, R. B., D. N. Bolam, G. Pell, H. J. Gilbert, and M. P. Williamson.** 2005. Structure of a mannan-specific family 35 carbohydrate-binding module: evidence for significant conformational changes upon ligand binding. J. Mol. Biol. **347:**287–296.

53. **Waldron, K., and C. Faulds.** 2007. Cell wall polysaccharides: composition and structure, p. 181–201. *In* J. Kamerling, G. J. Boons, Y. Lee, A. Suzuki, N. Taniguchi, and A. G. J. Voragen (ed.), Comprehensive glycoscience: analysis of glycans/polysaccharide functional properties, vol. 2. Elsevier B.V., Amsterdam, Netherlands.