Hydrolysis of softwood by *Aspergillus* mannanase: Role of a carbohydrate-binding module

Tuan Anh Pham [a,b,*], Jean Guy Berrin [a], Eric Record [a], Kim Anh To [b], Jean-Claude Sigoillot [a]

[a] INRA, UMR1163, Université Aix-Marseille I, II, Case 932, 163 avenue de Luminy, 13288 Marseille Cedex 09, France
[b] Hanoi University of Science and Technology, 1 Dai Co Viet, Hanoi, Viet Nam

ARTICLE INFO

ABSTRACT

Endo β-1,4-mannanases (β-mannanases, EC 3.2.1.78), belonging to CAZy GH5 and GH26 families, catalyze the hydrolysis of structurally different mannans. In this study, the mannanase encoding gene of *Aspergillus aculeatus* VN was expressed in *Aspergillus niger* D15#26 using pAN 52-4 vector, under the control of P*gpd*A promoter and T*trp*C terminator. In order to improve the hydrolytic capacity of this GH5 on lignocellulosic substrate, the family 1 carbohydrate-binding module (CBM1) of *Aspergillus niger* cellobiohydrolase B was artificially fused at the C-terminal end of this enzyme with a natural linker. Both mannanase and mannanase–CBM genes were successfully expressed in *A. niger* D15#26, producing proteins with molecular masses of 54 and 79 kDa, respectively. The Michaelis–Menten constants, pH activity profiles and temperature optima of three enzymes (wild-type mannanase, recombinant mannanase and recombinant mannanase–CBM) were similar, but the fused mannanase–CBM enzyme was more thermostable. Cross-comparison of the three enzymes for softwood hydrolysis in association with *Trichoderma reesei* enzymatic cocktail showed that mannanase–CBM improved the glucose yield compared to wild-type and recombinant mannanases.

1. Introduction

Lignocellulosic biomass is seen as the main raw-material source for future production of bioethanol. The main bottleneck in biomass conversion for biofuel production is the enzymatic depolymerization of cell wall polysaccharides into fermentable sugars (Demirbas, 2007; Sanchez and Cardona, 2008). The 3D structure of lignocellulose is formed by a network composed essentially of cellulose, hemicelluloses and lignin (Kirk and Cullen, 1988; Sanchez, 2009). Hemicellulose is a generic term covering various complex heteropolysaccharides including xylans and mannans (Kirk and Cullen, 1988). Mannans present in large amounts in wood as glucomannan or galactoglucomannan. Hardwood contains 2–5% glucomannan with a glucose/mannose ratio of 1:1.5 (or 2) (Timell, 1967; Wiedenhoeft and Miller, 2005). Galactoglucomannan is the main component of softwood hemicellulose (15–25% wood dry mass) (Timell, 1967). Its polymer backbone is composed of D-mannopyranose and D-glucopyranose. The residues in the main chain are partially substituted by α-1,6-linked D-galactosyl side group. The backbone polymer C-2 and C-3 positions can be acetyl-substituted. Molar galactose/glucose/mannose ratios are approximately 1/1/3 or 0.1/1/3 (or 4), for galactose-rich or galactose-poor softwood, respectively (Timell, 1967; Wiedenhoeft and Miller, 2005).

Cellulose and hemicelluloses of lignocellulosic substrate are depolymerized by a cocktail of hydrolytic enzymes mainly composed of cellulases and hemicellulases (Kirk and Cullen, 1988; De Vries and Visser, 2001). Hemicellulases present two main interests for the bioethanol production industry: (i) release of fermentable sugars such as mannose and xylose; (ii) synergy with cellulases to enhance depolymerization of the substrate. Such synergy was demonstrated by Murashima et al. (2003) between cellulases and xylanases, and was explained by a degradation of the xylan network allowing cellulases access to deeper structures.

Endo-1,4-β-D-mannanases (1,4-β-D-mannan mannohydrolases EC 3.2.1.78) are the major enzymes involved in hemicellulose degradation in wood. They catalyze the random hydrolysis of 1,4-β-D-mannopyranosyl linkages in the mannan backbone. Based on sequence similarities, these enzymes are grouped into glycoside hydrolase families 5 and 26 (Dhawan and Kaur, 2009; Cantarel et al., 2009). Both families contain β-mannanases from bacteria and filamentous fungi. Moreover, β-mannanases are also present in plants, where they play a role in seed germination (Yuan et al., 2007).

Complete depolymerization of mannans requires cooperation from enzymes acting on the main chain (β-mannosidase, β-mannanase and β-glucosidase) and enzymes acting on the side-chain substituents (acetyl mannan esterase and α-galactosidase) (Moreira and Filho, 2008).

Many plant cell wall degradation enzymes from a range of bacteria and fungi have a complex molecular architecture consisting of one or more catalytic domains and at least one non-catalytic carbohydrate-binding module (CBM) that function independently of the catalytic domains (Warren, 1996). The major function of CBMs is to facilitate the association of the parent enzyme with its substrate by increasing the local concentration of the enzyme and consequently enhancing its activity (Linder and Teeri, 1997; Shoseyov et al., 2006). In addition, some CBMs have been shown to directly assist hydrolysis by disrupting crystalline structure, thereby increasing substrate access (Shoseyov et al., 2006). CBMs have been classified into families according to amino-acid sequence similarities and 3D structures (Cantarel et al., 2009). Family 1 CBMs are exclusively found in fungi, and in many cases show to bind to cellulose, while CBMs belonging to different families can bind to chitin (family 12), xylan (family 22), mannan (family 27), or starch (family 26) (Cantarel et al., 2009).

The efficiency of complex molecular enzymes against insoluble substrates has been exploited to design chimeric enzymes. A synergistic effect was observed with the fusion between feruloyl esterase, xylanase and CBM1 in the release of ferulic acid from corn bran (Levasseur et al., 2005). We also recently demonstrated that the addition of a CBM1 to the *Pycnoporus cinnabarinus* laccase improved significantly the delignification of pulp (Ravalason et al., 2009).

Carbohydrate-binding modules have been found at either the C-terminus or N-terminus in β-mannanases (Benech et al., 2007; Hägglund et al., 2003; Sunna et al., 2000). Fungal mannanases from *A. niger*, *A. aculeatus* to date do not contain a CBM (Benech et al., 2007; Do et al., 2009). In the present study, we focused on a β-mannanase from an *A. aculeatus* VN strain previously isolated and selected from decaying wood samples collected from Viet Nam. The gene encoding this GH5 mannanase was cloned and expressed in *A. niger* D15#26 alone and artificially fused to the family 1 carbohydrate-binding module (CBM1) of *A. niger* CbhB to improve its enzymatic properties. Three enzymes (wild-type enzyme, recombinant enzyme and recombinant enzyme fused to CBM1) were purified and fully characterized in order to compare their efficiencies on softwood hydrolysis in association with *Trichoderma reesei* enzymatic cocktail.

2. Materials and methods

2.1. Strain and culture media

Escherichia coli JM109 (Promega, Charbonnières, France) was used for the construction and propagation of vectors. *Aspergillus niger* D15#26 (*pyrG* mutant) was used for homologous expression (Gordon et al., 2000). After transformation, *A. niger* transformants were grown on selective solid minimum medium (without uridine) containing $NaNO_3$ 70 mM, KCl 7 mM, KH_2HPO_4 11 mM, $MgSO_4$ 2 mM, glucose 1% (w/v), and 1 mL/L of element solution ($1000\times$ stock solution containing $ZnSO_4$ 76 mM, H_3BO_3 178 mM, $MnCl_2$ 25 mM, $FeSO_4$ 18 mM, $CoCl_2$ 7.1 mM, $CuSO_4$ 6.4 mM, Na_2MoO_4 6.2 mM, EDTA 174 mM). To screen the transformants for recombinant protein, 100 mL of culture medium containing $NaNO_3$ 70 mM, KCl 7 mM, Na_2HPO_4 200 mM, $MgSO_4$ 2 mM, 5% glucose (w/v) and 0.1 mL element solution, was inoculated with 1×10^6 spores/mL in 500 mL baffled flask (Record et al., 2002). *A. aculeatus* VN was cultivated in liquid medium containing guar gum (Sigma) 0.5%, yeast extract 1%, di-sodium tartrate 10 mM, di-ammonium tartrate

10 mM, KH_2PO_4 11 mM, $CaCl_2$ 5 mM, $MgSO_4$ 3 mM, and 30 mL/L of solution ($FeSO_4$ 8.5 mM, $ZnSO_4$ 5.6 mM, $MnSO_4$ 8.8 mM, $CuSO_4$ 0.96 mM).

2.2. Cloning and expression in Aspergillus niger D15#26

Cloning of the mannanase sequence: The mannanase encoding gene was amplified from genomic DNA of *A. aculeatus* VN. Two primers M-F1 forward primer 5′-AATGCGCGCCCTT-CCCCGGACGCCGAAC-3′ (BssHII) and M-R1 reverse primer 5′-GTAGGATCCTTAGTGGTGGTGGTGATGGTGCTTCGACTGCGCATTG-AT-3′ (BamHI) were designed according to *A. aculeatus* mannanase encoding sequence (GenBank accession no. L35487, Christgau et al., 1994). All PCR reactions were carried out using Expand high fidelity PCR system (Roche) according to the manufacturer's instructions. The amplification was initiated by a denaturation of 5 min at 95 °C, followed by 5 cycles of 1.5 min, 95 °C; 0.75 min, 60 °C; 1.5 min, 72 °C; followed by 30 cycles of 0.5 min, 94 °C; 0.5 min, 60 °C; 1.5 min, 72 °C; and then by a final elongation step of 7 min at 72 °C.

Construction of the fused mannanase–CBM sequence: The fusion of the mannanase encoding sequence with the linker-CBM (GenBank accession no. AF156269, Gielkens et al., 1999) was performed by the overlap extension PCR (Ho et al., 1989) involving three PCR reactions. The mannanase gene was amplified in the first PCR reaction using M-F1 forward primer 5′-AATG-CGCGCCCTTCCCCGGACGCCGAAC-3′ (BssHII) and MC-R1 reverse primer 5′-ACTGGAGTAAGTAGAGCCCTTAGACTGCGCATTGAT-3′ (see in Fig. 1). In the second PCR reaction, the linker-CBM region was amplified from plasmid pLac-CBM (Ravalason et al., 2009) containing linker-CBM of *A. niger* cellobiohydrolase B, using MC-F2 forward primer 5′-ATCAATGCGCAGTCTAAGGGCTCTACTTACTCCAGT-3′ and MC-R2 reverse primer 5′-ATGCGGATCCTTAATGGTGATGGTGATG-GTGCAGACACTGCGAGTAGTA-3′ (BamHI). Finally, the resulting overlapping fragments were mixed and the fused sequence was obtained in the third PCR reaction using M-F1 and MC-R2 external primers.

Mannanase and mannanase–CBM sequences were cloned into the pGEM-T vector (Promega) to obtain pGEM-T-Man and pGEM-T-ManCBM vectors and the cloned PCR products were checked by sequencing. Two vectors pGEM-T-Man and pGEM-T-ManCBM were digested with BssHII and BamHI restriction enzymes. Digested sequences were purified and cloned into BssHII–BamHI linearized and dephosphorylated pAN52-4 vector (GenBank accession no. Z32699) to obtain pMan and pManCBM expression vectors. In these cassettes, the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene promoter (P*gpd*A), the 5′ untranslated region of the *gpd*A mRNA, and *A. nidulans* terminator T*trp*C were used to drive the expression of enzyme encoding sequences. In addition, *A. niger* glucoamylase signal peptide (S*gla*A) was used to target protein secretion.

Aspergillus niger D15#26 strain was grown for 18 h at 30 °C, with shaking at 150 rpm in *A. niger* minimum medium supplemented with casamino acids 0.2%, yeast extract 0.5% and uridine 0.01 M. Then, protoplasts were prepared and fungal co-transformations were carried out as described by Punt and van den Hondel (1992) using expression vector and pAB4-1 plasmid containing the pyrG selection marker, in a 10:1 ratio. A control experiment was performed with *A. niger* D15#26 only transformed with pAB4-1 plasmid. Transformants were selected for uridine prototrophy on uridine-free minimal medium.

2.3. Screening for mannanase activity

The screenings were realized in two steps. In the first step, transformants were grown in 16-well plates containing 10 mL of

medium in each well, enzymatic activity was measured daily from day 5 (Alberto et al., 2009). In the second step, the 15 best transformants of rec-Man and rec-ManCBM were grown in 500 mL flasks containing 100 mL medium. Cultures were monitored for 12 days at 30 °C in a shaker incubator (130 rpm), pH was adjusted daily to 5.5 with 1 M citric acid.

2.4. Purification procedure

All purification procedures were realized at 4 °C. *A. aculeatus* VN culture medium was harvested and filtered (0.45 μm pore size) after 7 days of growth. Filtrate was concentrated by ultrafiltration through a polyethersulfone membrane (10 kDa cut-off, Millipore), dialyzed against citrate buffer (50 mM, pH 6) and loaded onto a DEAE-sepharose fast flow column (26 mm × 150 mm, Pharmacia). Elution was performed with a NaCl gradient from 0 to 500 mM NaCl in the same buffer at a flow rate of 1 mL/min. The active fractions were pooled, concentrated and dialyzed against citrate buffer (50 mM, pH 6, NaCl 150 mM). The second purification step was carried out using a Sephacryl S-200 column (16 mm × 700 mm, Pharmacia) at a flow rate of 0.3 mL/min. Fractions containing mannanase activity were pooled.

Cultures were harvested on day 12 for rec-Man transformants and day 9 for rec-ManCBM transformants. Culture media were filtered (0.45 μm pore size) and concentrated by ultrafiltration through a polyethersulfone membrane (10 kDa cut-off, Millipore). Media were further dialyzed against binding buffer (Tris–HCl 50 mM, pH 7.4, imidazole 10 mM, NaCl 150 mM). His-tagged proteins were purified using a 5 mL HisTrap FF (GE healthcare) at a flow rate of 5 mL/min. Elution was performed with elution buffer (Tris–HCl l50 mM, pH 7.4, imidazole 500 mM, NaCl 150 mM).

To visualize recombinant proteins, the small-scale purification were carried out using His-SpinTrap (GE Healthcare) according to the manufacturer's instructions.

2.5. Enzyme activity

Mannanase activity was determined using locust bean gum (galactomannan, Sigma) as substrate prepared at 0.5% (w/v) in citrate buffer (50 mM, pH 5). After 30 min incubation at 50 °C, the reducing sugars released were measured according to Miller (1959). One unit of enzyme activity is defined as the amount of enzyme releasing 1 μmol of mannose per min.

Exo-glucanase, endo-glucanase and xylanase activities were determined following Tabka et al. (2006).

2.6. Gel electrophoresis

The homogeneity and relative molecular mass of denatured proteins were checked by SDS-PAGE (10% acrylamide gels) using the Mini-PROTEAN system (BioRad). Proteins were stained using Coomassie blue and protein masses were estimated by cross-comparison against the protein molecular mass ladder (low molecular weight calibration kit, Amersham Biosciences). Deglycosylation experiments were performed using PNGase F (New England Biolabs) according to the manufacturer's instructions.

2.7. pH and temperature optima and stability

The effect of pH on enzyme stability was investigated by incubating the purified enzymes in 50 mM buffers at various pH ranging from 2 to 10 (hydrochloric-potassium chloride buffer pH 2; citrate buffer pH 3–6; phosphate buffer pH 6–8; Tris–HCl buffer pH 8–9, carbonate–bicarbonate buffer pH 9–10) at 50 °C. The remaining enzyme activity was then determined under standard

enzyme assay conditions. For the determination of thermal stability, aliquots of purified enzymes were incubated in citrate buffer (50 mM, pH 5) at various temperatures ranging from 50 to 75 °C. Thermal inactivation was stopped by cooling the samples on ice, and residual activity was determined under standard enzyme assay conditions. Temperature and pH optima were determined by changing the pH or temperature of the standard enzyme assay conditions.

2.8. Determination of kinetic parameters

The Michaelis–Menten constants for locust bean gum were determined by measuring the reaction rate using locust bean gum concentrations ranging from 0.5 to 8 mg/mL. Kinetic parameters (K_m and V_{max}) were calculated from the experimentally obtained data and non-linear regression analysis using "GraFit" software (Erithacus Software Ltd.).

2.9. Absorption experiments

Different amounts of rec-ManCBM enzyme were added into 2 mL Eppendorf tubes containing citrate buffer (50 mM, pH 5) in a final volume of 1 mL containing 20 mg of Avicel PH101 cellulose (Fluka). The mixtures were then incubated at 4 °C for 1 h. After centrifugation (8000 × g for 2 min, 4 °C), mannanase activity in the supernatant was measured and compared against the blank sample (substrate-free). The wt-Man and rec-Man were used as negative controls. The absorption experiments of enzymes to the softwood were realized in final volume of 10 mL containing 200 mg of softwood (mill refiner pulp kindly provided by the Pulp and Paper Research Institute CTP, Grenoble, France).

2.10. Enzymatic hydrolysis

Two enzymatic preparations containing hydrolases from *Trichoderma reesei* CL 847 were kindly provided by SAF-ISIS (Souston, France) from LESAFFRE group (Tabka et al., 2006). The cellulase preparation exhibited 0.06 U/mg of exo-glucanase, 9.75 U/mg of endo-glucanase, 1.12 U/mg of xylanase and 0.7 U/mg of mannanase. The second preparation presented mainly xylanase activity (8.7 U/mg) and other enzymes (0.02 U/mg of exo-glucanase, 2.75 U/mg of endo-glucanase, 0.5 U/mg of mannanase).

The softwood samples (mill refiner pulp) were pre-treated by NaOH at 15% dry weight (dw) at 131 °C for 1 h, and then washed until pH dropped to 7. Enzymatic hydrolysis was carried out in 150 mL flasks incubated at 50 °C and shaken at 100 rpm. The substrate was suspended to 3% dw in citrate buffer (50 mM, pH 5). Enzymes were applied at 60 U of mannanase/g dw and *T. reesei* enzymatic cocktail containing 0.66 U of exo-glucanase, 106 U of endo-glucanase, 39 U of xylanase, 8.6 U of mannanase and 25 U of β-glucosidase (Novozyme) per g dry mass of substrate. Sodium azide was added (0.1%) to prevent contamination. Samples were taken at 24 and 48 h to measure glucose (Glucose RTU kit, Biomérieux, France) and reducing-sugar release using DNS method (Miller, 1959). All experiments were performed independently in triplicate.

3. Results

3.1. Purification of wild-type mannanase (wt-Man)

A. aculeatus VN mannanase was purified in two steps: an anion exchange followed by a gel filtration. The wild-type enzyme was purified to homogeneity and showed a single band with relative molecular mass of 43 kDa on SDS-PAGE (see in Fig. 3, lane 5). Enzyme recovery was 7.6% (Table 1).

Table 1
Purification of wild-type mannanase (wt-Man) from *A. aculeatus* VN culture medium.

| Step	Total activity (U)	Proteins (mg)	Specific activity (U/mg)	Activity yield (%)
Culture medium	10,290	389.6	26.4	100
Ultrafiltration	8899	104.6	85.1	86.5
DEAE	3741	39.5	94.8	36.4
Ultrafiltration	3486	30.6	114	33.9
Gel filtration	781	5.7	138	7.6

3.2. Cloning of the mannanase gene

The mannanase gene was amplified from *A. aculeatus* VN genomic DNA using specific primers designed from the *A. aculeatus* mannanase coding sequence (GenBank accession no. L35487, Christgau et al., 1994). *A. aculeatus* VN mannanase gene contains 1206 nucleotides, including two introns of 63 nucleotides at positions 646 and 839. The deduced amino-acid sequence showed 100, 74 and 60% similarity to *A. aculeatus* (GenBank accession no. L35487), *A. niger* (GenBank accession no. ACJ06979.1) and *T. reesei* (GenBank accession no. L25310.1), respectively. The sequence alignment of this enzyme with GH5 mannanases above reveals that two catalytic residues Glu180 and Glu287 together with five residues Arg64, Asn179, His253, Tyr255 and Trp318 are strictly conserved in all GH5 mannanases (Hilge et al., 1998).

3.3. Expression and purification of two recombinant enzymes: mannanase (rec-Man) and mannanase–CBM (rec-ManCBM)

A. aculeatus VN mannanase encoding sequence was inserted into pAN52-4 expression vector (Fig. 1A). To improve the hydrolytic capacity of mannanase on lignocellulosic substrate, a second construction was obtained by fusing the CBM1 of *A. niger* CbhB at the C-terminal end of mannanase. The catalytic domain and CBM were fused by a native hyperglycosylated linker (Fig. 1B). Sixty transformants for each construction were selected for mannanase activity screening. In flask culture, the activity of the best rec-Man and rec-ManCBM transformants increased until day 12 (Fig. 2), reaching 20 U/mL and 17 U/mL, respectively. No mannanase activity was detected in the control culture (*A. niger* transformed with pyrG without expression vector).

In order to visualize recombinant mannanases in the best producing transformants, supernatants were loaded onto His-SpinTrap columns. Both flow-through and eluted fractions were subjected to SDS-PAGE analysis. *A. aculeatus* VN mannanase was used as a control. Single bands corresponding to relative molecular masses of 54 kDa and 79 kDa (Fig. 3, lanes 4 and 2) were observed in
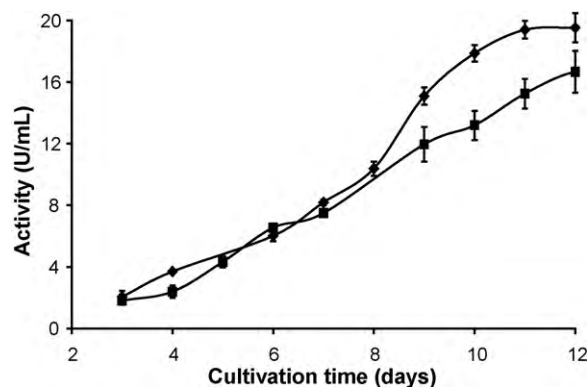

Fig. 2. Time-course of rec-Man and rec-ManCBM productions. (♦) rec-Man, (■) rec-ManCBM. Activity was expressed in U/mL and was monitored for 12 days.

the eluted fractions of rec-Man and rec-ManCBM, respectively. The masses of rec-Man and rec-ManCBM increased by 11 kDa and 36 kDa, respectively, compared with the mass of wild-type enzyme (43 kDa). Adding linker-CBM theoretically increases the relative mass by 8 kDa. Hence, these higher molecular weights of in both recombinant enzymes could be due to post-translational modification (*i.e.*, enzyme glycosylation). Deglycosylation experiments were performed in order to remove N-linker carbohrates. After deglycosylation, although wt-Man and rec-Man displayed the same relative molecular mass of 41 kDa, rec-ManCBM showed a band of 66 kDa higher than the theoretical mass (data not shown) which suggest that rec-ManCBM was mainly O-glycosylated. The analysis of rec-ManCBM amino-acid sequence by NecNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 3.1 (http://www.cbs.dtu.dk/services/NetOGlyc/) programs showed that this enzyme has 4 potential N-glycosylation sites located in the catalytic module and 28 potential O-glycosylation sites (1 site
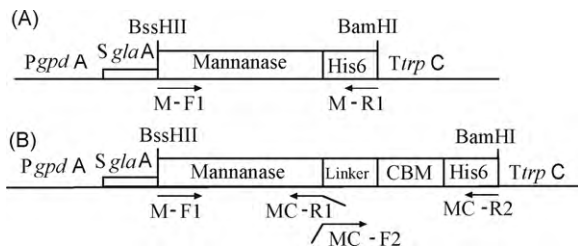
Fig. 1. Construction of two expression vectors pMan (A) and pManCBM (B). P*gpd*A: *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter gene; S*gla*A: *Aspergillus niger* glucoamylase signal peptide gene; T*trp*C: *Aspergillus nidulans* trpC terminator gene; BssHII, BamHI: restriction enzyme targets; M-F1 forward primer 5′-AATGCGCGCCCTTCCCCGGACGCCGAAC-3′, M-R1 reverse primer 5′-GTAGGATCCTTAGTGGTGGTGGTGATGGTGCTTCGACTGCGCATTGAT-3′; MC-R1 reverse primer 5′-ACTGGAGTAAGTAGAGCCCTTAGACTGCGCATTGAT-3′, MC-F2 forward primer 5′-ATCAATGCGCAGTCTAAGGGCTCTACTTACTCCAGT-3′, MC-R2 reverse primer 5′-ATGCGGATCCTTAATGGTGATGGTGATGGTGCAGACACTGCGAGTAGTA-3′.
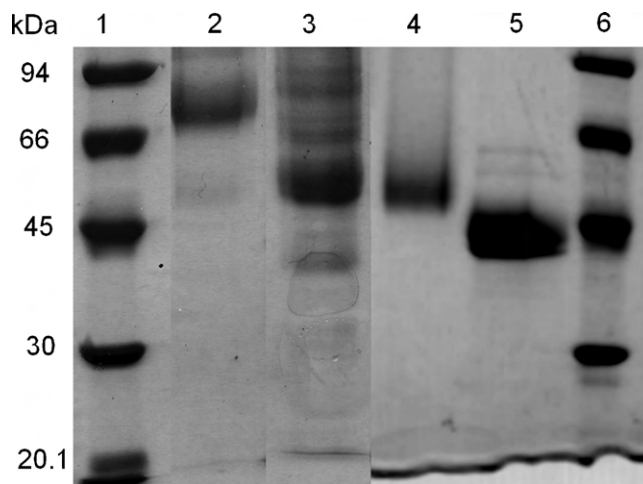
Fig. 3. SDS-PAGE gel of wt-Man, rec-Man and rec-ManCBM. SDS-PAGE gel was stained with Coomassie blue. Lanes 1 and 6: molecular weight standards. Lane 2: rec-ManCBM. Lane 3: degradation of rec-ManCBM. Lane 4: rec-Man. Lane 5: wt-Man.
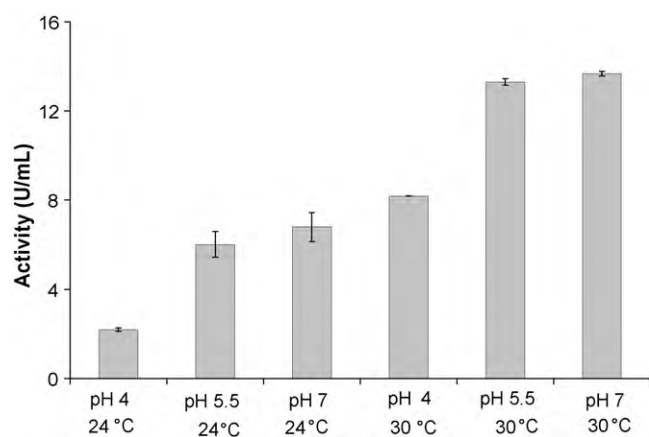
Fig. 4. Mannanase activity at day 11 of growth in different culture conditions. Temperature was maintained at 24 °C or 30 °C. pH was adjusted daily to 4, 5.5 or 7 with 1 M citric acid.

located in the catalytic module and 27 sites located in the linker). A major band of 54 kDa corresponding to the relative molecular mass of rec-Man was visualized in the flow-through fraction from rec-ManCBM purification (Fig. 3, lane 3). The high mannanase activity in this fraction led us to attribute this band to mannanase. Thus, fused rec-ManCBM was degraded in the culture medium. This degradation could be explained by the cleavage of the linker between mannanase and CBM, as previously demonstrated (Levasseur et al., 2005; Ravalason et al., 2009).

In order to reduce the degradation of fused enzyme, rec-ManCBM expression was optimized by cultivation in different conditions. Temperature was maintained at 24 °C or 30 °C, and pH of the medium was adjusted daily to 4, 5.5 or 7 with 1 M citric acid. Samples were taken at day 11 to measure mannanase activity (Fig. 4). Although, the highest activities were obtained at 30 °C and pH 5.5 and 7, SDS-PAGE analysis on each culture showed that rec-ManCBM was more stable at 24 °C and pH 4 (data not shown). However, at pH 4, rec-ManCBM activity was 3 times lower at 24 °C than 30 °C, prompting us to select pH 4 and 30 °C for the following experiments.

Large scale mannanase productions were carried out in 10 L reactor using the standard conditions (pH 5) for rec-Man and pH adjusted conditions for rec-ManCBM (pH 4). Two recombinant enzymes were purified further from the culture media by a single chromatographic step. Activity recoveries were 36% and 12% for rec-Man and rec-ManCBM, respectively.

3.4. Enzymatic characterization

The biochemical and kinetic parameters of the three enzymes wt-Man, rec-Man and rec-ManCBM were compared using locust bean gum as substrate. The three enzymes showed no significant differences in pH and temperature optima. The optimal temperature ranged between 70 and 75 °C (Fig. 5A) and optimal pH around 2.5 and 3 (Fig. 5B). Three enzymes were stable over a pH range between 4 and 7.

Focusing on temperature stability, all three enzymes were stable below 65 °C. Above this temperature, rec-ManCBM showed a higher stability than wt-Man and rec-Man. At 65 °C, wt-Man and rec-Man retained only 20% activity after 350 min whereas rec-ManCBM retained over 60% activity (Fig. 5C). The half-life times were 100, 103 and 693 min at 65 °C and 8, 9 and 27 min at 70 °C for wt-Man, rec-Man and rec-ManCBM, respectively. Concerning kinetics properties, the three enzymes displayed similar kinetic parameters on locust bean gum (Table 2).
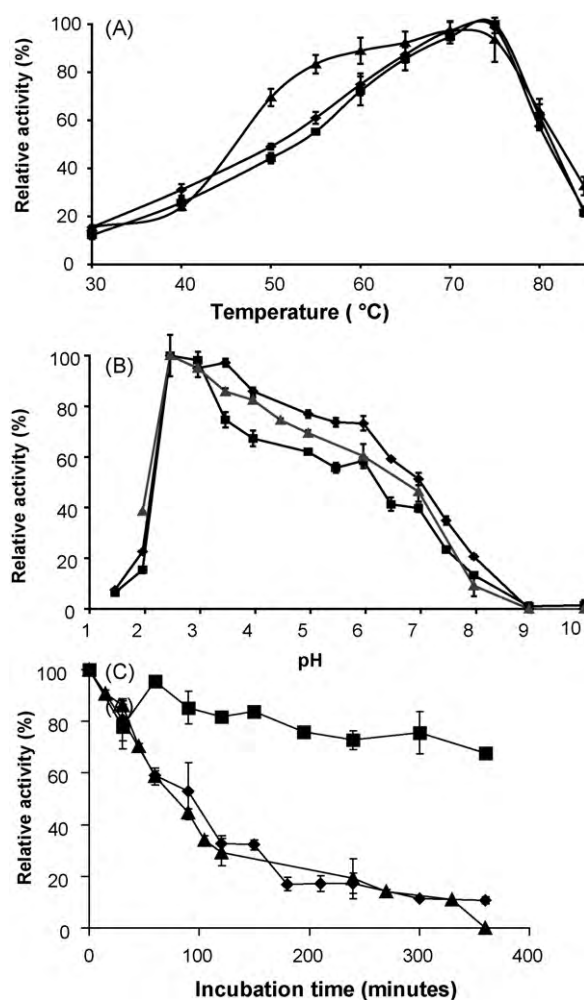
Fig. 5. Optimal temperature (A), optimal pH (B) and stability at 65 °C (C) of the three mannanases. (▲) wt-Man, (♦) rec-Man, (■) rec-ManCBM.

3.5. Absorption of rec-ManCBM

The cellulose-binding affinity of rec-ManCBM was evaluated on microcrystalline cellulose Avicel PH101 and pre-treated softwood. The fused enzyme was mixed with substrate at different ratios (Fig. 6). The supernatant mannanase activity was measured after incubation for 1 h at 4 °C. Maximum bindings (B_{max}) were 750 and 480 μg enzyme per g Avicel or g softwood, respectively. No significant binding of wt-Man and rec-Man was detected.

3.6. Hydrolysis of softwood

Mannanases were combined with the cellulase and xylanase of *Trichoderma reesei* and β-glucosidase in order to improve the hydrolysis of softwood. Adding rec-ManCBM to the enzyme cock-

Table 2
Comparison of kinetic parameters between the three mannanases.

	wt-Man[a]	rec-Man[b]	rec-ManCBM[c]
K_m (mg/mL)	1.2 ± 0.2	1.3 ± 0.2	0.9 ± 0.1
V_{max} (U/mg)	296.7 ± 12.8	385.3 ± 12.4	248.6 ± 8.3
k_{cat} (1/s)	212	346	327

[a] Mannanase from *A. acculeatus* VN.

[b] Recombinant mannanase expressed in *A. niger* D15#26.

[c] Recombinant mannanase with addition of the linker-CBM expressed in *A. niger* D15#26.
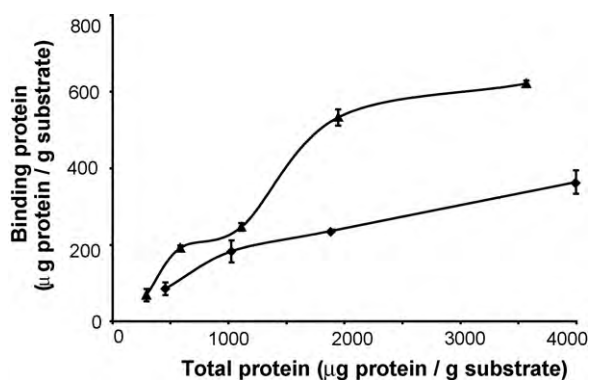
Fig. 6. Adsorption isotherms of rec-ManCBM on Avicel cellulose and softwood. (▲) Avicel cellulose, (♦) softwood. Enzyme was incubated with substrate at 4 °C for 1 h in citrate buffer 50 mM, pH 5.

Table 3
Saccharification of pre-treated softwood.

Enzyme	Glucose (mg/mL)	Reducing sugars (mg/mL)
Enzymatic cocktail	2.27 ± 0.04	3.26 ± 0.06
Enzymatic cocktail + wt-Man	2.31 ± 0.07	3.43 ± 0.10
Enzymatic cocktail + rec-Man	2.30 ± 0.07	3.36 ± 0.04
Enzymatic cocktail + rec-ManCBM	2.56 ± 0.03	3.63 ± 0.08

Basic treatment at 130 °C for 1 h and incubation with *T. reesei* enzymatic cocktail. Mannanase: 60 U/g dw. Sodium azide 0.1%. 50 °C, 100 rpm, 48 h.

tail led to a significant 13% increase in glucose amount released, against only a 1–3% increase for wt-Man or rec-Man. Release of reducing sugars increased more than 12% by adding rec-ManCBM and 3–5% by adding rec-Man or wt-Man (Table 3). Experiments were carried out independently in triplicate.

4. Discussion

Mannanase and mannanase–CBM enzymes were successfully expressed in *A. niger* D15#26, yielding 20 U/mL and 17 U/mL in the respective culture media.

4.1. Linker stability – effect of protease

The rec-ManCBM was artificially constructed, with the catalytic and binding domains connected by the 46 amino-acids hyper-O-glycosylation linker rich in serine and threonine (Gielkens et al., 1999). This linker provided the necessary distance between the two functional domains to fold independently and to conserve a conformational freedom relative to one another (Srisodsuk et al., 1993; Quentin et al., 2002). Furthermore, the high degree of glycosylation could increase protein sequence stability by protecting the linker against proteases (Langsford et al., 1987; Quentin et al., 2002). In fact, the linker region of many enzymes is sensitive to proteolytic digestion (Linder and Teeri, 1997). In this study, the linker was cleaved in rec-ManCBM culture medium. Similar observations have been reported for the fused laccase–CBM (Ravalason et al., 2009) or the bifunctional enzyme feruloyl esterase–xylanase–CBM (Levasseur et al., 2005), both expressed into *A. niger* D15#26. Specific proteases which can cleave the linker in enzyme–CBM had previously been found in cellulolytic organism media (Gilkes et al., 1988; Langsford et al., 1987). To explain this proteolytic cleavage, Linder and Teeri (1997) suggested that cellulases with CBMs are only required in the early stages of cellulose degradation when the main proportion of substrate remains insoluble. At a later stage, when the substrate has been largely converted into oligosaccha-

rides, enzymes without CBM operating in the liquid phase may be more effective.

Different ways to stabilize fused enzymes were experimented. Gustavsson et al. (2001) tested the stability with different linker lengths (4–44 residues) and with the presence of potential glycosylation sites. Jahic et al. (2003) decreased protease levels by changing the culture conditions, including pH and temperature. Adding casamino acid to the medium as competitive substrate has been applied as a strategy to reduce proteolytic enzymes in shake-flask culture (Sreekrishna et al., 1997). In this study, adding casamino acid led to an increase in mannanase activity in the culture medium but had no effect on rec-ManCBM stability (data not shown), consistently with Jahic et al. (2003). The change of culture conditions influenced the stability of rec-ManCBM. This enzyme was more stable in acid medium than in neutral or basic media. In fact, different culture conditions influence the microbial endogenous protein secretion, particularly proteases. *Aspergillus* can produce different types of proteases, including aspartic proteases (pH activity 3–4), metalloproteases (pH activity 6–9) and serine proteases (pH activity 8–9) (Monod et al., 2002). The stability of rec-ManCBM in acid medium suggests that rec-ManCBM could be cleaved by neutral and/or basic proteases but remain stable against acid proteases. There is another potential explanation for the stability at pH 4. *A. niger* D15#26 host transformants is a mutant derived from a protease-deficient *A. niger* AB1.13 strain that does not acidify the culture medium (Gordon et al., 2000). Consequently, culture pH is usually around neutrality or slightly basic. Such culture medium at pH 4 was not the suitable culture condition for *A. niger* D15#26. In this condition, small quantities of proteases were released, and the enzyme remained stable. Similarly, Jahic et al. (2003) reported that decreasing pH from 5.0 to 4.0 resulted in a 40–90% increase in the full-length lipase–CBM product.

4.2. Effect of CBM on enzymatic properties

Although the fusion of mannanase to CBM did not modify the temperature and pH optima (Fig. 5A and B) or the pH stability of the enzyme, rec-ManCBM displayed a higher thermal stability than wt-Man and rec-Man (Fig. 5C). Based on data from literature, adding CBMs could affect the enzyme stability profile, but it is difficult to predict their effect on enzyme characteristics. Adding a CBM at the C-terminal end of a chimerical bifunctional feruloyl esterase–xylanase enzyme had no effect on enzyme characteristics (Levasseur et al., 2005). Laccase–CBM exhibited a higher optimal temperature but less thermostability than the wild-type and recombinant laccases (Ravalason et al., 2009). Mamo et al. (2007) reported a drop in thermal stability after fusing a CBM to a xylanase from *Bacillus halodurans*.

4.3. Binding ability

Avicel and softwood were used for studying adsorption kinetics. Avicel show a strong polymerization of 100–240 glucopyranose units and 50–60% crystallinity (Wood, 1988). The binding of rec-ManCBM to Avicel confirmed that the structure of CBM1 was apparently not perturbed, and the CBM conserved its function in the modular enzyme. Rec-ManCBM was shown a higher binding capacity to Avicel than to softwood (Fig. 6). This difference is reflecting the different ratio of cellulose in Avicel and softwood, furthermore softwood cellulose is embedded in lignin and hemicelluloses.

4.4. Effect of CBM on hydrolysis of softwood

Galactoglucomannan is abundant in softwood (10–15% dry mass) and its concentration varies with type of softwood and location in the plant cell wall: normal wood contains higher

galactoglucomannan content (18%) than compression wood (9%), and the plant cell wall is richer in glucomannan in the S2 layer than the S1 or S3 layer (Wiedenhoeft and Miller, 2005). According to Salmén (2004), softwood glucomannan is more closely associated with cellulose microfibrils whereas xylan is more associated with lignin. Some of the glucomannans in softwood are incorporated into aggregates of cellulose. Adjacent to these aggregates, most glucomannans are arranged in parallel with cellulose fibrils, to which they are tightly connected. This structure should inhibit the cellulase approach to cellulose fibrils during saccharification. Adding mannanase to the enzymatic hydrolytic cocktail promote cellulase contact with cellulose fibrils by removing glucomannans at the surface. In this study, the addition of wt-Man or rec-Man slightly increased glucose and reduced sugar release (Table 3).

It is generally acknowledged that adding a CBM to the enzymatic catalytic domain increases the enzyme concentration at the substrate surface and thus increases the efficacy of enzyme (Linder and Teeri, 1997). The presence of CBM in *T. reesei* mannanase increased by 5-fold the mannan hydrolysis of a cellulose–mannan complex (Hägglund et al., 2003). Moreover, the mobility of CBMs on the surface of crystalline cellulose (Jervis et al., 1997) can induce mannanase movement on the substrate surface, and thus enhances the removal of glucomannan from the cellulose surface. In addition, CBM alters the binding between cellulose layers, making the substrate more accessible (Shoseyov et al., 2006). As a result, rec-ManCBM facilitated the action of cellulases and led to a 13% increase in the amount of glucose released versus a 1–3% increase in glucose release using wt-Man or rec-Man (Table 3).

In conclusion, this study demonstrated that mannanase enzymes can be useful in the destructuration of softwood and suggested that they could be used as enzyme helpers (*i.e.*, in association with *T. reesei* enzymatic cocktail) for efficient hydrolysis of lignocellulosic substrate. A number of *Aspergillus* species are naturally capable of producing mannanase, but expression of the mannanase gene in the industrial *A. niger* D15#26 strain could improve enzyme productivity. In addition, fusion of the enzyme with CBM improves the hydrolytic capacity against lignocelluloses, and adding this chimerical enzyme to the hydrolysis enzyme cocktail of *Trichoderma reesei* appears to improve hydrolysis yield.

References

Alberto, F., Navarro, D., de Vries, R.P., Asther, M., Record, E., 2009. Technical advance in fungal biotechnology: development of a miniaturized culture method and an automated high-throughput screening. Lett. Appl. Microbiol. 49, 278–282.

Benech, R.O., Li, X., Patton, D., Powlowski, J., Storms, R., Bourbonnais, R., Paice, M., Tsang, A., 2007. Recombinant expression, characterization, and pulp prebleaching property of a *Phanerochaete chrysosporium* endo-β-1,4-mannanase. Enzyme Microb. Technol. 41, 740–747.

Cantarel, B.L., Coutinho, P.M., Rancurel, C., Bernard, T., Lombard, V., Henrissat, B., 2009. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res. 37, 233–238.

Christgau, S., Kauppine, S., Vind, J., Kofod, L.V., Dalbøge, H., 1994. Expression cloning, purification and characterization of a beta-1,4-mannanase from *Aspergillus aculeatus*. Biochem. Mol. Biol. Int. 33, 917–925.

Do, B.C., Dang, T.T., Berrin, J.G., Haltrich, D., To, K.A., Sigoillot, J.C., Yamabhai, M., 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, 59–70.

Demirbas, A., 2007. Progress and recent trends in biofuels. Prog. Energy Combust. Sci. 33, 1–18.

De Vries, R.P., Visser, J., 2001. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. Microbiol. Mol. Biol. Rev. 65, 497–522.

Dhawan, S., Kaur, J., 2009. Microbial mannanases: an overview of production and applications. Crit. Rev. Biotechnol. 27, 197–216.

Gilkes, N.R., Warren, R.A.J., Miller, R.C., Kilburn, D.G., 1988. Precise excision of the cellulose binding domains from two *Cellulomonas fimi* cellulases by a homologous protease and the effect on catalysis. J. Biol. Chem. 263, 10401–10407.

Gielkens, M.M.C., Dekkers, E., Visser, J., de Graaff, L.H., 1999. Two cellobiohydrolase-encoding genes from *Aspergillus niger* require D-xylose and the xylanolytic transcriptional activator XlnR for their expression. Appl. Environ. Microbiol. 65, 4340–4345.

Gordon, C.L., Khalaj, V., Ram, A.F.J., Archer, D.B., Brookman, J.L., Trinci, A.P.J., Jeenes, D.J., Doonan, J.H., Wells, B., Punt, P.J., van den Hodel, C.A.M.J.J., Robson, G.D., 2000. Glucoamylase: green fluorescent protein fusion to monitor protein secretion in *Aspergillus niger*. Microbiology 146, 415–426.

Gustavsson, M., Lehtiö, J., Denman, S., Teeri, T.T., Hult, K., Martinelle, M., 2001. Stable linker peptides for a cellulose-binding domain–lipase fusion protein expressed in *Pichia pastoris*. Protein Eng. 14, 711–715.

Hägglund, P., Eriksson, T., Collén, A., Nerinckx, W., Claeyssens, M., Stålbrand, H., 2003. A cellulose-binding module of the *Trichoderma reesei* β-mannanase Man5A increases the mannan-hydrolysis of complex substrates. J. Biotechnol. 101, 37–48.

Hilge, M., Gloor, S.M., Rypniewski, W., Sauer, O., Heightman, T.D., Zimmermann, W., Winterhalter, K., Piontek, K., 1998. High-resolution native and complex structures of thermostable β-mannanase from *Thermomonospora fusca* – substrate specificity in glycosyl hydrolase family. Structure 6, 1433–1444.

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., Pease, L.R., 1989. Site-directed mutagenesis by overlap extension using polymerase chain reaction. Gene 77, 51–59.

Jahic, M., Gustavsson, M., Jansen, A.K., Martinelle, M., Enfors, S.O., 2003. Analysis and control of proteolysis of a fusion protein in *Pichia pastoris* fed-batch processes. J. Biotechnol. 102, 45–53.

Jervis, E.J., Haynes, C.A., Kilburn, D.G., 1997. Surface diffusion of cellulases and their isolated binding domains on cellulose. J. Biol. Chem. 272, 24016–24023.

Kirk, T.K., Cullen, D., 1988. Enzymology and molecular genetics of wood degradation by white-rot fungi. In: Yong, R.A., Akhtar, M. (Eds.), Environmentally Friendly Technology for the Pulp and Paper Industry. John Wiley & Sons, Inc, New York, pp. 273–307.

Langsford, M.L., Gilkes, N.R., Singh, B., Moser, B., Miller, R.C.J., Warren, R.A.J., Kilburn, D.G., 1987. Glycosylation of bacterial cellulase prevents proteolytic cleavage between functional domains. FEBS Lett. 225, 163–167.

Levasseur, A., Navarro, D., Punt, P.J., Belaïch, J.P., Asther, M., Record, E., 2005. Construction of engineered bifunctional enzymes and their overproduction in *Aspergillus niger* for improved enzymatic tools to degrade agricultural by-products. Appl. Environ. Microbiol. 71, 8132–8140.

Linder, M., Teeri, T.T., 1997. The role and function of cellulose-binding domains. J. Biotechnol. 57, 15–28.

Mamo, G., Hatti-Kaul, R., Mattiasson, B., 2007. Fusion of carbohydrate binding modules from *Thermotoga neapolitana* with a family 10 xylanase from *Bacillus halodurans* S7. Extremophiles 11, 169–177.

Monod, M., Capoccia, S., Léchenne, B., Zaugg, C., Holdom, M., Jousson, O., 2002. Secreted proteases from pathogenic fungi. Int. J. Med. Microbiol. 292, 405–419.

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

Murashima, K., Kosugi, A., Doi, R.H., 2003. Synergistic effects of cellulosomal xylanase and cellulases from *Clostridium cellulovorans* on plant cell wall degradation. J. Bacteriol. 185, 1518–1524.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428.

Punt, P.J., van den Hondel, C.A., 1992. Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. Methods Enzymol. 216, 447–457.

Quentin, M., Ebbelaar, M., Derksen, J., Mariani, C., van der Valk, H., 2002. Description of a cellulose-binding domain and a linker sequence from *Aspergillus* fungi. Appl. Microbiol. Biotechnol. 58, 658–662.

Ravalason, H., Herpoël-Gimbert, I., Record, E., Bertaud, F., Grisel, S., de Weerte, S., van den Hondel, C.A.M.J.J., Asther, M., Petit-Conil, M., Sigoillot, J.C., 2009. Fusion of a family 1 carbohydrate binding module of *Aspergillus niger* to the *Pycnoporus cinnabarinus* laccase for efficient softwood kraft pulp biobleaching. J. Biotechnol. 142, 220–226.

Record, E., Punt, P.J., Chamkha, M., Labat, M., van den Hondel, C.A.M.J.J., Asther, M., 2002. Expression of the *Pycnoporus cinnabarinus* laccase gene in *Aspergillus niger* and characterization of the recombinant enzyme. Eur. J. Biochem. 269, 602–609.

Salmén, L., 2004. Micromechanical understanding of the cell-wall structure. C. R. Biol. 327, 873–880.

Sanchez, O.J., Cardona, C.A., 2008. Trends in biotechnology production of fuel ethanol from different feedstocks. Bioresour. Technol. 99, 5270–5295.

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

Shoseyov, O., Shani, Z., Levy, I., 2006. Carbohydrate-binding modules: biochemical properties and novel applications. Microbiol. Mol. Biol. Rev. 70, 283–295.

Sreekrishna, K., Brankamp, R.G., Kropp, K.E., Blankenship, D.T., Tsay, J.T., Smith, P.L., Wierschke, J.D., Subramaniam, A., Birkenberger, L.A., 1997. Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia patoris*. Gene 190, 55–62.

Srisodsuk, M., Reinikainen, T., Penttilä, M., Teeri, T.T., 1993. Role of the interdomain linker peptide of *Trichoderma reesei* cellobiohydrolase I in its interaction with crystalline cellulose. J. Biol. Chem. 268, 20756–20761.

Sunna, A., Gibbs, M.D., Chin, C.W.J., Nelson, P.J., Bergquist, P.L., 2000. A gene encoding a novel multidomain β-1,4-mannanase from *Caldibacillus cellulovorans* and action of the recombinant enzyme on kraft pulp. Appl. Environ. Microbiol. 66, 664–670.

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

Timell, T.E., 1967. Recent progress in the chemistry of wood hemicelluloses. Wood Sci. Technol. 1, 45–70.

Warren, R.A.J., 1996. Microbial hydrolysis of polysaccharides. Annu. Rev. Microbiol. 50, 183–212.

Wiedenhoeft, A.C., Miller, R.B., 2005. Structure and function of wood. In: Rowell, R.M. (Ed.), Handbook of Wood Chemistry and Wood Composites. CRC Press, pp. 9–33.

Wood, T.M., 1988. Preparation of crystalline, amorphous and dyed cellulase substrates. Methods Enzymol. 160, 19–25.

Yuan, J.S., Yang, X., Lai, J., Lin, H., Cheng, Z.M., Nonogaki, H., Chen, F., 2007. The endo-β-mannanase gene families in *Arabidopsis*, rice and poplar. Funct. Integr. Genomics 7, 1–16.