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Fusarium verticillioides secretome as a source of auxiliary enzymes to enhance saccharification of wheat straw

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A B S T R A C T

Fusarium verticillioides secretes enzymes (secretome), some of which might be potentially useful for saccharification of lignocellulosic biomass since supplementation of commercial cellulases from *Trichoderma reesei* with the *F. verticillioides* secretome improved the enzymatic release of glucose, xylose and arabinose from wheat straw by 24%, 88% and 68%, respectively. Determination of enzymatic activities revealed a broad range of hemicellulases and pectinases poorly represented in commercial cocktails. Proteomics approaches identified 57 proteins potentially involved in lignocellulose breakdown among a total of 166 secreted proteins. This analysis highlighted the presence of carbohydrate-active enzymes (CAZymes) targeting pectin (from glycoside hydrolase families GH5, GH27, GH28, GH43, GH51, GH54, GH62, GH88 and GH93, polysaccharide lyase family PL4 and carbohydrate esterase family CE8) and hemicelluloses (from glycoside hydrolase families GH3, GH10, GH11, GH30, GH39, GH43 and GH67). These data provide a first step towards the identification of candidates to supplement *T. reesei* enzyme preparations for lignocellulose hydrolysis.

Keywords:

Fusarium verticillioides
Secretome
Saccharification
Wheat straw
Glycoside hydrolases

1. Introduction

Second generation bioethanol produced from various lignocellulosic materials, such as wood, agricultural or forestry residues, is one of the most promising renewable energies. Wheat straw is a feedstock of particular interest since it represents a potentially large and readily available agricultural residue resource of low value. Current costs of enzymatic saccharification are among the largest contributors to the overall cost of lignocellulosic ethanol production. A major challenge is the development of an efficient and economically viable hydrolysis process step (Margeot et al., 2009).

The filamentous fungus *Trichoderma reesei* remains one of the most effective producers of cellulases. For the conversion of cellulose into glucose, three types of cellulolytic enzymes are secreted by *T. reesei*: endoglucanases (EG, EC 3.2.1.4), cellobiohydrolases (CBH, EC 3.2.1.91) and β -glucosidases (BGL, EC 3.2.1.21). Compared to other filamentous fungi, this model fungus was shown to be lacking many hemicellulase and pectinase families (Martinez et al.,

2008). In order to overcome the recalcitrant structure of lignocellulose and release the locked polysaccharides, enzymes altering the interaction between lignin, hemicellulose and cellulose or those involved in degradation of hemicellulose and lignin are required. Previous studies demonstrated that supplementation of cellulases with various hemicellulases or other polysaccharide- and lignin-modifying enzymes can have a beneficial effect on enzymatic hydrolysis, presumably by improving cellulose accessibility (Couturier et al., 2011; Gao et al., 2011).

Many filamentous fungi gain nutrition from the breakdown and the decay of plant biomass and therefore are particularly interesting producers of cell wall-degrading enzymes (CWDEs) including cellulases, hemicellulases, ligninases and pectinases (Van den Brink and de Vries, 2011). Exploration of fungal biodiversity for additional auxiliary enzymes that could be added to *T. reesei* cellulases preparations to improve biomass degradation is of great interest. With the increasing availability of fungal genome sequences and the development of proteomic methods, it has become possible to identify the secreted enzyme pools involved in lignocellulosic biomass degradation. Several descriptive and/or differential analyses of secreted proteins by fungi grown on lignocellulose and related carbon sources have been reported (for a review see Bouws et al. (2008)). However, only a few studies have highlighted the relationships between distribution and prevalence

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of plant CWDEs and efficiency of lignocellulose breakdown (Ravalason et al., 2008; Sharma et al., 2011; Couturier et al., 2012).

In the present study, the secretome of *Fusarium verticillioides*, an ascomycete filamentous fungus well-known as a corn pathogen, was demonstrated to enhance the saccharification of wheat straw in supplementation to commercial cellulases. Taking advantage of the availability of genomic data for this fungus (Ma et al., 2010), the secretome was analyzed and it is discussed how the CWDEs found in the *F. verticillioides* secretome increase the hydrolysis efficiency of *T. reesei* cellulases.

2. Methods

2.1. Raw material

Dried wheat straw (*Triticum aestivum* var. Samuraï,) was obtained from ARD (Pomacle, France) and reduced to particles of 1–2 mm using an analytical mill (IKA A11, IKA-Werke, Staufen, Germany). The composition of untreated wheat straw was 35.6% cellulose, 22.6% xylan, 1.5% galactan, 3.3% arabinan and 17.2% lignin (on a dry weight basis).

2.2. Fungal strain

F. verticillioides strain 7600 (obtained from the Fungal Genetics Stock Center, FGSC, Kansas City, USA) was maintained in the “Centre International de Ressources Microbiennes” culture collection dedicated to filamentous fungi of biotechnological interest (CIRM-CF; <http://www.inra.fr/crb-cirm/>), National Institute of Agricultural Research (INRA), Marseille, France. It was selected from a panel of fungal strains previously screened for their capacity to improve the hydrolysis of wheat straw in combination with the commercial cellulases GC220 (Genencor Danisco, NY, USA; data not shown).

2.3. Growth conditions

F. verticillioides was grown in a medium containing (g per liter), autoclaved fraction of maize bran (AFMB) (Bonnin et al., 2001), 5; glucose, 5; yeast extract, 1; ammonium tartrate, 1.84; KH_2PO_4 , 1.3; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.035; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.046; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.007; 1 mL of the vitamin solution as described by Tatum et al. (1950). Cultures were carried out in 500-mL baffled flasks, containing 100 mL medium. Five-millimeters malt agar disks of mycelium from a Petri dish (4 per flask) ground in a FastPrep-24 sample grinder (Q-BIOgene, Irvine, CA) at 4 m/s for 1 min in 1 mL of sterilised purified water (MilliQ, Millipore) were used as inoculums. Incubation took place at 30 °C in a rotary shaker (Infors AG, Switzerland) at 110 rpm. After 7 days, the culture was stopped and the culture medium (secretome) filtered through a 0.45 μm -pore size PVDF membrane (Millipore, Guyancourt, France) and stored at 4 °C until use.

2.4. Enzymatic hydrolysis of wheat straw

The crude secretome of *F. verticillioides* grown on AFMB was used for wheat straw hydrolysis in the presence of *T. reesei* cellulases. Experiments were carried out in 50-mL screw-cap tubes containing the reaction mixture including 1 g of ground wheat straw (dry weight) and 40 mL fungal secretome adjusted at pH 4.8 with HCl (2 M). The suspension was further supplemented with 6.2 FPU/g substrate (dry weight) of commercial cellulases GC220 from *T. reesei* and 22.6 U/g substrate (dry weight) of β -glucosidase Novozyme SP188 from *Aspergillus niger* (Novozymes, Bagsvaerd, Denmark). Commercial enzyme loadings refer to enzymatic

activities measured at 45 °C and in a citrate–phosphate buffer (pH 4.8) (see Section 2.5). Tetracycline (12.5 g/L) and cycloheximide (10 g/L) were added to prevent microbial contamination. Enzymatic hydrolysis was performed at 45 °C and 110 rpm in a rotary shaker (Infors AG, Switzerland) for 72 h. Control experiments were carried out under the same conditions using the commercial enzymes and secretome individually, and enzymes without substrate.

Samples were taken from the reaction mixture and the monosaccharides released after hydrolysis were analyzed by High Performance Anion Exchange Chromatography (HPAEC) coupled with PAD (ICS3000; Dionex, Sunnyvale, CA) equipped with a Carbo-Pac PA-1 analytical column (250 by 4 mm) as described by Couturier et al. (2011). Enzymatic reactions were stopped by the addition of 18 mM NaOH before injection (5 μL) into the HPAEC system. Elution (1 mL/min) was carried out in 18 mM NaOH. Calibration curves were plotted using glucose, xylose, arabinose, galactose and cellobiose standards (Sigma–Aldrich, Lyon, France) and response factors were calculated (Chromeleon program, Dionex). All assays were carried out in triplicate. The concentration of reducing sugars in the hydrolysates was analyzed with the dinitrosalicylic acid method using glucose as a standard, as described by Navarro et al. (2010).

2.5. Enzyme activity assays and protein determination

Unless otherwise indicated, all enzymatic activities were measured in citrate–phosphate buffer (50 mM, pH 4.8) and at 45 °C.

Overall cellulase activity was determined using filter paper (FP). Whatman No. 1 filter paper disks (6 mm diameter) were incubated with the enzyme solutions for 2 h, in a final reaction volume of 150 μL . The reducing sugars released were measured using the dinitrosalicylic acid method with glucose as the standard. One unit of filter paper activity was defined as the amount of protein that released 1 μmol of reducing sugar per min.

The activities of enzymes towards carboxymethylcellulose (CMC), microcrystalline cellulose (avicel PH-101), low viscosity wheat arabinoxylan, insoluble wheat arabinoxylan, birchwood xylan, citrus pectin, sugar beet arabinan and larch arabinogalactan were measured by using the dinitrosalicylic acid method, as described by Couturier et al. (2012). Briefly, 20 μL of enzymes were mixed with 100 μL of substrates at 1% concentration and incubated for 1 h. The reaction was terminated by the addition of dinitrosalicylic acid reagent (1% w/v) and boiled for 10 min. The absorbance at 540 nm was measured relative to a glucose standard curve. One unit of enzyme activity was defined as the amount of protein that released 1 μmol of reducing sugar per min.

β -Glucosidase, β -xylosidase, α -arabinofuranosidase and α -galactosidase activities were assayed by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl- β -D-glucopyranoside (pNP-Glu), *p*-nitrophenyl- β -D-xylopyranoside (pNP-X), *p*-nitrophenyl- α -L-arabinofuranoside (pNP-A), and *p*-nitrophenyl- α -D-galactopyranoside (pNP-Gal), respectively, as described by Couturier et al. (2012). Briefly, 20 μL of enzymes were mixed with 100 μL of substrate (1 mM) and incubated for 20 min. The reaction was stopped by adding 130 μL of 1 M sodium carbonate (pH 11.5), and the release of 4-nitrophenol was quantified at 410 nm using a 4-nitrophenol standard curve. One unit of enzyme activity was defined as the amount of protein that released 1 μmol of *p*-nitrophenol per min.

Laccase and cellobiose dehydrogenase (CDH) activities in the culture supernatant were assayed as described in Sigoillot et al. (2002). One unit of enzyme activity was defined as the amount of protein that oxidizes 1 μmol of substrate per min.

Lignin and manganese peroxidase activities were determined spectrophotometrically as described by Ravalason et al. (2008).

One unit of enzyme activity was equivalent to 1 μmol of product formed per min

Protein concentration was determined by using the BioRad protein assay kit with bovine serum albumin as the standard (BioRad, Marnes-la-Coquette, France).

All the experiments were performed in triplicate and the standard deviation was lower than 10% of the mean.

2.6. Identification of proteins by LC-MS/MS analysis

Proteins (40 μg) were separated by one-dimensional (1D) electrophoresis (Precasted Tris-Glycine 10% SDS-PAGE, BioRad, Marnes-la-Coquette, France) and stained with Coomassie blue (BioRad, Marnes-la-Coquette, France). Each 1D electrophoresis lane was cut into 24 pieces of gel (2 mm in width) and protein identification was performed using PAPPISO platform facilities (<http://pappiso.inra.fr>). In-gel digestion was carried out with the Progest system (Genomic Solution) according to a standard trypsinolysis protocol. Gel pieces were first washed twice with 50% (v/v) acetonitrile, 25 mM ammonium carbonate in water. Each sample was supplemented with 10 μL of 10 mM DTT and incubated for 1 h at 56 °C. After cooling, the supernatant was removed and the samples were incubated in the presence of 10 μL of 55 mM iodoacetamide at room temperature in the dark. Gel plugs were washed with acetonitrile and then dried in a vacuum speed concentrator. Digestion was performed for 6 h at 37 °C with 100 ng of modified trypsin (Promega) dissolved in 25 mM NH_4CO_3 . Tryptic peptides were first extracted with 50% (v/v) acetonitrile, 0.5% trifluoroacetic acid in water, and then with pure acetonitrile. Both peptide extracts were pooled, dried in a vacuum speed concentrator and suspended in 25 μL of 2% (v/v) acetonitrile, 0.08% (v/v) trifluoroacetic acid in water.

LC-MS/MS analysis was performed using the method described by Couturier et al. (2012). The raw mass data obtained were first converted to mzXML format with the ReAdW software (<http://tools.proteomecenter.org/software.php>). Protein identification was performed querying MS/MS data against the *F. verticilloides* 7600 protein database (*Fusarium* Comparative Sequencing Project, Broad Institute of Harvard and MIT (<http://www.broadinstitute.org/>), 2011.01.28) together with an in-house contaminant database, using the X!Tandem software (X!Tandem Tornado 2008.02.01.3, <http://www.thegpm.org>) with the following parameters: one trypsin missed cleavage allowed, alkylation of cysteine and conditional oxidation of methionine, precursor and fragment ion set at 10 ppm and 0.5 Da, respectively. A refined search was added with similar parameters except that semitryptic peptides and possible N-terminal acetylation of proteins were searched. All peptides matched with an *E*-value lower than 0.01 were parsed with X!Tandem pipeline software (<http://pappiso.inra.fr/bioinfo/xtandempipeline/>). Proteins identified with at least two unique peptides and a log (*E*-value) lower than 4 were validated.

3. Results and discussion

3.1. Effect of *F. verticilloides* secretome supplementation on the hydrolysis of wheat straw

In order to evaluate the potential of *F. verticilloides* secretome to improve the enzymatic hydrolysis of untreated wheat straw, the fungal strain was grown on a medium containing Autoclaved Fraction of Maize Bran (AFMB) as carbon supplement. AFMB is constituted of soluble and highly substituted arabinoxylan that is known to promote the production of CWDEs (Bonnin et al., 2001; Couturier et al., 2012). The effect of the resulting fungal secretome on wheat straw saccharification was studied by determining the

kinetics of glucose, xylose, arabinose and reducing sugars released during a 72 h hydrolysis (Fig. 1). All enzymatic systems were supplemented with β -glucosidase (Novozyme SP188) to prevent end-product inhibition from cellobiose accumulation for efficient and extensive hydrolysis. Cellulose to glucose conversion yield reached 24% with GC220 alone (Fig. 1A). This value is in accordance with those of previous studies on enzymatic hydrolysis of untreated wheat straw (Rémond et al., 2010). Although lower amounts of glucose were released with *F. verticilloides* secretome alone, supplementation of GC220 with the secretome resulted in a 24% increase in cellulose conversion, indicating a synergism between both enzyme systems. A more striking effect was observed for xylose and arabinose release, as the addition of *F. verticilloides* secretome to GC220 led to an 88% increase in xylan conversion (Fig. 1B) and a 68% increase in arabinan conversion (Fig. 1C). According to these results, the highest release of reducing sugars was obtained using the combination of *F. verticilloides* secretome and GC220, compared to any of the single enzymes systems (Fig. 1D). It should be noted that the total amount of reducing sugars released was higher than the sum of the individual monosaccharides glucose, xylose and arabinose (Table 1). These findings suggest the release of other sugars from wheat straw, either in monomeric or oligomeric form. The impact of protein loading on the hydrolysis efficiency was also evaluated (Table 1). Interestingly, proteins from *F. verticilloides* crude secretome supplementing GC220 accounted for only approximately 20% of the total protein load. Furthermore, by using a twofold protein load for the *F. verticilloides* secretome, no significant additional benefits to cellulose and xylan conversion were observed. An increase in GC220 load did not impact glucose release and only led to a slight increase in xylose, arabinose and reducing sugars.

To characterize the enzymatic system produced by *F. verticilloides* in comparison to GC220 and Novozyme SP188, cellulose-, hemicellulose-, and pectin-degrading enzymes activities were measured using commercial substrates. Results were expressed as activity loading used for enzymatic hydrolysis in relation to wheat straw dry weight (Table 2). Cellulase activity on filter paper, Avicel PH101 and CMC arose mainly from GC220, and β -glucosidase activity was similar in both GC220 and the *F. verticilloides* secretome. As expected, Novozyme SP188 contained mainly β -glucosidase activity. Furthermore, the main auxiliary activities measured in GC220 were xylan-related activities, while the *F. verticilloides* secretome accounted for a broader set of enzymes, including other hemicellulases and pectinases. It should be pointed out that α -arabinofuranosidase, β -xylosidase and pectin-related activities were in significantly lower amounts in the commercial cocktails than in the *F. verticilloides* secretome.

Due to the low amount of cellulolytic activities of the *F. verticilloides* secretome, saccharification enhancement was not thought to be due to cellulolytic enzymes. Likewise, doubling the commercial cellulases loading did not result in an increase in cellulose conversion (Table 1). Since no cellobiose was detected in the hydrolysis products (data not shown), β -glucosidase activity was not a limiting factor for cellulose conversion. Consequently, further addition of β -glucosidase, provided by the *F. verticilloides* secretome, was not thought to result in improved hydrolysis. Saccharification improvement might therefore be explained by the action of enzymes not directly targeting the cellulose backbone, such as hemicellulases. It is well known that one of the bottlenecks in enzymatic saccharification is the accessibility of cellulose adversely affected by hemicelluloses acting as a physical barrier (Alvira et al., 2010). Wheat straw hemicellulose consists mainly of arabinoxylan substituted by α -L-arabinofuranose, 4-O-methylglucuronic acid and acetyl groups (Sun et al., 2005). Complete arabinoxylan hydrolysis therefore requires xylanase and β -xylosidase activities for backbone biodegradation, but also auxiliary

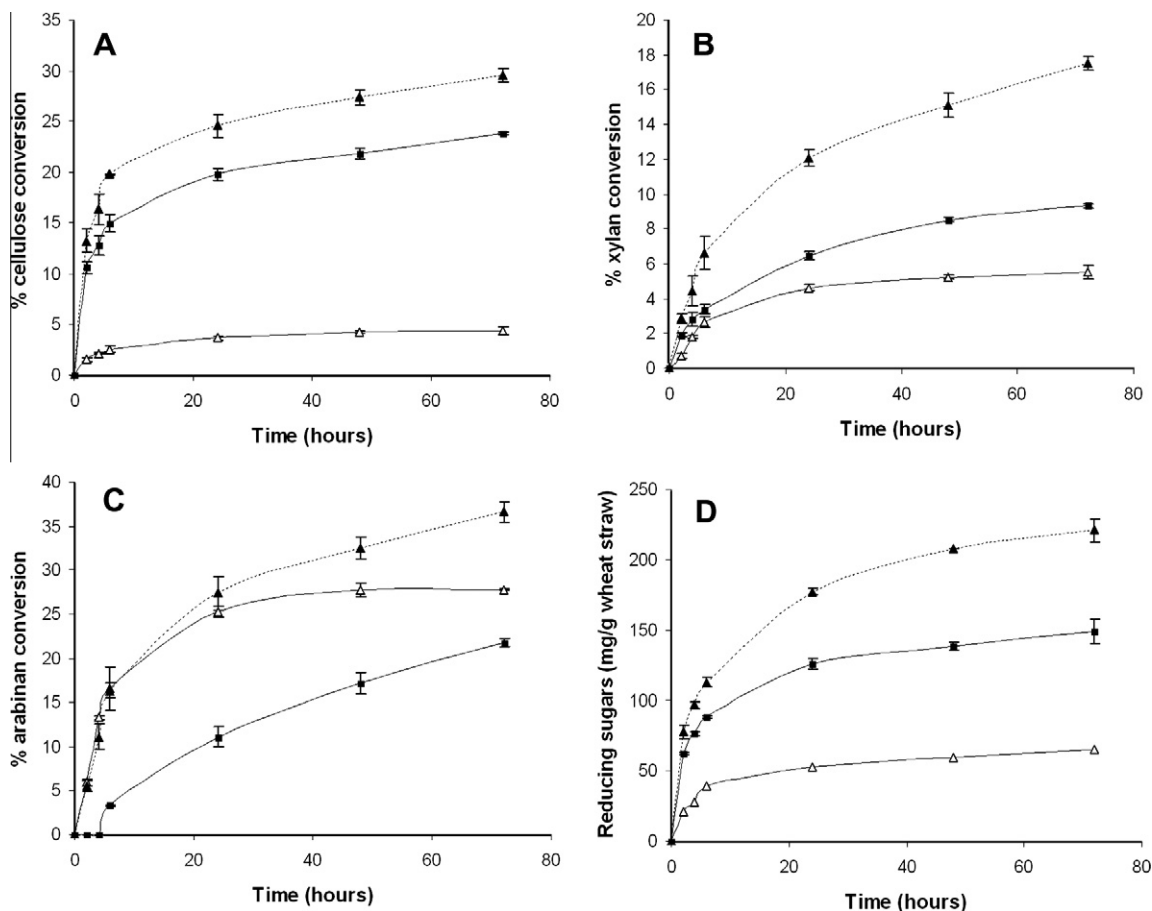


Fig. 1. Enzymatic hydrolysis of wheat straw. Time course of cellulose conversion (A), xylan conversion (B), arabinan conversion (C) and reducing sugar release (D) by *F. verticillioides* extracellular enzymes (Δ), *T. reesei* commercial cellulases (\blacksquare) and both used in combination (\blacktriangle). Polysaccharides conversion yields are expressed as the percentage of maximal theoretical yields. Assays were performed with *F. verticillioides* crude secretome and/or 6.2 FPU/g substrate commercial cellulases (GC220), supplemented with 22.6 U/g substrate commercial β -glucosidase (Novozyme SP188), for 72 h at pH 4.8 and 45 °C. All experiments were conducted in triplicate, and the error bars indicate the standard deviations.

Table 1
 Glucose, xylose, arabinose and reducing sugars released after 72 h hydrolysis of wheat straw by *F. verticillioides* crude secretome and commercial enzyme preparations.

Enzymes and protein loading (mg/g wheat straw)	Sugars released (mg/g wheat straw)			
	Glucose	Xylose	Arabinose	Reducing sugars
GC220 (8.3 mg/g) ^a	94.0 ± 0.4	23.4 ± 0.3	8.0 ± 0.2	156.8 ± 5.2
Novozyme SP188 (3.7 mg/g) ^b				
<i>F. verticillioides</i> (3.5 mg/g)	17.6 ± 1.6	5.5 ± 0.7	10.3 ± 0.1	65.4 ± 0.6
Novozyme SP188 (3.7 mg/g)				
<i>F. verticillioides</i> (3.5 mg/g)	116.8 ± 2.6	44.0 ± 1.0	13.5 ± 0.4	223.3 ± 7.3
GC220 (8.3 mg/g)				
Novozyme SP188 (3.7 mg/g)				
GC220 (16.6 mg/g)	93.2 ± 4.4	32.8 ± 3.6	9.6 ± 1.0	173.8 ± 6.2
Novozyme SP188 (3.7 mg/g)				
<i>F. verticillioides</i> (7.0 mg/g)	110.4 ± 3.2	44.4 ± 1.2	14.2 ± 0.3	219.1 ± 7.0
GC220 (8.3 mg/g)				
Novozyme SP188 (3.7 mg/g)				

^a Protein loading corresponding to 6.2 FPU per g of wheat straw.

^b Protein loading corresponding to 22.6 U of β -glucosidase per g of wheat straw.

enzymes such as α -arabinofuranosidases, α -glucuronidases and acetyl xylan esterases acting cooperatively to release side chains residues (Van den Brink and de Vries, 2011). The high xylanase activities measured in GC220 could explain that 9.3% of xylan was converted to xylose after 72 h of hydrolysis (Table 1). However, the xylose release was twofold higher when GC220 was supplemented with *F. verticillioides* secretome, whereas xylanase activity was threefold lower than that provided by GC220. In addition,

when doubling the GC220 loading, xylose and reducing sugars release remained lower than that obtained by applying GC220 in combination with the *F. verticillioides* secretome (Table 1). Moreover, the highest release of arabinose was obtained using a combination of enzymatic systems, which might be correlated to the high α -arabinofuranosidase activity present in the secretome. These results suggest that the activities related to the boosting effect might arise from more efficient or different xylanases and α -

Table 2

CWDE activities (per g dry matter) of *F. verticillioides* crude secretome and commercial enzyme preparations used in wheat straw hydrolysis experiments.

Enzymes	Cellulose-degrading enzymes (U/g wheat straw)				Xylan-degrading enzymes (U/g wheat straw)					Pectin-degrading enzymes (U/g wheat straw)			
	FP	Avicel	CMC	pNP-Glu ^a	Birch Xylan	Wheat xylan (soluble)	Wheat xylan (insoluble)	pNP-X ^a	pNP-A ^{a,b}	Pectin	Arabinan	Arabinogalactan	pNP-Gal ^a
<i>F. verticillioides</i> secretome	0.1	0.0	0.7	36.1	39.4	55.9	12.0	2.2	5.6	19.8	2.2	4.6	7.2
Commercial enzymes	6.2	7.5	51.8	32.9	84.8	180.4	32.0	0.2	0.1	2.9	0.1	1.1	0.1
GC220	0.2	0.0	0.0	22.6	5.3	4.0	0.1	0.2	1.4	4.2	0.1	0.3	4.2
Novozyme SP188													

^a pNP-Glu: *p*-nitrophenyl- β -D-glucopyranoside. pNP-X: *p*-nitrophenyl- β -D-xylopyranoside. pNP-A: *p*-nitrophenyl- α -L-arabinofuranoside. pNP-Gal: *p*-nitrophenyl- α -D-galactopyranoside.

^b α -Arabinofuranosidases can also release arabinose residues from pectin.

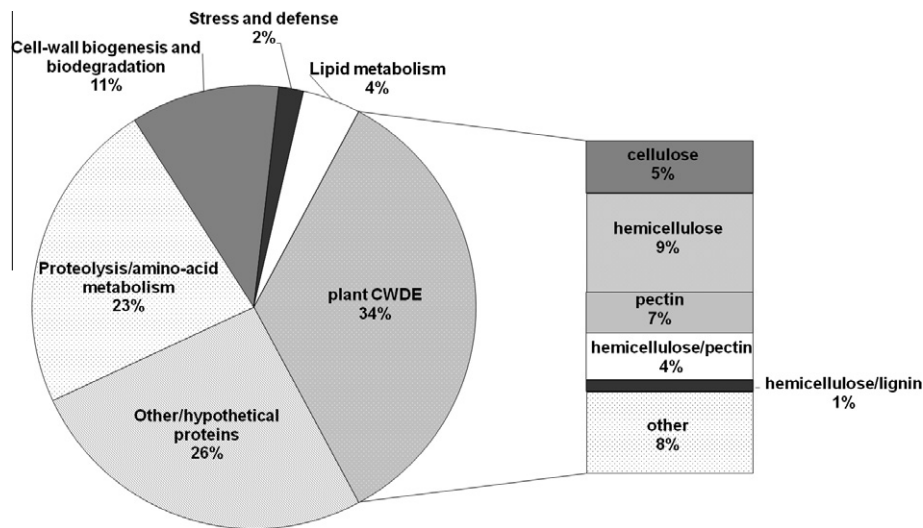


Fig. 2. Functional classification of the 166 proteins detected in *F. verticillioides* secretome grown on autoclaved fraction of maize bran.

arabinofuranosidases than those found in GC220, and/or from other auxiliary hemicellulolytic enzymes absent or different in this commercial cocktail.

3.2. Identification of CWDEs secreted by *F. verticillioides*

Most of the studies on *F. verticillioides* have been devoted to the understanding of mycotoxin biosynthesis, pathogenesis of fungal-plant interactions and the development of new fungal control strategies (Brown et al., 2006). Here, this fungus was demonstrated to produce extracellular enzymes able to improve sugar conversion by *T. reesei* cellulases. Plant pathogenic fungi, in particular fungi belonging to the genus *Fusarium*, have already been shown to represent potent sources of novel enzymes for efficient lignocellulose degradation. Indeed, they are able to secrete a large number and diversity of CWDEs involved in the breakdown of plant cell walls and plant tissue colonization (Phalip et al., 2009; King et al., 2011; Couturier et al., 2012). In order to provide a qualitative global analysis of the *F. verticillioides* secretome, especially of proteins involved in biomass degradation, a proteomic approach was adopted. Using one-dimensional electrophoresis followed by LC-MS/MS, 166 proteins were detected and identified with high confidence. Since only 17% of the proteins detected in the secretome were annotated in the *F. verticillioides* genome database, BlastP was used to assign putative functions by homology searches against all protein sequences in the NCBI nr database ([http://](http://www.ncbi.nlm.nih.gov/blast)

www.ncbi.nlm.nih.gov/blast) using an *e*-value threshold of *e*-40. These analyses led to the identification of 151 proteins representing a 90% identification rate. Of these, 57 proteins (34%) were identified as putative plant CWDEs. The remaining 94 identified proteins included proteins putatively involved in (i) proteolysis and amino-acid metabolism (23%), (ii) fungal cell-wall biogenesis and biodegradation (11%), (iii) lipid metabolism (4%) and (iv) defense and stress response (2%) (Fig. 2). Unsurprisingly, the closest relatives in the databases were mainly proteins from fungi phylogenetically related to *F. verticillioides*, such as *Fusarium graminearum*, *Fusarium oxysporum* and *Nectria haematococca* (*Fusarium solani*). Similarities were high, since the large majority of proteins displayed more than 70% identity with *F. verticillioides* proteins.

The plant CWDEs identified in the *F. verticillioides* secretome included cellulose-, hemicellulose-, pectin-, lignin-degrading and other carbohydrate-modifying enzymes (Table 3). Families were assigned using the CAZy database (<http://www.cazy.org>; Cantarel et al., 2009). Forty-seven glycoside hydrolases (GH) classified in 27 families, two carbohydrate esterases (CE) and one polysaccharide lyase (PL) were identified. Seven proteins harbored a carbohydrate-binding module (CBM) from families CBM1, CBM4, CBM6 and CBM9 that target the plant cell wall. Putative CWDEs were also detected, including proteins of unknown function containing a CBM, or proteins for which no clear putative function could be assigned. The number of proteins corresponding to characterized enzymes from *F. verticillioides* was relatively small, i.e., one GH28

Table 3
 CWDE identified in *F. verticillioides* secretome grown on autoclaved fraction of maize bran.

F. verticillioides ORF ^a	Total number of spectra detected	Number of unique peptides	Predicted protein function	Predicted family ^b	Predicted MW (kDa)/ predicted pI	Closest informative homolog (BlastP)		
						GenBank Accession number	Organism	E value
<i>Cellulose-degrading enzymes</i>								
FVEG_05521	196	25	β -1,4-Glucosidase	GH3	97/5.3	XP386781	<i>F. graminearum</i>	0
FVEG_13391	147	23	β -1,4-Glucosidase	GH3	65/5.2	XP003052401	<i>N. haematococca</i>	0
FVEG_09772	16	10	β -1,4-Glucosidase	GH1	70/5.0	XP003049770	<i>N. haematococca</i>	0
FVEG_04722	16	6	Endo- β -1,4-glucanase	GH61	41/5.2	XP386263	<i>F. graminearum</i>	5.00E-157
FVEG_13055	9	8	β -1,4-Glucosidase	GH3	69/4.8	XP003043120	<i>N. haematococca</i>	0
FVEG_01037	5	4	Endo- β -1,4-glucanase	GH7	55/5.7	AAA65586	<i>F. oxysporum</i>	0
FVEG_12423	5	5	Endo- β -1,4-glucanase	GH5 - CBM1	50/5.6	XP391242	<i>F. graminearum</i>	0
FVEG_08890	4	3	β -1,4-Glucosidase	GH3	88/5.3	XP003043563	<i>N. haematococca</i>	0
FVEG_08825	2	2	Endo- β -1,4-glucanase	GH6 - CBM1	49/5.3	AAA65585	<i>F. oxysporum</i>	0
<i>Xylan-degrading enzymes</i>								
FVEG_09861	86	25	β -1,4-Xylosidase	GH3	72/6.6	XP388169	<i>F. graminearum</i>	0
FVEG_07702	58	11	β -1,4-Xylosidase	GH39	49/7.1	XP381924	<i>F. graminearum</i>	2.00E-165
FVEG_13578	46	9	Endo- β -1,4-xylanase	GH30	52/6.1	ADG62369	<i>Bispora sp.</i>	6.00E-141
FVEG_08605	40	9	Endo- β -1,4-xylanase	GH43	63/8.0	AAV98256	<i>F. graminearum</i>	0
FVEG_08824	39	12	α -Glucuronidase	GH67	94/7.3	CAA92949	<i>T. reesei</i>	0
FVEG_08345	16	7	β -1,4-Xylosidase	GH43	56/8.8	XP387871	<i>F. graminearum</i>	0
FVEG_12502	14	8	Endo- β -1,4-xylanase	GH10	39/8.9	AAV98254	<i>F. graminearum</i>	6.00E-167
FVEG_10625	9	7	β -1,4-Xylosidase	GH43	37/7.5	XP383179	<i>F. graminearum</i>	3.00E-164
FVEG_10098	8	5	Endo- β -1,4-xylanase	GH43	46/5.6	XP384081	<i>F. graminearum</i>	9.00E-167
FVEG_13426	7	4	β -1,4-xylosidase	GH43	40/7.7	XP388169	<i>F. graminearum</i>	3.00E-134
FVEG_08344	6	3	Endo- β -1,4-xylanase	GH43 - CBM6	49/5.8	AAT84252	<i>F. graminearum</i>	0
FVEG_08828	4	2	Endo- β -1,4-xylanase	GH11	25/9.0	XP003050975	<i>F. graminearum</i>	1.00E-95
FVEG_13343	3	2	Endo- β -1,4-xylanase	GH10	36/8.9	AAC06240.1	<i>F. oxysporum</i>	0
FVEG_13553	3	2	Endo- β -1,4-xylanase	GH43	40/4.6	XP384081	<i>F. graminearum</i>	3.00E-101
FVEG_11825	2	2		GH10	26/6.5	XP391434	<i>F. graminearum</i>	4.00E-105
<i>Pectin-degrading enzymes</i>								
FVEG_13077	174	29	α -Galactopyranosidase/ β -arabinopyranosidase	GH27	59/4.9	BAH10648	<i>F. oxysporum</i>	0
FVEG_12289	69	15	α -Galactopyranosidase/ β -arabinopyranosidase	GH27	63/8.8	BAH10649	<i>F. oxysporum</i>	0
FVEG_12299	46	15	Endo- β -1,6-galactanase	GH5	41/5.1	BAF42338	<i>F. oxysporum</i>	0
FVEG_13262	45	14	Exo- α -1,5-arabinanase	GH93	41/6.7	CAJ83998	<i>F. oxysporum</i>	0
FVEG_07518	15	9	α -Galactosidase	GH27	45/7.6	BAJ23877	<i>F. oxysporum</i>	0
FVEG_08342	14	4	Δ -4,5-Unsaturated-glucuronyl hydrolase	GH88	42/4.4	XP383319	<i>F. graminearum</i>	3.00E-162
FVEG_04751	6	2	Rhamnogalacturonan lyase	PL4	75/6.2	XP003050105	<i>N. haematococca</i>	0
FVEG_12766	6	2	Pectin methyl esterase	CE8	42/8.4	XP003041606	<i>N. haematococca</i>	1.00E-168
FVEG_10735	4	2	Exo- α -1,5-arabinanase	GH93	43/8.8	CAX17703	<i>F. graminearum</i>	0
FVEG_13516	4	2	Endo- α -1,4-polygalacturonase	GH28	39/6.1	ABQ11230	<i>F. verticillioides</i>	0
FVEG_12744	3	2	Exo- α -1,4-polygalacturonase	GH28	51/8.1	BAE97083	<i>F. oxysporum</i>	0
<i>Xylan/pectin-degrading enzymes</i>								
FVEG_12952	65	15	α -Arabinofuranosidase	GH54	51/5.9	BAG80559	<i>F. oxysporum</i>	0
FVEG_05689	33	14	α -Arabinofuranosidase	GH51	73/8.3	XP003045662	<i>N. haematococca</i>	0
FVEG_13328	12	5	α -Arabinofuranosidase	GH43	49/5.5	BAG80558	<i>F. oxysporum</i>	0
FVEG_12666	2	2	α -Arabinofuranosidase	GH62	35/8.4	AAV66346	<i>F. graminearum</i>	2.00E-143
<i>Lignin and xylan/lignin degrading enzymes</i>								
FVEG_10901	41	14	Feruloyl esterase	CE1	61/7.5	CAC83933	<i>A. niger</i>	2.00E-116
FVEG_01031	16	4	Superoxide dismutase		28/5.5	XP_380752	<i>F. graminearum</i>	9.00E-105
<i>Xyloglucan-degrading enzymes</i>								
FVEG_12553	159	26	α -Fucosidase	GH29	65/5.2	CBX99860	<i>L. maculans</i>	9.00E-180
FVEG_12189	96	23	α -Fucosidase	GH29	67/5.3	XP391430	<i>F. graminearum</i>	0
<i>Starch-degrading enzymes</i>								
FVEG_13849	109	27	Glucosylase	GH15 - CBM20	63/8.8	ABY89281	<i>F. verticillioides</i>	0
FVEG_13659	106	31	α -Glucosidase	GH31	116/5.8	XM003040737	<i>N. haematococca</i>	0
FVEG_04947	51	14	Glucosylase	GH15 - CBM20	69/5.6	ABY89280	<i>F. verticillioides</i>	0
FVEG_12957	27	11	α -Amylase	GH13	51/5.4	AAZ73168	<i>F. verticillioides</i>	0
<i>Other carbohydrate-modifying enzymes</i>								
FVEG_07688	50	13	Galactose mutarotase	GH32	44/5.7	XP382005	<i>F. graminearum</i>	0
FVEG_11933	5	5	β -Fructofuranosidase		57/4.7	XP003037462	<i>S. commune</i>	3.00E-167
FVEG_01938	3	3	Transaldolase		36/5.2	XP388899	<i>F. graminearum</i>	9.00E-176
FVEG_12482	2	2	Glucose/sorbose dehydrogenase		45/4.7	XP384041	<i>F. graminearum</i>	0

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Table 3 (continued)

F. verticillioides ORF ^a	Total number of spectra detected	Number of unique peptides	Predicted protein function	Predicted family ^b	Predicted MW (kDa)/ predicted pI	Closest informative homolog (BlastP)		
						GenBank Accession number	Organism	E value
<i>Putative cell-wall modifying enzymes</i>								
FVEG_06728	48	7	β-1,4-Glucosidase/avenacinase	GH3	88/4.9	XP003050575	<i>N. haematococca</i>	0
FVEG_12856	19	5	Glycoside hydrolase	Related to GH61	37/9.6	XP003047765	<i>N. haematococca</i>	9.00E-120
FVEG_12107	8	3	Hypothetical protein	CBM9	25/5.3	XP388097	<i>F. graminearum</i>	2.00E-100
FVEG_03820	8	4	Related to ice-nucleation protein	GH16	45/4.8	AAQ14297	<i>F. acuminatum</i>	3.00E-176
FVEG_10100	7	3	Hypothetical protein	CBM4	28/4.3	XP380274	<i>F. graminearum</i>	1.00E-48
FVEG_10606	4	2	β-Glucuronidase/heparanase	GH79	50/5.7	XP003040950	<i>N. haematococca</i>	0

^a ORF number provided by the Broad Institute *F. verticillioides* database.

^b Families were assigned according to the CAZy database. GH, glycoside hydrolase; PL, polysaccharide lyase; CE, carbohydrate esterase; CBM, carbohydrate binding module.

endo-polygalacturonase (GenBank Accession No. ABQ11230; Raiola et al., 2008), two GH15 glucoamylases (ABY89280 and ABY89281; Bluhm et al., 2008) and one GH13 α-amylase (AAZ73168; Bluhm and Woloshuk, 2005). Interestingly, some of the proteins identified, most particularly xylanases, β-xylosidases and α-arabinofuranosidases, were closely related to enzymes detected in secretome studies of *F. graminearum* grown on hop and wheat (Phalip et al., 2005; Paper et al., 2007).

It should be noted that proteomic data were generally in agreement with the enzymatic activities detected in the *F. verticillioides* secretome. Concerning lignin-degrading enzymes, *Fusarium* species, including *F. verticillioides*, were shown to secrete lignin peroxidase, manganese peroxidase and laccase (Pant and Adholeya, 2007; Li et al., 2008). Only a low level of laccase activity was detected in the secretome (data not shown), which could match to a peptide corresponding to a putative multicopper oxidase (protein accession number FVEG_12358 in the *F. verticillioides* genome database).

The presence of some of the enzymes detected could explain the efficiency of the *F. verticillioides* secretome to potentiate *T. reesei* cellulase cocktail. Hence, even if *T. reesei* is considered as an efficient biomass degrader, genome analysis surprisingly revealed the presence of a relatively low diversity of carbohydrate-active enzymes, especially when compared to phytopathogenic fungi (Martinez et al., 2008). In particular *T. reesei* was shown to have only a limited number of hemicellulases encoding-genes. Among them only 5 xylanases, belonging to GH10 and GH11 families, and a limited number of β-xylosidases and α-arabinofuranosidases found in GH3, GH43, GH54 and GH62 families are represented. In comparison, the *F. verticillioides* secretome analysis revealed the presence of a great diversity of GH families assigned to hemicellulases, representing the most numerous class of the CWDEs detected (40% of the proteins). Among them, eight members of the GH43 family, including putative xylanases, β-xylosidases and α-arabinofuranosidases, were identified, while only two GH43 are represented in *T. reesei*. Furthermore, the detection of eight xylanases (from GH10, GH11, GH30 and GH43 families), five β-xylosidases (from GH30, GH39 and GH43 families), and one member of almost all α-arabinofuranosidases GH families (GH43, GH51, GH54 and GH62) in *F. verticillioides* secretome strongly suggested a better ability for this fungus to degrade hemicellulose than *T. reesei*. These results correlate with the fact that very few hemicellulose-degrading enzymes are generally detected in *T. reesei* secretomes or commercial enzyme preparations (Herpœl-Gimbert et al., 2008; Nagendran et al., 2009; Couturier et al., 2012). Furthermore, wheat straw xylan conversion yield with GC220 remained quite low despite its relatively high xylanase activity. Therefore,

as suggested by both enzymatic activities and proteomic data, it is likely that *F. verticillioides* α-arabinofuranosidases and β-xylosidases, acting synergistically with xylanases, were partially responsible for the hydrolysis improvement. These auxiliary enzymes have already been shown to increase the efficiency of lignocellulosic biomass degradation (Couturier et al., 2011; Gao et al., 2011). Besides hemicellulases, a set of pectinases were also detected in the *F. verticillioides* secretome. It is worth noting that among the enzymes detected, two GH93 exo-α-1,5-arabinanases, one PL4 rhamnogalacturonan lyase and one CE8 pectin methyl esterase are absent from *T. reesei* genome. It is well known that *T. reesei* has only a few pectin-degrading enzymes, mainly represented by four GH28 polygalacturonases and one GH105 unsaturated rhamnogalacturononol hydrolase, indicating that this fungus is more adapted to degrade decaying biomass than intact or living plants (Kubicek et al., 2011). In contrast, plant pathogenic fungi are known to produce many pectinases, considered as key virulence factors. In particular, wheat infection with *Fusaria* is characterized by the production of pectin-degrading enzymes as main CWDEs in the early stage of plant invasion (Kang and Buchenauer, 2000; Tomassini et al., 2009). Pectinases are thought to promote plant cell wall penetration and tissue colonization during the infection process. The presence of pectin, even in small amounts, acts as a glue for other polysaccharides and influences cell wall plasticity. It has been shown that reduction of pectin in several plant tissues (including wheat) improved saccharification yields (Lionetti et al., 2010). Furthermore, pectinase supplementation to commercial cellulase and β-glucosidase preparations has been reported to significantly enhance the hydrolysis of wheat straw (Saha et al., 2005). The present data suggest that pectin-degrading enzymes from the *F. verticillioides* secretome participate in the depolymerisation of pectin, leading to the weakening of the surrounding cell wall and thus revealing other plant cell wall polysaccharides.

The increase in sugar conversion when the *T. reesei* enzymatic cocktail was supplemented with the *F. verticillioides* secretome could therefore be explained by the presence of CWDEs absent in *T. reesei*, and/or by a greater diversity of enzymes targeting plant polysaccharides, particularly arabinoxylans and pectin.

4. Conclusion

The addition of crude *F. verticillioides* secretome improves the hydrolytic efficiency of the commercial cellulases cocktail GC220 from *T. reesei* on untreated wheat straw. The *F. verticillioides* secretome contains other and/or more active CWDEs than GC220, as revealed by its lignocellulolytic enzymes activity pattern in relation

to its capacity to improve hydrolysis. Using proteomics, the first secretome characterization of the *F. verticillioides* strain for which genomic data were available was performed. This approach allowed the identification of candidate enzymes that could complement *T. reesei* enzyme preparations to develop enzyme mixtures better adapted for lignocellulose hydrolysis.

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