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Secretomic Survey of *Trichoderma harzianum* Grown on Plant Biomass Substrates

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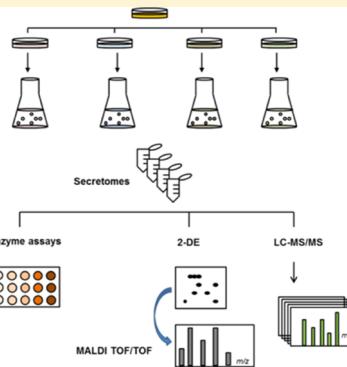
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Supporting Information

ABSTRACT: The present work aims at characterizing *T. harzianum* secretome when the fungus is grown in synthetic medium supplemented with one of the four substrates: glucose, cellulose, xylan, and sugarcane bagasse (SB). The characterization was done by enzymatic assays and proteomic analysis using 2-DE/MALDI-TOF and gel-free shotgun LC-MS/MS. The results showed that SB induced the highest cellulolytic and xylanolytic activities when compared with the other substrates, while remarkable differences in terms of number and distribution of protein spots in 2-DE gels were also observed among the samples. Additionally, treatment of the secretomes with PNGase F revealed that most spot trails in 2-DE gels corresponded to N-glycosylated proteoforms. The LC-MS/MS analysis of the samples identified 626 different protein groups, including carbohydrate-active enzymes and accessory, noncatalytic, and cell-wall-associated proteins. Although the SB-induced secretome displayed the highest cellulolytic and xylanolytic activities, it did not correspond to a higher proteome complexity because CM-cellulose-induced secretome was significantly more diverse. Among the identified proteins, 73% were exclusive to one condition, while only 5% were present in all samples. Therefore, this study disclosed the variation of *T. harzianum* secretome in response to different substrates and revealed the diversity of the fungus enzymatic toolbox.

KEYWORDS: carbohydrate-active enzymes, secretome, shotgun proteomics, *Trichoderma harzianum*



INTRODUCTION

The free-living filamentous fungi of the genus *Trichoderma* (phylum Ascomycota) are the predominant agents of mycobiota in soil, rhizosphere of plants, and decomposing cellulosic materials.^{1,2} Their wide distribution is favored by the ability to produce and secrete hydrolytic enzymes and secondary metabolites, making them effective decomposing agents of organic matter and active antagonists against mycoparasitic fungi and nematodes.^{3,4} The success in the colonization of different habitats is also due to the fast response of the fungus to different surrounding stimuli such as light, temperature, and starvation through the regulation of growth and biosynthesis of specific molecules.^{2,5}

The secretion of hydrolytic enzymes is vital for filamentous fungi because the enzymatic degradation of polymers such as cellulose and hemicelluloses is necessary to produce simpler molecules that can be used as metabolic fuels. During the process of secretion, enzymes are released through the plasma membrane to the periplasmic space to be incorporated into the

cell wall or secreted to the extracellular medium.^{6,7} Non-enzymatic structural proteins are incorporated into plasma membrane and cell wall, where they are involved in recognition and interaction processes with other fungi.⁸

Recently, transcriptomic and proteomic studies have allowed the identification of carbohydrate-active enzymes (CAZymes) present in fungal secretions.^{9–12} The group of CAZymes comprises proteins that degrade, modify, or create glycosidic bonds and have been categorized in the CAZy database (<http://www.cazy.org>) into glycoside hydrolases (GHs), glycosyltransferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), and redox enzymes grouped in the Auxiliary Activities (AA) family.¹³ Additionally, the CAZy database includes to date 67 families of carbohydrate-binding modules (CBMs) because a large number of CAZymes have a

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modular structure in which the CBM plays a key role in the recognition of substrate and adhesion of the catalytic module.¹⁴

Numerous CAZymes produced by *Trichoderma* sp., including cellulases, pectinases, and xylanases, have traditionally been used in the processing of textiles, paper, feed, food, and beverages.^{15,16} Lately, the cellulolytic potential of some species such as *T. reesei* has been employed in the bioconversion of plant biomass into fermentable sugars for the production of bioethanol.¹⁷

Trichoderma harzianum, the most common species of the genus *Trichoderma*, is currently used as the active ingredient of several commercial biofungicide formulations. Its biocontrol activity depends on the secretion of chitinases, glucanases, mannosidases, and proteases that act in the degradation of the cell wall of plant pathogenic fungi.^{18–20} In addition to mycopathogenic ability, *T. harzianum* is able to secrete a wide range of hydrolytic proteins such as cellulases, xylanases, mannanases, and laccases whose production and characterization have been addressed by different groups.^{21–24}

In a previous study, the analysis of *T. harzianum* secretome, when a preculture mycelia was grown in medium added with cellulose, was carried out, revealing the presence of several glycoside hydrolases, mainly represented by chitinases and endochitinases as well as hypothetical proteins.²⁵ Another study from the same group, using BN-PAGE analysis, revealed the presence of cellulases and hemicellulases working as multi-enzymatic complexes in *T. harzianum* secretome when sugarcane bagasse (SB) was used as carbon source.²⁶

The aim of the present work was to characterize by means of enzymatic and proteomic approaches the variation of *T. harzianum* secretome composition when the microorganism was cultivated in media supplemented with carbon sources of different complexity, that is, the monosaccharide glucose, two polysaccharides (CM-cellulose and xylan), and an agricultural residue (sugarcane bagasse).

MATERIALS AND METHODS

Strain and Cultivation Conditions

Trichoderma harzianum T 4 was provided by I. S. Melo (Embrapa/CNPMA/Brazil)²⁷ and is maintained in the fungi culture collection of the Laboratory of Enzymology (University of Brasilia/Brazil). For the spore production, the fungus was cultured on solid synthetic medium (SM) (0.68g KH₂PO₄, 0.87g K₂HPO₄, 1.7 g (NH₄)₂SO₄, 0.2g KCl, 0.2g CaCl₂, 0.2g MgSO₄·7 H₂O, 2 mg FeSO₄, 2 mg MnSO₄, and 2 mg ZnSO₄) in 1 L of distilled water, pH 6.0²⁸ containing 1% (w/v) of one of the following substrates as carbon source: glucose, carboxy methyl cellulose (CMC), oat spelt xylan (all substrates purchased from Sigma-Aldrich, St. Louis, MO) or pretreated SB. Prior to use in the culture medium, SB was thoroughly washed with water, autoclaved at 121 °C/30 min, dried at 60 °C, and milled. Prior to secretome production, *T. harzianum* was cultivated in agar plates containing one of the four carbon sources used in this study. After 6 days of growth, a suspension of spores (10⁸ spores/mL) was prepared and used to inoculate 30 mL of SM liquid medium (100 mL Erlenmeyer flask) containing 1% (w/v) of each carbon source and incubated in a rotatory shaker at 28 °C and 150 rpm for 9 days. The secretome samples were obtained from supernatant by filtration of each fungi culture using Whatman No. 1 paper. The filtrates containing the secretome samples were then dialyzed, lyophilized, and stored at -20 °C. Three independent

biological replicates of fungi growth were cultivated in each carbon source under the same conditions. For 2-DE and deglycosylation experiments, the biological triplicates of each condition were combined, generating pooled samples. For LC–MS/MS experiments, the biological triplicates were analyzed separately.

Enzyme Activity Assays

The endoglucanase (CMCase) and xylanase activities were determined by mixing 5 µL of each secretome sample with 10 µL of each substrate (CMC or xylan from birchwood) solution at a concentration of 1% (w/v) in 50 mM sodium phosphate buffer, pH 7.0 followed by incubation at 50 °C for 30 min. The release of the reducing sugar was measured using the DNS method.²⁹ Filter paper activity (FPase) was determined by measuring the reducing sugars liberated from the hydrolysis of a piece of Whatman No. 1 filter paper (1 × 0.6 cm) after incubation with 15 µL of secretome for 60 min at 50 °C.^{30,31} The reducing sugar concentrations were calculated after reading the absorbance at 540 nm against a standard curve of the equivalent glucose or xylose. All activities were expressed in International Units (IU), corresponding to the quantity of enzyme able to hydrolyze one micromole of reducing sugars (glucose, xylose) per 1 min. Triplicates of each sample were analyzed. Protein concentration was determined by BCA protein assay kit (Pierce, Biotechnology, Rockford, IL) using bovine serum albumin (BSA) as standard.

Deglycosylation of Secretome Proteins

For N-deglycosylation, lyophilized samples, corresponding to 1 mL of secretome, were reconstituted in 100 µL of digestion buffer consisting of 50 mM sodium phosphate buffer pH 7.5, 0.2% (w/v) SDS, 0.01% (v/v) β-mercaptoethanol, and 0.01 % (v/v) tergitol NP40. Subsequently, the samples were heated to 100 °C for 10 min. Immediately after cooling, 1 U of PNGase F (EC3.5.1.52) (Sigma-Aldrich) was added to each sample, followed by incubation for 18 h at 37 °C. Control samples were prepared by treating the secretomes under the same conditions described before but without the addition of PNGase F. The glycoprotein RNase B (EC 3.1.27.5) (Sigma-Aldrich) was used as PNGase F standard substrate.

To investigate the presence of possible O-glycosylation in *T. harzianum* secretome proteins, we treated the samples by incubation with a cocktail of enzymes composed of α-2-(3,6,8,9) neuraminidase, O-glycosidase, β (1–4)-galactosidase, and β-N-acetylglucosaminidase from the deglycosylation kit E-DEGLY (Sigma-Aldrich) following manufacturer's instructions. For the reaction of O-deglycosylation, bovine fetuin (Sigma-Aldrich) was used as standard substrate.

Two-Dimensional Gel Electrophoresis (2-DE)

For 2-DE analysis, lyophilized pooled samples (from biological triplicates) corresponding to a total volume of 3.5 mL of secretome were reconstituted in water and subsequently precipitated using 2-D Clean-Up Kit (GE Healthcare, Little Chalfont, U.K.). The resulting pellets were solubilized in 350 µL of rehydration buffer (7 M urea, 2 M thiourea, 85 mM DTT, 2.5% (v/v) Triton x-100, 0.5% (v/v) Pharmalyte, 10% (v/v) isopropanol, and 0.002% (w/v) bromophenol blue). Samples were homogenized during 1 h and centrifuged during 15 min before application onto 18 cm 4–7 IPG strips (GE Healthcare, Uppsala, Sweden) by in-gel rehydration.³² IEF was carried out using an in Ettan IPGphor 3 apparatus (GE Healthcare) at 20 °C with 75 µA constant current for total 33

680 Vh according to the following protocol: 500 V for 1 h (step), 1000 V for 1h (gradient), 10 000 V for 3 h (gradient), and 10 000 V for 1.5 h (step). After IEF, IPG strips were incubated for 40 min in a reducing solution (50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate, and 125 mM DTT) followed by protein alkylation with acrylamide incubating the IPG strips in a solution of 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate, and 300 mM acrylamide. The IPG strips were applied onto an 8–15%T gradient (18 cm × 18 cm) SDS-PAGE gel and kept in place with 3 mL of 0.3% (w/v) agarose solution with traces of bromophenol blue. Second dimension was performed using Protean II Xi cell system (Bio-Rad, Hercules, CA) at 25 mAmp 18 °C and terminated once bromophenol blue dye reached the bottom of the gel. The gels were fixed and stained with CBB-G250.³³ The 2-DE procedures were done in triplicate.

In-Gel Digestion of Protein Spots

The protocol was modified from ref 34. In brief, protein spots from each 2-DE gel were excised and washed twice with 50% ACN/H₂O Milli-Q. Spots were then rinsed with 50 mM NH₄HCO₃ and dehydrated with 100% ACN. After vacuum drying, spots were rehydrated with a digestion buffer (25 mM NH₄HCO₃, 5 mM CaCl₂ containing 12.5 ng/μL trypsin (Promega, Madison, WI)). Proteolytic digestion was performed overnight at 37 °C. The tryptic peptides were directly applied onto an AnchorChip target plate (Bruker, Daltonics, Bremen, Germany), followed by addition of 0.5 μL of matrix solution (5 μg/μL HCCA in 50% (v/v) ACN, 0.1% (v/v) TFA: 5 μg/μL DHB in 30% (v/v) ACN, 0.1% (v/v) TFA, 1:1).

MALDI-TOF

MALDI-TOF-MS was performed using an Autoflex II TOF/TOF (Bruker, Daltonics, Karlsruhe, Germany) mass spectrometer. MS spectra were acquired in positive ion reflector mode, *m/z* range 800–3500 Da, using 300 laser shots per spot. The spectra were externally calibrated using a peptide mass standard (Bruker, Daltonics) and internally calibrated using known trypsin and keratin peaks. MS and MS/MS spectra were analyzed using the FlexAnalysis 2.4 program. PMF and PFF spectra searches were performed against NCBIInr database by means of Mascot search software (www.matrixscience.com), using taxonomy restrictions to “Fungi”, peptide mass tolerance of 100 ppm, maximum one missed cleavage for trypsin, “Propionamide” as fixed modification, “Methionine oxidation” as variable modification, and MS/MS tolerance of 0.5 Da. Identification of proteins was considered positive when the Mascot score was *p* < 0.05.

LC-MS/MS

Each dried secretome sample (100 μg of protein) was resuspended in 18 μL of 7 M urea/2 M thiourea and 2 μL of 1 M NH₄HCO₃ pH 8.0. The sample was then reduced by the addition of 2 μL of 100 mM DTT (Sigma-Aldrich) at 30 °C for 1 h, followed by alkylation using 2.5 μL of 400 mM IAA (Sigma-Aldrich) at room temperature for 30 min in the dark. Afterward, the concentration of urea and thiourea was decreased by dilution with 175 μL of H₂O milli-Q. Sequencing grade trypsin (Promega) was added to achieve a final concentration of 0.1 μg/μL, and the samples were incubated overnight at 39 °C. The tryptic peptides were acidified with 0.1% trifluoroacetic acid (TFA) and desalting using C18 Ultra-Micro Spin Columns (Harvard Apparatus, Holliston, MA). The

resulting peptides were dried in a vacuum centrifuge, reconstituted in 15 μL of 0.5% TFA solution, and analyzed on a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) interfaced with an EASY-nLC II nanoliquid chromatography (Proxeon, Denmark). The peptides were enriched using a trap column (200 μm × 2.0 cm) packed with ReproSil-Pur 120 C18-AQ (particle size 5 μm, Dr. Maish, Ammerbuch, Germany). After 4 min of washing with solvent A (95% (v/v) H₂O, 5% acetonitrile (v/v), 0.1% (v/v) formic acid), peptides were separated on an analytical column (200 μm × 10 cm) packed with ReproSil-Pur 120 C18-AQ (particle size 3 μm, Dr. Maish) at a flow rate of 200 nL·min⁻¹. The chromatographic separation used gradient elution of 5–20% of solvent B (5% (v/v) H₂O, 95% (v/v) acetonitrile, 0.1% (v/v) formic acid) for 120 min, 20–50% B for 45 min, 50–100% B for 5 min, and 100% B for 10 min. Peptides were eluted from the analytical column into the mass spectrometer via a nanospray probe (Thermo Scientific) using a nanospray emitter (20 μm i.d., 10 μm tip i.d.) (New Objective, Woburn, MA) with a spray voltage of 1.8 kV, and the transfer capillary temperature was set at 200 °C. The LTQ Orbitrap Velos was operated in Data-Dependent Acquisition (DDA) mode using the Xcalibur 2.1 software (Thermo Scientific) at a mass resolution of 60 000 at 400 *m/z* from 300 to 1800 *m/z* using one microscan and a target value of 10⁶ ions on the Orbitrap for MS1. The acquisition cycle consisted of the fragmentation of the 10 most intense precursor ions using higher energy dissociation (HCD) for fragmentation at 7500 mass resolution and 5 × 10⁴ charges for MS/MS. The ion selection threshold for MS/MS was set to 500 counts using a precursor isolation window of 4 amu. At least two LC-MS/MS analyses were performed for all three biological replicates of each condition.

Data Analysis

MS/MS spectra were identified using Proteome Discoverer software 1.3 (Thermo Scientific) with the SEQUEST search algorithm against a concatenated forward and reverse *Trichoderma* sequence database (<http://www.uniprot.org>). Search parameters included trypsin as the enzyme, a maximum of two missed cleavages allowed, carbamidomethylation of cysteines as a fixed modification, and oxidation of methionine as variable modification. Precursor mass tolerance was set to 10 ppm and product ions were set at 10 ppm. The maximum precursor ion charge state used for searching was 3. For protein identification, at least two peptides were considered, and the false discovery rate (FDR) was set to 1%.

RESULTS AND DISCUSSION

In this study, the effect of simple and complex carbon substrates in the secretome of the fungi *T. harzianum* was evaluated during submerged cultivation in media supplemented with glucose, CMC, xylan, or SB.

Therefore, glucose was used as a promoter of catabolite repression of enzyme, while the substrates CMC and xylan represented the main components of plant cell wall, cellulose, and hemicellulose. The use of SB tested the response of the fungus to a heterogeneous agricultural residue. SB received a prior treatment that included washing steps to eliminate or reduce the concentrations of sucrose and inhibitory compounds probably present in the bagasse as a result of agricultural practice. Also, the SB particle size was reduced by milling to raise the accessibility of cell-wall polysaccharides to the enzymatic attack. Pretreated SB has been successfully employed

as raw material for enzyme production to increase the productivity of endoglucanases (EGLs) and β -glucosidases (BGLs) of several Ascomycetes including the *T. harzianum* strains IOC-3844 and IOC-4038.^{23,24}

Enzymatic Profile of the Secretomes

The hydrolytic efficiency of xylanases and cellulases (FPase and CMCase) present in *T. harzianum* secretomes was evaluated, and the results are shown in Figure 1 and in the Supporting Information (SI-1).

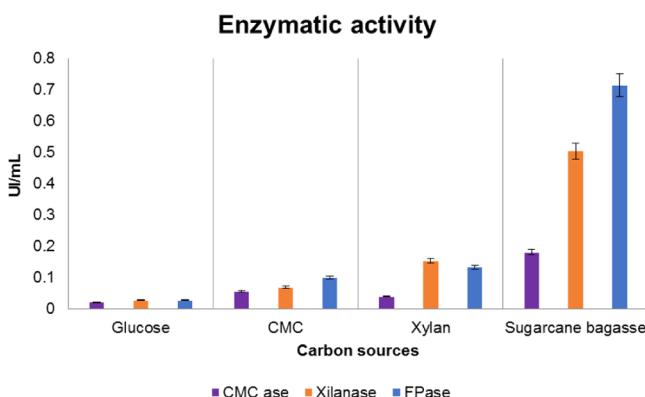


Figure 1. Enzymatic activity of the *T. harzianum* secretomes. Endoglucanase (CMCases), cellulase (FPases), and xylanase activities were assayed in the fungi secretomes coming from growth in synthetic medium to which 1% (w/v) glucose, CMC, xylan, or sugarcane bagasse was added.

As could be expected, the secretome produced in glucose media showed low levels of xylanase (0.0273 UI/mL), CMCase (0.0208 UI/mL), and FPase (0.0274 UI/mL) activities due to catabolite repression triggered by glucose.³⁵ Growth on CMC increased the activities of cellulases (0.0560 UI/mL for CMCase and 0.0993 UI/mL for FPase) and xylanases (0.0689 UI/mL). The use of xylan instead of CMC as carbon source enhanced xylanase activity (0.1530 UI/mL) and also improved the ability to hydrolyze filter paper (0.1328 UI/mL). Moreover, under SB-based cultivation, cellulolytic (0.1808 UI/mL for CMCase and 0.7152 UI/mL for FPase) and xylanolytic (0.5044 UI/mL) activities were strongly elevated compared with the other secretomes, probably due to the combined effect of polysaccharides of different natures. The results indicate that a rich source of complex polymers such as SB could be more efficient for the production of enzymes with biotechnological interest because it stimulates the secretion of various types of enzymes required to hydrolyze the plant biomass constituents.^{22,23}

Secretomic Analysis

The four *T. harzianum* secretome samples (from glucose, CM-cellulose, xylan, and SB-containing media) were subjected to 2-DE. Initially, wide range pH (3–10) 2-DE gels were used, and it was verified that most spots were located in the acidic part of the gel (data not shown). Thus, narrow pH (4–7) range 2-DE gels were used, resulting in substantial improvement of spot resolution. The resulting gels permitted us to visually verify remarkable differences in their protein profiles concerning the number, intensity, and distribution of protein spots (Figure 2).

Each 2-DE profile displayed an average of 50–60 spots/gel, most of which corresponded to trails of spots indicating different molecular forms:³⁶ MALDI TOF mass

spectrometry was used to identify protein spots including the proteoforms (Figure 2, Supporting Information SI-4). The 2-DE gel of the secretome from CMC-based medium displayed proteoforms in the high molecular region, corresponding to the enzyme β -N-acetyl glucosaminidase (NAG) and other hypothetical proteins that constitute most of the identified proteins (Figure 2, Supporting Information SI-4).

Regarding the 2-DE profile of xylan medium secretome, the most intense spots corresponded to high mass variants identified as β -xylosidase (BXL), hexosaminidase (HXM), and arabinofuranosidase (ABF) (Figure 2, Supporting Information SI-4). Finally, in the SB secretome, 2-DE profile and mass spectrometry identification revealed proteins common to the other carbon sources tested, such as NAG also secreted in media containing CMC and ABF, BXL, and HXM that were also secreted in the medium containing xylan (Figure 2, Supporting Information SI-4).

The presence of multiple forms of the same enzyme in the secretome is an event frequently observed in 2-DE maps of fungi secretomes.^{37,38} Several authors have demonstrated that this multiplicity can arise as a result of different phenomena such as differential processing of the mRNA and posttranslational modifications such as glycosylation.^{39,40}

The enzyme PNGase F responsible for the removal of oligosaccharides attached to asparagine residues (N-glycans) in glycoproteins as well as the O-glycosidases, α -2-(3,6,8,9) neuraminidase, β (1–4)-galactosidase, and β -N-acetyl glucosaminidase, which remove oligosaccharides attached to serine and threonine residues (O-glycans), were used to treat the secretome samples to examine if the occurrence of proteoforms observed in 2-DE profiles was the consequence of protein glycosylation (Figure 3). SDS-PAGE analysis showed changes in the position, intensity, and number of bands in all samples after the treatment with the enzyme PNGase F, in contrast with the treatment with O-glycosidases that resulted in no evidence of changes in protein profiles (Figure 3 B).

On the basis of the changes observed in 1-D SDS-PAGE profiles, the secretomes subjected to N-deglycosylation with PNGase F were also analyzed by 2-DE. Thus, the 2-DE profiles revealed that the number of protein spots corresponding to possible proteoforms was remarkably reduced compared with the control samples that received no treatment (Figure 4). Also, several spots corresponding to proteoforms of high molecular mass changed the position in function of pI such as N-acetyl glucosaminidase (gil1911745) and β -xylosidase (gil119494343), as observed in CMC medium secretome (Figure 4).

The existence of N-glycosylation in glycoside hydrolases identified in 2D gels was predicted in the protein sequences using NetNGlyc v. 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc>),⁴¹ indicating the presence of one or more glycosylation sites for each protein and confirming the results observed in changes caused by the N-deglycosylation treatment of samples (Figure 4).

Glycosylation in secreted enzymes such as cellobiohydrolases (CBHs) and EGLs has been observed in several filamentous fungi including *Aspergillus* sp., *Penicillium*, and *T. reesei*.^{42–45} Microheterogeneity due to glycosylation in cellulose Cel 7A secreted by *T. reesei* was verified when the fungus was grown under different conditions. The microheterogeneity was shown to be due mainly to the variable ratio of single N-acetylglucosamine over high-mannose structures on the N-glycosylation sites and in the presence or absence of phosphate.⁴⁶ N-Glycosylation was also shown to affect the

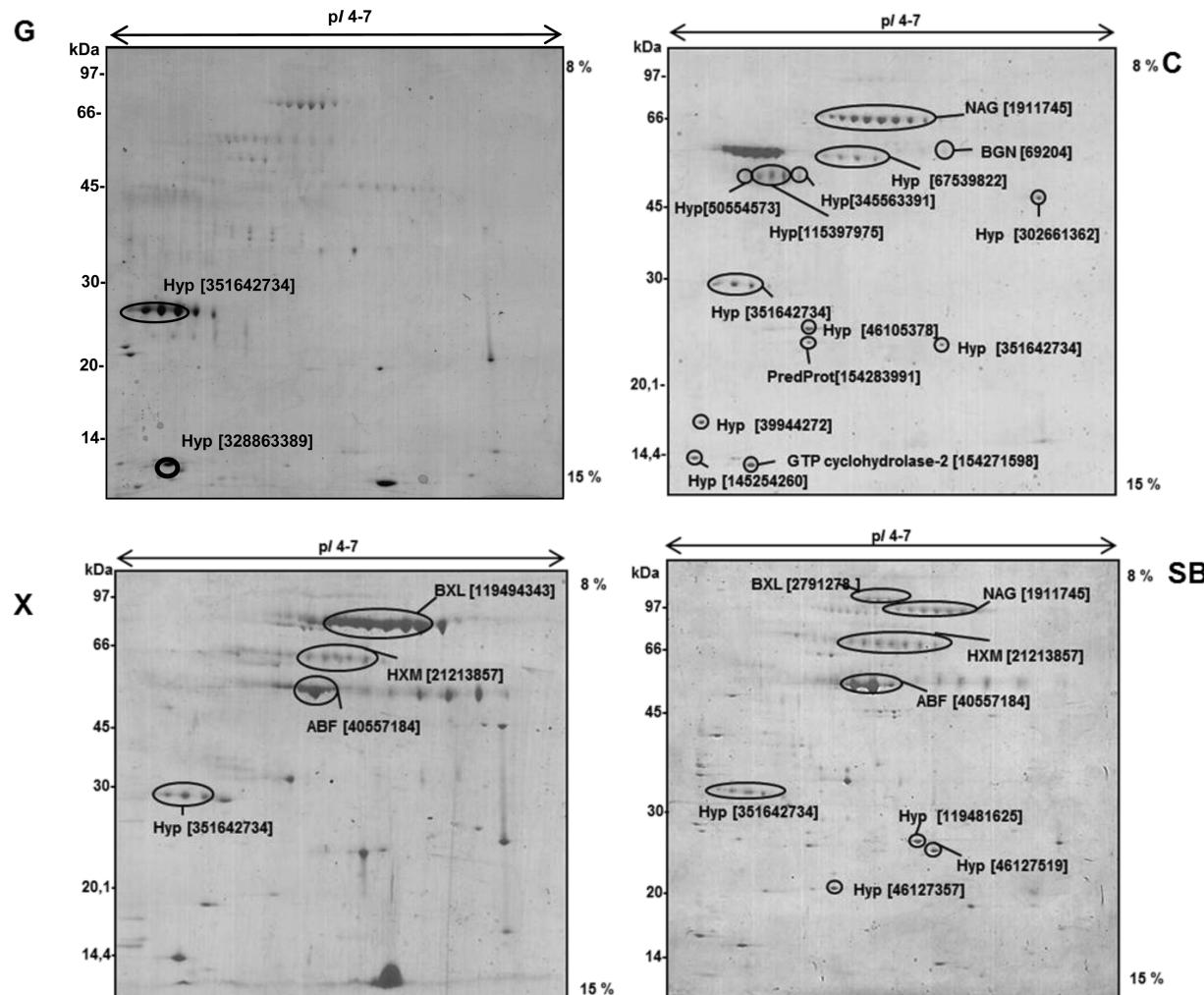


Figure 2. Comparison of the 2-DE profiles of *T. harzianum* secretome. Secretomes coming from growth in medium containing (G) glucose, (C) cellulose, (X) xylan, and (SB) sugarcane bagasse were examined by 2-DE. IPG strips (18 cm) pH 4–7 were used in the first dimension and in the second dimension gradient SDS-PAGE 8–15%. Proteins were visualized after staining with CBB G-250 and then identified using MALDI-MS mass spectrometry, as described in the Materials and Methods.

function of fungi-secreted enzymes. For instance, studies on the recombinant *T. reesei* cellulase Cel7A expressed in *A. niger var. awamori* demonstrated that it contained six times the amount of N-linked glycans than the enzyme purified from a commercial *T. reesei* enzyme preparation. The comparison of the activities of both enzymes as well as treatment with PNGase F showed that the increased glycosylation level of the recombinant protein resulted in a reduced activity.⁴⁷ Likewise, a more recent research revealed that the activity of the CBH I secreted by *P. decumbens* is affected by the levels of N-glycosylation, the N-glycosylation site, and the N-glycan structure of the glycoforms.⁴⁸

The analysis of *T. harzianum* secretomes by 2-DE demonstrated that the four secretomes show remarkable differences in terms of protein profile. This methodology was also useful to reveal that several proteins present in the secretome are N-glycosylated. However, the 2-DE-based strategy provided a limited number of identified proteins. Therefore, the four *T. harzianum* secretome samples were also subjected to the gel-free proteomic approach, based on high resolution LTQ-Orbitrap LC-MS/MS. By using this strategy, a total of 626 different protein groups were identified in the secretomes of *T. harzianum*. This number is comparable to the

number of genes predicted for extracellular proteins in other *Trichoderma* species. For instance, in *T. reesei*, *T. atroviride*, and *T. virens*, the number of predicted extracellular proteins were estimated to comprise 747, 968, and 947, respectively, and ~15% of their secretome corresponds to CAZymes.^{49,50}

Figure 5 shows a Venn diagram representing the distribution of the identified proteins among the four *T. harzianum* secretome samples as a result of duplicate technical LC-MS/MS analysis of each biological replicate. The vast majority (73%) of the secreted proteins were exclusively identified under just one condition. The comparison of the four secretomes showed that 21.72% of the identified proteins were common to two or three conditions and that only 33 proteins were present in the four samples, corresponding to 5.27% of all identifications. This last group of common proteins included 7 families of hemicellulases, 7 proteins associated with the fungal cell-wall and morphogenesis, 1 chitinase, and 17 putative proteins (Supporting Information S.I-4). In all common proteins, a signal peptide sequence was verified by means of Signal P 4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>), confirming that common proteins were all secreted via classical ER/Golgi-dependent secretory pathway (Supporting Information S.I-4).

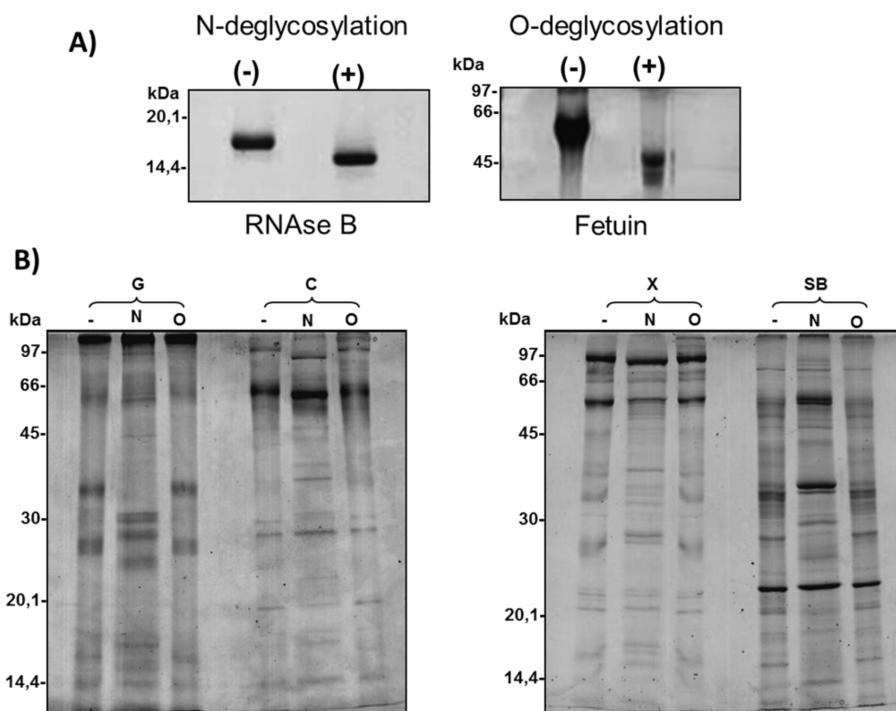


Figure 3. Effect of deglycosylation on SDS-PAGE secretome profiles. (A) Standard proteins (RNase B and fetuin) untreated (–) and after treatment with PNGase F for N-deglycosylation or E-DEGLY Kit (Sigma) for O-deglycosylation, respectively, (+) were used as controls. (B) Secretome samples produced in media supplemented with glucose(G), CMC(C), xylan(X), and sugarcane bagasse(SB) were examined after N-deglycosylation(N) and O-deglycosylation(O) or without treatment(–).

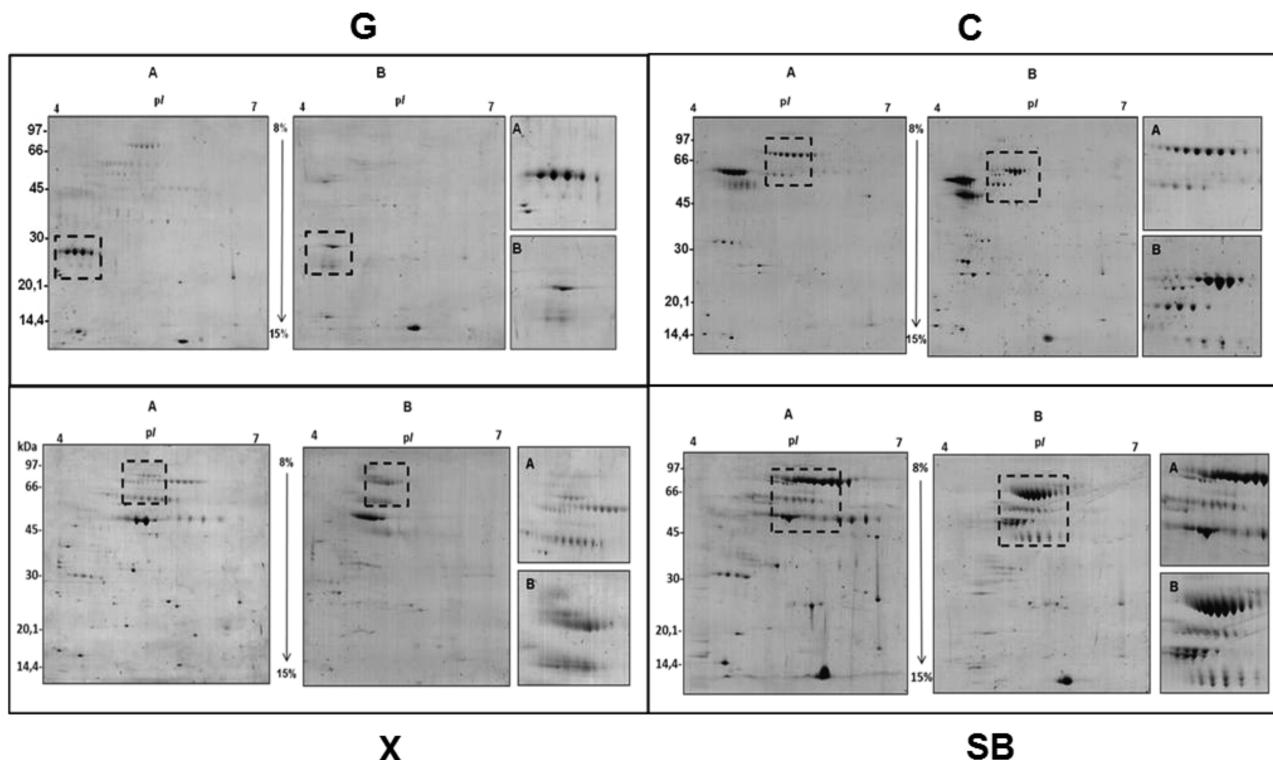


Figure 4. Effect of N-deglycosylation on 2-DE profile of *T. harzianum* secretome samples produced in media supplemented with glucose(G), CMC(C), xylan(X), and sugarcane bagasse(SB). (A) Sample untreated and (B) sample treated with the enzyme PNGase F. 2-DE gels were made in the first dimension in IPG strips (18 cm) pH 4–7 and in the second dimension SDS-PAGE 8–15%. The dotted boxes highlight gel areas that displayed major changes after N-deglycosylation. Gels were stained with CBB-G250 as described in the Materials and Methods.

The identified proteins were classified as cellulases, xylanases and other hemicellulases, pectinases, ligninases, biological

control-related proteins, cell-wall associated proteins, putative, predicted proteins, and others listed in Supporting Information

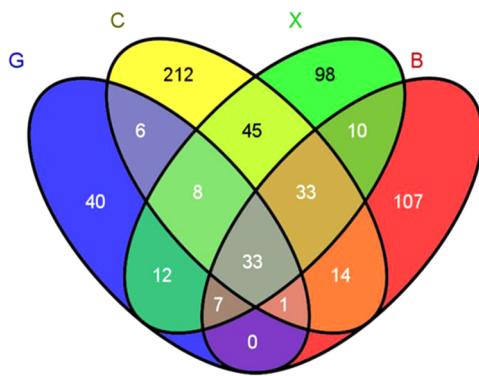


Figure 5. Venn diagram representing unique and common protein identified in each *T. harzianum* secretome. A total 626 nonredundant protein groups were identified in the secretomes resulted from growth in media added with G, glucose; C, CMC; X, xylan; and B, sugarcane bagasse by means of gel-free LC–MS/MS strategy. The Venn diagram was prepared by means of tool Venny (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>).

S.I-2. The number of identified proteins in all categories was plotted against each carbon source (Figure 6), revealing that the putative and predicted protein corresponds to a large proportion of the *T. harzianum* secretome similar to that observed in secretomic analysis of the fungi *Thermobifida fusca*, *Aspergillus niger*, and *T. reesei*.^{51–53}

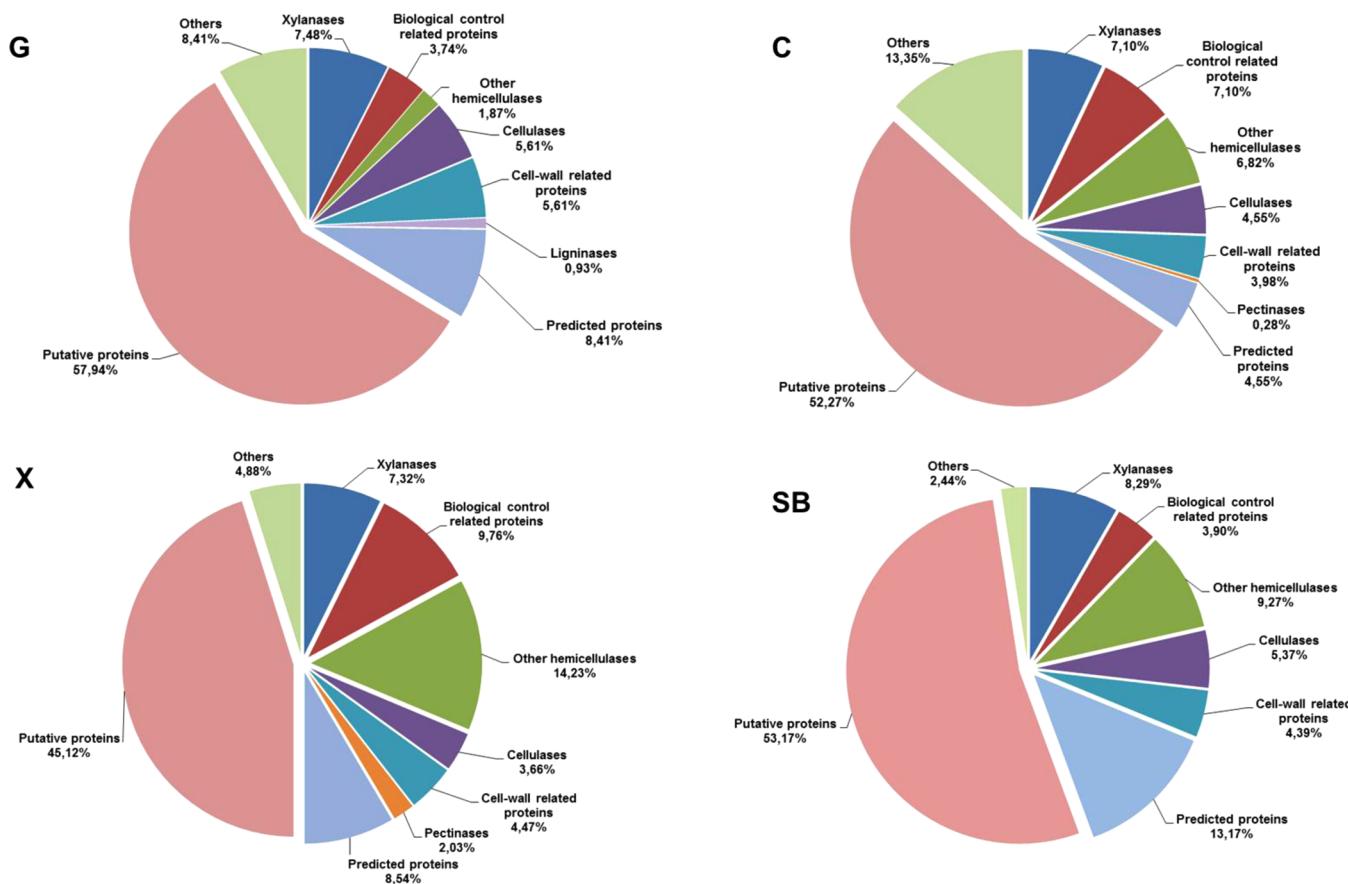


Figure 6. Functional classification of proteins identified in the *T. harzianum* secretomes. The proteins identified in the shotgun LC–MS/MS analysis of the secretome produced from growth in medium containing (G) glucose, (C) CMC, (X) xylan, and (SB) sugarcane bagasse were classified according their biological function.

Blast analysis of the putative and predicted proteins identified in the four samples was performed using the program Blast2GO⁵⁴(www.blast2go.com) against NCBI nr sequence database. Blastp annotation results indicated that the proteins identified have at least one homologous gene or protein mainly from *Trichoderma* species with several sequence descriptions in the current NCBI database (Supporting Information S.I-S).

The growth in medium containing 1% (w/v) glucose resulted in the secretion of 107 protein groups of which 40 were exclusively identified in this carbon source (Figure 5, Supporting Information S.I-2). The unique proteins included the lignin-degrading enzyme laccase (C5H3G0), phospholipase C (PLC-E) (Q157RS), and glycoside hydrolases families GHs 72 (G9N0W8-homologous to β-1,3-glycanosiltransferase), GH 18 (G9NKD3-homologous to chitin), and GH 54 (homologous to G9MZ65-arabinofuranosidase) as well as a variety of putative proteins. Interestingly, cellulases including cellobiohydrolases (CBHs) and BGLs as well as the xylanolytic enzymes, endoxylanase (XYNs), ABFs, and BXLs were verified under this condition (Figure 6), confirming the detection of cellulolytic and xylanolytic activities in this secretome (Figure 1).

The secretion of cellulases and xylanases in the presence of glucose could be the consequence of low constitutive enzyme expression sufficient to initiate the formation of soluble sugar inducers. Thus, the carbon catabolite regulator CreA/CRE₁, a transcription factor identified in several fungi, interferes with

the induction of genes encoding lignocellulolytic enzymes but not with their basal transcription.^{55,56}

In the secretome obtained from CMC-based media, 352 protein groups were identified and 212 were unique to this carbon substrate (Figure 5, Supporting Information S.I-2). The identified proteins included the three major cellulases, EGLs, CBHs, and BGLs, as well as auxiliary enzymes related to the cellulose hydrolysis and CBMs comprising 4.55% of this secretome (Figure 6). Biological control-related enzymes endochitinases (Q4JQJ0, O43111, Q12735, G9P606) were also secreted under cellulose-induced conditions and correspond to the 7.10% of the secretome (Figure 6). Within proteins exclusively expressed in this secretome stands out the xylanases endo-1,4- β -xylanase (D2XV89) and GH 43 (homologous to G9P412-arabinofuranosidase) and CE 5 (G9NJW6-homologous to acetyl xylan esterase (AXE)), CE 15 (G0RV93 4-O-methyl-glucuronil methyl esterase), CE 1 (G9N0G0), and CE 3 (G9NU00) (Supporting Information S.I-3).

In the secretome coming from media supplemented with xylan, 246 protein groups were identified, 98 only secreted in this condition (Figure 5). Besides the putative proteins, other hemicellulases constitute the major group of secreted enzymes (14, 23%) (Figure.6). Furthermore, enzyme fucosidases that act on fucose (6-deoxy-galactose), a component of glycoproteins found in plant cell-wall polymers, were solely induced in this carbon source (Supporting Information S.I-2). In addition, this secretome displayed the greatest proportion of pectinases (2.03%) among the secretomes (Figure.6).

The secretome produced by *T. harzianum* in the presence of SB showed a total of 205 protein groups of which 107 were exclusively identified in this sample (Figure.5). The exclusive proteins included CE family 1 (G9NSZ2), expansin-like protein (G9MY29), polysaccharide lyase family 1 (G9P185), endoglucanase (Q8NJY4) and GHs families 62 (G9MV83), 71 (G9MWV6), 92 (G9MLE1), and others (Supporting Information S.I-2).

Carbohydrate-Active Enzymes Related to Cellulose Conversion

Degradation of the cellulosic portion of plant biomass by filamentous fungi depends on the secretion and synergistic action of EGLs and CBHs. CBHs, which are part of GH families 6, 7, and 74 and act in the early steps of cellulose hydrolysis, were detected in all samples (Q6QNU5, Q8NJY4, G9NTY1, G9MUN0, G9NFV6). They generate cellobiose molecules that are subsequently converted by BGLs into glucose. BGLs, identified in the four secretomes (G9P180, G9P6W2 G9MUU, G9NS06), can also function as inducer-providing enzymes via transglycosylation of cellobiose in sophorose via trans/acting activator Xyr 1, which simultaneously affects the CBHs transcription. This Xyr 1-dependent mechanism was initially recognized in *T. reesei* and could explain the secretion of cellulases even under glucose repression conditions.⁵⁷

An ideal conversion of cellulose includes the synergistic combination of endo- and exocellulases as well as auxiliary proteins with noncatalytic function such as expansin-like proteins, which were found in all secretomes (G9PBA9, G9MY29). The action of expansins consists of the disruption of hydrogen bonds found in cellulose microfibrils, increasing the cavities of plant cell wall and in consequence facilitating the entrance of the hydrolytic enzymes deeper in the structure.⁵⁸ One of the best known expansions is swollenin (SWO), a

protein originally isolated from *T. reesei* as a nonhydrolytic component of the cellulolytic system,⁵⁹ which was also identified in the present study in the secretome obtained from growth on CMC and SB-supplemented media (B3FRAS).

Beyond expansins, a novel group of CAZymes recently categorized as AA that could enhance the decomposition of the cellulose were identified in the secretomes coming from cultures added with xylan or SB as carbon substrates. The polysaccharide copper-dependent mono-oxygenases (PMOs) (G9NS04, G9NE55) belong to this new group of redox enzymes and act synergistically with CBHs, BGLs, and other cellobiose dehydrogenases, catalyzing an oxidative cleavage at the interior of the cellulose polymer to generate celodextrins.⁶⁰

Another accessory protein, the cellulose-induced protein (CIP 1), was identified only in CMC and SB-containing secretomes (Q7Z9M9). CIP 1 and 2 were originally identified in transcriptional analysis of *T. reesei* upon conditions of cellulase induction.⁶¹ CIPs have CBMs and are coregulated with other cellulases. Its function is not yet well-established, although recent studies have indicated that CIPs display low activity on the synthetic substrate *p*-nitrophenyl- β -D cellobioside and synergism with PMOs and swollenin. It has been suggested that both CIP 1 and CIP 2 could have applicability in textile, detergent, and treatment of paper pulp.⁶¹

Hydrolytic enzymes secreted by filamentous fungi often have a modular architecture comprised by a catalytic module linked to one or more CBMs. In this study, CBMs belonging to the family 13 (G9NKM4, G9NUY8) were identified only in secretomes from CMC and xylan-based media. CBMs approximate the enzyme molecule to the target substrate, increasing its concentration at the substrate surface and also favor conformational changes in the structure of polysaccharides to enhance the catalytic activity of enzymes.¹⁴

Carbohydrate-Active Enzymes Related to Hemicellulose Conversion

The degradation of hemicelluloses requires the synergistic action of different enzymes including xylanases once xylan is the major component of the hemicellulose.⁶² In the cell wall, xyans are strongly bound to the cellulose microfibrils, and thus the enzymatic attack of the xylan could be the initial step in the lignocellulose bioconversion.

In the present study, the major components of the hemicellulolytic machinery were identified in the secretome of *T. harzianum* such as endoxylanases (XLNs) identified in the four secretomes (Q9UVF9, G9NQ12, D2XV89, Q6QNU8, Q7Z8Q3, G9NE77) and the β -xylosidases (BXLs) (Q92458) required to complete the hydrolysis to monomeric units of xylose.⁶² Complete xylan conversion requires the aid of accessory enzymes such as α -L-arabinofuranosidases (ABF-G9NNW8, G9NYH4, G9MZ65, G9NKQ7), CEs, AXE, and ferulic acid esterase (FAE). The accessory enzymes play an important role in increasing the solubility of the xylan because they catalyze the removal of arabinofuranosidic ends, acetylated groups, and ferulic acid, yielding molecules of xylose and xylobiose, thereby facilitating the action of xylanases. Likewise, α -glucuronidases (Q99024, G9NF15) that cleave the α -1,2-glycosidic bonds between the 4-O-methyl- α -D-glucuronic and xylose⁶³ were also detected in the secretome.

The hemicellulose is abundant in xyloglucans, polysaccharides formed by a backbone of β -(1,4)-D-glucopyranose modified by side chains of galactose, fucose, and arabinose residues.⁶⁴ Fucosidases hydrolyze fucose (6-deoxygalactose) to

complete the deconstruction of some xyloglucans and was exclusively found in the secretome produced in xylan-added medium (G9NTF2, G9MVW6, G9NI38, G9NLB0). Interestingly, fucosidases were not found in the SB secretome, possibly because the pretreatment removed part of xyloglucans of the SB. Fucosidases were also found in *T. reesei* secretome and were characterized in filamentous fungi species *Fusarium oxysporum* and *F. graminearum* after the addition of 1% fucose to the culture media.^{65,66}

Mannans are also part of the hemicelluloses and are important structural components of the plant cell wall.⁶⁷ In this study, mannosidases (G9P5N3, G9NK86, G9P297, G9NZE2, G9MZ53), members of the GH 76 family, galactosidases (G9NPC7), and GH 2 and GH 92 families were detected in secretomes coming from CMC and xylan-based media.

Cell-Wall-Related Proteins

The secretome of *T. harzianum* also contains extracellular proteins that remain assembled to the outer layer of the membrane. These proteins are involved in substrate recognition as well as biosynthesis and regeneration processes of the fungal cell wall. Therefore, the GH 17 glycan 1,3- β -glycosidase (G9MZ09, G9P2J8, G9NN5, G9N8G8) can participate in the degradation of β -glycan, while GPI-anchored glucanoyl transferases (G9NSK7, G9P756, G9PBD3, G0RQW0, G9NIE1, G9NI23, G0RJJ2, G9N0W8) catalyze the linear elongation of the β -(1-3) glycan, promoting the formation of branching in the hyphae.^{68,69}

Moreover, proteins enabling aerial growth, conidiation, and attachment to surfaces were common to the four secretomes, such as hydrophobins (Q4F6W8) amphiphilic proteins expressed in the surface of the aerial structures of filamentous fungi that have applicability as surfactant compound. These proteins are also frequently associated with sporulation and growth under starvation conditions.^{70,71}

In the same way, an eliciting plant response-like protein (EPL) (G9PB60) related to hydrophobins was also identified in the four secretomes. EPLs belong to the family of cerato-platanin proteins and were detected in other *Trichoderma* species such as *T. atroviridis* and *T. virens*. Traditionally, its function was related to the induction of defense mechanisms that some filamentous fungi yield in plants. However, current reports associated the EPL1 with substrate binding processes because it increases the polarity of aqueous solutions and surfaces.⁷² The secretion of EPLs has been demonstrated in fungi such as *H. atroviridis* and *Botrytis cinerea* in culture media containing several carbon sources (glucose, glycerol, L-arabinose, D-xylose, colloidal chitin, and cell wall of the plant pathogenic fungus *Rhizoctonia solani*) as well as in osmotic stress and limiting carbon and nitrogen conditions.^{73,74}

Carbohydrate-Active Enzymes Related to Biological Control

The fungal cell wall is a complex structure typically composed of chitin, β -1,6, and β -1,3 glycans, mannans, and proteins. Hydrolytic enzymes associated with cell wall are involved in the morphogenesis processes such as expansion and cell division, spores germination, formation of septa and branching hyphae as previously discussed.⁷⁵ In the same way, chitinases and glycanases have a key role in the mycoparasitic attack of the *Trichoderma* species on other fungi.³

In this study, some of the main biological control-related enzymes from *T. harzianum* were identified, such as

endochitinases (I3NN23, D9MWV9, Q12735, O43111, Q9UV49, Q4IQJ0) found in media containing CMC, xylan, and SBN-acetyl glucosaminidases (NAG) (P87258, Q0ZLH7, Q8NIN7) identified under the four culture conditions and finally chitosanases as exo- β -D-glicosaminidase members of GH 2 (G9P602, 9MZV2) that are involved in the final step of the hydrolysis of chitin because it renders D-glucosamine residues of the chitosan a derivative of the chitin.⁷⁶⁻⁷⁸

Interestingly, a preceding work of the group which *T. harzianum* was grown previously in glucose- and peptone-enriched medium (TLE), and the mycelia that was transferred to CMC synthetic media showed different secretome constitution, especially in the number of enzymes related to biological control²⁶ in comparison with the current methodology used here. This previous work showed *T. harzianum* producing a considerable amount of proteases and many chitinases from GH18 (31.80% of the total) and GH 20 (7.10% of the total) families; in total of 38.90% of the secreted proteins were identified as being the 42 kDa endochitinase (11 proteoforms) and N-acetyl- β -glucosaminidase (two proteoforms) as the most abundant.²⁶

The large quantity of 42 kDa endochitinase could be explained by its role in autodigestion processes of hyphae in older cultures and the recent demonstration that chi42 is not directly induced by chitin, in contrast with the significant expression that resulted after prolonged periods of physiological stress and starvation.⁷⁹

The comparison of those two studies (from mycelium and conidial inoculum) indicates that the direct spore inoculation on CMC synthetic medium drives the fungus to produce hemicellulolytic enzymes and minimizes the production of enzymes related to stress, as endochitinases and chitinases. The mycelia transfer from a rich medium to another medium containing cellulose as the only carbon source clearly stresses the fungus due to its forthwith inability to breakdown the cellulose. The differences in *T. harzianum* secretome composition observed between this and the previous work show that not only does the nature of the carbon source produce differences in the expression of glycosyl hydrolases but also other parameters such as stage of development of the preculture, type of fermentation, inoculum size, presence of growth cofactors, and morphological state of the organism may influence the set of hydrolytic enzymes that are secreted to the extracellular medium.

Other types of cell-wall-degrading enzymes were identified. The β -glycanases 1-3 (G9MX73, G9NEC2, G9NTC3) found in GH families 55, 64, and 71 play a key role because the β -glycan is the most abundant component of the fungal cell wall in processes that include remodeling, extension, and autolysis events; however, some β -glycanases can be secreted by the fungus in response to nutrient starvation or as part of the mycoparasitic attack.⁸⁰

In addition to the CAZymes previously mentioned, the pectinase endopolygalacturonase (EPG) from GH family 28 (G9NQQ1, G9NE75) that catalyzes the hydrolytic cleavage of the α -glycosidic linkages in the 1-4 D-glucuronic acid of pectin was exclusively identified in the SB secretome. Additionally, the pectin esterase (G9PB37, G9MMP9) that hydrolyzes the ester bonds of the pectin yielding as product methanol and pectinic acid⁸¹ was identified in SB and xylan secretomes.

A laccase (CSH3G0) was exclusively encountered in the glucose secretome. Laccases may be related to the degradation of lignocellulosic material as well as the physiological events

involving the oxidation of organic compounds as a synthesis of melanin-like pigments in conidia and induction of the formation of fruiting bodies.⁸²

Finally, some intracellular proteins were present in all secretomes, probably as a consequence of cell lysis events during the samples preparation, cell death, or nonconventional mechanisms of protein secretion.

CONCLUSIONS

The activity of plant biomass-degrading enzymes as well as the composition of *T. harzianum* secretome displayed remarkable variation in response to the carbon source used for fungal growth. In all four samples, 2-DE profiles demonstrated the presence of proteoforms that were partially due to N-glycosylations. The existence of O-glycosylations was not evident in the secretomes. The abundance of acidic and high molecular weight proteoforms in 2-DE gels, probably N-glycosylated, suggests that this type of post-translational modification may have an important role in the regulation of secretome and protein variability.

Enzymatic assays demonstrated that SB provided the highest levels of cellulolytic and hemicellulolytic activities among the samples, even compared with secretome samples that used pure CM-cellulose or xylanase as carbon source. However, the high activity of SB secretome does not correlate to a high variability in proteome composition because CM-cellulose secretome was shown to be significantly more diverse according to LC-MS/MS data. Somehow the molecular complexity of SB contributed to the production of a more efficient, although not the most diverse, set of plant cell wall degrading enzymes. The analysis of *T. harzianum* secretome using four different substrates, including a catabolite repressor, two polysaccharides, and a complex agricultural residue, made possible the identification of a much more comprehensive set of secretome proteins when compared with any study using only one condition. This observation is evident because the vast majority (72%) of the identified proteins was found exclusively in just one of the secretome samples and only 5% of the identified proteins were common to the four secretomes. Nonetheless, we believe that future efforts to investigate the differential expression of the common proteins using quantitative proteomic analysis tools (isotopic labeling or label free methodologies) will certainly contribute to a better understanding of the *T. harzianum* secretome.

The data presented here also demonstrated that *T. harzianum* secretome CAZymes, cell-wall-related proteins, and redox enzymes are differentially secreted according to the carbon substrate added to culture media, suggesting that the secretory pathways of certain protein families such as laccases, pectinases, and fucosidases are strongly dependent on the nature of the carbon source. Moreover, a carbon-source-independent mechanism could explain the secretion of proteins common to all conditions such as cell-wall-related proteins whose auxiliary function might not be directly related to biomass degradation but to the increase in the substrate solubility and proximity that would indirectly enhance enzymatic efficiency. Finally, this work provides evidence that the secretome of *T. harzianum* offers new elements of the cellulolytic system of filamentous fungus as part of its natural toolbox, making it a good candidate for clean transformation of the agricultural byproduct such as SB under the appropriate conditions.

ASSOCIATED CONTENT

Supporting Information

Data from enzymatic assays, effect of the N- and O-deglycosylation of secretomes, table of proteins identified by LC-MS/MS, and details of identified proteins by MALDI TOF/TOF. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ABF, arabinofuranosidase; BXL, β -xylosidase; CAZymes, carbohydrate-active-enzymes; CBH, cellobiohydrolase; CBM, carbohydrate-binding module; EGL, endoglucanase; GH, glycoside hydrolase; PMO, polysaccharide copper-dependent mono-oxygenases; SB, sugarcane bagasse; SWO, swollenin; XLN, xylanase

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