RESEARCH ARTICLE

Comparative Secretome Analysis of Trichoderma reesei and Aspergillus niger during Growth on Sugarcane Biomass

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Abstract

Background

Our dependence on fossil fuel sources and concern about the environment has generated a worldwide interest in establishing new sources of fuel and energy. Thus, the use of ethanol as a fuel is advantageous because it is an inexhaustible energy source and has minimal environmental impact. Currently, Brazil is the world’s second largest producer of ethanol, which is produced from sugarcane juice fermentation. However, several studies suggest that Brazil could double its production per hectare by using sugarcane bagasse and straw, known as second-generation (2G) bioethanol. Nevertheless, the use of this biomass presents a challenge because the plant cell wall structure, which is composed of complex sugars (cellulose and hemicelluloses), must be broken down into fermentable sugar, such as glucose and xylose. To achieve this goal, several types of hydrolytic enzymes are necessary, and these enzymes represent the majority of the cost associated with 2G bioethanol processing. Reducing the cost of the saccharification process can be achieved via a comprehensive understanding of the hydrolytic mechanisms and enzyme secretion of polysaccharide-hydrolyzing microorganisms. In many natural habitats, several microorganisms degrade lignocellulosic biomass through a set of enzymes that act synergistically. In this study, two fungal species, Aspergillus niger and Trichoderma reesei, were grown on sugarcane biomass with two levels of cell wall complexity, culm in natura and pre-treated bagasse. The production of enzymes related to biomass degradation was monitored using secretome analyses after 6, 12 and 24 hours. Concurrently, we analyzed the sugars in the supernatant.
Results
Analyzing the concentration of monosaccharides in the supernatant, we observed that both species are able to disassemble the polysaccharides of sugarcane cell walls since 6 hours post-inoculation. The sugars from the polysaccharides such as arabinoxylan and β-glucan (that compose the most external part of the cell wall in sugarcane) are likely the first to be released and assimilated by both species of fungi. At all time points tested, A. niger produced more enzymes (quantitatively and qualitatively) than T. reesei. However, the most important enzymes related to biomass degradation, including celllobiohydrolases, endoglucanases, β-glucosidases, β-xilosidases, endoxylanases, xyloglucanases, and α-arabinofuranosidases, were identified in both secretomes. We also noticed that the both fungi produce more enzymes when grown in culm as a single carbon source.

Conclusion
Our work provides a detailed qualitative and semi-quantitative secretome analysis of A. niger and T. reesei grown on sugarcane biomass. Our data indicate that a combination of enzymes from both fungi is an interesting option to increase saccharification efficiency. In other words, these two fungal species might be combined for their usage in industrial processes.

Introduction
The increasing demand for sustainable energy has promoted considerable efforts to replace fossil fuels with biofuels. As the second world’s largest producer and exporter of ethanol from sugarcane, approximately half of Brazil’s fuel supply is produced from renewable energy sources [1]. Currently, Brazilian production relies on the fermentation of sucrose, known as first-generation (1G) bioethanol. If second—generation (2G) bioethanol was commercialized, Brazil could increase bioethanol production by approximately 40% [2]. To reach this level in industrial processes, the obstacle of cell wall recalcitrance must be overcome. Cell wall recalcitrance is a phenomenon directly related to the enormous complexity of the plant cell wall [3]. In the case of sugarcane, de Souza et al. [4] proposed a model for the architecture of polymers within the cell walls of the leaf and culm (the stem of the sugarcane) that included the structural complexity of hemicelluloses, such as arabinoxylan, xyloglucan, and mixed-linkage-β-glucans, as well as pectins, such as homogalacturonnan and arabinogalactans. They found that sugarcane tissues are composed of ca. 30% of cellulose and 60% hemicelluloses, with pectins and lignin accounting for the rest of the biomass [4].

The biomass of sugarcane displaying these features is transformed into bagasse, a major residue from the Brazilian agroindustry (280 kg per 1 ton of sugarcane crushed) [5]. Bagasse is obtained from a process that crushes and washes biomass (to obtain sucrose), changing the composition in relation to culm. Bagasse is composed of cellulose (40–50%), hemicellulose (25–35%), and lignin (15–20%) [6, 7], highlighting the fact that a portion of hemicelluloses and pectins are washed out during sucrose extraction. Thus, the compositions of sugarcane culms in natura and bagasse are considerably different, with the former displaying higher complexity and proportionally higher levels of soluble polymers that belong to the classes of hemicelluloses and pectins.

Although considerable progress has been made in the saccharification of recalcitrant plant biomass, the cost of 2G bioethanol will not become economically competitive unless the full
conversion of lignocellulose biomass can be reached. Complete hydrolysis of cellulose yields glucose, whereas hemicellulose hydrolysis can produce monomers of xylose, arabinose, mannose, glucose and galactose. In order to break down the cell wall and release these monomers, pre-treatment with enzymatic cocktails is necessary prior to hydrolysis, and this step constitutes the majority of the cost in 2G bioethanol processing [8]. A better understanding of the hydrolytic mechanisms and enzyme secretion of polysaccharide-hydrolyzing microorganisms is needed to overcome the cost associated with enzyme pretreatments. In many natural habitats, plant biomass is degraded by a variety of lignocellulolytic microorganisms that work together to break down the recalcitrant structure of lignocellulosic materials. Although bacteria and yeast (the latter more rarely) produce hydrolytic enzymes [9, 10], most enzymes used in commercial cocktails are derived from fungi, such as Aspergillus niger and Trichoderma reesei, due to their efficiency in producing and secreting a broad range of cellulases and hemicellulases.

A. niger is industrially used to produce many pectinases [11, 12] and hemicellulases [13, 14]. A sequencing effort reported that A. niger contains 14,056 genes [15], and it has one of the most remarkable sets of genes encoding hydrolytic enzymes among sequenced fungal genomes. According to the Carbohydrate-Active Enzymes (CAZY) database (http://www.cazy.org/), A. niger has more than 250 glycoside hydrolases (GHs). Another hyper producer of cellulolytic enzymes is T. reesei RUT-C30. This strain was obtained from the wild-type strain, QM6a, after three rounds of random mutagenesis, with the aim of increasing cellulase production. Due to their great potential for producing hydrolytic enzymes, both of these fungi have been the focus of several studies on GH discovery and there is a marked effort to understand the regulation of the expression of genes that encoding them. To date, only one master carbon repression regulator has been described (CreA/Cre1). The A. niger transcription factor, XlnR (and the T. reesei orthologues Xyr1), is a major positive transcriptional regulator of xylanases and cellulases encoding genes for this species. In A. niger, the expression of most cellulases and hemicellulases is co-regulated by the same inducer (xylose), but for T. reesei, at least four different inducers have been described (xylose, xylobiose, sophorose and lactose) [16, 17]. Several differences in the regulation of GH production between these two fungi have been already described [16, 18, 19], but comparative studies could provide a more comprehensive overview of how these important industrial species sense and produce hydrolytic enzymes.

Although many secretome studies have been performed using A. niger and T. reesei [20–24], very few were based on sugarcane culm and/or bagasse [25, 26]. In the present work, these two fungal species were grown on sugarcane biomass with two levels of cell wall complexity: culm in natura and pretreated bagasse. The production of enzymes related to biomass degradation was monitored using secretome analyses after 6, 12 and 24 hours. Concomitantly, we analyzed the sugars released in the supernatant. Our experiments demonstrate that both species degrade biomass after 6 hours post-inoculation, but comparative secretome analysis of A. niger and T. reesei revealed that it can occur through different mechanisms. This study provides a better understanding of the saccharification process, and it can be used as a basis for the production of optimized enzymatic cocktails.

**Materials and Methods**

**Fungi strains and media**

The species used in this work were the filamentous fungi Trichoderma reesei RUT-C30 and Aspergillus niger N402. Both strains were maintained on potato dextrose agar (PDA) at 29°C and 30°C, respectively. The basic culture medium (BCM) (pH 5.5) was composed of 0.05% yeast extract (w/v), 50 mL/L salt solution (6 g/L NaNO₃, 1.5 g/L KH₂PO₄, 0.5 g/L KCl and 0.5 g/L MgSO₄), 200 μL/L trace elements (10 g/L ethylenediaminetetraacetic acid, 4.4 g/L ZnSO₄, 7
H₂O, 1.0 g/L MnCl₂·4H₂O, 0.32 g/L CoCl₂·6H₂O, 0.315 g/L CuSO₄·5H₂O, 0.22 g/L (NH₄)₆Mo₇O₂₄·4H₂O), 1.47 g/L CaCl₂·2H₂O and 1 g/L FeSO₄·7H₂O) and a predetermined concentration of carbon source, according to our experimental conditions (see below).

The exploded bagasse was prepared as described by Souza et al. [25]. Briefly, sugarcane bagasse in natura was treated with 14 kg/cm² water steam, washed exhaustively with distilled water until reducing sugars were not detected by dinitrosalicylic acid (DNS) [27] and dried at 40°C for several days. The culm was ground into particles with a 2-mm diameter, 3 g of culm particles were washed six times with 25 mL of 80% (v/v) ethanol at 80°C for 20 min, washed with distilled water to remove ethanol, and dried. After drying, both sugarcane exploded bagasse (SEB) and sugarcane culm (SC) were sifted on a 600-μm industrial sieve.

Substrate-based induction conditions

T. reesei and A. niger spores were harvested from fresh potato dextrose agar (PDA) plates by adding 1 mL of sterile distilled water. The spore suspensions were inoculated in triplicate to a final concentration of 1 × 10⁶ spores per 30 mL of BCM (pH5.5) in a 250-mL Erlenmeyer flask containing 1% fructose (w/v) as the sole carbon source. T. reesei and A. niger spores were grown at 29°C and 30°C, respectively, for 24 hours (A. niger) on a rotary shaker with agitation at 200 rpm. T. reesei was also grown on a rotary shaker with agitation of 200 rpm, but it was grown for 48 hours to achieve an initial mycelial mass similar to that of A. niger. After, mycelia were removed by filtration through Whatman grade 1 filters (GE Healthcare), and they were washed with sterile water and transferred to 30 mL of fresh BCM media (without yeast extract) containing 0.5% of SEB or SC (w/v) as the sole carbon source for 6, 12 or 24 hours. T. reesei cultures were grown in a controlled environmental growth chamber under constant illumination with white light.

The mycelia and biomass used as carbon sources were harvested by filtration, washed thoroughly with sterile water and quickly frozen in liquid nitrogen for further cell wall monosaccharide composition analyses. The supernatant was stored at -20°C for enzymatic, soluble supernatant sugar and mass spectrometry analyses.

Fungal growth

Nitrogen content, an indirect measure of fungal growth, was measured based on the Pregl-Dumas’ classical method [28]. The mycelial mass of A. niger and T. reesei grown on BCM with bagasse or culm for 6, 12 or 24 hours was rinsed with distilled water to remove traces of medium, and it was dried at 80°C for 4 h. The sample was macerated, and 2 mg (weighed with a digital electronic balance) was burned at approximately 975°C in the presence of pure oxygen. The process released nitrogen, carbon dioxide and water, which were passed through special columns that absorbed the carbon dioxide and water. A column carrying a thermal conductivity detector separated the nitrogen from any other residue, and the resulting nitrogen content was measured. The instrument (PerkinElmer, model 2400, series II) was previously calibrated by analyzing a pure standard of known nitrogen, and the amount of nitrogen in each sample was given as a percentage in relation to the initial mass.

Monosaccharide Analyses in Culture Supernatant

Monosaccharide analysis was performed on the supernatant. Each sample (1.8 mL) was completely dried using a Refrigerated CentriVap Concentrator (LABCONCO), resuspended in 500 μL of sterile deionized water and filtered through a 0.45-U pore size, 13-mm diameter (Durapore, Millex). The samples were subsequently analyzed by HPAEC-PAD on a CarboPac PA-1 column (DX-500 system, Dionex). The elution of carbohydrates occurred in a gradient
mixture of water and 200 mM sodium hydroxide at a flow rate of 0.8 mL/min for 50 min. Sugars were identified and quantified by comparing the retention times and ratios of sample peak area to internal standard peak area in relation to ratios determined for external standards using a Chromeloe 6.8 Chromatography Data System software.

**Supernatant preparation and SDS-PAGE analysis**

To analyze the secretome profiles of *T. reesei* and *A. niger*, triplicate supernatants (~90 mL) containing enzymes from each time-point were pooled and clarified by filtration through a 0.22-μm filter (Hydrophilic Millex, Millipore). The clarified supernatant was concentrated using a 3-kDa membrane (Vivaspin 20, GE HealthCare) to a final volume of 200 μL, and 20 μL was separated by 10% SDS-PAGE (110 V, 90 min). Three independent biological replicates of pooled supernatants were performed for the secretome experiments. The proteins were visualized by staining with 0.1% Coomassie Brilliant Blue R250 (w/v), followed by destaining with 45% methanol and 10% acetic acid solution (v/v). All bands from triplicate SDS-PAGE gels were manually excised, reduced, alkylated and digested in gel with trypsin-modified sequencing-grade reagents (Promega), according to a previously described method [29]. S1 Fig shows SDS-PAGEs with one replicate of each sample, before the concentration step.

**Mass spectrometry and protein identification**

Peptides were resuspended in 0.1% formic acid (v/v), and an aliquot (4.5 μL) was analyzed on an ETD-enabled LTQ Velos Orbitrap Mass Spectrometer (Thermo Fisher Scientific) coupled to a nanoflow liquid chromatography column (LC-MS/MS) via an EASY-nLC System (Proxeon Biosystem) through a Proxeon nanoelectrospray ion source. Peptides were separated by a 2–90% acetonitrile gradient in 0.1% formic acid using an analytical column PicoFrit Column (20 cm x ID75 μm, 5-μm particle size, New Objective), with a flow of 300 nL/min over 27 min. The nanoelectrospray voltage was set to 2.2 kV, and the source temperature was 275°C. All instrument methods for the LTQ Orbitrap Velos were set up in the data-dependent analysis (DDA) mode. Full scan MS spectra (m/z 300–1,600) were acquired in the Orbitrap analyzer after accumulation to a target value of 1e6. The resolution in the Orbitrap was set to r = 60,000. The 20 most intense peptide ions with charge states ≥ 2 were sequentially isolated to a target value of 5,000 and fragmented in the linear ion trap by low-energy CID (normalized collision energy of 35%). The signal threshold for triggering an MS/MS event was set to 1,000 counts. Dynamic exclusion was enabled with an exclusion size list of 500 and exclusion duration of 60 s. The activation Q-value was 0.25 and the activation time was 10 ms.

Data were acquired using the Xcalibur software package, and the raw data files were converted to a peak list format (mgf), without summing the scans, using Mascot Distiller v.2.3.2.0 (Matrix Science Ltd.). The database search was performed against the *Trichoderma* (13,808 proteins) and *Aspergillus niger* (36,414 proteins) from the NCBI database using the Mascot v2.3.02 engine (Matrix Science Ltd.), with carbamidomethylation as a fixed modification, oxidation of methionine as a variable modification and one trypsin missed cleavage. The precursor mass tolerance was set to 10 ppm, and the fragment mass tolerance was set to 0.1 Da. For protein identification, the resulting search data were analyzed in Scaffold 3.5.1 (Proteome Software). The defined parameters were: minimum protein probability of 80%, minimum peptide probability of 90% and unique different minimum peptide of 1. We accepted proteins with up to 10% FDR protein and 5% FDR peptide. CAZymes with only one unique peptide detected were considered in the present manuscript as less confidently quantified than those proteins with multiple peptides detected. All the single protein matches were further checked if the Mascot MS/MS ion score was greater than 25 (significance threshold p < 0.05), giving better
confidence to protein identification. To access the signal peptide presence, we used the signal peptide prediction program, SignalP version 4.1 (http://www.cbs.dtu.dk/services/SignalP/). The CAZy database (http://www.cazy.org/) was used to classify the identified proteins according to their families.

Enzymatic assays

Enzymatic activity was determined from the amount of reducing sugar liberated from different polysaccharide substrates by the 3,5-dinitrosalicylic acid (DNS) method [27] using glucose as standard. First, 30 mL of supernatant from the samples induced with SEB or SC for 24 hours was concentrated using a centrifugal concentrator (Vivaspin 20, 10 kDa, GE HealthCare) to a final volume of 5 mL. The supernatant activity was assayed using xylan from beechwood, β-glucan, debranched arabinan from sugar beet, xyloglucan from tamarind and carboxymethylcellulose (CMC) (purchased from Sigma—Aldrich and Megazyme) as substrates at a 0.5% final concentration. Briefly, activity was measured using 50 μL of the substrate solution, and 20 μL of supernatant was diluted in 100 mM sodium acetate buffer (pH 5.5) to achieve a final volume of 100 μL. The reaction was incubated at 40°C for 5 min for xylan and β-glucan and for 10, 70, and 180 min, for xyloglucan, CMC and debranched arabinan, respectively. The reaction was stopped by adding 100 μL of DNS. One unit (U) of enzymatic activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute.

Results and Discussion

Saccharification of sugarcane biomass

To identify when the degradation of sugarcane exploded bagasse (SEB) and culm (SC) were initiated, we analyzed the sugars that were released into the A. niger and T. reesei culture supernatants using high-performance liquid chromatography (HPAEC-PAD), over a time course of 6, 12 and 24 hours post-inoculation. The supernatant from each time point was also collected from control samples that were not inoculated with fungi. Fig 1 shows the changes in the proportions of glucose, cellobiose, xylose, arabinose and galactose over the course of the experiment. We observed bagasse degradation after 6 hours post-inoculation since the total concentrations of glucose and xylose were higher than in the control samples (Fig 1a and 1c). In culm, this feature was more noticeable after 12 hours (Fig 1b and 1d).

The changes in the proportion of sugars (Fig 1) led us to conclude that both cellulose and hemicellulose were being degraded. We observed only galactose in the samples with fungi that used bagasse and culm as carbon source, suggesting that the degradation of branched hemicelluloses (mainly composed of xylose and galactose) started within 6 hours. It is also likely that the fungi were consuming sugars derived from arabinoxylan, since we detected changes in arabinose concentration. Together with pectin and β-glucan, arabinoxylans form the most water-soluble and accessible part of the cell wall in sugarcane [4]. Therefore, these sugars are readily released by the fungal species as soon as they come into contact with the substrate due to the actions of multiple enzymes, such as α-arabinofuranosidases (GH3, GH43, GH51, GH54 and GH62), which remove the residues of arabinose, and β-1,4-endoxylanase (GH10, GH11, GH30) and β-1,4-xylosidase (GH3, GH43), which hydrolyze xylose from the xylan backbone [30].

Despite the higher recalcitrance of the culm, we also noticed that the samples from fungi grown on this substrate presented the highest amount of sugars in the supernatant (Fig 1b and 1d). These results suggest that both T. reesei and A. niger secreted more enzymes using culm as a carbon source compared to bagasse, since there were no differences in growth between the two substrates (indirectly measured by nitrogen content, Fig 2). Because both fungi consume sugars, these values do not represent the real rate of sugar release; however, they suggest that
both fungi released different amounts of monosaccharides and/or have different rates of sugar uptake.

**CAZymes profiling of** \( T. \text{reesei} \) **and** \( A. \text{niger} \) **secretomes**

Because the sugars released are dependent on secreted hydrolases, we investigated the \( T. \text{reesei} \) and \( A. \text{niger} \) secretomes. These hydrolysis systems appear dependent on the microorganism and carbon source used [23, 31, 32]. To date, few studies have utilized lignocellulosic biomass as a major carbon source, especially with sugarcane biomass [25, 26]. Our data showed that \( A. \text{niger} \) secreted a large number of CAZymes at all time points/carbon sources In total, we identified 45 different CAZymes in \( T. \text{reesei} \) (Table 1) and 89 in \( A. \text{niger} \) (Table 2) (Glycosyl Transferases, GTs, were not included). S1 Table lists all these enzymes and their respective molecular weights, numbers of identified peptides, peptide sequences, mass-to-charge ratios (m/z), numbers of unique peptides, and presence/absence of a signal peptide. S2 Table lists all other secreted proteins (not CAZymes, with signal peptide) found in both secretomes with their respective informations.

Compared to previous studies, we identified a higher number of CAZymes in \( A. \text{niger} \). Adav et al. [20] identified 30 enzymes produced while culturing \( A. \text{niger} \) in a bioflo fermenter containing minimal medium and glucose. Oliveira et al. [33] identified 40 hydrolytic enzymes in
the secretome of *A. niger* grown on a bioreactor containing minimal medium added of D-xylose (an inducer of cellulases and hemicellulases) or D-maltose as the sole carbon sources. However, in those reports, the inducers were simple carbon sources, and not complex carbon sources such as sugarcane bagasse and culm. An exception is the work from Souza et al. [25] that used SEB as carbon source, but they identified only 17 proteins using a much less refined proteomics method.

Interestingly, only one enzyme was reported in common to all these studies, the acid α-amylase (AamA). This enzyme belongs to the GH13 family and it cleaves internal α-(1,4)-glycosidic bonds in starch and glycogen [34]. However, we identified the most important enzymes related to biomass degradation. To hydrolyze the cellulose chains into monomers, the main chain must be cleaved internally, and this event is performed by endoglucanases (GH5, GH12). Likewise, the release cellobiose occurs via the action of exoglucanases (GH6, GH7), and it is subsequently converted into glucose by β-glucosidases (GH3) [35]. The hemicellulose fraction is formed by arabinoxylan, β-glucan and xyloglucan, and due to this variety of substrates, the enzymatic mixture required to break it down is more diverse, including endoxylanases (GH10, GH11), β-xylosidase (GH3), arabinofuranosidases (GH43, GH51, GH54), galactosidases (GH35) and others [22]. In the *A. niger* secretome, all of these enzymes were present at the earliest time point examined in this study (6 h).

We observed some differences for *T. reesei* grown on other carbon sources since the number of *T. reesei* CAZymes was higher than that reported by Herpoël-Gimbert et al. [22]; they identified 22 hydrolytic enzymes using lactose and xylose as carbon sources. However, Adav et al. [24] identified over 90 CAZymes using a quantitative proteomic approach, the iTRAQ system. Moreover, a recent study using untreated sugarcane bagasse investigated the secretome of two *Trichoderma* strains using solid-state fermentation [26]. In this study, they identified 39 GHs, and other proteins that play an important role in biomass degradation (for example, swollenin). These differences could be due either to the carbon source, culture conditions or...
Table 1. CAZymes detected in the secretome of *Trichoderma reesei*.

| CAZy Family | Predicted Protein | JGI Protein ID | Substrate/Time point | Possible Polysaccharide Substrate/Classification |
|-------------|-------------------|----------------|----------------------|--------------------------------------------------|
| GH17        | Candidate glucan 1,3-β-glucosidase | 24326          | B24h, C6h, C24h       | 1,3-1,4-β-Glucan                                  |
| GH17        | Candidate glucan endo-1,3-β- glucosidase | 110434          | C12h, C24h            | 1,3-1,4-β-Glucan                                  |
| GH55        | Exo-1,3-b-gluconan | 25104          | B12h, C6h, C12h, C24h | 1,3-1,4-β-Glucan                                  |
| GH55        | β-1,3-gluconan   | 93142          | B6h, B12h, B24h, C6h, C12h, C24h | 1,3-1,4-β-Glucan                                  |
| GH1         | β-glucosidase CEL1A, bgI2 | 127115          | B12h, B24h            | Cellulose                                         |
| GH3         | β-glucosidase, CEL3B | 25095          | B6h, B12h, B24h, C24h | Cellulose                                         |
| GH3         | β-glucosidase, bgI3i | 109567         | B24h, C6h, C24h       | Cellulose                                         |
| GH3         | β-glucosidase, bgI1, cel3a | 136547         | B24h, C24h            | Cellulose                                         |
| GH5         | Endoglucanase CEL5A | 72489          | C24h                  | Cellulose                                         |
| GH6         | Cellobiohydrolase CEL6A, cbh2 | 122470         | B24h, C12h, C24h      | Cellulose                                         |
| GH7         | Endoglucanase CEL7B, egl1 | 5304           | C24h                  | Cellulose                                         |
| GH7         | Cellobiohydrolase CEL7A, cbh1 | 125125         | B6h, B12h, B24h, C6h, C12h, C24h | Cellulose                                         |
| AA9 (GH61)  | Copper-dependent monoxygenase | 139633         | B24h, C24h            | Cellulose                                         |
| CBM1        | Swollenin         | 104220         | B12, B24, C12, C24    | Cellulose                                         |
| GH5         | Endo-β-1,4-mannosidase, man5a | 122377         | C12h, C24h            | Mannan                                            |
| GH79        | Candidate β-glucuronidase | 69609          | C12h                  | Xylan/Arabinobioxyan                               |
| GH43        | Candidate β-xylosidase/arabinosidase | 133200         | C6h, C12h, C24h       | Xylan/Arabinobioxyan                               |
| GH3         | B-xylosidase, bx1 | 140746         | B6h, B12h, B24h, C24h | Xylan/Arabinobioxyan                               |
| GH11        | Xylanase, xyn2    | 124931         | B12h, B24h, C6h       | Xylan/Arabinobioxyan                               |
| GH30        | Endo-β-1,4-xylanase, xyn4 | 90847          | B6h, B12h, B24h, C6h  | Xylan/Arabinobioxyan                               |
| CE5         | Acetyl xylan esterase, axe2 | 94461          | C6h, C12h, C24h       | Xylan/Arabinobioxyan                               |
| CE5         | Acetyl xylan esterase, axe1 | 139631         | B24h                  | Xylan/Arabinobioxyan                               |
| GH54        | α-L-arabinofuranosidase, abf3 | 72252          | C24h                  | Xylan/Arabinobioxyan                               |
| GH74        | Xyloglucanase, CEL74A | 111943         | B24h, C12h, C24h      | Xyloglucan                                        |
| GH16        | Cell wall glucanase | 96805          | B6h, B12h, B24h, C6h, C12h, C24h | Carbohydrate transport and metabolism                            |
| GH27        | α-D-galactosidase | 75420          | B12h                  | Carbohydrate transport and metabolism              |
| GH3         | Candidate β-N-acetylglucosaminidase | 12475          | B6h                  | Carbohydrate transport and metabolism              |
| GH30        | Candidate endo-β-1,4-xylanase | 93498          | B24h                  | Carbohydrate transport and metabolism              |
| GH31        | Candidate α-glucosidase | 104546         | B6h, B24h, C12h, C24h | Carbohydrate transport and metabolism              |
| GH37        | Candidate α,α-trehalase | 102372         | C6h, C12h, C24h       | Carbohydrate transport and metabolism              |
| GH65        | Candidate α,α-trehalase | 139320         | B24h, C12h, C24h      | Carbohydrate transport and metabolism              |
| GH72        | Candidate β-1,3-glucanosyltransferase | 98936         | C6h, C12h, C24h      | Carbohydrate transport and metabolism              |
| GT20        | Bifunctional trehalose-6-phosphate synthase | 72420         | B6h, B12h            | Carbohydrate transport and metabolism              |
| GT20        | Bifunctional trehalose-6-phosphate synthase | 67350         | B12h                  | Carbohydrate transport and metabolism              |
| GT31        | Distantly related to β- glycosyltransferases | 101599         | B12h                  | Carbohydrate transport and metabolism              |
| GT35        | Glycosyl transferase | 23636          | B6h, B12h, B24h, C6h  | Carbohydrate transport and metabolism              |
| GH16        | Candidate glucanosyltransferase | 66752          | B6h, B12h, B24h, C6h, C12h, C24h | Cell wall biosynthesis and morphogenesis            |
| GH72        | Candidate β-1,3-glucanosyltransferase | 9074           | B6h, B12h, C6h, C12h, C24h | Cell wall biosynthesis and morphogenesis            |
| GH72        | Candidate β- 1,3-glucanosyltransglycosylase | 124639       | B6h, B12h, B24h, C6h, C12h, C24h | Cell wall biosynthesis and morphogenesis            |

(Continued)
experimental design. However, in contrast to \textit{A. niger}, the most important proteins found in this study were also present in the aforementioned reports, such as cellobiohydrolases (Cbh1/Cbh2), endoglucanases (Eg1, Eg2 and Eg4) and $\beta$-glucosidases (Bgl1/Bgl2). We also identified hemicellulases involved in the cleavage of the main chain of xylan and xyloglucan (endoxylanase and xyloglucanase) and the side chain of hemicelluloses, including $\beta$-xylosidase, $\alpha$-arabinofuranosidase and $\alpha$-galactosidase, and acetylxyylan esterase.

Differences in the enzyme secretion in distinct sugarcane biomass

An additional goal of this study was to better understand the differences in enzyme patterns in distinct biomass types. Thus, in addition to bagasse, culm \textit{in natura} was used as a sole carbon source. Fig 3 shows a heat-map representing the number of enzymes identified for each class of enzymes (CAZymes classification was used) in each family of glycosyl hydrolase directly related to biomass degradation (see Tables 1 and 2) in \textit{A. niger} and \textit{T. reesei} after 6, 12 and 24 hours growing on untreated sugarcane culm and pretreated bagasse.

The pattern of enzyme production observed for \textit{A. niger} was similar for culm and bagasse. However, analyzing the enzymes secreted exclusively when \textit{A. niger} was grown on culm, we observed a range of pectinases (Fig 3, Table 2), which have a variety of biotechnological and industrial applications, and several of them have been cloned, characterized, or have had their production optimized \cite{12, 36–38}. Although seven pectinases were identified as being secreted using both carbon sources, twelve pectinases were secreted exclusively when \textit{A. niger} was grown on culm (Fig 3, Table 2). It is likely that the fraction containing more soluble pectins was lost during the sugarcane crushing (probably because of the hot water treatment), rendering bagasse with proportionally less pectin compared to culm. Therefore, the induction of these enzymes is higher when the fungi are grown using culm as a carbon source. Curiously, although \textit{T. reesei} secreted several enzymes when growing on culm and bagasse, none were related to pectin, even though its genome encodes several pectinases.

Despite the variety of enzymes produced by both fungi, a comparative analysis of the total number of peptides, which may be indicative of protein/enzyme abundance \cite{39, 40} (Fig 4), suggested that \textit{A. niger} and \textit{T. reesei} secreted more enzymes when grown on culm. As mentioned previously, there were no differences in fungi growth between the two substrates (Fig 2). Thus, the higher abundance of peptides produced by fungi growing on culm correlates with the greater amount of sugars in the supernatant (Fig 1b and 1d). This result could be related to differences in the recalcitrance of substrates, which reflects the different levels of complexity of the cell walls, as culm did not receive any pretreatment. However, how fungi sense these differences remains unclear.
Table 2. CAZymes detected in the secretome of *Aspergillus niger*.

| CAZy Family | Predicted Protein | JGI Protein ID | Substrate/Time point | Possible Polysaccharide Substrate/Classification |
|-------------|-------------------|----------------|----------------------|-----------------------------------------------|
| GH17        | Glucan endo-1,3-β-glucosidase (egl) | 158521         | B6h, B12h, B24h, C6h, C12h, C24h | 1,3–1,4-β-Glucan |
| GH55        | Exo-β,1,3-glucanase (bxga, exgo) | 156270         | B12h, B24h, C6h, C12h, C24h | 1,3–1,4-β-Glucan |
| GH55        | β-1,3-exoglucanase | 157838         | B12h | 1,3–1,4-β-Glucan |
| GH3         | β-D-glucoside glucohydrolase M   | 163273         | B6h, B12h, B24h, C12h | Cellulose |
| GH3         | β-glucosidase 2 | 163842         | B12h, B24h | Cellulose |
| GH3         | β-glucosidase | 165962         | C24h | Cellulose |
| GH3         | β-glucosidase | 168801         | B6h, B12h, B24h, C6h, C12h | Cellulose |
| GH5         | Endo-β,1,4-glucanase A (egla) | 156195         | B6h, B12h, B24h, C12h, C24h | Cellulose |
| GH5         | Endo-β,1,4-glucanase B (eng1) | 161114         | B6h, B12h, B24h, C6h, C12h, C24h | Cellulose |
| GH5         | Endoglucanase B (eglb) | 167967         | B6h, B12h, C6h, C12h | Cellulose |
| GH6         | Glucan 1,3-β-glucosidase A (exga) | 168853         | B6h, B12h | Cellulose |
| GH6         | Exocellobiohydrolase | 161440         | B12h, B24h, C6h, C12h | Cellulose |
| GH6         | β-glucanocellobiohydrolase C | 164557         | B6h, B12h, B24h, C6h | Cellulose |
| GH7         | Cellobiohydrolase B (cbhb) | 156194         | B6h, B12h, B24h, C6h, C12h, C24h | Cellulose |
| GH7         | Cellobiohydrolase A (cbha) | 161153         | B6h, B12h, B24h, C6h, C12h, C24h | Cellulose |
| GH30        | Glucan endo-1,6-β-glucosidase | 158067         | B6h, B12h, B24h, C6h, C12h, C24h | Cellulose |
| AA9 (GH61)  | Endoglucanase IV | 161785         | B6h, B12h, C6h, C12h | Cellulose |
| AA9 (GH61)  | Endoglucanase 4 | 166052         | B24h | Cellulose |
| AA9 (GH61)  | Putative endoglucanase IV | 166976         | B6h, B12h, B24h | Cellulose |
| CE8         | Pectinesterase | 157769         | C6h, C12h, C24h | Pectin |
| CE8         | Pectin methylsterase A (pmea) | 158617         | B6h, C6h, C12h | Pectin |
| CE8         | Pectin methylsterase A (pmea) | 159650         | B6h, B12h, C6h, C12h, C24h | Pectin |
| CE12        | Putative rhamnogalacturonan acetyl esterase | 159617        | C6h, C12h, C24h | Pectin |
| CE12        | Rhamnogalacturonan acetyl esterase | 162676        | C6h, C12h | Pectin |
| CE16        | Putative pectin acetyl esterase | 156782         | C6h | Pectin |
| GH5         | Endo-β,1,6-galactanase | 158118         | B6h, B12h, B24h, C6h, C12h | Pectin |
| GH28        | Endopolygalacturonase-1 | 156180         | C12h | Pectin |
| GH28        | Endopolygalacturonase B (pgab) | 157015         | B6h, B12h, B24h, C6h | Pectin |
| GH28        | Exopolygalacturonase (pgxb) | 158660         | C6h, C12h, C24h | Pectin |
| GH28        | Endo-xylagalacturonan hydrolase A (xgha) | 159651        | B12h, B24h, C6h, C12h, C24h | Pectin |
| GH28        | Endo-polygalacturonase D (pgad) | 162788         | C6h, C12h | Pectin |
| GH28        | Exo-xylagalacturonan hydrolase (pgxa) | 163648        | C12h | Pectin |
| GH28        | Rhamnogalacturonan (rhga) | 164433         | C12h | Pectin |
| GH28        | Exopolygalacturonase X (pgax) | 165048         | C6h | Pectin |
| GH28        | Rhamnogalacturonase B (rgb) | 166203         | C6h, C12h | Pectin |
| GH28        | Rhamnogalacturonan α-galacturonohydrolase | 168924        | C12h | Pectin |
| GH53        | Arabinogalactan endo-1,4-β-galactosidase | 169030        | B24h, C6h | Pectin |
| PL1         | Pectin lyase A (pela) | 166220         | B12h, C6h, C12h | Pectin |
| CE1         | Feruloyl esterase A (falea) | 162483         | B6h, B12h, B24h, C6h, C12h, C24h | Phenylpropanoids |

(Continued)
Table 2. (Continued)

| CAZy Family | Predicted Protein | JGI Protein ID | Substrate/Time point | Possible Polysaccharide Substrate/Classification |
|-------------|-------------------|----------------|----------------------|-----------------------------------------------|
| CE1         | Feruloyl esterase C (faec) | 164585 | B6h, B12h | Phenylpropanoids |
| CE1         | Feruloyl esterase (faeb) | 165335 | B6h, B12h, B24h, C6h, C12h, C24h | Phenylpropanoids |
| CE1         | Acetyl xylan esterase (axea, acea) | 164821 | B24h, C6h, C12h | Xylan/Arabinoxylan |
| CE16        | Acetylxylanase | 161113 | B6h, B12h, C6h, C12h | Xylan/Arabinoxylan |
| GH3         | Exo-1,4-β-xylanase (xind) | 156034 | B6h, B12h, B24h, C6h, C12h | Xylan/Arabinoxylan |
| GH3         | Bifunctional xylosidase-arabinosidase | 168244 | B24h | Xylan/Arabinoxylan |
| GH10        | Endo-1,4-β-xylanase C | 158107 | B6h, B12h, B24h, C12h | Xylan/Arabinoxylan |
| GH11        | Xylanase 2 / B (xynb, xlnb) | 155137 | B6h, B12h, B24h, C6h, C12h, C24h | Xylan/Arabinoxylan |
| GH11        | Endo-1,4-β-xylanase B | 166974 | C6h | Xylan/Arabinoxylan |
| GH43        | Arabinin endo-1,5-α-l-arabinosidase C | 157571 | B6h, B12h, B24h, C6h, C12h, C24h | Xylan/Arabinoxylan |
| GH43        | Xylan β-xylanosidase | 161454 | B12h, B24h | Xylan/Arabinoxylan |
| GH43        | Glycosyl hydrolase family 43 protein | 162327 | B12h, B24h, C6h, C12h, C24h | Xylan/Arabinoxylan |
| GH43        | Arabinin endo-1,5-L-arabinosidase A | 162583 | B6h, C6h, C24h | Xylan/Arabinoxylan |
| GH43        | Endo-arabinase | 166877 | B12h | Xylan/Arabinoxylan |
| GH51        | α-L-arabinofuranosidase A (abfa, exoa) | 155097 | C6h, C12h, C24h | Xylan/Arabinoxylan |
| GH51        | α-L-arabinofuranosidase E | 162554 | B6h, B24h, C12h | Xylan/Arabinoxylan |
| GH54        | α-L-arabinofuranosidase B | 166753 | B6h, B12h, B24h, C6h, C12h, C24h | Xylan/Arabinoxylan |
| GH62        | α-L-arabinofuranosidase (axha) | 158109 | B6h, B12h, B24h, C6h, C12h, C24h | Xylan/Arabinoxylan |
| GH67        | α-glucuronidase A | 166362 | B6h, B12h, B24h | Xylan/Arabinoxylan |
| GH35        | β-galactosidase A (laca) | 156240 | B6h, B12h | Xylan/Arabinoxylan |
| GH12        | Xyloglucan-endo-β-1,4-glucanase | 155384 | B6h, C6h, C12h | Xyloglucan |
| GH12        | Endo-β-1,4-glucanase | 158544 | B6h, B12h, B24h, C12h, C24h | Xyloglucan |
| GH12        | Endo-β-1,4-glucanase (egla) | 166061 | B6h, B12h, B24h, C6h, C12h, C24h | Xyloglucan |
| GH74        | Xyloglucanase | 155242 | B12h, B24h | Xyloglucan |
| GH32        | Invertase (suca, suc1) | 162354 | B24h, C6h | Carbohydrate transport and metabolism |
| GH32        | Exo-inulinase E (inue, inu1) | 165128 | C6h, C12h, C24h | Carbohydrate transport and metabolism |
| GH65        | α-othrehalase | 155210 | B6h, B12h, B24h | Carbohydrate transport and metabolism |
| GH95        | Glycosyl hydrolase | 167353 | B24h | Carbohydrate transport and metabolism |
| GH27        | α-galactosidase II (aglb)/melibiase | 157631 | B12h, C12h | Carbohydrate transport and metabolism |
| GH27        | α-galactosidase A (aglbA/agla) | 159990 | B12h, B24h, C12h | Carbohydrate transport and metabolism |
| GH27        | α-galactosidase D (agld) | 165965 | B6h, C12 | Carbohydrate transport and metabolism |
| GH16        | β-glucanase | 155502 | B24h, C24h | Cell wall biosynthesis and morphogenesis |
| GH16        | Glycogenase crf1 | 156136 | B6h, B12h, B24h, C6h, C12h, C24h | Cell wall biosynthesis and morphogenesis |
| GH16        | GPI-anchored glucosyltransferase | 160973 | B6h, C6h | Cell wall biosynthesis and morphogenesis |
| GH16        | β-glucanase | 163407 | B6h, B12h, B24h, C6h, C12h, C24h | Cell wall biosynthesis and morphogenesis |
| GH17        | 1,3-β-glucanosyltransferase (bgt1) | 161620 | B6h, B12h, B24h | Cell wall biosynthesis and morphogenesis |
| GH72        | 1,3-β-glucanosyltransferase | 156831 | B12h, C12h | Cell wall biosynthesis and morphogenesis |
| GH72        | 1,3-β-glucanosyltransferase | 161995 | C6h | Cell wall biosynthesis and morphogenesis |
| GH72        | 1,3-β-glucanosyltransferase | 162537 | B6h, B24h, C6h, C12h, C24h | Cell wall biosynthesis and morphogenesis |
| GH72        | 1,3-β-glucanosyltransferase (gel1) | 163189 | B6h, B12h | Cell wall biosynthesis and morphogenesis |

(Continued)
Table 2. (Continued)

| CAZy Family | Predicted Protein | JGI Protein ID | Substrate/Time point | Possible Polysaccharide Substrate/Classification |
|-------------|-------------------|---------------|----------------------|-----------------------------------------------|
| GH1         | Glucan endo-1,3-β-D-glucosidase | 155359 | B12h, B24h | Cell wall biosynthesis and morphogenesis |
| GH13        | Cell-wall 4-α-glucanotransferase (agta) | 162772 | B6h, B12h | Starch |
| GH13        | Acid α-amylase (aama) | 163584 | B6h, B12h, B24h, C6h, C12h, C24h | Starch |
| GH15        | Glucoamylase (glaa) | 158641 | B6h, B12h, B24h, C6h, C12h, C24h | Starch |
| GH18        | Class V endochitinase (chib) | 157223 | B12h, B24h | Chitin |
| GH18        | Exo-chitinase (cfc) | 157878 | B12h | Chitin |
| GH20        | N-Acetyl-β-glucosaminidase (nag1) | 162684 | B24h | Chitin |
| GH5         | β-mannanase (mana) | 159852 | B6h, B12h, B24h | Mannan |
| GH47        | α-1,2-mannosidase | 156279 | B6h, B12h, B24h | Mannan |
| GH92        | Glycosyl hydrolase | 166207 | B12h, B24h | Mannan |

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Fig 3. Graphical representation of secreted CAZymes. Heat-map of the number of enzymes of each CAZY family secreted by A. niger and T. reesei after 6, 12 and 24 hours (h) growth on sugarcane culm and bagasse. This map includes only enzymes/proteins related to biomass degradation (Tables 1 and 2).
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Comparison between *T. reesei* and *A. niger* secretomes

The secretome profiles of these two fungi were compared, and our data indicates they differed considerably (Fig 3, Tables 1 and 2). As mentioned before, the assembly, modification and breakdown of complex carbohydrate and glycoconjugates are carried out by the CAZymes [41]. However glycosyl transferases and the carbohydrate esterases from the family CE10 genes are not involved in degradation of plant cell walls. In the *A. niger* supernatant, we detected 65 enzymes directly related to biomass degradation, approximately 22% of the total of carbohydrate-active enzymes encoded by its genome (292 proteins, excluding GTs), whereas *T. reesei* secreted 24 enzymes (approximately 10% of its 228 CAZymes, excluding GTs). GHs found exclusively in the *A. niger* secretome were related to hemicellulose degradation, such as GH10 (endo-1,4-β-xylanase), GH51 and GH62 (both α-L-arabinofuranosidases), and pectin, including endopolygalacturonase (GH28) and endo-1,4-β galactosidase (GH53) (Fig 3). Important esterases were also present in *A. niger*, but not *T. reesei*, such as the acetyl xylan esterase (CE1) and acetylesterase (CE16), that catalyzes the hydrolysis of acetyl groups from hemicellulose, and pectin methyl esterase (CE8), acetyl esterase (CE12), that have activity against pectin.

*T. reesei* produces the most commercially used cellulases [42], and among the GHs related to biomass degradation found exclusively on its secretome, we identified a β-glucosidase from the GH1 family. At earlier time points, the mode by which *T. reesei* attacks arabinoxylans seems to differ from that of *A. niger*, with an exclusive acetyl xylan esterase (CES) and endoxylanase (GH30). No evidence of the presence of laccases were found for any of the species studied, suggesting that the hydrolytic attack of these fungi toward sugarcane biomass does not involve lignin degradation, at least on the time points tested.

We also performed a comparative analysis of the total number of peptides. In agreement with previous studies [23, 24], the celllobiohydrolase Cel7A displayed the highest number of peptides in the *T. reesei* secretome (S1 Table). The celllobiohydrolase Cel6A, was the third most
abundant enzyme; together with CEI7A, these enzymes represent 80% of the peptides from cellulases (S1 Table), as reported by other authors [22, 24]. An enzyme from the GH16, a putative glucanosyl transferase (ID 66752) related to cell wall biosynthesis and morphogenesis, was the second most abundant protein. Unlike T. reesei, the relative analysis of abundance revealed that the peptides from α-L-arabinofuranosidase B (GH54) and xylanase 2 (GH11) were the most abundant peptides in A. niger. Although Adav et al. [20] described the same arabinofuranosidase as the one of most abundant proteins after D-xylose induction, xylanase 2 was barely detected. This xylanase was induced by culm and bagasse in A. niger, but it was less prevalent in T. reesei (Fig 3, S1 Table).

Interestingly, when we compared the growth of both fungi, T. reesei had a larger mycelia mass compared to A. niger (indirectly measured by nitrogen content, Fig 2). Considering the abundance of secreted enzymes in both fungi, A. niger produced not only a wider range of enzymes but also secreted higher quantities compared to T. reesei (Fig 4). To verify whether the abundance of peptides reflected the abundance of enzymes, we performed enzymatic assays using beechwood, β-glucan, debranched arabinan from sugar beet, xyloglucan from tamarind and CMC as substrates. Given the low sensitivity of the assay and the small amount of protein, we measured the enzymatic activities at 24 hours, the time point with the highest enzyme production (Tables 1 and 2). Supernatants derived from both cultures were capable of hydrolyzing hemicelluloses, such as β-glucan, xylan (Fig 5a), xyloglucan (Fig 5b), arabinan (Fig 5c), and

Fig 5. Estimation of enzyme activities. Enzymatic activities (U/mL) against different substrates of A. niger and T. reesei after 24 hours (h) growth on sugarcane culm (C) and bagasse (B). a) B-glucan and xylan b) Xyloglucan, and c) CMC and Arabinan. Each bar represents the mean and the standard deviation of values from three independent experiments.

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cellulose: CMC (Fig 5c), which is in agreement with the identified enzymes. Moreover, enzymes derived from the supernatant fraction of A. niger were able to hydrolyze the majority of substrates more effectively than T. reesei, corroborating the peptide proportion data presented in showed in Fig 4. Furthermore, enzymatic activity toward CMC was higher in T. reesei, likely due to its remarkable cellulolytic ability.

Therefore, we evaluated secreted enzymes involved in cell wall degradation by both fungi over the experimental time course and tried to correlate the monosaccharides released in the supernatant and enzymes related to the degradation of their polysaccharides. As we mentioned before, our data suggests that arabinoxylan is been consumed within the first 6 hours after attack by both fungal species. Hypothetically, at least for A. niger, the attack of feruloyl esterases and the acetyl esterases might allow for arabinobinoxylan degradation. At early time points, the general performance of the enzyme production system of A. niger corroborated the model of sugarcane cell wall hydrolysis proposed by De Souza et al. [4] that reported that sugarcane cell walls are composed of domains that can be extracted with progressively higher concentrations of alkali. Pectins (ca. 10% of the wall) are more soluble, followed by 1,3 and 1,4-β-glucans (ca.10% of the wall) and highly interactive arabinobinoxylans (ca. 40% of the wall) and a xyloglucan/xylan-cellulose domain (ca. 40% of the wall) that is less soluble. Thus pectinases and esterases should be the first enzymes to act on the walls, opening the way for the hemicellulases and cellulases (Fig 6). Observing the appearance of enzymes related to biomass degradation (Fig 3), we found that the carbohydrate esterases (feruloyl and acetyl xylan esterases (CE1), pectin methyltransferase (CE8), rhamnogalacturonan acetyl esterase (CE12), pectin and acetyl esterase (CE16)) were some of the first enzymes secreted by A. niger. These enzymes have molecular weights below the threshold that is considered the pore size of the cell wall, 35–40 Angstrons [43]. Thus, these enzymes are likely to penetrate the cell wall matrix before other glycosyl hydrolases that will attach polymer decorations and main chains. Thus, by de-esterifying polysaccharides, they make the main chains of these polymers more accessible to endo-enzymes.

From the proteomics point of view, T. reesei displays a different strategy to disassemble sugarcane cell walls compared with A. niger (Fig 6). The former species produced relatively low quantities and variety of enzymes, and it did not produce pectinases at all. The fact that no feruloyl esterase is present in T. reesei extracts suggests that this species employs a mechanism that uses proportionally fewer debranching enzymes in the early stages of biomass degradation and that it is able to attack cellulose microfibrils without a prior attack to the phenylpropanoids of the cell wall. On the other hand, swollenin was detected in T. reesei after 12 hours in culm and bagasse. This swollenin isolated from T. reesei (the protein was named SWO1) behaves like a plant expansin. When purified, SWO1 disrupted the cell wall structure without the production of free glucose [44]. Swollenin and the acetyl-xylan-esterase (CE5–30.5 KDA), which were present only in the T. reesei genome and secretome (Fig 3, Table 1), likely act by disrupting the cell wall architecture and loosening polymer-polymer interaction in a way that polysaccharides become more accessible to glycosyl hydrolases, such as 1,3–1,4-β-glucanases, arabinobinoxylanases and cellulases (Fig 6).

Despite the distinct modes of attack to the biomass, both fungi are able to break down the biomass cell wall since the earliest time points, but we can suggest that A. niger invests more in cell wall hydrolysis in terms of number of enzymes and enzyme activities related to cell wall hydrolysis than T. reesei, which secreted fewer enzymes. These two different behaviors are probably associated with the biology of each species. Another important aspect that seems to influence the observed behavior of both fungi is that they are limited, to a certain extent, by their respective genomes, i.e., the enzyme arsenals to address biomass [15, 45].
Notwithstanding, it must be taken into consideration that *T. reesei* was submitted to several rounds of random mutagenesis to obtain the hypercellulolytic strain RUT-C30.

**Conclusions**

Here, we provide the first comparative secretome analysis of the most important lignocellulolytic fungal species, *A. niger* and *T. reesei*, growing on sugarcane biomass. This secretome study indicates that biomass degradation begins within the first 6 hours of fungal growth. A proteomic approach was used to analyze the secretome profiles of *A. niger* and *T. reesei*, and our data indicated that the two fungal species have different modes of attacking the same biomass, at least within 24 hours of the saccharification process. Thus, we advanced our understanding of the synergic mode of attack of swollenin, esterases, and glycosyl hydrolases in the context of...
enzyme cocktails and architecture of the plant cell wall. Using a semi-quantitative method based on peptide counts, we estimated the relative differences in the amount of extracellular enzyme production. We noticed that the induction of hydrolytic enzymes is higher when both fungi were growing using culm as a carbon source, probably due to the higher recalcitrance of this substrate. At the time points measured, *A. niger* produced more enzymes (quantitatively and qualitatively) than *T. reesei*, but both species were able to disassemble the carbohydrate portion of sugarcane cell walls. Considering that *T. reesei* and *A. niger* have different mechanisms for degrading biomass, these data suggest that a combination of enzyme from the two species might be an interesting option to increase saccharification efficiency. In other words, the two fungal species might be combined for use in industrial processes.

**Supporting Information**

**S1 Fig.** SDS-PAGEs of the secretome of *A. niger* and *T. reesei* grown on sugarcane biomass. Proteins secreted by *A. niger* (A) and *T. reesei* (B) after 6, 12 and 24 hours (h) growing on sugarcane culm (C) and bagasse (B). Lane M: molecular weight marker. (TIF)

**S1 Table.** CAZymes identified in the secretome of *A. niger* and *T. reesei*. This file includes all hydrolytic enzymes identified in the secretome of *A. niger* and *T. reesei*, their respective molecular weights, number of identified peptides, peptide sequences, mass-to-charge ratios (m/z), number of unique peptides, and presence/absence of signal peptide. (XLSX)

**S2 Table.** Proteins with signal peptide (not CAZymes) identified in both secretomes. This file includes other proteins identified in the secretome of *A. niger* (A) and *T. reesei* (B), their respective molecular weights, number of identified peptides, peptide sequences, mass-to-charge ratios (m/z) and number of unique peptides. (XLSX)

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**Author Contributions**

Conceived and designed the experiments: JVCO. Performed the experiments: GPB CCS ESS ATS APS. Analyzed the data: GPB CCS ESS JVCO. Contributed reagents/materials/analysis tools: AFPL FMS. Wrote the paper: JVCO MB GHG.

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