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Lignocellulolytic characterization and comparative secretome analysis of a *Trichoderma erinaceum* strain isolated from decaying sugarcane straw

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ABSTRACT

The fungus *Trichoderma reesei* is employed in the production of most enzyme cocktails used by the lignocellulosic biofuels industry today. Despite significant improvements, the cost of the required enzyme preparations remains high, representing a major obstacle for the industrial production of these alternative fuels. In this study, a new *Trichoderma erinaceum* strain was isolated from decaying sugarcane straw. The enzyme cocktail secreted by the new isolate during growth in pretreated sugarcane straw-containing medium presented higher specific activities of β-glucosidase, endoxylanase, β-xylosidase and α-galactosidase than the cocktail of a wild *T. reesei* strain and yielded more glucose in the hydrolysis of pretreated sugarcane straw. A proteomic analysis of the two strains' secretomes identified a total of 86 proteins, of which 48 were exclusive to *T. erinaceum*, 35 were exclusive to *T. reesei* and only 3 were common to both strains. The secretome of *T. erinaceum* also displayed a higher number of carbohydrate-active enzymes than that of *T. reesei* (37 and 27 enzymes, respectively). Altogether, these results reveal the significant potential of the *T. erinaceum* species for the production of lignocellulases, both as a possible source of enzymes for the supplementation of industrial cocktails and as a candidate chassis for enzyme production.

1. Introduction

Plant-derived biomass is the most abundant source of carbohydrates on Earth, and its conversion into biofuels has the potential to significantly reduce our dependence on fossil fuels (Hood, 2016). The commercial-scale production of these environment-friendly fuels depends, however, on the availability of cost-efficient technologies for the release of sugar monomers from the polysaccharide chains that make up plant cell walls. The cell walls of plants are largely composed of lignocellulose, a recalcitrant matrix of biopolymers containing mainly cellulose (30–50 %), hemicellulose (20–35 %) and lignin (10–25 %) (Houston et al., 2016; Szczerbowski et al., 2014). Deconstruction of lignocellulosic (LC) biomasses for the production of biofuels is generally accomplished in two stages. First, a pretreatment step works to increase the porosity of the plant material, making the cellulose and hemicellulose polysaccharides more accessible to enzymes. Next, the pretreated material is subjected to an enzymatic hydrolysis that

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decomposes the exposed sugar polymers into monomers. The obtained sugar molecules can then be converted into biofuels, such as bioethanol, and into value-added chemicals that may serve as economic drivers for biorefineries (Balan, 2014; Carroll and Somerville, 2009; Limayem and Ricke, 2012).

The enzymatic hydrolysis step represents one of the major bottlenecks for the industrial-scale production of LC biofuels. The required enzyme cocktails are expensive, accounting for about a third of the overall production costs (Klein-Marcuschamer et al., 2012). These cocktails must contain a diverse arsenal of lignocellulases (ligninases, cellulases and hemicellulases) in order to efficiently break down the complex structure of LC substrates (Giacobbe et al., 2016; Sun et al., 2015) and, unless their composition is customized for each type of biomass and pretreatment method employed in a given process, there may be significant differences in the observed hydrolysis efficiencies (Monschein and Nidetzky, 2016; Raud et al., 2016).

The industrial production of lignocellulases relies greatly on saprotrophic fungi and in their natural ability to secrete biomass-degrading enzymes (Eichlerová et al., 2015). This is particularly true for the *Trichoderma reesei* species, which has given origin to several industrial strains and is utilized in the production of most enzymatic formulations used by LC biorefineries. In fact, the production of about 80 % of all LC ethanol manufactured worldwide relies on *T. reesei*-derived enzymes (Bischof et al., 2016). Throughout the years, several limitations have been identified for *T. reesei*'s secretome, as for instance, its low β-glucosidase activity (Harris et al., 2010; Laothanachareon et al., 2015; Pham et al., 2010; Szczodrak, 1989). Still, despite the observed deficiencies and the fact that other fungi produce more efficient lignocellulolytic cocktails (reviewed in (Gusakov, 2011; Wang et al., 2012)), *T. reesei* remains the fungus of choice for industrial lignocellulase production. The knowledge gained through over seventy years of study and the technologies available for its industrial application favor the continued use of this fungal workhorse (Bischof et al., 2016; Druzhinina and Kubicek, 2017). Nevertheless, the advancement of the LC biofuels industry hinges on improving the efficiency and reducing the cost of available enzyme cocktails. It is, therefore, crucial to discover novel enzymes that may complement *T. reesei*'s secretome and to study other fungal species that can potentially replace it as an enzyme production platform.

A virtually unexplored source of biodiversity with untapped potential for the production of lignocellulases are all the other species of the genus *Trichoderma*. This group comprises over 80 species that inhabit a wide range of habitats and display very distinct phenotypes (Samuels, 2006). Yet, only a few of them have been investigated for their lignocellulolytic capabilities (Azin et al., 2007; Delabona et al., 2013; Kovács et al., 2009; Lan et al., 2013; Marx et al., 2013). For instance, the *Trichoderma erinaceum* species was first described over a decade ago (Bissett et al., 2003) but is yet to be studied for the production of lignocellulases.

Trichoderma isolates and other saprotrophic fungi are commonly found in soil samples and rotting organic materials (Bissett et al., 2003; Blaszczyk et al., 2011; Cabero et al., 2012; Hoyos-Carvajal et al., 2009). Considering that the enzyme arsenals of these microorganisms correlate with their microhabitats (Schneider et al., 2012; Treseder and Lennon, 2015), a feedstock used in the production of LC biofuels would be a suitable starting point in the search for isolates and enzymes of interest to the industry. A prime example is sugarcane straw, which is one of the main feedstocks considered for the production of LC ethanol (Oliveira et al., 2013; Pereira et al., 2015). Due to the seasonality of sugarcane cropping and the logistics involved in the production of LC ethanol, the straw must be stored near the biorefineries to ensure continuity to the process (Jonker et al., 2015). At these storage facilities the straw is usually kept in open air, being exposed to the weather and creating a hospitable environment for the proliferation of biomass-degrading microorganisms.

In the present study, a new *Trichoderma erinaceum* strain was isolated from decaying sugarcane straw collected at the straw storage facility of a LC ethanol biorefinery located in northeastern Brazil. With the aim of determining if the *T. erinaceum* species can potentially contribute to the production of lignocellulases, we set out to characterize the lignocellulolytic capabilities of the newly isolated strain. To this purpose, the *T. erinaceum* isolate and a wild *T. reesei* strain were cultured in pretreated sugarcane straw-containing medium and the secreted enzyme cocktails were compared through enzyme activity assays, hydrolysis assays of pretreated sugarcane straw and liquid chromatography tandem-mass spectrometry (LC-MS/MS)-based proteomic analyses.

2. Materials and methods

2.1. Strains, isolation and molecular taxonomic identification of fungi

A wild *Trichoderma reesei* strain (CBS 130855) was used as a reference control in all experiments. Additional fungal strains were isolated from decaying sugarcane straw collected at GranBio's straw storage facility, located in Barra de São Miguel, Alagoas state, Brazil (GranBio, 2011). Several samples of the decaying straw were collected in sterile plastic bags and transported to the laboratory for further processing. Each sample was immersed in 0.1 % peptone water for approximately 15 min, placed on Malt Extract Agar (MEA) medium (Sigma–Aldrich, St. Louis, USA) and incubated at 28 °C for 20 d. The plates were monitored daily and observed fungal hyphae were transferred to new MEA plates for isolation. For taxonomic identification, the isolated fungi were grown on Potato Dextrose Agar (PDA, Merck Millipore, Burlington, USA), a medium on which better sporulation rates had been observed. Spores from each isolate were suspended in 0.1 % triton to a final concentration of 10^7 spores mL^{-1}. 100 μL of each spore suspension were inoculated into 50 mL of potato dextrose broth (BD Difco, Franklin Lakes, USA) and incubated at 30 °C and 200 rpm for 48 h. Genomic DNA was extracted as previously described (Al-Samarrai and Schmid, 2000). The Internal Transcribed Spacers (ITS) 1 and 2 sequences were PCR-amplified with the universal primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990) and the Phusion® high-fidelity DNA polymerase (New England Biolabs, Ipswich, USA). The obtained DNA fragments were Sanger sequenced with the same primers. Sequencing was conducted by the Life Sciences Core Facility (LaCTAD) staff at the State University of Campinas (UNICAMP). The genera of the isolated fungal strains were determined by performing pairwise sequence alignments of the obtained sequences against the following web-based curated reference databases: International Society for Human and Animal Mycology (ISHAM) Barcoding Database (Irinyi et al., 2016), CBS Fungal Barcoding Database (2012) and the Michigan State University Ribosomal Database Project (RDP) Classifier tool with the UNITE Fungal ITS trainset (Wang et al., 2007). The F3 and F7 isolates were identified to the species level using the *Trichoderma*-specific ITS barcode identification program TrichOKEY2 (Druzhinina et al., 2005). The species identity of the F3 strain was confirmed by amplification and sequencing of a 5′ end fragment of the *tef* (eEF1a1) gene that contains three introns. The degenerate primers tef71f (CAAAATGGGTAAGGAGGASAAGAC) and tef997R (CAGTACCGGCRGCRATRATSAG) were used for both PCR and Sanger sequencing (Shoukouhi and Bissett, 2009). The *Tricho*BLAST web-based program (Kopchinskiy et al., 2005) was then used to search

the obtained sequence against a multiloci database of phylogenetic markers that includes only vouchered entries.

2.2. Phylogenetic analysis

The taxonomic identification of the fungal isolates was validated through the construction of a phylogenetic tree. The obtained ITS sequences were first aligned with sequences from various fungal species which were downloaded from curated reference databases. ITS sequences from *Trichoderma* species were downloaded from the International Subcommission on *Trichoderma* and *Hypocrea* (ISTH) Multiloci Database website (Druzhinina and Kopchinskiy, 2004). All other fungal ITS sequences were downloaded from the CBS Fungal Barcoding Database (2012). The ITS sequences were aligned with the MAFFT (Multiple Alignment using Fast Fourier Transform) web-based software (Li et al., 2015). The default parameter values for Gap Open Penalty (1.53), Gap Extension (0.123) and Maxiterate - the maximum number of iterations to perform when refining the alignment – (2) were used to perform the alignment. The aligned sequences were then used to infer a phylogenetic tree by maximum likelihood with the IQ-TREE web-based software (Nguyen et al., 2015). The substitution model TIM2 + F + I + G4 was automatically selected with Model Finder (Kalyaanamoorthy et al., 2017). An Ultrafast Bootstrap branch support analysis was performed with 10^4 alignments, 10^4 iterations and a minimum correlation coefficient of 0.99 (Minh et al., 2013). The default IQ-TREE search parameter values for perturbation strength (0.5) and IQ-TREE stopping rule (100) were used.

2.3. Biomass pretreatment and characterization

The pretreated sugarcane straw (PTSS1) used in the initial screening, enzymatic assays and hydrolysis experiments was provided by GranBio (2011). This material was subjected to a steam explosion pretreatment using proprietary technology at GranBio's lignocellulosic ethanol factory BioFlex 1, located in São Miguel dos Campos, Brazil. The carbohydrate and lignin content of the pretreated biomass was determined following the National Renewable Energy Laboratory (NREL) analytical procedure (Sluiter et al., 2012). In short, the pretreated biomass was fractionated using a two-step acid hydrolysis. This procedure caused the lignin to fractionate into acid soluble and acid insoluble material. The acid soluble lignin was quantified by UV–Vis spectroscopy. The insoluble material was quantified gravimetrically. The polysaccharides present were hydrolyzed into monomers, becoming soluble, and were then quantified by HPLC. The acetyl content of the liquid was also determined by HPLC. The pretreated sugarcane straw (PTSS2) utilized in the proteomic experiments was prepared from straw collected at GranBio's straw storage facility. The pretreatment process was done in two stages in a 20 L batch steam explosion reactor. First, the biomass was mixed with water at a mass ratio of 1:6 and subjected to a steam explosion at 200 °C and 16–17 bar for 5 min. The resultant liquid fraction was discarded, and the solid fraction was subjected to a second steam explosion at 165 °C and 8–9 bar for five minutes. The recovered solid fraction was then washed five times with distilled water for the removal of soluble residual sugars and inhibitors produced during the process. The washed biomass was dried in an oven at 50 °C for 72 h. The moisture content of both pretreated materials was determined with a benchtop moisture meter.

2.4. Enzyme extracts preparation

The isolated fungi and the control strain were grown on Potato Dextrose Agar (PDA) medium (Merck Millipore, Burlington, USA) until sporulation. Spores were suspended in 0.1 % triton solution to a final concentration of 10^7 spores mL^{-1} (three replicates per strain). 500 μL of these suspensions were inoculated into 200 mL of Mandels Andreotti medium containing (per liter) 1.4 g $(NH_4)2SO_4$, 2.0 g KH_2PO_4, 1.6 g $MgSO_4{}^*7H_2O$, 0.3 g $CaCl_2{}^*2H_2O$, 0.3 g urea, 1 g peptone, 5 mg $FeSO_4{}^*7H_2O$, 1.6 mg $MnSO_4{}^*H_2O$, 1.4 mg $ZnSO_4{}^*7H_2O$, 2.0 mg $CoCl_2{}^*2H_2O$ and 10.0 g of an additional carbon source. For the initial fungal screening assay, the Mandels Andreotti medium was supplemented with either 10.0 g of carboxymethyl cellulose (CMC - Sigma–Aldrich, St. Louis, USA), 10.0 g of Avicel® (Sigma–Aldrich, St. Louis, USA) or 5.0 g of PTSS1 (dry-basis) plus 5.0 g of soybean meal as carbon sources. For the remaining assays with *T. erinaceum* and *T. reesei*, the medium was supplemented with 10.0 g L^{-1} (dry-basis) of either PTSS1 (enzymatic assays and hydrolysis) or PTSS2 (proteomic analyses). The cultures were kept in a rotary shaker at 30 °C and 200 rpm for 144 h, a period after which the cultures were found to be fully grown. The cultures were then centrifuged for collection of the liquid fraction. The protein concentration of collected supernatants was determined through the Bradford method (Bradford, 1976) using Bovine Serum Albumin (BSA, BioRad, Hercules, USA). These crude enzyme extracts were used directly in enzymatic assays or concentrated for the hydrolysis and proteomics experiments using Vivaspin® 15 columns (Sartorius, Göttingen, Germany) with a molecular weight cut-off (MWCO) of 10 kDa.

2.5. Enzymatic assays

The Filter Paper Activity (FPA) assay was conducted as described by Ghose (1987) with a 10× scale-down of the reaction volume. Each reaction was comprised of 50 μL of the enzyme extract, 100 μL of 50 mM citrate sodium buffer pH 5.0 and 5.0 mg of Whatman® nº 1 filter paper. The reactions were allowed to proceed for 60 min at 50 °C. Endoglucanase activity assays were based on the protocol described by Ghose (1987). Each reaction was comprised of 180 μL of 2.0 % (w/v) of the substrate carboxymethyl cellulose, pH 5.0 (CMC, Sigma–Aldrich, St. Louis, USA) and 20 μL of the enzyme extract. The reaction was incubated at 50 °C for 30 min. The xylanase activity assay protocol was adapted from Ghose and Bisaria (1987). Each reaction was comprised of 180 μL of 2.0 % (w/v) beechwood xylan, pH 5.0 (CMC, Sigma–Aldrich, St. Louis, USA) and 20 μL of the enzyme extract and was incubated at 50 °C for 10 min. The concentration of reducing sugars released in the FPA, endoglucanase and xylanase assays was determined through the addition of 300 μL of 3,5-dinitrosalicylic acid (DNS) solution (Miller, 1959) to each reaction and heating in boiling water for 5 min for color development. Absorbances were measured at 540 nm. Standard curves for sugar concentrations were obtained by substituting the enzyme extract volume in the reactions with glucose or xylose solutions of known concentrations. β-glucosidase, β-xylosidase, α-arabinofuranosidase and α-galactosidase activities were determined using the p-nitrophenol (p-NP)-based substrates *p*-nitrophenyl β-D-glucopyranoside (p-NPG), p-nitrophenyl β-D-xylopyranoside (p-NPX), p-nitrophenyl α-L-arabinofuranoside (p-NPAra) and p-nitrophenyl α-D-galactopyranoside (p-NPGal), respectively. Each reaction was comprised of 20 μL of the enzyme extract and 80 μL of a 1 mM solution of the respective substrate. The substrate solutions were prepared by dissolving the appropriate amount of each substrate in 50 mM sodium citrate buffer, pH 5.0. The reactions were incubated at 50 °C for 10 min and stopped with the addition of 100 μL of a 1 M sodium carbonate solution. Absorbances were measured at 405 nm and standard curves were obtained by substituting the enzyme extract volume in the reactions with p-NP solutions of known concentrations. The measured

concentrations of enzymatic products were used to calculate the units of enzymatic activity (U) per mg of protein present in the enzyme extracts, where one U is defined as the amount of enzyme that released 1 μmol of enzymatic product per minute during the reactions. Statistical analyses of obtained results were performed with the SigmaPlot software (Systat Software, San Jose, CA).

2.6. Hydrolysis of pretreated sugarcane straw

Hydrolysis reactions were set up in 50 mL Falcon tubes and kept at 50 °C and 50 rpm in a rotisserie incubator for 72 h. The pH of the pretreated sugarcane straw substrate was adjusted to 5.0 with a 28 % (v/v) ammonium hydroxide solution. Each individual reaction was set up by adding to a tube: 1 g of pretreated biomass (dry-basis), sodium acetate buffer to a final concentration of 100 mM, enzyme extracts to a final concentration of 2 mg protein/g dry biomass and distilled water to a final total mass of 20 g. The commercial enzyme cocktail Celluclast (Novozymes, Bagsværd, Denmark) was used as a reference enzyme preparation at the same protein concentration as the fungal enzyme extracts. Following the incubation period, reaction tubes were centrifuged, and the supernatants removed and stored at −20 °C until analytical processing. The following equations were used to calculate the hydrolysis yield of glucose and xylose from the biomass glucans and xylans, respectively, as described by Zhu et al. (2011):

$$Glucose\ yield\ \% = \frac{glucose\ in\ hydrolysate\ (g) * 0.9}{total\ glucans\ in\ starting\ biomass} \times 100\%$$

$$Xylose\ yield\ \% = \frac{xylose\ in\ hydrolysate\ (g) * 0.88}{total\ xylans\ in\ starting\ biomass} \times 100\%$$

Statistical analyses of obtained results were performed with the SigmaPlot software (Systat Software, San Jose, CA).

2.7. HPAEC-PAD and HPLC analyses

Cellobiose and xylobiose concentrations were analyzed by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) in a CarboPac PA-10 guard column (4 × 50 mm) attached to a CarboPac PA-10 analytical column (4 × 250 mm) at 30 °C (Thermo Scientific Dionex ICS-5000 + system) using a mixture of 50 % water and 50 % (v/v) 200 mM NaOH as eluent (1.0 mL min^{-1}) for 30 min. Glucose and xylose concentrations were analyzed by High-Performance Liquid Chromatography (HPLC) with a Waters e2795 system connected to refractive index detector (Waters 2414 system), equipped with an Aminex HPX-87H column (300 × 7.8 mm - BioRad) at 35 °C using 0.5 mM H_2SO_4 (Sigma–Aldrich, St. Louis, USA) as a mobile phase and a flow rate of 0.6 mL min^{-1} for 65 min. All samples were properly diluted and filtered through a 0.45 mm syringe filter to remove particles before analysis.

2.8. One-dimensional SDS-polyacrylamide gel electrophoresis

A polyacrylamide gel (15 %) was loaded with 10 μg of each protein sample resuspended in loading buffer and heated for 5 min at 95 °C for protein denaturation. The Page Ruler Prestained Protein Ladder, 10 kDa–180 kDa (Thermo Scientific, Waltham, USA), was used as a protein size standard. The run was conducted in a vertical electrophoresis tank at a fixed voltage of 120 V. The gel was stained with Coomassie Blue, destained and photographed (Sasse and Gallagher, 2009).

2.9. Liquid chromatography tandem-mass spectrometry analysis

Biological triplicates of the enzyme extracts from *T. erinaceum* and *T. reesei* were analyzed by mass spectrometry. Following previously described protocols, 10 μg of proteins from the concentrated extracts were subjected to a process of alkylation, reduction, trypsin digestion in-solution (Ribeiro et al., 2012) and desalting (Rappsilber et al., 2007). The samples (10 μg) were dried in a vacuum concentrator and reconstituted in 0.1 % formic acid. An aliquot containing 40 ng of peptide mixture was analyzed in an EASY-nLC system (Proxeon Biosystem, West Palm Beach, USA) connected to LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, USA) equipped with a Proxeon nanoelectrospray ion source. Peptides were separated by a 2–90 % acetonitrile gradient in 0.1 % formic acid using a PicoFrit® analytical column (20 cm × ID75 μm, 5 μm particle size, New Objective) at a flow rate of 300 nL min^{-1} over 85 min. The nanoelectrospray voltage was set to 2.2 kV and the source temperature was 275 °C. All instrument methods were set up in the data dependent acquisition mode (DDA). The full scan MS spectra (m/z 300–1600) were acquired in the Orbitrap analyzer after accumulation to a target value of 1×10^6. The resolution in the Orbitrap was set to r = 60 000 and the 20 most intense peptide ions with charge states ≥2 were sequentially isolated to a target value of 5000 and fragmented in the linear ion trap using low-energy CID (normalized collision energy of 35 %). The signal threshold for triggering an MS/MS event was set to 1000 counts. Dynamic exclusion was enabled with an exclusion size list of 500, duration of 60 s and a repeat count of 1. The spectra were acquired using the Thermo Xcalibur v.2.1 software (Thermo Fisher Scientific, Waltham, USA) and the files with preliminary data converted to a list of peaks without summing the scans with the Mascot Distiller v.2.3.2.0 software (Matrix Science, London, UK). A search was conducted against all the 70,317 sequences of *Trichoderma* sp. available at the Uniprot database (The UniProt Consortium, 2017). These sequences were downloaded on Jan. 9, 2017 and contained 32,779,763 residues. The search parameters used were as follows: a maximum of one missed trypsin cleavage site, carbamidomethylation as a fixed modification, oxidation of methionine as variable modification, fragment mass tolerance of 1 Da and peptide mass tolerance of 10 ppm. The obtained data was further processed using the Scaffold software (Proteome Software, Portland, USA) with a default FDR value of 0.01 for protein identification. Only proteins matched by unique peptides in at least 2 of the 3 biological replicates were considered. The identified proteins were then analyzed for the presence of a signal peptide in order to control for a possible contamination with non-secreted proteins. This analysis was performed with the web-based SignalP 4.1 software (Petersen et al., 2011). Only proteins that were positively identified for the presence of a signal peptide were included in the presented results. The proteins were annotated for the presence of CAZyme domains with the web-based software dbCAN (database of Carbohydrate-active enzyme Annotation) (Huang et al., 2018). The functional annotation was performed with the web-based PANNZER (Protein ANNotation with Z-scoRE) 2 software (Toronen et al., 2018). Each protein was annotated with the top scoring gene ontology (GO) term observed.

3. Results and discussion

3.1. Isolation, preliminary screening and identification of fungal strains

A total of 8 fungal strains were isolated from decaying sugarcane straw collected at the straw storage facility of a LC ethanol biorefinery. The cellulolytic capabilities of the newly isolated fungi

were first assessed in a preliminary screening where the isolates were cultured in Mandels Andreotti medium supplemented with three different carbon sources (Fig. S1). The supernatants produced by the new strains presented comparable levels of Filter Paper Activity (FPA). An assessment of secreted β-glucosidase activity during growth in pretreated sugarcane straw-containing medium, however, indicated an advantage for isolates F3 and F7 in comparison to the other isolates and to a control *T. reesei* strain (Fig. S2).

Following the initial screening, the new isolates were identified to the genus level through sequencing of the Internal Transcribed Spacers (ITS) 1 and 2. The obtained sequences (GenBank accession numbers MK027216-MK027223) were searched against reference curated databases. The sequence matches indicated that isolates F1, F2 and F4 belonged to the genus *Fusarium*, isolates F5, F6 and F8 to the genus *Aspergillus* and isolates F3 and F7 to the genus *Trichoderma*. Isolates F3 and F7 were further identified as *T. erinaceum* strains with the *Trichoderma*-specific ITS barcode identification program TrichOKEY2 (Druzhinina et al., 2005), which has been widely used for the identification of *Trichoderma* isolates (Blaszczyk et al., 2011; Hoyos-Carvajal et al., 2009; Jiang et al., 2016; Lopes et al., 2012). The taxonomic identities of the fungal isolates were further validated through the construction of a phylogenetic tree (Fig. 1). The obtained ITS sequences were aligned with ITS sequences from various species of the genera *Fusarium*, *Aspergillus*, *Trichoderma* and other related taxa. The aligned sequences were then used to infer a phylogenetic tree by maximum likelihood. An ultrafast bootstrap (UFBoot) branch support analysis was performed with 10^4 bootstrap alignments, where values ≥95 % indicate reliable clades (Minh et al., 2013). The inferred phylogeny supported the previously obtained taxonomic identities. Isolates F1, F2 and F4 grouped in a cluster with all the included *Fusarium* species. This clade had a support value of 96 % in the UFBoot analysis. The same is true for isolates F5, F6 and F8, which clustered with all the included *Aspergillus* isolates. This clade presented a

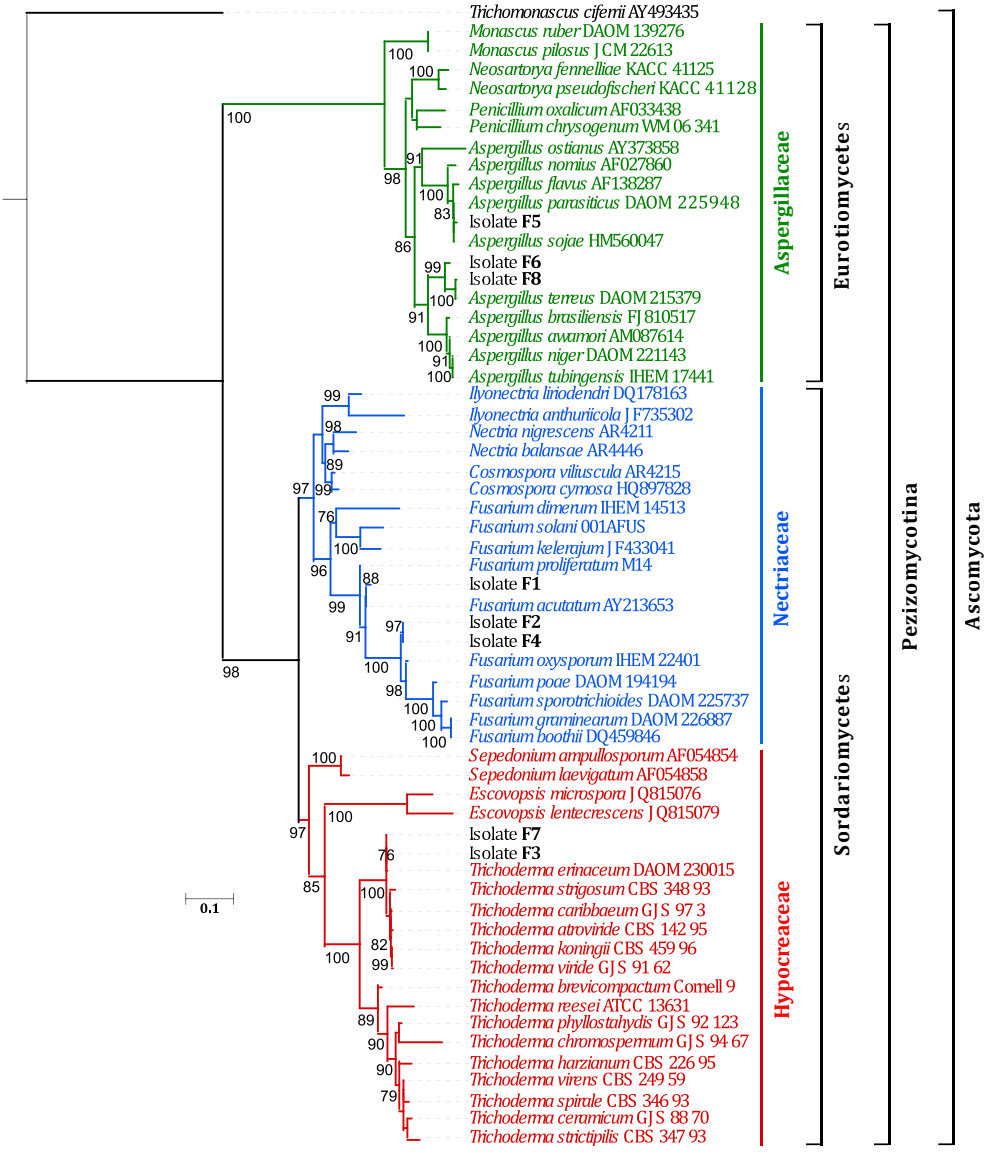

Fig. 1. Phylogenetic tree based on ITS1-5.8S-ITS2 sequences. The tree was inferred through a maximum-likelihood analysis of aligned ITS1-5.8S-ITS2 sequences from 60 fungal isolates. These included 16 species of Aspergillaceae, 16 species of Nectriaceae, 19 species of Hypocreaceae, the 8 isolates described in this study and one *Trichomonascus ciferrii* isolate (Family: Trichomonascaceae; Order: Saccharomycetales; Class: Saccharomycetes; Subdivision: Saccharomycotina), which was included as an outgroup taxon (Kurtzman and Robnett, 2007). The tree shown is a consensus tree constructed from 10^4 bootstrap trees. Log-likelihood of consensus tree: −8562.13. Ultrafast bootstrap (UFBoot) values (%) are given as branch support values (only values ≥75 % are shown). UFBoot values ≥95 % indicate reliable clades. The scale bar indicates 0.1 substitutions per site.

lower UFBoot support value of 86 %. Within this clade, however, Isolate F5 formed a cluster with 4 *Aspergillus* isolates with 100 % UFBoot support and isolates F6 and F8 formed a cluster with the *Aspergillus terreus* DAOM 215379 strain with 99 % UFBoot support. The identification of isolates F3 and F7 as belonging to the *Trichoderma* genus was also supported by the obtained phylogeny. These two isolates formed a clade with all the included *Trichoderma* species with 100 % UFBoot support. Within this clade, the two isolates formed a cluster with the *T. erinaceum* DAOM 230015 strain which presented a UFBoot support value of 76 %.

The *T. erinaceum* species was first described in 2003 (Bissett et al., 2003) and since then has been identified in several *Trichoderma* biodiversity studies (Hoyos-Carvajal et al., 2009; Jiang et al., 2016; Lopes et al., 2012). *T. erinaceum* isolates have been studied for their antagonistic activity against plant pathogenic fungi (Affokpon et al., 2011; Herath et al., 2015) and for the secretion of compounds with potential medicinal applications (Joo and Yun, 2005; Xie et al., 2013). However, to the best of our knowledge, the lignocellulolytic profile of this fungal species has not been previously characterized. Considering this and the fact that high levels of β-glucosidase activity are a key factor for the efficient hydrolysis of lignocellulose (Nakazawa et al., 2012), one of the new *T. erinaceum* strains, the isolate F3, was selected for further characterization. Before proceeding, the species identity of this isolate, hereinafter referred to as *T. erinaceum* F3, was further confirmed through sequencing of a fragment of the *tef* (eEF1a1) gene. The obtained sequence (GenBank accession number MK124760) was searched against a reference database with the *Tricho*BLAST web-based program (Kopchinskiy et al., 2005). The software identified two introns used as phylogenetic markers (*tef1_int4* and *tef1_int5*), both of which matched *T. erinaceum* sequences.

3.2. Lignocellulolytic enzyme activities of T. erinaceum F3

To better evaluate the lignocellulolytic profile of the *T. erinaceum* F3 strain, the fungus was grown in Mandels Andreotti medium containing 10 g L^{-1} of steam-explosion pretreated sugarcane straw (PTSS1) as a carbon source. A wild *T. reesei* strain was used as a control in this and all subsequent experiments. Following a period of 144 h, culture supernatants from both strains were collected and used as crude enzyme extracts. No significant difference in protein content was observed between *T. erinaceum* F3's (0.060 ± 0.006 mg mL^{-1}) and *T. reesei*'s (0.071 ± 0.007 mg mL^{-1}) extracts (two-tailed t-test; $n = 3$; $p = 0.128$). Enzyme activity assays were divided in three groups according to the lignocellulose fraction targeted: cellulases, main chain hemicellulases and side chain hemicellulases (Fig. 2). Among the cellulase activity assays, a significant difference ($p < 0.001$) was observed for β-glucosidase, with the enzyme extract produced by *T. erinaceum* F3 presenting a specific activity (62 ± 8 IU mg^{-1}) about 3.9× higher than that observed for the *T. reesei* strain (16 ± 2 IU mg^{-1}). The F3 strain also presented significantly higher activities than the *T. reesei* strain in the main chain hemicellulase assays of endoxylanase ($p < 0.01$) and β-xylosidase ($p < 0.0001$). In the endoxylanase assay, the specific activity presented by *T. erinaceum* F3 (18,000 ± 2,000 IU mg^{-1}) was approximately 2.9× higher than the observed for *T. reesei* (6,200 ± 600 IU mg^{-1}). An even greater difference was observed in the β-xylosidase assay where the F3 strain presented a specific activity (35 ± 2 IU mg^{-1}) 5× higher than that of *T. reesei* (7 ± 2 IU mg^{-1}). A significant advantage was also displayed by *T. erinaceum* F3 in the side chain hemicellulase activity assay of α-galactosidase ($p < 0.05$). The F3 strain presented a specific activity of 20 ± 3 IU mg^{-1} whereas *T. reesei* measured activity had a value of 14 ± 1 IU mg^{-1}. No significant differences between the two strains were observed in the remaining enzyme activity assays.

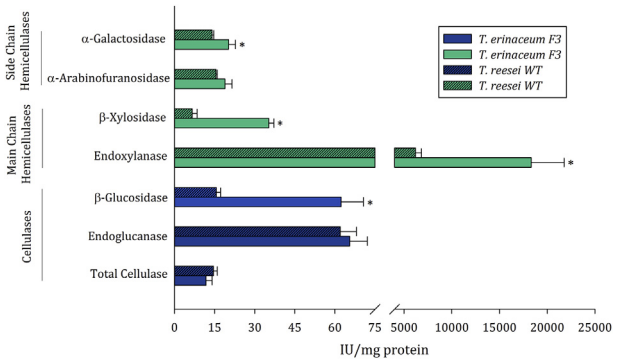

Fig. 2. Comparison of hemicellulolytic (green) and cellulolytic (blue) activities from *T. erinaceum* F3 and *T. reesei*. The two strains were cultivated in biological triplicates in medium containing pretreated sugarcane straw (PTSS1) for a period of 144 h. The cultures were then centrifuged and the supernatants collected for enzymatic assays. Error bars denote standard deviations from the mean values ($n = 3$). Two-tailed *t* tests were performed to assess the significance of differences between specific activities measured for the two strains (*, significant difference [p-value, < 0.05]; WT, wild type). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The higher β-glucosidase activity detected in the enzyme cocktail secreted by the *T. erinaceum* F3 strain is of particular interest given the well described low β-glucosidase activity of *T. reesei*'s secretome (Szczodrak, 1989). Together with the advantage observed in 3 of the 4 hemicellulase activity assays performed, this result suggested that the enzymes produced by the new strain may perform well in the hydrolysis of LC substrates.

3.3. Hydrolysis of steam-explosion pretreated sugarcane straw

To investigate the performance of the enzymes secreted by the *T. erinaceum* strain in the breakdown of LC biomass, a hydrolysis assay was performed using steam-explosion pretreated sugarcane straw as a substrate. The PTSS1 substrate had its composition determined (Table 1) and was used at a concentration of 5 % total solids. The crude enzyme extracts from *T. erinaceum* F3 and *T. reesei* used in the enzyme activity assays were concentrated and added to hydrolysis reactions at a final concentration of 2 mg protein/g dry biomass. The commercial enzyme cocktail Celluclast®, which is based on enzymes from *T. reesei* (Henrissat et al., 1985), was used as a reference control at the same concentration as the fungal extracts. As described in Fig. 3A, the *T. erinaceum* F3 extract released 45 % and 13 % more glucose than the *T. reesei* extract and the Celluclast cocktail, respectively. Nevertheless, it released significantly less xylose than both enzymatic solutions and overall less sugars than the Celluclast cocktail. Due to the low xylose yield, the calculated total conversion of insoluble sugars was slightly lower for the F3 strain (29 %) than for *T. reesei* (31 %) and Celluclast (32 %).

Low glucose and xylose yields during the hydrolysis of lignocellulosic substrates have been previously associated with the accumulation of cellobiose and xylobiose, respectively (Sipos et al., 2010). The quantification of these disaccharides showed that, in fact, the hydrolysis with *T. reesei* enzymes, which resulted in lower glucose yields, generated a significantly higher concentration of cellobiose (Fig. 3B). In a similar fashion, the hydrolysis with *T. erinaceum* F3 enzymes, which had a lower xylose yield, led to the accumulation of xylobiose.

The higher glucose yield observed for *T. erinaceum* F3 in the hydrolysis assay is likely linked to the nearly four-fold higher levels of β-glucosidase specific activity detected for the new strain in comparison to *T. reesei*. As aforementioned, the accumulation of cellobiose during the hydrolysis of LC biomass with *T. reesei*'s

Table 1
Composition of steam-explosion pretreated sugarcane straw (PTSS1).

Components	Glucan	Xylan	Insoluble lignin	Soluble lignin	Ashes	Others
% Dry weight (w/w)	43.11	9.72	23.72	2.23	5.76	15.46

enzymes is well documented and can be avoided by increasing the β-glucosidase activity of the cocktail (Antonov et al., 2016; Dashtban and Qin, 2012; Szczodrak, 1989). It is worth noting that this gain in activity could potentially be attained by supplementation of *T. reeseiś* enzymatic extract with the enzymes produced by the *T. erinaceum* F3 strain. Despite the higher hemicellulase activity levels observed for *T. erinaceum* F3, hydrolysis of pretreated sugarcane straw with this extract yielded less xylose than with that from *T. reesei* or with Celluclast. It also led to the accumulation of xylobiose, a particularly unexpected result considering the higher β-xylosidase specific activity detected for the new strain's secretome. β-xylosidase enzymes, which catalyze the cleavage of the disaccharide xylobiose into two xylose molecules, are known to be inhibited to different degrees by xylose accumulation (Fan et al., 2010; Yan et al., 2008). Therefore, one possible explanation for the observed results would be that the enzymes with β-xylosidase activity secreted by *T. erinaceum* F3 are more inhibited by xylose than those secreted by the *T. reesei* strain. Another possibility is that these enzymes have different activities towards xylobiose than towards the artificial substrate used in the β-xylosidase activity assays, β-D-xylopyranoside (p-NPX), as previously observed for

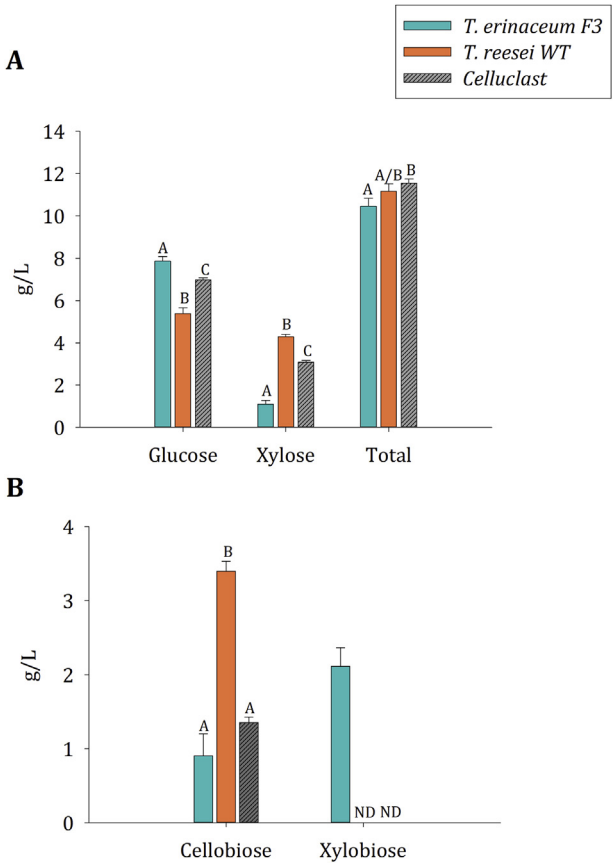

Fig. 3. Hydrolysis of pretreated sugarcane straw. Supernatants produced by the *T. erinaceum* F3 and *T. reesei* strains during growth in medium containing pretreated sugarcane straw (PTSS1) were collected, concentrated and used for the hydrolysis of the same pretreated biomass. Values indicate the final concentrations of (A) the monosaccharides glucose, xylose and (B) the disaccharides cellobiose and xylobiose in the hydrolysis reactions following the incubation period (ND, non-detectable; WT, wild type). Error bars denote standard deviations from the mean values (n = 3). Different capital letters above the bars indicate significantly different sugar concentrations for the respective groups (by the Tukey HSD test with 95 % simultaneous confidence intervals).

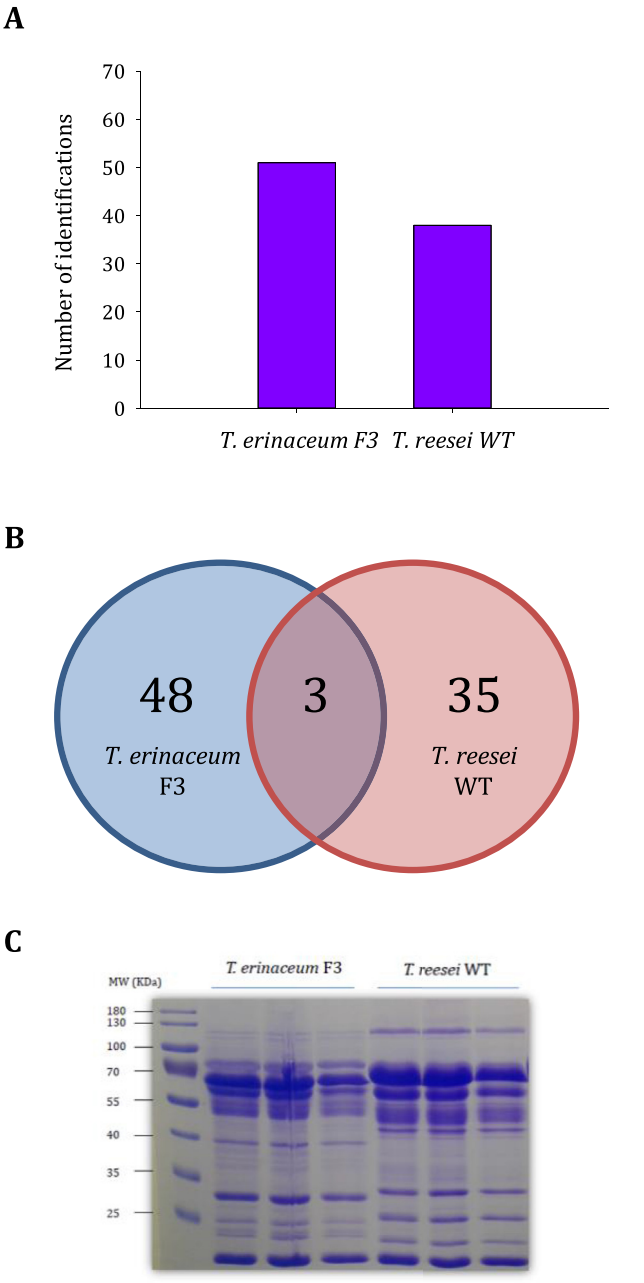

Fig. 4. Protein identification in the secretomes of *T. erinaceum* F3 and *T. reesei*. The two fungi were cultured in medium containing pretreated sugarcane straw (PTSS2) for 144 h. Following this period, culture supernatants were collected, concentrated and processed for LC-MS/MS analysis. Values indicate (A) the total number of proteins identified for each strain and (B) the number of exclusive and shared proteins between the two strains. (C) The concentrated protein samples were also analyzed through one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE). The polyacrylamide gel (15 %) was loaded with 10 μg of each protein sample (WT, wild type).

some enzymes (Campos et al., 2014; Kitpreechavanich et al., 1986). Still, further investigation is required to test these hypotheses.

3.4. *Proteomic analysis of* T. erinaceum *F3 and* T. reesei *secretomes*

To gain insight into the proteomic basis of the lignocellulolytic differences observed between *T. erinaceum* F3 and the *T. reesei* strain, individual proteins secreted by the two fungi during cultivation in medium containing steam-explosion pretreated sugarcane straw (PTSS2) were identified through a LC-MS/MS analysis. A total of 86 distinct proteins were collectively identified for the two strains (Tables S1 and S2), with 48 proteins being exclusive to *T. erinaceum* F3, 35 exclusive to *T. reesei* and only 3 common to both strains (Fig. 4A,B). The largely divergent protein composition of the two strains' secretomes was also apparent in the one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE) analysis performed, where very distinct band patterns were observed (Fig. 4C).

The identified proteins were annotated according to their biological process function, as defined by the Gene Ontology (GO) Consortium (Ashburner et al., 2000). The obtained results are summarized in Table 2. Although most of the proteins identified for the two strains are exclusive to each of them, remarkable similarities were observed in their functional distribution. A large fraction of the enzymes secreted by both strains were found to be related to lignocellulose hydrolysis, as might be expected for saprotrophic fungi (Eichlerová et al., 2015). The GO categories 'carbohydrate metabolism', 'cellulose catabolism' and 'polysaccharide catabolism', together with the hemicellulose depolymerization-related categories 'xylan catabolism' and 'L-arabinose metabolism' were common to both strains and accounted for about half of all the proteins identified for each fungus. Other categories likely related to lignocellulose depolymerization where observed for only one of the strains and had a single protein representative. These included the categories 'glucan metabolism' and 'arabinan metabolism' for *T. erinaceum* F3 and 'carbohydrate catabolism' for *T. reesei*. Another similarity observed in the analysis was that about 10 % of the proteins identified for each of the strains were linked to cell wall biosynthesis and organization. It is likely that these enzymes act to remodel the fungus own cell wall or to enable mycoparasitic activity towards other species (Gruber and Seidl-Seiboth, 2012). The same is true for a single protein from *T. erinaceum* F3 which was annotated in the 'chitin metabolism' category. Another group of proteins possibly related to the antagonistic abilities of the studied strains against other fungi are those annotated in the 'proteolysis' category, which had 3 representatives from *T. erinaceum* F3 and 1 from *T. reesei*. The secretion of proteases by *Trichoderma* strains has often been associated with their ability to attack other fungi (Gruber and Seidl-Seiboth, 2012; Szekeres et al., 2004).

In order to further comprehend the differences between the lignocellulolytic profiles of the two strains, their secretomes were analyzed with regards to their Carbohydrate-Active Enzymes (CAZymes) content, as defined by Henrissat and colleagues (Lombard et al., 2014). A similar total CAZyme content was observed for the two strains, with 72.5 % for the F3 strain and 71.1 % for *T. reesei* (Fig. 5). Similar values were also seen for the secretomes' content of proteins annotated as belonging to specific CAZyme classes. As shown in Fig. 5, the secretome of *T. erinaceum* F3 presented 54.9 % of proteins annotated as Glycoside Hydrolases (GHs) while *T. reesei*'s had 57.9 %. The content of proteins belonging to the Auxiliary Activity (AA) class was found to be 11.8 % for *T. erinaceum* F3 and 13.2 % for *T. reesei*. A significant difference between the two strains was observed with regards to the content of Carbohydrate Esterases (CEs). The *T. erinaceum* F3 isolate had 5.9 %

Table 2
Functional annotation of identified proteins.

GO ID	GO Biological Process Category	*T. erinaceum* F3	*T. reesei*
5975	carbohydrate metabolism	12	11
30245	cellulose catabolism	5	3
272	polysaccharide catabolism	3	2
6508	proteolysis	3	1
46373	L-arabinose metabolism	2	1
45493	xylan catabolism	2	2
71555	cell wall organization	2	2
3824	catalytic activity	2	–
44042	glucan metabolism	1	–
31221	arabinan metabolism	1	–
34411	cell wall (1->3)-beta-D-glucan biosynthetic process	1	2
71970	fungal-type cell wall (1->3)-beta-D-glucan biosynthetic process	1	–
70880	fungal-type cell wall beta-glucan biosynthetic process	1	–
6030	chitin metabolism	1	–
128	flocculation	1	–
42744	hydrogen peroxide catabolism	1	1
52051	interaction with host via protein secreted by type II secretion system	1	–
33609	oxalate metabolism	1	–
55114	oxidation-reduction process	1	–
6979	response to oxidative stress	1	1
6517	protein deglycosylation	1	–
55085	transmembrane transport	1	1
9058	biosynthetic process	1	1
90502	RNA phosphodiester bond hydrolysis, endonucleolytic	–	1
6487	protein N-linked glycosylation	–	1
51723	protein methylesterase activity	–	1
6482	protein demethylation	–	1
9166	nucleotide catabolic process	–	1
16052	carbohydrate catabolism	–	1
–	non-determined	5	4

The proteins identified in the secretomes of the *T. erinaceum* F3 and *T. reesei* strains were annotated according to their biological process functions. The numbers shown indicate the total protein count in each Gene Ontology (GO) category for the respective strains.

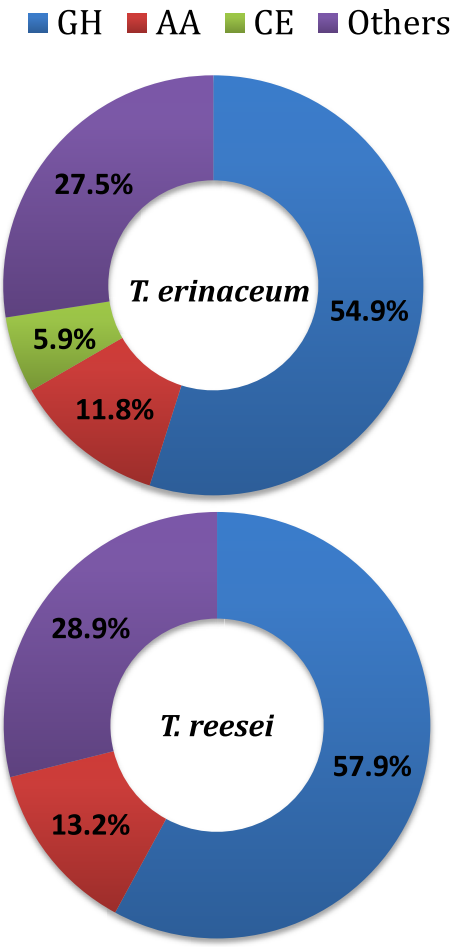

Fig. 5. Distribution of proteins identified as CAZymes in the secretomes of *T. erinaceum* F3 and *T. reesei*. The proteins identified in the studied secretomes were annotated for the presence of CAZyme domains. Values indicate the percentage of proteins identified for each fungus that belong to specific CAZyme classes (GH, glycoside hydrolase; AA, auxiliary activity; CE, carbohydrate esterase).

of its proteins annotated as belonging to this class, whereas no representative was detected for the *T. reesei* strain. No enzyme annotated as Glycosyl Transferase (GT) or as Polysaccharide Lyase (PL) was detected for either strain.

The glycoside hydrolases identified in the two secretomes were then sorted according to the GH family to which they belong (Fig. 6). Among the 21 GH families detected, the following 5 families were exclusive to the *T. erinaceum* F3 strain: GH3 (β-glucosidase), GH13 (α-amylase), GH54 (α-L-arabinofuranosidase), GH92 (α-mannosidase) and GH127 (β-L-arabinofuranosidase). Conversely, GH families 28 (polygalacturonase), 45 (endoglucanase) and 76 (α-1,6-mannanase) were exclusively detected for *T. reesei*. A higher number of proteins were detected for *T. erinaceum* F3 in 4 of the 21 detected families, these being GH5 (endo-β-1,4-glucanase), GH10 (endoxylanase), GH55 (endo-β-1,3-glucanase) and GH62 (α-L-arabinofuranosidase). The *T. reesei* strain, on the other hand, had a higher protein count in the GH18 family (chitinase). Moreover, proteins annotated as belonging to 4 AA families were detected in the studied secretomes. These included AA families 2 (lignin peroxidase), 5 (galactose oxidase), 7 (chitooligosaccharide oxidase) and 9 (Lytic Polysaccharide Monooxygenases – LPMO). An equal number of representatives of family AA2 (2 proteins) were identified for each of the two fungi. A single AA7 representative was detected for *T. erinaceum* F3 while none was detected for *T. reesei*. The opposite was true for the AA5 family, with a single representative being annotated for *T. reesei* and none for the F3 strain. A count advantage was also observed for the *T. erinaceum* F3 strain in the AA9 family, having 3 proteins identified versus 2 for *T. reesei*. The proteins annotated as carbohydrate esterases, which were detected only in the secretome of the *T. erinaceum* strain, were distributed in 2 CE families. The CE family 1 (feruloyl esterase) had one representative protein and the CE family 5 (acetyl xylan esterase) had 2 proteins detected. Lastly, one protein annotated as swollenin was identified in *T. reesei*'s secretome (Saloheimo et al., 2002). Although not included in the CAZyme classification, these proteins have been shown to have cellulose hydrolase activity (Andberg et al., 2015).

Mass spectrometry-based methods have been an invaluable tool for deciphering the protein composition of fungal secretomes and,

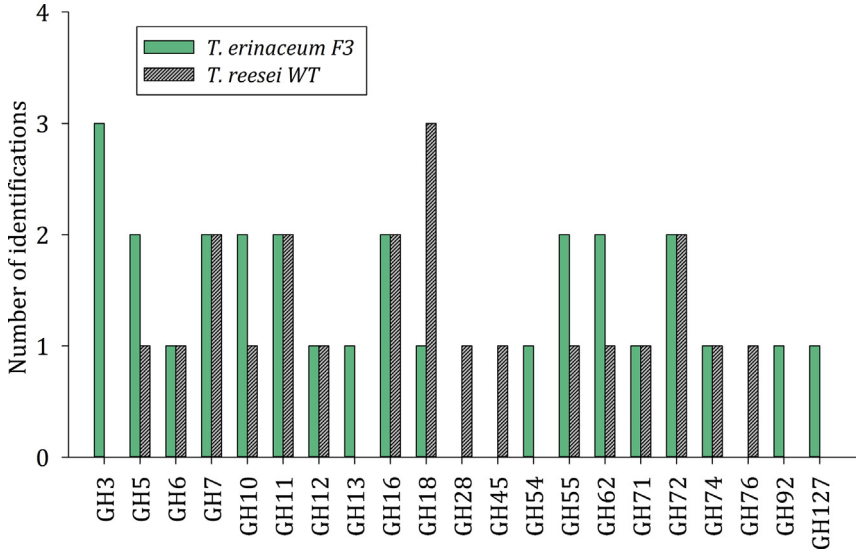

Fig. 6. Grouping and distribution of identified glycoside hydrolases (GHs) within specific GH families. Values indicate the number of proteins identified as belonging to specific GH families for each strain.

consequently, to better understand the enzyme requirements for the hydrolysis of LC biomasses. The performed LC-MS/MS-based proteomic analysis provided significant insight into the compositional differences between the secretomes of *T. erinaceum* F3 and *T. reesei*. The analysis showed that, when grown in medium containing pretreated sugarcane straw, the *T. erinaceum* F3 strain produced a more complex enzyme cocktail with a higher number of carbohydrate-active enzymes than the *T. reesei* strain. The advantage presented by *T. erinaceum* F3 with regards to the quantity and diversity of glycoside hydrolases correlated, in some cases, with observed differences in enzymatic activity. For instance, 3 predicted β-glucosidases belonging to the GH3 family were detected for the *T. erinaceum* F3 isolate whereas none was detected for *T. reesei* and, as described, the newly isolated strain yielded higher β-glucosidase activity than *T. reesei.* The same is true for endoxylanases, with *T. erinaceum* F3 presenting higher endoxylanase activity levels and a higher number of predicted enzymes belonging to the GH10 family than *T. reesei* (2 versus 1, respectively). Another noteworthy difference between the two analyzed secretomes was the higher number of predicted LPMO enzymes belonging to the AA9 family detected for *T. erinaceum* F3. These oxidative enzymes, which were originally classified as glycoside hydrolases (GH61) and later placed in the AA9 family of the CAZy database (Levasseur et al., 2013), have been intensively studied since they were first characterized and are of particular interest to the LC biofuels industry due to their potential in boosting the overall enzymatic hydrolysis of LC biomass (Harris et al., 2010; Johansen, 2016).

4. Conclusions

In conclusion, the *T. erinaceum* F3 strain, when grown in medium containing pretreated sugarcane straw, secreted an enzyme cocktail with higher lignocellulolytic activities than *T. reesei* and which yielded more glucose during the hydrolysis of pretreated sugarcane straw. The proteomic analysis of the new strain's secretome revealed a more complex enzyme system with a higher number of carbohydrate-active enzymes than that of *T. reesei.* These results reveal the potential of the *T. erinaceum* species for the LC biofuels industry, both as a possible source of genes for the genetic engineering of industrial lignocellulase production strains and as a candidate platform for enzyme production. The described data also support further studies, which may include genome and transcriptome sequencing, the characterization of individual enzymes and strain improvement for increased enzyme production.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.01.007.

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