Transcriptome Profile of *Trichoderma harzianum* IOC-3844 Induced by Sugarcane Bagasse

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Abstract

Profiling the transcriptome that underlies biomass degradation by the fungus *Trichoderma harzianum* allows the identification of gene sequences with potential application in enzymatic hydrolysis processing. In the present study, the transcriptome of *T. harzianum* IOC-3844 was analyzed using RNA-seq technology. The sequencing generated 14.7 Gbp for downstream analyses. De novo assembly resulted in 32,396 contigs, which were submitted for identification and classified according to their identities. This analysis allowed us to define a principal set of *T. harzianum* genes that are involved in the degradation of cellulose and hemicellulose and the accessory genes that are involved in the depolymerization of biomass. An additional analysis of expression levels identified a set of carbohydrate-active enzymes that are upregulated under different conditions. The present study provides valuable information for future studies on biomass degradation and contributes to a better understanding of the role of the genes that are involved in this process.

Introduction

The fungus *Trichoderma harzianum* is a well-known biocontrol agent [1],[2]. Most previously published genetic studies concerning this organism have explored its molecular mechanisms of biocontrol. This biocontrol ability enables the fungus to identify and degrade cell walls, and the mechanisms that underlie these processes were explored in the present study.

Several studies have suggested that *T. harzianum* may be utilized for the production of hydrolytic enzymes from a cellulolytic complex [3],[4],[5],[6], due to its ability to produce high levels of both β-glucosidase and endoglucanases [7]. These studies have demonstrated that this fungus is a potential source of hydrolytic enzymes and may aid in understanding the transcriptional regulation of biomass degradation by filamentous fungi. The utilization of sugarcane bagasse as a biomass for the production of second-generation ethanol requires its degradation into monomeric carbohydrates and small oligosaccharides that may be metabolized by ethanol-producing yeast. The major bottleneck for this process is the enzymatic hydrolysis of sugarcane bagasse [8]. The hydrolytic effectiveness of an enzymatic mixture is highly dependent on the feedstock and any pretreatment it has received [9]. A strategic issue to be considered during the development of enzymatic mixtures optimized for second-generation ethanol production is the cultivation of microorganisms utilizing the lignocellulosic material that will be hydrolyzed. This cultivation method may select for enzymes that are optimal for the hydrolysis of a specific feedstock [9],[10]. One of the primary mechanisms of the adaptive processes of cells in a complex medium is the alteration of transcription levels, which can lead to the production of specialized proteins, differences in membrane composition and other changes in cellular machinery [11].

A large variety of enzymes with different specificities are required to degrade the components of lignocellulose [10],[12],[13],[14]. However, many other proteins may also contribute to lignocellulose degradation in ways that are not yet clearly understood, such as the glycoside hydrolase family 61 proteins, the expansins and the swollenins [10],[14],[15]. Three types of enzymes are required to hydrolyze cellulose into glucose monomers: exo-1,4-β-glucanases, such as EC 3.2.1.91 and EC 3.2.1.176 (cellbiohydrolase); endo-1,4-β-glucanases, such as EC 3.2.1.4; and β-glucosidases, such as EC 3.2.1.21 (cellobiases) [10],[16]. Cellbiohydrolases attack the reducing or nonreducing ends of the cellulose chains, whereas endo-glucanases cleave these chains in the middle and reduce the degree of polymerization [10],[17]. The composition of hemicellulose is more variable than that of cellulose; therefore, more enzymes are required for its effective hydrolysis. The enzymes that degrade hemicellulose can be divided into depolymerizing enzymes, which cleave the backbone of the molecule, and enzymes that remove the

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substrate of the molecule, which may sterically hinder the depolymerization enzymes. The core enzymes for the degradation of xylan to monomers are the endo-xylanases, which cleave the xylan backbone into shorter oligosaccharides, and β-xylosidase, which cleaves short xylo-oligosaccharides into xylose. Similarly, the core enzymes for the degradation of mannan are endo-mannanase and β-mannosidase. However, xylans and mannans generally contain a number of different substituents linked to their main backbones, including arabinose, acetyl groups, galactose and glucose. A host of ancillary enzymes are required to remove these substituents and allow the core enzymes to degrade the xylan and mannan backbones. These ancillary enzymes include the α-L-arabinofuranosidases, α-glucuronidase, fucolactic acid esterase, α-galactosidase, feruloyl esterase, acetyl xylanesterase and acetyl mannan esterase. The fucolactic acid esterases specifically cleave the linkages between hemicellulose and lignin. The α-L-arabinofuranosidases also possess different specificities; some cleave 1,2 linkages or 1,3 linkages, whereas others cleave doubly substituted arabinose residues from arabinoxyylan [10],[18].

Fungi from the genera Trichoderma, Penicillium, Aspergillus and Humicola grisea var. thermoida degrade lignocellulose components, including sugarcane bagasse [8]. These fungi can degrade cellulose, hemicellulose and lignin in decaying plants using a complex set of excreted hydrolytic and oxidative enzymes, including glycosyl hydrolases from different families [10]. Although many studies have been conducted to characterize the action of the enzymes involved in lignocellulose degradation, little is known regarding the transcription and genomic regulation of the genes that encode these enzymes. Trichoderma reesei is the major industrial source of the cellulases and hemicellulases that are utilized in the depolymerization of biomass to simple sugars, which are then further converted into chemical intermediates and biofuels. Unexpectedly, despite the industrial utility and effectiveness of the carbohydrate-active enzymes of T. reesei, the genome of this species encodes fewer cellulases and hemicellulases than that of any other sequenced fungus that can hydrolyze plant cell wall polysaccharides [19],[20]. Thus, a better understanding of the genetic mechanisms of this fungus is necessary to explore its extraordinary biotechnological potential. The present study analyzes the transcriptome of T. harzianum IOC-3844 grown in a sugarcane bagasse-based culture medium and the induction of hydrolytic activity in this medium, with particular emphasis on the potential contributions of the fungus to fuel biotechnology and other industrial applications. This organism is available in public databases, containing the “DSB” library was generated from a preculture medium that contained DSB, the “CEL” library was generated from a preculture medium that contained crystalline cellulose, and the “LAC” library was generated from a preculture medium that contained lactose. This latter condition was designated as the control.

Preculture and fermentation
Conidial suspensions were prepared through the addition of sterilized distilled water and Tween 80 to the PDA plates, which resulted in conidial suspensions of 9 × 10⁵ spores mL⁻¹. After preparation, 4.0 mL of each conidial suspension was transferred to Erlenmeyer flasks containing 600 mL of each preculture medium, and the flasks were incubated at 72 h at 29°C on a rotary shaker at 200 rpm. A volume of 30 mL of each medium was transferred to individual Erlenmeyer flasks containing 270 mL of the production medium. The flasks were incubated at 29°C for 129 h on a rotary shaker at 200 rpm. Samples of the mycelia and the fermentation extracts were removed to determine the enzymatic activity and to conduct the transcriptome analyses.

Analytical measurements
The filter paper activity (FPase) was determined as described by Ghose (1987) [23], with modifications to diminish the scale of the procedure by a factor of 10. All statistical comparisons were done using Student’s t test (P<0.05).

RNA extraction and transcriptome sequencing
The mycelial samples from the LAC, CEL and DSB conditions were extracted after 96 h of fermentation, stored at −70°C and analyzed using the Stratagene GX-96 reaction thermocycler (Agilent Technologies, Santa Clara, CA, USA). The RNA was sequenced using an Illumina HiSeq platform at BGI (Beijing Genomics Institute, Shenzhen, China).
used for RNA extraction. The fungal mRNA was isolated according to Jones et al. (1985) [24] with some modifications. Two grams of each mycelial sample was frozen using liquid nitrogen in a mortar and ground with a pestle into a fine powder. Next, NTES buffer (4.5 mL, 0.1 M NaCl, 0.01 M MTris-HCl at pH 7.5, 1 mM EDTA and 1% SDS) and phenol/chloroform/isoamyl alcohol [3 mL of a 1:1:1 mixture] were added, and the sample was ground until the mixture had thawed. After vortexing for 10 min, the solution was centrifuged at 8,000 rpm for 10 min at 4°C. To the aqueous phase, 3 mL of phenol/chloroform/isoamyl alcohol [1:1:1 mixture] was added, and the solution was centrifuged at 8,000 rpm for 10 min. The aqueous phase was then removed, and the nucleic acid was precipitated through the addition of a 0.1 volume aliquot of 2 M NaAc, pH 4.5, and two volumes of 100% ethanol. The precipitate was centrifuged at 8,000 rpm for 10 min, and the pellet was resuspended in 2.5 mL of sterile water. To remove the DNA, 2.5 mL of 4 M LiAc was added, and the solution was incubated for 48 h at −20°C. The precipitate was collected by centrifugation at 8,000 rpm for 10 min, then washed with 70% ethanol, resuspended in 2.5 mL of sterile water and stored at 4°C.

The RNA samples were quantitated using a fluorescence-based method, and their quality was determined using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The libraries were constructed using 4 μg of each RNA sample and the TruSeq RNA sample preparation kit (Illumina Inc., San Diego, CA) according to the manufacturer’s instructions. The expected target sizes were confirmed using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), and the libraries were quantified using qPCR. The average insertion size was 260 bp. The clustering was conducted using 10 μM of each library and a TruSeq PE Cluster Kit on cBot (Illumina Inc., San Diego, CA). The sequencing was performed on the Illumina Genome Analyzer IIx, which is a next-generation high-throughput sequencer (Illumina Inc., San Diego, CA), according to the manufacturer’s specifications for paired-end reads of 72 bp in individual lanes.

Download and processing the sequence data

After the sequencing was completed, the data were transferred to a local high-performance computing server at CBMEG (University of Campinas, Campinas, Brazil). The results were submitted to NCBI under accession number SRX189214, and the raw sequences (archives of paired and paired-end sequences) were submitted to the NCBI Sequence Read Archive (SRA) under accession numbers SRR579379, SRR631745 and SRR631746 for the DSB, CEL and LAC libraries, respectively. Initially, all sequences were trimmed. We utilized the CLC Genomics Workbench (v4.0; Finlandsgade, Dk) to perform the reads trimming, and parameters were set to: quality limit: 0.03; ambiguous limit: 2; minimum number of nucleotides in reads: 65; phred scale: 15. De novo assembly was conducted using CLC Genomics Workbench (v4.0; Finlandsgade, Dk) with the following parameters: similarity = 0.98 and length fraction = 0.9. The resulting contigs were compared with the NCBI non-redundant protein database (NR) using BLAST to identify homologous sequences [25], with an E-value cutoff of ≤1 e−5. The sequences were functionally annotated according to Gene Ontology terms [26], and the annotations were compared with the Kyoto Encyclopedia of Genes and Genomes [28] to establish biochemical pathway associations using Blast2Go, which is a universal web-based annotation application [27]. The sequences were aligned against the Carbohydrate-Active Enzymes (CAZymes) database to identify glycosyl hydrolases [29],[30],[31],[32],[33], glycosyltransferases [34],[35], carbohydrate-binding modules [36] and carbohydrate esterases [37]. The T. harzianum IOC-3844 genome was provided by Dr. Reginaldo M. Kuroshu (University of São Paulo, São Carlos, Brazil). Only the best alignments showing expectation values lower than 1×10−3 were considered for functional gene annotation. To compare the transcript sequences with the genome and CAZyme datasets, CLC Genomics Workbench was used. The archive of the assembly scaffolds for T. harzianum CBS 225.95, which is available on JGI (sequence data produced by the US Department of Energy Joint Genome Institute in collaboration with the user community) [30], was used to calculate the similarity between the data.

Expression pattern

A paired Kal’s t-test was conducted on the log2-transformed data to determine whether significant differences existed between the expression ratios found in each treatment and the control. Contigs were identified as being differentially expressed in upregulated groups when significance was detected with a false discovery rate lower than 1×10−3. Hierarchical clustering analysis and K-means clustering were performed on the CAZymes that were identified as being differentially expressed. Clustering was performed using Euclidean distance as the distance metric in three partitions according to the cluster features, on the transformed expression values.

Real-Time PCR analysis

To validate the expression profiles of the assembled genes obtained through sequencing data analysis, quantitative real-time (RT-qPCR) was performed for selected genes. Genes associated with biomass degradation processes were selected and are shown in Table S1, together with the primers and annealing temperatures.

Quantification of gene expression was performed by continuously monitoring SYBR Green fluorescence. The reactions were performed in triplicate in a total volume of 6.25 μL. Each reaction included 3.12 μL of SYBR Green Master Mix (Invitrogen, Carlsbad, CA), 1.0 μL of direct and reverse primers, 0.5 μL of cDNA and 1.6 μL of water. The reactions were assembled in 384-well plates. PCR amplification-based expression profiling of the selected genes was performed using a gene for squalene-epoxidase as endogenous control. Four genes were tested as endogenous control: genes for actin, beta-tubulin, glyceraldehyde 3-phosphate dehydrogenase, and squalene-epoxidase. The last one had the best performance in RT-qPCR analysis, remaining constant in all treatments. The enzyme squalene-epoxidase catalyses the conversion of squalene to 2,3-(S) oxidosqualene, which is an intermediate in the synthesis of the fungal cell membrane component ergosterol. RT-qPCR was conducted in an ABI PRISM 7500 HT (Applied Biosystems, Foster City, CA). Gene expression was calculated via the ΔCT method [47]. All statistical comparisons were done using Student’s t test (P<0.05). The obtained RT-qPCR results were in agreement with the RNA expression analyses of the generated assemblies. The same expression profile was observed for the genes encoding GH16, GH10, CE5, and GH5. Figure 1 shows the expression of the selected genes.
Results

Enzymatic Activity Profile

The FPase was evaluated to determine the enzymatic activity profile of the cellulases during 129 h of fermentation (Figure 2) using DSB as a carbon source. RNA was isolated from the mycelia at 96 h of cultivation; this time point was associated with a significant production of FPase (0.53 FPU mL$^{-1}$) (Figure 2). This 96 h cultivation period included a 48 h adaptation phase. Previous studies have indicated that the enzymatic activity of this fungus, as measured by cellulase production, is lower when grown on soluble carbon sources than when grown on DSB, and these results are in agreement with the present study [4]. The substrate acts as both an adhesion surface and as fermentable biomass for the fungi, and it activates the synthesis of hydrolytic complexes.

The increased FPase activity indicated the enhanced ability of the fungus to metabolize and degrade compounds in the biomass. The maximum activity was observed between 72 and 96 h, after which the activity was repressed due to the formation of degradation products.

To identify the origin of the enzymatic activity in the extracts, we evaluated the transcriptome expression at 96 h of fermentation.

Sequencing assembly

In total, 246 million raw sequencing reads were generated with a target length of 72 bp (Table 1). After quality trimming, 84.11% of the data were retained for a total of 14.7 Gbp of sequencing data. De novo assembly using trimmed reads from all libraries resulted in 32,494 contigs, with an N50 of 1,251 bp. The assembled transcripts redundancy was determined through CD-HIT-EST. After this analysis, the final number of contigs was 32,396.

Analysis of the transcriptome under the influence of sugarcane bagasse as a substrate

Transcript profiling is an important strategy for studying the expression of large gene sets under particular conditions. To determine the influence of the complex sugarcane bagasse substrate on gene expression, the contigs generated from the de...
assembly of the transcriptome were analyzed. The generated assembly was compared with the archive of the assembly scaffolds for *T. harzianum** CBS 226.95** [38], which demonstrated a similarity of 96% with the contigs derived from the assembly of the transcriptome. To identify the responses of the transcriptome under the different conditions tested, the contigs were annotated and classified according to their predicted functions (Figure 3).

The high number of sequences generated in this study, which were produced only through the use of next-generation sequencing, allows a precise overview of the different biological processes that occur in an organism at a given moment, and classifying these sequences allows for analysis of the genes that may be involved in biomass degradation. Sequences that were classified as possessing catalytic activity (6,975) or regulating enzymatic activity (143) may be involved in biomass degradation. When analyzed according to biological processes, the majority of the annotations were classified as participating in metabolic processes (7,393), followed by cellular processes (6,294). Regarding molecular functions, binding and catalytic activity were the most frequent classifications. Concerning cellular components, genes involved in cellular (5,184) and organelle (2,665) components and the membrane (2,143) were the most abundant.

For the hydrolysis of complex substrates such as sugarcane bagasse, a microorganism must produce an array of specialized enzymes that can hydrolyze lignocelluloses. The interaction between different classes of enzymes has been extensively studied [12],[13] and was observed in the present study. Figure 4 summarizes several of the enzyme classes that are potentially involved in biomass degradation and the number of contigs assigned to each of them. Of the contigs formed after assembly,
Figure 3. Molecular functions, biological process distribution and cellular localization of the transcriptome assembly. Contigs were assigned putative classifications based on homology and evaluated for their predicted involvement in molecular functions (A), biological processes (B) and cellular localization (C).

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Figure 4. Identified sequences that catalyze reactions that are potentially involved in biomass degradation. The results of identification based on homology using the NCBI NR database indicate the presence of genes that are related to the depolymerization of biomass in the transcriptome.

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were identified from the following glycoside hydrolase families: ing genes involved in hemicellulose depolymerization, 22 genes sequences that encoded carbohydrate-binding modules. Regard-

GH1, 10 genes as GH3, one gene as GH6 and one gene as GH61. as GH7, two genes as GH12, two genes as GH45, three genes as CAZymes, we identified 23 genes that encoded proteins of the results). We identified 487 predicted CAZymes in the transcrip-

hydrolysis of terminal (1→4)-linked α-D-glucose residues from the nonreducing ends of the chains. Both of these steps release β-D-glucose, which is the monomer that is further metabolized. Hemicellulose possesses a more varied composition than cellulose and requires enzymes to be effectively hydrolyzed. Sequences were classified as being involved in the degradation of xylan to monomers, including both endo-xylanases (EC 3.2.1.8), which cleave the xylanbackbone into shorter oligosaccharides, and β-xylosidase (EC 3.2.1.37), which cleaves short xylo-oligosaccharides into xylose. Similarly, sequences were related to mannan degradation (EC 3.2.1.113, EC 3.2.1.25, EC 3.2.1.24, EC 3.2.1.78 and EC 3.2.1.101). Several ancillary enzymes were also identified, including α-glucuronidase (EC 3.2.1.139), α-galactosidase (EC 3.2.1.22) and arabinofuranosidase (EC 3.2.1.55).

Systematic synergisms between the different enzyme classes could be observed for specific metabolic pathways in the T. harzianum transcriptome. These pathways included the metabolism of different sugars, which are associated with the depolymerization of biomass and were classified according to specific criteria of the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Table 2) [28]. In this manner, different classes of enzymes that act together to degrade the cellulose backbone were identified. The application of enzymes to catalyze the degradation of cellulose to glucose and heteroxylans to pentose is now considered to be the most viable strategy for providing cost-efficient second-generation ethanol processes [39],[18], and the present study confirms that a variety of different metabolic pathways are necessary for sugar degradation in this yeast.

Classification according to CAZymes

To determine the number of encoded genes related to biomass degradation in the transcriptome, we searched for the following carbohydrate-active enzyme groups: glycoside hydrolases (GHs), glycosyltransferases (GTs), carbohydrate esterases (CEs) and the corresponding carbohydrate-binding modules (CBMs). We compared the transcriptome with a group of annotated sequences from the CAZymes database, including the annotated sequences for 17 cellulases from T. reesei. To identify CAZymes in T. harzianum IOC-3844, the assembled transcriptome was aligned against the specific CAZy dataset, and only the best alignment was considered for each gene sequence. A total of 527 CAZymes were identified in the T. harzianum IOC-3844 predicted gene set (unpublished results). We identified 487 predicted CAZymes in the transcriptome using a cutoff E-value of 1×10^{-5}. From the predicted CAZymes, we identified 23 genes that encoded proteins of the glycoside hydrolyase families that are involved in cellulose depolymerization: three genes were classified as GH5, one gene as GH7, two genes as GH12, two genes as GH5, three genes as GH1, 10 genes as GH3, one gene as GH6 and one gene as GH61. In the cellulose depolymerization group, we found 10 different sequences that encoded carbohydrate-binding modules. Regarding genes involved in hemicellulose depolymerization, 22 genes were identified from the following glycoside hydrolyase families: three genes from GH10, three genes from GH11, two genes from GH26, three genes from GH43, three genes from GH54, one gene from GH62, two genes from GH67, one gene from GH74 and four genes from GH95. Six carbohydrate-binding module sequences were classified as belonging to enzymes that degrade hemicellulose (Figure 5).

Comparative expression analysis

To analyze the differences in expression levels among the tested growth conditions, we compared the total assembly generated from all of the sequenced transcriptome libraries (DSB+CEL=LAC library) with each individual transcriptome assembly. The mapping results are shown in Table 1.

To identify the transcriptomic responses under each condition, we analyzed the distribution of the genes that were identified as being differentially expressed. Pairwise comparisons of the subsets indicated the total number of genes that were overexpressed under each condition. The classification of differentially expressed contigs allowed us to determine the set of genes for carbohydrate-active enzymes that were upregulated in each group (Table 3, Figure 6 and 7).

Different genes corresponded to different glycoside hydrolase families involved in carbohydrate metabolism in the different upregulated groups. According to the Carbohydrate-Active Enzymes database [40], the glycoside hydrolases of family 1 include enzymes that possess β-glucosidase (EC 3.2.1.21), β-galactosidase (EC 3.2.1.23) and β-mannosidase (EC 3.2.1.25) activities; the glycoside hydrolases of family 3 exhibit chitinase (EC 3.2.1.14) activity; the glycoside hydrolases of family 55 exhibit exo-b-1,3-glucanase (EC 3.2.1.58) and endo-b-1,3-glucanase (EC 3.2.1.39) activities; the glycoside hydrolases of family 3 exhibit β-glucosidase (EC 3.2.1.21) and xylan 1,4-β-xylosidase (EC 3.2.1.37) activities; the glycoside hydrolases of family 5 possess chitosanase (EC 3.2.1.132), b-mannosidase (EC 3.2.1.25), endo-b-1,4-gluca-

nase/cellulase (EC 3.2.1.4) and glucan b-1,3-glucosidase (EC 3.2.1.58) activities; the glycoside hydrolases of family 11 present endo-1,4-β-xylanase (EC 3.2.1.8) activity; and the glycoside hydrolases of family 16 exhibit endo-1,3-β-glucanase (EC 3.2.1.39) or endo-1,3(4)-β-glucanase (EC 3.2.1.6) activity. The LAC library contained 33 classified genes, whereas the CEL library contained 23 genes and the DSB library contained 22 genes. These gene classifications included glycosyltransferases (GTs), which catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules to form glycosidic bonds; carbohydrate esterases (CEs); and the corresponding carbohydrate-binding modules (CBMs). Glycosyltransferases can be classified as either retaining or inverting enzymes according to the stereochemistry of their substrates and reaction products. The glycosyltransferases of family 2 (GT2) exhibit cellulose synthase (EC 2.4.1.12) and chitin synthase (EC 2.4.1.16) activities and appear in all three libraries. The glycosyltransferases of family 4 (GT4) exhibit sucrose synthase (EC 2.4.1.13) and sucrose-phosphate synthase (EC 2.4.1.14) activities. Therefore, some of the genes that are responsible for biomass degradation reactions are highly expressed, whereas others, though not highly expressed, may also confer the ability to degrade organic compounds for energy in this fungus. Thus, the fungus can adapt its cellulolytic system to the composition of its medium by increasing or decreasing the expression of certain genes, as observed in the present study.

Discussion

The ability of filamentous fungi to efficiently degrade plant polymers is an important aspect of microbial ecology and may
| Metabolic Pathway                        | Library | Enzyme Code Classification                                                                 |
|-----------------------------------------|---------|--------------------------------------------------------------------------------------------|
| Fructose and mannose metabolism         | DSB     | EC 1.1.1.17: 5-dehydrogenase                                                                 |
|                                         | CEL     | EC 3.2.1.78: endo-1,4-β-mannosidase                                                          |
| Lysine biosynthesis                      | DSB     | EC 1.2.1.31: dehydrogenase                                                                  |
| Lysine degradation                       | DSB     | EC 1.2.1.31: dehydrogenase                                                                  |
| Purine metabolism                        | CEL     | EC 2.7.7.7: DNA polymerase                                                                  |
|                                         | CEL     | EC 3.6.1.3: adenylypyrophosphatase                                                           |
|                                         | CEL     | EC 3.6.1.15: nucleoside triphosphate phosphohydrolase                                        |
|                                         | LAC     | EC 3.6.1.3: adenylypyrophosphatase                                                           |
| Thiamine metabolism                      | CEL     | EC 3.6.1.15: nucleoside triphosphate phosphohydrolase                                        |
| Methane metabolism                       | CEL     | EC 1.14.13.8: monoxygenase                                                                  |
|                                         | CEL     | EC 1.11.1.7: lactoperoxidase                                                                 |
| Phenylpropanoid biosynthesis             | CEL     | EC 1.11.1.7: lactoperoxidase                                                                 |
| Phenylalanine metabolism                 | CEL     | EC 1.11.1.7: lactoperoxidase                                                                 |
| Glycerophospholipid metabolism           | CEL     | EC 4.1.1.65: decarboxylase                                                                    |
|                                         | LAC     | EC 1.1.1.8: dehydrogenase (NAD+)                                                            |
| Pyrimidine metabolism                    | CEL     | EC 2.7.7.7: DNA polymerase                                                                  |
| Starch and sucrose metabolism            | CEL     | EC 3.2.1.1: endo-1,4-β-D-glucanase                                                           |
|                                         | LAC     | EC 3.2.1.37: 1,4-β-xylidase                                                                  |
| Drug metabolism: cytochrome P450          | CEL     | EC 1.14.13.8: monoxygenase                                                                  |
| Drug metabolism: other enzymes           | LAC     | EC 3.1.1.1: aliesterase                                                                      |
| Riboflavin metabolism                    | CEL     | EC 1.1.1.193: reductase                                                                      |
| Fructose and mannose metabolism          | CEL     | EC 3.2.1.78: endo-1,4-β-mannosidase                                                          |
| Amino sugar and nucleotide sugar metabolism | LAC | EC 3.2.1.37: 1,4-β-xylidase                                                                  |
|                                         | LAC     | EC 3.2.1.55: arabinosidase                                                                   |
| Pyruvate metabolism                      | LAC     | EC 2.3.3.9: synthase                                                                        |
| Nitrogen metabolism                      | LAC     | EC 1.9.3.1: oxidase                                                                          |
| Other glycan degradation                  | LAC     | EC 3.2.1.45: psychosine hydrolase                                                            |
| Glycerolipid metabolism                  | LAC     | EC 1.1.1.72: dehydrogenase (NADP⁺)                                                           |
| Oxidative phosphorylation                | LAC     | EC 1.1.9.3.1: oxidase                                                                        |
| Glyoxylate and dicarboxylate metabolism  | LAC     | EC 2.3.3.9: synthase                                                                        |
| Sphingolipid metabolism                  | LAC     | EC 3.2.1.45: psychosine hydrolase                                                            |
| Fructose and mannose metabolism          | DSB     | EC 1.1.1.17: 5-dehydrogenase                                                                  |
|                                         | CEL     | EC 3.2.1.78: endo-1,4-β-mannosidase                                                          |
| Lysine biosynthesis                      | DSB     | EC 1.2.1.31: dehydrogenase                                                                  |
| Lysine degradation                       | DSB     | EC 1.2.1.31: dehydrogenase                                                                  |
| Purine metabolism                        | CEL     | EC 2.7.7.7: DNA polymerase                                                                  |
|                                         | CEL     | EC 3.6.1.3: adenylypyrophosphatase                                                           |
|                                         | CEL     | EC 3.6.1.15: nucleoside triphosphate phosphohydrolase                                        |
|                                         | LAC     | EC 3.6.1.3: adenylypyrophosphatase                                                           |
| Thiamine metabolism                      | CEL     | EC 3.6.1.15: nucleoside triphosphate phosphohydrolase                                        |
| Methane metabolism                       | CEL     | EC 1.14.13.8: monoxygenase                                                                  |
|                                         | CEL     | EC 1.11.1.7: lactoperoxidase                                                                 |
| Phenylpropanoid biosynthesis             | CEL     | EC 1.11.1.7: lactoperoxidase                                                                 |
| Phenylalanine metabolism                 | CEL     | EC 1.11.1.7: lactoperoxidase                                                                 |
| Glycerophospholipid metabolism           | CEL     | EC 4.1.1.65: decarboxylase                                                                    |
|                                         | LAC     | EC 1.1.1.8: dehydrogenase (NAD⁺)                                                             |
| Pyrimidine metabolism                    | CEL     | EC 2.7.7.7: DNA polymerase                                                                  |
| Starch and sucrose metabolism            | CEL     | EC 3.2.1.1: endo-1,4-β-D-glucanase                                                           |
|                                         | LAC     | EC 3.2.1.37: 1,4-β-xylidase                                                                  |
afford many potential industrial applications. The fungal strain *T. harzianum* demonstrates promising results for on-site cellulase production and is a potential candidate for the production of hydrolytic enzymes [4],[6].

To evaluate the cellulase activity of this fungus on pretreated sugarcane bagasse, we measured FPase, which reflects the overall activity of multicomponent enzyme complexes for cellulose hydrolysis [41]. An increase in cellulose activity is observed over the course of cultivation until 96 h, which represents the maximum cellulolytic activity (Figure 2). The DSB sample, which was used as the inducer, initiated fermentation at a level 4-fold greater than cellulolytic activity, which is most likely due to previous adaptation of the fungus to the substrate during the production of mycelia (during the preculture). In this case, the set of genes that were activated during the induction of mycelial growth was identical to the set used in fermentation, which allowed for a higher rate of fermentation in the first 24 h. In the first 48 to 96 h, the cellulolytic activity profile of the sample induced with DSB maintained a growth profile and FPase that was statistically similar to that of samples induced with cellulose. This

![Figure 5. Encoded genes related to cellulose and hemicellulose depolymerization.](image)

Figure 5. Encoded genes related to cellulose and hemicellulose depolymerization. Genes classified in the transcriptome analysis of *T. harzianum* fermentation on sugarcane bagasse. doi:10.1371/journal.pone.0088689.g005
result indicates that the set of genes that were active after the adaptation phase of fermentation may have been similar between the samples; however, the sample induced with DSB must have differentially expressed some genes in the first 24 h of growth because it reached a higher peak of cellulolytic activity (0.2 ± 0.01 FPU mL⁻¹) compared with the samples induced with cellulose and lactose. The sample that used lactose as the inducer of mycelial growth maintained lower levels of activity throughout the fermentation. Notably, in the first 24 h of fermentation, the CEL and LAC samples both achieved similar (0.05 ± 0.004 and 0.02 ± 0.002 FPU mL⁻¹, respectively) levels of FPase, suggesting that the set of genes that were activated during the preculture phase generated similar rates of cellulose-degrading enzymatic activity.

To elucidate how the complex sugarcane bagasse substrate influences the set of fungal gene transcripts that conferred enzymatic activity, we analyzed the transcription profiles of the samples. The results represent the first characterization of global gene expression in *T. harzianum* grown on a complex substrate (Figure 3). In the analysis of 32,494 contigs from the cDNA library, 6,975 sequences were classified as possessing catalytic activity (21.46% of total contigs), of which 2,555 possess hydrolase activity and act on chemical bonds such as ester, carbon-nitrogen and carbon-carbon bonds (Table 4). The high number of identified hydrolase sequences allowed us to determine the gene sequences that were related to specific degradation reactions. A similar annotation profile, which was generated using Gene Ontology (GO), was described by Steindorff et al. (2012) [5] for an EST sequencing library of 2,927 high-quality sequences. In both experiments, catalytic activity and binding represented the major classified molecular functions, with metabolic and cellular processes being the most prevalent classifications, and the cell and organelle category constituting the most common cellular localization.

The current study identified genes that were upregulated by different substrates in the preculture phase (Table 3). The DSB library contained 792 classified contigs, 514 of which were homologous to the *T. harzianum* genome and 22 of which were related to the CAZyme library. Among the 377 classified contigs in the CEL library, 243 were related to the genome, and 23 were identified among the CAZyme. Among the 299 classified contigs in the LAC library, 272 genes were related to the genome, and 33 were identified in the CAZyme dataset (Table 3). Therefore, according to the CAZyme classification, 79 genes were differentially expressed between two conditions and exhibited an expression level that was measurable in the other conditions. In this analysis, the gene expression values fell into three profiles after K-means clustering (Figure 6). Cluster 1 (Figure 7A) contained the genes (members of the glycoside hydrolase family) that were most highly expressed in the DSB library; cluster 2 (Figure 7B) contained the most highly expressed genes in the CEL library, and cluster 3 (Figure 7C) contained the most highly expressed genes in the LAC library. A difference observed between the set group of GHs could be related to the influence of the different substrates. Several contigs were analyzed further in terms of their expression values and similarity.

Among the differentially expressed genes, we identified genes related to extracellular degradative enzymes that play an important role in pathogenesis. These enzymes include the carbohydrate esterase family 5 protein, whose cutinase domain (contig 25106, classified based on CAZ similarity as EHK47149.1, IPR000675) hydrolyzes cutin and facilitates fungal penetration.
through the cuticle. Inhibition of this enzyme can prevent fungal infection through intact cuticles. When cutin monomers are released from the cuticle due to small amounts of cutinase on fungal spore surfaces, these monomers can greatly increase the amount of cutinase secreted by the spore, although the mechanism underlying this process remains unknown. Another, more highly expressed, contig was classified as a member of the GH11 family (contig 20286). The overwhelming majority of the glycoside hydrolases of this family are xylanases. These enzymes carry out the endohydrolysis of \((1\rightarrow4)\)-beta-D-xylosidic linkages in xylans and random hydrolysis of \((1\rightarrow3)\)-beta-D-glycosidic linkages in \((1\rightarrow3)\)-beta-D-xylans. Contig 27456 exhibited similarity to family GH71, which includes \(\alpha\)-1,3-glucanase (EC 3.2.1.59). \(\alpha\)-Glycosyl hydrolases (EC 3.2.1.) are a widespread group of enzymes that hydrolyze glycosidic bonds between two or more carbohydrates or between a carbohydrate and a noncarbohydrate moiety (IPR005197), and they are also related to CBM24 (\(\alpha\)-1,3-glucan (mutant)-binding function) [42].

In the DSB library, contig 20009 was found to be differentially expressed at a significant RPKM level (103.86) and was similar to a GH16 protein from *Trichoderma reesei* (EHK18881.1, IPR000757). The GH16 family contains a variety of enzymes with a range of known activities. Lichenase (EC 3.2.1.73), xylolucan xylolucosyltransferase (EC 2.4.1.207), agarase (EC 3.2.1.81), kappa-carrageenase (EC 3.2.1.83), endo-\(\beta\)-1,3-glucanase (EC 3.2.1.39), endo-\(\beta\)-1,3-1,4-glucanase (EC 3.2.1.6) and endo-\(\beta\)-galactosidase (EC 3.2.1.103) are all members of this family.

In this study, we identified sequences related to different classes of enzymes that act on the cellulose backbone, such as GH5, which exhibits endo-\(\beta\)-1,4-glucanase activity in *T. reesei* (EC 3.2.1.4) and is responsible for the hydrolysis of the \((1\rightarrow4)\)-beta-D-glucosidic linkages in cellulose. The GH3 family exhibits \(\beta\)-glucosidase activity in *T. reesei* (EC 3.2.1.21), where it hydrolyzes terminal, nonreducing \(\beta\)-D-glucosyl residues and releases \(\beta\)-D-glucose. This monomer can enter into the eukaryotic energy pathway of glycolysis. Glycolysis produces energy and requires an input of two ATP molecules. This input is used to generate four new ATP molecules, resulting in a net gain of two ATP molecules. Two NADH molecules are also produced; these molecules serve as electron carriers for other biochemical reactions in the cell. The enzymes that are necessary to catalyze the degradation of glucose molecules are expressed throughout the growth of the fungus on the complex substrate, possibly to produce energy through glycolysis and support cell survival and reproduction. The enzymes that act in biomass degradation were the focus of this work, and this analysis allowed us to identify a set of enzymes that are involved in carbohydrate metabolism based on expression profiles.

Regarding expression differences, the LAC library contained numerous genes receiving CAZyme classifications. Lactose, an
Table 3. Classification of sequences present in upregulated groups, according to the CAZyme database.

| Upregulated group | Cluster | Contig | Length (bp) | Lowest E-value | CAZy     | RPKM   |
|-------------------|---------|--------|-------------|---------------|----------|--------|
|                   |         |        |             |               |          |        |
| **DSB 1**         | 6765    | 1,521  | 0.00        | GT69          | 58.25    | 28.90  | 29.42  |
|                   | 7294    | 1,155  | 3.72E-33    | GT39          | 84.76    | 51.53  | 42.58  |
|                   | 14029   | 3,726  | 0.00        | GT2           | 97.16    | 35.91  | 58.69  |
|                   | 15954   | 665    | 3.74E-13    | GH28          | 64.39    | 28.07  | 32.29  |
|                   | 16314   | 1,044  | 0.00        | GH76          | 144.84   | 88.02  | 98.48  |
|                   | 19203   | 218    | 1.86E-34    | GT2           | 293.39   | 163.56 | 212.91 |
|                   | 19252   | 218    | 1.37E-47    | GT2           | 81.63    | 42.09  | 33.30  |
|                   | 19677   | 250    | 1.52E-24    | GT2           | 58.78    | 31.54  | 24.40  |
|                   | 20009   | 209    | 5.02E-35    | GT16          | 103.86   | 68.61  | 58.86  |
|                   | 21947   | 1,631  | 0.00        | GT2           | 194.11   | 123.70 | 142.81 |
|                   | 22621   | 1,184  | 0.00        | GH125         | 151.61   | 80.77  | 97.54  |
|                   | 23959   | 698    | 0.00        | GT20          | 251.35   | 144.52 | 151.95 |
|                   | 24020   | 330    | 2.94E-173   | GT4           | 359.77   | 167.88 | 260.13 |
|                   | 24053   | 1,141  | 0.00        | GT48          | 210.54   | 152.82 | 151.10 |
|                   | 24197   | 823    | 1.37E-36    | CE11          | 156.59   | 115.13 | 101.66 |
|                   | 24491   | 696    | 0.00        | GH128         | 332.95   | 226.17 | 240.71 |
|                   | 25186   | 416    | 2.98E-6     | CE11          | 228.13   | 132.09 | 154.75 |
|                   | 27900   | 309    | 5.9E-42     | GH78          | 373.50   | 176.02 | 247.26 |
|                   | 28105   | 267    | 2.72E-97    | GH27          | 201.54   | 119.69 | 131.08 |
|                   | 28257   | 262    | 1.35E-19    | GH1           | 70.75    | 37.95  | 31.09  |
|                   | 29726   | 1,876  | 0.00        | GH5           | 118.25   | 83.09  | 68.79  |
|                   | 31930   | 361    | 3.11E-150   | GH43          | 447.09   | 355.16 | 205.51 |
| **CEL 2**         | 15484   | 1,124  | 0.00        | GT24          | 37.62    | 129.12 | 77.71  |
|                   | 15510   | 596    | 0.00        | GHS/CBM1      | 49.83    | 239.69 | 88.81  |
|                   | 15808   | 642    | 1.32E-64    | GH5           | 54.56    | 95.81  | 60.99  |
|                   | 17441   | 1,93   | 5.04E-34    | GH10          | 83.44    | 257.14 | 120.21 |
|                   | 19410   | 217    | 2.19E-39    | GH3           | 24.20    | 49.84  | 17.90  |
|                   | 19509   | 227    | 3.56E-37    | GT2           | 287.20   | 425.57 | 320.97 |
|                   | 19636   | 212    | 5.34E-29    | GT2           | 38.56    | 103.35 | 54.65  |
|                   | 19662   | 220    | 7.85E-39    | GT2           | 115.65   | 220.27 | 135.39 |
|                   | 20286   | 203    | 2.77E-26    | GH11          | 954.08   | 2397.6 | 1835.5 |
|                   | 20620   | 1,993  | 9.99E-31    | CE15          | 16.83    | 58.90  | 22.72  |
|                   | 23663   | 557    | 0.00        | CBM20         | 257.18   | 433.50 | 330.20 |
|                   | 23934   | 1,045  | 0.00        | GT2           | 221.66   | 416.88 | 256.88 |
|                   | 24061   | 1,256  | 0.00        | GT2           | 115.31   | 164.11 | 77.22  |
|                   | 24114   | 526    | 0.00        | GT48          | 363.92   | 467.54 | 372.60 |
|                   | 24258   | 1,118  | 0.00        | GT2           | 111.80   | 187.01 | 75.47  |
|                   | 24717   | 1,283  | 0.00        | GT2           | 279.09   | 550.73 | 376.59 |
|                   | 25735   | 222    | 5.84E-52    | GT2           | 211.60   | 375.59 | 260.66 |
|                   | 26247   | 262    | 2.93E-56    | GH18          | 153.47   | 228.37 | 111.86 |
|                   | 28387   | 203    | 2.13E-33    | GH18          | 71.69    | 119.10 | 73.19  |
|                   | 31105   | 817    | 1.17E-141   | GH10          | 47.92    | 166.37 | 67.24  |
|                   | 32239   | 223    | 3.49E-37    | GT48          | 36.66    | 74.47  | 42.48  |
|                   | 5331    | 2,885  | 0.00        | GH2/GH27      | 34.36    | 80.30  | 42.64  |
|                   | 6707    | 1,307  | 0.00        | GH10          | 80.29    | 185.68 | 77.10  |
| **LAC 3**         | 7215    | 1,907  | 0.00        | GH43          | 78.81    | 91.29  | 130.49 |
|                   | 10859   | 1,914  | 0.00        | GH47          | 193.40   | 198.30 | 294.08 |
|                   | 18143   | 324    | 2.36E-87    | GH76          | 79.40    | 74.62  | 126.43 |
inexpensive, soluble substrate, leads to reasonably good induction for cellulase production [43],[44]. The fungus does not directly take up lactose but instead hydrolyzes the compound to galactose and glucose. Cellulase synthesis cannot be induced by galactose, and the addition of galactose to the medium decreases FPase levels in the supernatant [45], as reported in this work. Karaffa et al. [43] reported that lactose induces significantly higher cellulase levels compared to galactose, but galactose induces cellulase gene expression at low growth rates in T. reesei [194]. In this study, the highest degradation rate would have occurred in the fungi precultured on the lactose medium, followed by cellulose and DSB, due to the complexity of the substrate and the stability of the organic chains involved. The presence of lactose in the early stages of the experiment would have induced genes that are sensitive to lactose and galactose, which may explain the low level of FPase observed in the sample that used lactose as the inducer of hydrolytic systems (even in the fermentation step). As shown in the results, sugarcane bagasse was able to activate the expression of a different set of genes that were differentially expressed compared with the control, and this difference was associated with an increase in cellulose enzymatic activity during fermentation. This strain of T. harzianum demonstrates a complex and efficient genetic mechanism for biomass degradation. The use of RNA-Seq technology was shown to be an efficient strategy for the discovery and selection of potential target genes. The results reported here are valuable for further studies on the expression, purification and characterization of recombinant enzymes for efficient cellulose degradation.

| Upregulated group | Cluster | Contig | Length (bp) | Lowest E-value | CAZy | RPKM |
|------------------|---------|--------|-------------|---------------|------|------|
|                  |         |        |             |               | DSB  | CEL  | LAC  |
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Table 4. Contig sequences classified according to their putative hydrolytic activity.

| GO ID     | TERM                                                        | Number of classified sequences |
|-----------|-------------------------------------------------------------|---------------------------------|
| GO:0016787| hydrolase activity                                          | 2,555                           |
| GO:0004553| hydrolase activity, hydrolyzing O-glycosyl compounds        | 336                            |
| GO:0016788| hydrolase activity, acting on ester bonds                   | 494                            |
| GO:0016798| hydrolase activity, acting on glycosyl bonds                | 373                            |
| GO:0016818| hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides | 778                            |
| GO:0016798| hydrolase activity, acting on glycosyl bonds                | 373                            |
| GO:0016818| hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides | 778                            |
| GO:0016817| hydrolase activity, acting on acid anhydrides               | 789                            |
| GO:0016810| hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds | 140                            |
| GO:0016820| hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances | 174                            |
| GO:0042578| phosphoric ester hydrolase activity                         | 155                            |
| GO:0052689| carboxylic ester hydrolase activity                         | 77                             |
| GO:0017171| serine hydrolase activity                                   | 113                            |
| GO:0008081| phosphoric diester hydrolase activity                       | 54                             |
| GO:0016811| hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds in linear amides | 40                             |
| GO:0016790| thiolester hydrolase activity                               | 45                             |
| GO:0008484| sulfuric ester hydrolase activity                            | 17                             |
| GO:0016813| hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds in linear amides | 17                             |
| GO:0016814| hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds in cyclic amides | 25                             |
| GO:0047617| acyl-CoA hydrolase activity                                 | 9                              |
| GO:0033961| cis-stilbene-oxide hydrolase activity                       | 8                              |
| GO:0016803| ether hydrolase activity                                    | 10                             |
| GO:0016289| CoA hydrolase activity                                      | 9                              |
| GO:0016801| hydrolase activity, acting on ether bonds                   | 11                             |
| GO:0019238| cyclohydrolase activity                                     | 9                              |
| GO:0004416| hydroxyacylglutathione hydrolase activity                   | 4                              |
| GO:0016799| hydrolase activity, hydrolyzing N-glycosyl compounds        | 5                              |
| GO:0019120| hydrolase activity, acting on acid halide bonds, in C-halide compounds | 3                             |
| GO:0003933| GTP cyclohydrolase activity                                 | 5                              |
| GO:0004848| ureidoglycolate hydrolase activity                           | 3                              |
| GO:0003935| GTP cyclohydrolase II activity                              | 3                              |
| GO:0004477| methenyltetrahydrofolate cyclohydrolase activity            | 2                              |
| GO:0004045| aminoacyl-tRNA hydrolase activity                            | 2                              |
| GO:0003934| GTP cyclohydrolase I activity                               | 2                              |
| GO:0008474| palmitoyl-(protein) hydrolase activity                      | 2                              |
| GO:0004463| leukotriene-A4 hydrolase activity                            | 2                              |
| GO:0016823| hydrolase activity, acting on acid carbon-carbon bonds in ketonic substances | 3                             |
| GO:0016824| hydrolase activity, acting on acid halide bonds             | 3                              |
| GO:0016822| hydrolase activity, acting on acid carbon-carbon bonds      | 3                              |
| GO:0004039| allophanate hydrolase activity                              | 1                              |
| GO:0004635| phosphoribosyl-AMP cyclohydrolase activity                  | 1                              |
| GO:0004649| poly(ADP-ribose) glycohydrolase activity                    | 1                              |
| GO:0033971| hydroxyisourate hydrolase activity                          | 1                              |
| GO:0018738| S-formylglutathione hydrolase activity                      | 1                              |
| GO:0004301| epoxide hydrolase activity                                  | 1                              |
| GO:0033699| DNA 5′-adenosine monophosphate hydrolase activity           | 1                              |
| GO:0003937| IMP cyclohydrolase activity                                 | 1                              |
### Supporting Information

#### Table S1

| GO ID     | TERM                                      | Number of classified sequences |
|-----------|--------------------------------------------|-------------------------------|
| GO:0016802 | trialklysufonium hydrolase activity       | 1                             |
| GO:0016815 | hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds in nitriles | 1                             |

**References**

1. Hermosa R, Viterbo A, Chet I, Monte E (2012) Plant-beneficial effects of Trichoderma and of its genes. Microbiology [Reading, England] 158: 17–25.
2. Vinale F, Sevastianparam K, Ghosalberti EL, Marra R, Woo SL, et al. (2008) Trichoderma plant-pathogen interactions. Soil Biology and Biochemistry 40: 1–10.
3. Maeda RN, Serpa VI, Rocha VAL, Mesquita RAA, Anna LMM, et al. (2011) Enzymatic hydrolysis of pretreated sugar cane bagasse using *Penicillium ficuum* and *Trichoderma harzianum* cellulases. Process Biochemistry 46: 1196–1201.
4. Delabona PDS, Farina CS, da Silva MR, Azzoni SF, Pradella JGDC (2012) Use of a new *Trichoderma harzianum* strain isolated from the Amazon rainforest with putative roles in mycoparasitism against *Fusarium oxysporum*. Biological Control 61: 134–140.
5. Steinföld AS, Silva RDN, Coelho ASG, Nagata T, Norntha EF, et al. (2012) *Trichoderma harzianum* expressed sequence tags for identification of genes with putative roles in mycoparasitism against *Fusarium oxysporum*. Biological Control 61: 134–140.
6. Colussi F (2011) Purification, and Biochemical and Biophysical Characterization of Cellulohydrolase I from *Trichoderma harzianum* IOC 3044. Journal of Microbiology and Biotechnology 21: 883–887.
7..de Castro AM, Pedro KCNR, da Cruz JC, Ferreira MC, Leite SFG, et al. (2010) *Trichoderma harzianum* IOC-4038: A promising strain for the production of a cellulolytic complex with significant β-glucosidase activity from sugarcane bagasse cellulose. Applied biochemistry and biotechnology 162: 2111–2122.
8. Soocor CR, Vandenbergh LDPDS, Medeiro APB, Karp SG, Buckeider M, et al. (2010) Bioethanol from lignocellulosas: Status and perspectives in Brazil. Bioresource technology 101: 4029–4025.
9. Suorsen A, Teller PJ, Lubeck PS, Ahring BK (2011) Osmic enzyme production during bioethanol production from biomass: screening for suitable fungal strains. Applied biochemistry and biotechnology 164: 1058–1070.
10. Van Dyk JS, Pletchick BI (2012) A review of lignocellulose biocconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-Factors affecting enzymes, conversion and synergy. Biotechnology advances 30: 1458–1460.
11. Van Viet AHM. (2010) Next generation sequencing of microbial transcripts: challenges and opportunities. FFMS, microbiology letters 392: 1–7.
12. Banerjee G, Car S, Scott-Craig GS, Borusnch MS, Walton JD (2010) Rapid optimization of enzyme mixtures for deconstruction of diverse pretreatment/biomass feedstock combinations. Biotechnology for biofuels 3: 22.
13. Gilbert HJ (2010) The biochemistry and structural biology of plant cell wall deconstruction. Plant physiology 153: 444–455.
14. Banerjee G, Scott-Craig GS, Walton JD (2010) Improving Enzymes for Biomass Conversion: A Basic Research Perspective. BioEnergy Research 3: 82–92.
15. Salohmeo I, Palohmeo I, Haka I, Per J, Swason B, et al. (2002) Swollenin, a *Trichoderma reesi* protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials. European Journal of Biochemistry 269: 4202–4211.
16. Eriksson T, Karlsson J, Tjernhan D (2002) A Model Explaining Declining Rate in Hydrolysis of Lignocellulose Substrates with Cellulohydrolase I (Cel7A) and Endoglucanase I (Cel7B) of *Trichoderma reesi*. Applied biochemistry and biotechnology 101: 41–60.
17. Teeri T (1997) Crystalline cellulose degradation: new insight into the function of cellobiohydrolases G3. Trends in Biotechnology 15: 160–167.
18. Meyer AS, Rosgaard L, Stersen HR (2009) The minimal enzyme cocktail concept for biomass processing. Journal of Cereal Science. 50: 337–344.
19. Martinez D, Berka RM, Henniars B, Salomheo I, Arcas M, et al. (2000) Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesi* (syn. *Hypocrea jecorina*). Nature biotechnology 26: 553–560.
20. Liu H-Q, Feng Y, Zhao D-Q, Jiang J-N (2011) Evaluation of cellulases produced from four fungi cultured on furfural residues and microcrystalline cellulose. Biodegradation 23: 463–472.
21. Mandal M, Weber J (1969) The production of cellulases. In: Hajny GJ, Reese ET editors. Advances in Chemistry, AMERICAN CHEMICAL SOCIETY; Washington, DC. DOI: 10.1021/ba-1969-0095.ch023.
22. Rocha GJM, Gonçalves AR, Oliveira BR, Olivares EG, Rossell CEV (2012) Steam explosion pretreatment reproducibility and alkaline delignification reactions performed on a pilot scale with sugarcane bagasse for bioethanol production. Industrial Crops and Products 33: 274–279.
23. Ghose TK (1987) Measurement of cellulase activities. Pure Appl Chem 59: 257–268.
24. Jones JD, Daumuir P, Bedbrook J (1985) High level expression of introduced chimeric genes in regenerated transformed plants. The EMBO journal 4: 2411–2418.
25. Pearson WR (2013) An introduction to sequence similarity (“homology”) searching. Current protocols in bioinformatics 42: 3.1.1–3.1.13.
26. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, et al. (2000) Gene Ontology: tool for the unification of biology. Nature America Inc 25: 23–29.
27. Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, et al. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21: 3674–3676.
28. Kanehisa M, Goto S (2000) KEGG: kyoto encyclopedia of genes and genomes. Nucleic acids research 28: 27–30.
29. Henriassat B, Vegetales M, Grenoble F (1991) A classification of glycosyl hydrolases based on enzyme sequence similarities. Biochem J 280: 509–516.
30. Henriassat B, Bairoch A (1995) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 293: 781–786.
31. Henriassat B, Bairoch A (1996) Updating the sequence-based classification of glycosyl hydrolases. Biochem J 316: 695–696.
32. Henriassat B, Davies G (1997) Structural and sequence-based classification of glycoside hydrolases. Curr Opin Struct Biol 7: 637–644.
33. Davies G, Henriassat B (1995) Structures and mechanisms of glycosyl hydrolases. Structure 3: 853–859.
34. Campbell J, Davies G, Bulone V, Henriassat B (1997) A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. The Biochemical journal 326: 929–939.
35. Coutinho PM, Deleury E, Davies GJ, Henriassat B (2003) An Evolving Hierarchical Family Classification for Glycosyltransferases. Journal of Molecular Biology 320: 307–317.
36. Boraston AB, Bolam DN, Gilbert HJ, Davies GJ (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. The Biochemical journal 379: 769–781.
37. Lombard V, Bernard T, Rancurel C, Brunner H, Coutinho PM, et al. (2010) A hierarchical classification of polysaccharide lyases for glycoconjugomics. The Biochemical journal 432: 437–444.
38. Grögerer IV, Nordberg H, Shahalov I, Aerts A, Cantor M, et al. (2012) The genome portal of the Department of Energy Joint Genome Institute. Nucleic acids research 40: D26–32.
39. Hummel ME, Ding S-Y, Johnson DK, Adney WS, Nimlos MR, et al. (2007) Biomass Recalification: Engineering Plants and Enzymes for Biofuels Production. Science 315: 804–807.
40. Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, et al. (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycoconjugomics. Nucleic Acids Res 37:D233–238.
41. Urb K, Szak G, Tengerdy RP (2000) Standardization of the filter paper activity assay for solid substrate fermentation. Biotechnology Letters 22: 65–69.
42. Fuglsang CC (2000) Biochemical Analysis of Recombinant Fungal Mutanases. A new family of alpha 1,3-glucanases with novel carbohydrate-binding domains. Journal of Biological Chemistry 275: 2009–2018.

43. Karaffa L, Fekete E, Gamauf G, Szentirmai A, Kubicek CP, et al. (2006) D-Galactose induces cellulase gene expression in *Hypocrea jecorina* at low growth rates. Microbiology 152: 1507–1514.

44. Lo C-M, Zhang Q, Callow NV, Ju L-K (2010) Roles of extracellular lactose hydrolysis in cellulase production by *Trichoderma reesei* Rut C30 using lactose as inducing substrate. Process Biochemistry 45: 1494–1503.

45. Fang X, Yano S, Inoue H, Sawayama S (2008) Lactose enhances cellulase production by the filamentous fungus *Acremonium cellulolyticus*. Journal of bioscience and bioengineering 106: 115–120.

46. Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M (2012) KEGG for integration and interpretation of large-scale molecular data sets. Nucleic acids research 40: D109–14.

47. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 25: 402–408.