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Quantitative Proteomic Study of *Aspergillus Fumigatus* Secretome Revealed Deamidation of Secretory Enzymes

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Abstract

Aspergillus sp. plays an essential role in lignocellulosic biomass recycling and is also exploited as cell factories for the production of industrial enzymes. This study profiled the secretome of *Aspergillus fumigatus* when grown with cellulose, xylan and starch by high throughput quantitative proteomics using isobaric tags for relative and absolute quantification (iTRAQ). Post translational modifications (PTMs) of proteins play a critical role in protein functions. However, our understanding of the PTMs in secretory proteins is limited. Here, we present the identification of PTMs such as deamidation of secreted proteins of *A. fumigatus*. This study quantified diverse groups of extracellular secreted enzymes and their functional classification revealed cellulases and glycoside hydrolases (32.9%), amylases (0.9%), hemicellulases (16.2%), lignin degrading enzymes (8.1%), peptidases and proteases (11.7%), chitinases, lipases and phosphatases (7.6%), and proteins with unknown function (22.5%). The comparison of quantitative iTRAQ results revealed that cellulose and xylan stimulates expression of specific cellulases and hemicellulases, and their abundance level as a function of substrate. In-depth data analysis revealed deamidation as a major PTM of key cellulose hydrolyzing enzymes like endoglucanases, cellobiohydrolases and glucosidases. Hemicellulose degrading endo-1,4-beta-xylanase, monosidases, xylosidases; lignin degrading laccase, isoamyl alcohol oxidase and oxidoreductases were also found to be deamidated.

Introduction

Lignocellulosic biomass comprising mainly of cellulose, hemicellulose, and lignin is the largest sink of global carbon. With the growing demand and shortage of fuel, exhausting fossil fuel reservoirs, rising CO_2 emissions and natural environmental deterioration; replacement of fossil fuels by renewable energy sources is an urgent global agenda. Biomasses such as agricultural wastes, crop residues, forest wastes etc. constitute potential sources of feedstock and could be targeted as a potential raw material for biofuels and new biomaterials portfolios including feed, specialty chemicals, and other value-added products [1]. These biomasses are natural, abundant, and renewable resources of carbon. Their full exploitation remains critical to the development of a sustainable global economy. Carbohydrate moieties of biomass such as cellulose, hemicellulose and pectin can be hydrolyzed into monomeric sugars and subsequently fermented into bioethanol, biogases, organic acids, chemicals, or biofuel, while the aromatic compounds, vanillin and gallic acids, can also be produced from lignin [2]. Xylitol and furfural can be derived from xylose [3]. In the biofuel production sector, the USA has developed a relatively mature technology for the production of corn ethanol and they produce more than 52.6 billion liters of ethanol per year [4, 5].

The carbohydrate fractions of the biomass can be converted into fermentable monomeric sugars through enzymatic (hemicellulase/cellulase) reactions. It has been exploited to produce ethanol, xylitol, and 2, 3-butanediol via microbial fermentation processes [6]. The conventional conversion of starch to glucose requires a two-step process, namely, liquefaction and saccharification, but this process is energy-intensive [5]. On the contrary, raw starch degrading enzymes (RSDE) can directly degrade raw starch granules below the gelatinization temperature of starch and thus significantly reduce

energy requirements, simplifying the process in the industry [7]. As reviewed by Sun et al, [5] several strains of *Aspergillus* sp. has potential to produce RSDE. To explore the cellulose and lignocellulosic biomass hydrolysis potential and to investigate the abundance of lignocellulolytic enzymes, the proteomic analysis of secretome by biomass degrading microbes such as *Thermobifida fusca* [8], *Trichoderma reesei* [9-11], *Phanerochaete chrysosporium* [12, 13], *Botrytis cinerea* [14, 15], *Postia placenta* [16], *Aspergillus niger* [17] *Fusarium graminearum* [18] and many more have been documented. However, the post translational modifications (PTMs) of these proteins that could result in a huge impact on their functions have not been studied so far. Although, proteomic analysis of secretomes from biomass degrading microbes has been performed, but very limited literature exists on PTMs of lignocellulolytic enzymes.

The fungal species like *T. reesei* is extensively studied and also used in the industry as a source of cellulases and hemicellulases. However, with a total of 200 glycosyl hydrolases (GH) encoding genes, *T. reesei* has fewer GHs than ascomycetes fungi of the eurotiomycetes lineage such as *A. nidulans* (247GHs), *A. fumigatus* (263 GHs). *A. oryzae* (185 GHs) has fewer GHs than *T. reesei* [19]. Similarly, with 16 hemicellulase genes, *T. reesei* has the smallest set of hemicellulases in comparison to other fungi like *A. nidulans* (35 genes), *A. fumigatus* (36 genes), *A. oryzae* (40 genes). *Aspergillus sp.* therefore represents a family of fungi with more diverse range of lignocellulosic hydrolyzing enzymes for industrial application. iTRAQ based proteomic analysis of the secretome of thermophilc *A. fumigatus* Z5 in the presence of different carbon sources (glucose, Avicel and rice straw) revealed the carbon source dependent production of cellulases and hemicellulases [20]. *Aspergillus niger* NS-2 grown in

presence of wheat bran following solid state fermentation techniques produced good yields of lignocellulolytic enzymes [21]. Thus, studies of *Aspergillus* sp. are gaining more attention due to its ability to secrete lignocellulolytic enzymes that are useful in lignocellulosic biorefinery. However, recent research has mostly focused on the evaluation of lignocellulolytic enzyme production or purification of the main components [22, 23], and limited literature exits on quantitative profiling of the secretome. Thermostable enzymes from thermophilic fungi has its own industrial advantages since thermostable enzymes use shorter reaction times for the complete saccharification of plant polysaccharides as compared to hydrolytic enzymes of mesophilic fungi [24-26]. Thermal pretreatment of biomass loosens biomass structure and enhance penetration of hydrolytic enzymes [27]. The comprehensive quantitative profiling of lignocellulolytic enzymes of thermophilic fungi on different carbon sources could be a practical approach for understanding unique enzyme systems. Coupling of thermal biomass treatment with thermotolerant enzymes could enhance biomass hydrolysis significantly. It is also noted that most of glycosyl hydrolases undergo modification like glycosylation, deamidation, phosphorylation etc. and these PTMs play important role in enzyme function, stability and interaction with lignocellulose [28]. PTMs like glycosylation and sites of modification in endoglucanase I, II and exoglucanase I, II from *T. reesei* and some biomass degrading enzymes of *Phanerochaete chrysosporium etc.* have been documented [29-32]. Most of these studies indicated that glycosylation plays an important role in glycosyl hydrolase function, biomass conversion and is influenced by culture conditions. Thus, information on PTMs of lignocellulolytic enzymes will make it possible to interpret their biological role, their expression regulation, interaction with substrates and

their role in regulating metabolism with new insights. Therefore, the main aim of this study was to explore the quantitative production of lignocellulolytic enzymes by *A. fumigatus* LF9 in presence of different carbon sources by adopting isobaric tag for relative and absolute quantitation (iTRAQ)-based quantitative proteomics using liquid chromatography tandem mass spectrometry (LC–MS/MS). Furthermore, this study focused on the characterization of deamidation of quantified enzymes.

MATERIALS AND METHODS

Microorganism Isolation, Cultivation and Secretome Extraction

The fungal strains were isolated from biofertilizer compost from Singapore. The procedure adopted for the isolation and identification of strain has been detailed in our earlier publication that investigated abundances of lignocellulolytic enzymes at different temperatures using saw dust as a substrate [33]. The culture of *A. fumigatus* LF9 was prepared by using potato dextrose broth composed of 20 g L^{-1} D-glucose and 4 g L^{-1} potato extract (Sigma, St. Louis, MO, USA) at 40º C. Cell biomass was collected by centrifugation at 4º C(Beckman Coulter, Brea, CA, USA), washed with sterilized Milli-Q water and inoculated in flask containing medium with the following composition: ammonium sulfate 3.1 g L^{-1} , sodium chloride 1.5 g L^{-1} , dipotassium phosphate 1.2 g L^{-1}, monopotassium phosphate 0.9 g L^{-1}, magnesium sulfate 0.3 g L^{-1} and 5.0 g L^{-1} glucose. The isolate was grown at 50º C, and the biomass was collected by centrifugation and inoculated into test flasks containing medium as stated above but with 5 g L^{-1} glucose, cellulose, xylan and starch was used as a carbon source. The test flasks were incubated at 50º C at 100 rpm and growth was monitored by analyzing total protein

content to determine the harvesting period in mid exponential phase (4 days). For proteomic analysis of the secretome from glucose, cellulose, xylan and starch condition, three flaks for each carbon source was used. The secretome was collected by centrifugation at 7500 g at 4 °C (Beckman Coulter, Brea, CA, USA) for 7 min, mycelium was removed and supernatant (secretome) was filtered through 0.25 μm filters. The supernatant (10−15 mL from ~200 mL) was retained and stored at −80°C for the enzyme assay. The remaining secretome (~180 mL) was concentrated by lyophilization. Proteins were precipitated by ice-cold acetone and protein content was determined by the Bradford method [34]. Biosafety guidelines were strictly followed while working with this fungal isolate.

Protein Separation, Protein Digestion, and Peptide Extraction

The protein digestion, peptide extraction, and LC-MS/MS analysis were performed as described previously [8, 10, 35]. Briefly, proteins (200μg) from each test sample were separated on 10% SDS-PAGE at 100 V, and protein bands were visualized by staining with Coomassie Brillant blue G-250. Each sample lane was sliced and separately washed with 75% acetonitrile (ACN) containing Triethylammonium bicarbonate buffer (TEAB, 25mM), and then destained using TEAB (25 mM) alone and TEAB with 50% ACN. Following destaining, gel pieces were reduced with Tris 2-carboxyethyl phosphine hydrochloride (5 mM) and alkylated with methyl methanethiosulfonate (10 mM). The gel pieces were then washed with TEAB (25 mM) alone and then dehydrated using 100% ACN. Protein digestion was performed by subjecting dehydrated gel pieces to sequencing grade modified trypsin (Promega,

Madison, WI) at 37 °C for 12 h. The peptides were extracted using 50% ACN, 5% acetic acid. Tryptic peptides derived from each lane were combined separately and concentrated using a concentrator (Eppendrof AG, Hamburg, Germany) for further iTRAQ labeling.

iTRAQ Labeling, LC-MS/MS Analysis and data analysis

The iTRAQ labeling of peptides from test samples from different carbon sources were performed using a 4-plex iTRAQ reagent multiplex kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The peptides from each test condition were labeled with respective isobaric tags, incubated for 2 h, quenched with water, combined together and vacuum-centrifuged to dryness. The main advantage of iTRAQ technique is the multiplex comparison of four to eight different conditions in single mass spectrometry-based experiment. The iTRAQ labeling for different conditions were assigned as follows- 113: *A. fumigatus* LF9 glucose (control); 114: *A. fumigatus* LF9 cellulose; 115: *A. fumigatus* LF9 xylan; 116: *A. fumigatus* LF9 starch. The iTRAQ ratios of 114/113 (cellulose/glucose), 115/113 (xylan/glucose), and 116/113 (starch/glucose) are presented in this study. The iTRAQ labeled peptides were reconstituted in buffer A (10 mM ammonium acetate, 85% acetonitrile, 0.1% formic acid) and fractionated using ERLIC column (200 × 4.6 mm, 5μm particle size, 300 Å pore size) by a HPLC system (Shimadzu, Japan) at flow rate of 1.0 mL min^{-1} using our previously optimized protocol [36]. The HPLC chromatograms were recorded at 280 nm, and fractions were collected using an automated fraction collector and vacuum dried. The vacuum dried peptides were reconstituted in 3% ACN with 0.1% formic acid for LC-MS/MS analysis. For proteomic analysis of secretome, the experimental design included

three flaks for each carbon source. Set 1 sample was analyzed in duplicate (technical replicates). The samples of set 2 and set 3 were prepared as stated above, analyzed individually and mean data with standard deviation were reported.

The LC-MS/MS analysis of labeled peptides was performed using a QStar Elite mass spectrometer (Applied Biosystems/MDS Sciex) coupled with online microflow HPLC system (Shimadzu). The labeled peptides were separated on a home-packed nanobored C18 column with a picofrit nanospray tip (New Objectives, Wubrun, MA) coupled to the LC-MS/MS system at a constant flow rate of 300 nl min^{-1}. Mobile phase A (0.1% formic acid in H_2O) and mobile phase B (0.1% formic acid in 80% ACN) were used to establish the 90 min gradient comprising 15 min of 12−23% B, 55 min 23-40% B, 5 min 40-65% B, 3 min 65-90% B, maintained at 90% B for 2 min and finally re-equilibration at 12% B for 10 min. The mass spectrometer was set in positive ion mode using Analyst QS 2.0 software (Applied Biosystems) and data was acquired with a selected mass range of 300 –1600m/z. The peptides with +2 and above were selected for MS/MS. The three most abundant peptides above a five-count threshold were selected for MS/MS, and dynamically excluded for 30 s with ±30 mDa mass tolerance. Smart information-dependent acquisition (IDA) was activated with automatic collision energy and automatic MS/MS accumulation. The fragment intensity multiplier was set to 20 and maximum accumulation time was 2 s. The peak areas of the iTRAQ reporter ions reflect the relative abundance of the proteins in the samples.

The data acquisition was performed with Analyst QS 2.0 software (Applied Biosystems/MDS SCIEX). The peak list generation, protein identification, and peptide quantification were performed using ProteinPilot™ software 3.0 (revision number

114732; Applied Biosystems, Foster City, CA). The Paragon algorithm in the ProteinPilot™ software was used for the peptide identification. The data was further processed by Pro Group algorithm where isoform-specific quantification was adopted to trace the differences between expressions of various isoforms. The defined parameters were as follows: (i) Sample Type, iTRAQ 4-plex (Peptide Labeled); (ii) Cysteine alkylation, MMTS; (iii) Digestion, Trypsin; (iv) Instrument, QSTAR Elite ESI; (v) Special factors, None; (vi) Species, None; (vii) Specify Processing, Quantitate; (viii) ID Focus, biological modifications, amino acid substitutions; (ix) Database, concatenated JGI downloaded from (http://www.aspgd.org/, http://genome.jgi.doe. gov/Aspfu1/ Aspfu1.home.html); (x) Search effort, thorough. The default precursors and fragment mass tolerances for QStar Elite MS instrument were adopted by the software. ProteinPilot™ software automatically determined various PTMs based on the HUPO-PSI Modification Nomenclature for Mass Spectrometry standard (http://www.unimod.org). The peptides for iTRAQ quantitation were automatically selected by Pro Group algorithm (at least one peptide with 99% confidence) for calculations of the reporter peak area, p-value etc. The resulting data set was auto bias-corrected to get rid of any variations imparted due to the unequal mixing during combining different labeled samples. A strict cut-off of unused ProteinScore ≥ 2, which corresponds to a confidence limit of 99%, and at least two peptides with 95% confidence were considered for protein identifications. False discovery rate (FDR) was calculated using in-house developed program as $FDR = 2 \times Md / (Md + Mt)$, where Md represents the number of decoy matches, and Mt is the number of target matches. Proteins identified with$\leq$1% FDR was further analyzed in this study.

Zymogram Development and Enzyme Assays

The zymographic analysis of test samples was performed using 10% SDS-PAGE gel that contained 1% carboxymethyl cellulose as described earlier [13]. In brief, SDS-PAGE gel was loaded with 10 μg of test protein and electrophoresis was performed at 90 V for 120 min. The gels were washed overnight with 50 mM phosphate buffer (pH 7.2±0.2) containing 25% isopropanol, incubated at 50 °C for 1 h and stained with 0.1% (w/v) Congo red for 15 min. The gel was washed with 1 M NaCl until bands became visible. The enzyme activities were determined on the supernatant samples using a protocol reported by Ghose [37]. The endo-1,4-β-glucanase, exo-1,4-β-glucanase, and β-glucosidase were assayed using carboxymethyl cellulose (CMC), Avicel, and salicin (β-salicyl alcohol glucoside) as substrates respectively. Xylan (from birchwood (Sigma, CAS: 9014-63-5) were used as substrate for xylanase activity. Reducing sugars were determined by using 3,5-dinitrosalicylic acid (DNSA)[38] One international unit (IU) of enzyme activity was defined as the amount of enzyme releasing 1μmol of reducing sugar per minute.

Results

This work focused on the secretome of *A. fumigatus* LF9 during cellulose, xylan and starch degradation following iTRAQ labeling. With a 99% confidence level (cut off of unused protein score ≥2.0) and false discovery rate of ≤1.0, 429±26 proteins were identified. These proteins were sorted using N-terminal Sec-dependant secretion signal using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) [39]. This study identified

some intracellular proteins, suggesting cell lysis or secretion through unknown mechanism. Based on the iTRAQ ratio distribution frequency and percentage variation of reporter ion intensities, the significance threshold for proteins with iTRAQ ratios ≤ 0.7 was set for down- and ≥1.4 for up-regulation. To gain biological insights into regulated proteins, the iTRAQ quantified proteins were functionally classified. The functional classification revealed 32.9, 0.9, 16.2, 8.1, 11.7, 7.6 and 22.5% cellulases and glycoside hydrolases, amylases, hemicellulases, lignin degrading enzymes, peptidases and proteases, chitinases,lipases and phosphatases, and proteins with unknown function respectively (Fig 1).

Abundance of cellulases and glycoside hydrolases

This iTRAQ based study quantified 73 proteins belonging to cellulase, glycoside hydrolase and amylases. Their comparative iTRAQ ratios were listed in Table1. The analysis of these proteins showed up-regulation of 27 (36.9%), 41 (56.1%) and 30 (41.1%) of 73 proteins when cellulose, xylan and starch were used as substrates respectively. When cellulose was used as a major substrate, enzymes like GH12, GH5 GH45 endoglucanase, (Afu7g06150, Afu5g01830, Afu7g06740), GH7 cellobiohydrolase D (Afu6g07070), GH3 beta-D-glucoside glucohydrolase (Afu7g06140), GH7 1,4-beta-D-glucan-cellobiohydrolyase (Afu6g11610), GH3 beta-glucosidase (Afu5g07080, Afu1g17410), GH30 beta-1,6-glucanase (Afu8g07120), GH15glucan1,4-alpha-glucosidase (Afu2g00690) were significantly up-regulated with iTRAQ ratios ranging between 7.05 and 1.81. Thus, analysis of the secretome of *A. fumigatus* LF9 revealed a significant up-regulation of celluloytic enzymes. Some enzymes like GH16 endo-1,3(4)-

beta-glucanase (Afu1g05290), GH16 cell wall glucanase (Afu6g08510) etc. were down-regulated when the strain was cultivated in cellulosic medium.

When cellulose was used as a main carbon source, iTRAQ ratios of GH12, GH5, and GH45 endoglucanase were 7.05, 6.57 and 3.96 respectively. Enzymes GH7 cellobiohydrolase D (Afu6g07070) and 1-4-beta-D-glucan-cellobiohydrolyase (Afu6g11610) were quantified with iTRAQ ratios of 3.97 and 3.29. GH3 beta-glucosidases (Afu7g06140, Afu5g07080 and Afu1g17410) were quantified with iTRAQ ratios between 3.34 and 1.81. Taken together, endoglucanases, exocellobiohydrolases and beta-glucosidases were significantly up-regulated when cellulose was used as a substrate, suggesting the cellulose degradation potential of this isolated strain. Cluster analysis of the proteins having cellulolytic activities showed five different clusters (Fig 2). Proteins clustered under cluster C3 and C5 were up-regulated when cellulose was used as a substrate.

When xylan was used as the main carbon source, enzymes like GH61, GH5 endoglucanase (Afu4g07850, Afu2g09520, Afu6g11600), GH7 endoglucanase (Afu6g01800), GH61 endo-1,4-beta-glucanase (Afu3g03870), GH5 glucanase, (Afu7g05610), GH71 alpha-1,3-glucanase (Afu2g03980), GH62, GH31 GH43,GH10, GH2, GH76 glycosyl hydrolase (Afu2g00920, Afu1g03140, Afu3g01660, Afu3g15210, Afu2g14520, Afu3g00340), GH3, GH1, GH3, GH35 beta glucosidase, (Afu6g08700, Afu5g07080, Afu1g14710, Afu1g05770, Afu1g17410, Afu1g16700), GH7 1,4-beta-D-glucan-cellobiohydrolyase (Afu6g11610), cellobiose dehydrogenase (Afu2g17620), GH72 1,3-beta-glucanosyltransferase (Afu2g05340) were significantly up-regulated, with iTRAQ ratios in the range of 6.86 to 2.02. Interestingly, these enzymes except GH7 1,4-

beta-D-glucan-cellobiohydrolyase (Afu6g11610) were not up-regulated when cellulose was use as a substrate. This suggests that xylan can stimulate the expression of cellulases and revealed the abundance level of enzymes as a function of substrate used. The cluster analysis of cellulolytic proteins as presented in Fig 2 suggested that the proteins clustered under cluster C2 were significantly up-regulated when xylan was used as a substrate. Enzymes like GH31, GH1 glucosidase, GH81 endo-1,3-beta-glucanase,GH76 glycosyl hydrolase were up-regulated when starch was used as a substrate.

Abundance of Hemicellulases

A particularly noteworthy observation is that 26 of the 36 iTRAQ quantified (or 72.2%) hemicellulases were significantly up-regulated when xylan was used as substrate. Fascinatingly, enzymes GH10 endo-1,4-beta-xylanase (Afu4g09480, Afu6g13610), GH11 endo-1,4-beta-xylanase (Afu3g00320, Afu3g00470), GH43 xylosidase (Afu8g04710), acetyl xylan esterase (Afu3g00420), GH43 xylosidase/arabinosidase, (Afu2g04480), alpha-L-arabinofuranosidase (Afu6g14620, Afu2g15160), alpha-galactosidase (Afu4g03580) GH3 beta-xylosidase (Afu3g02090), cell wall galactomannoprotein (Afu4g03240) GH62 arabinofuranosidase (Afu2g12770) were the most significantly up-regulated with iTRAQ ratios between 7.06 and 3.36, when xylan was used as a substrate (Table S1, supplementary). Again, with iTRAQ ratios ranging between 2.7 to 1.9, enzymes including carboxylesterase, (Afu3g09070), GH92 alpha-1,2-mannosidase (Afu6g13760), GH35 beta-galactosidase, (Afu1g14170) GH43 beta-xylosidase (Afu2g00930), GH2 beta-mannosidase (Afu6g08840), alpha-galactosidase (Afu6g02560), GH68 arabinosidase (Afu2g00650) were up-regulated in xylan containing culture condition (Fig 3 and 4). Thus, based on the abundance level and regulation of

hemicellulases, *A. fumigatus* LF9 has the potential to hydrolyze hemicellulose completely. The hierarchical clustering of iTRAQ quantified hemicellulolytic proteins demonstrated four different clusters. The proteins of cluster H3 were significantly expressed in the xylan containing culture media (Fig 5).

Hemicellulases such as GH28 extracellular polygalacturonase, (Afu1g17220), alpha-L-arabinofuranosidase (Afu6g14620), GH11 endo-1,4-beta-xylanase (Afu3g00320), GH10 extracellular endo-1,4-beta-xylanase,(Afu4g09480), acetyl xylan esterase (Afu3g00420), GH11 endo-1,4-beta-xylanase, (Afu3g00470) were up-regulated when cellulose was used as a substrate. The iTRAQ ratios of these enzymes were in the range of 8.53 to 1.51. Enzymes such as GH2 beta-mannosidase (Afu6g08840), GH47 alpha-mannosidase (Afu1g14560), GH92 alpha-1,2-mannosidase (Afu5g10520, Afu6g13760), GH43 xylosidase/arabinosidase (Afu2g04480), alpha-galactosidase (Afu6g02560), esterase (Afu5g09860), GH68 arabinosidase (Afu2g00650) were up-regulated when starch was used as a substrate. Thus, cellulose and starch can provoke expression of specific hemicellulases. Proteins clustered under cluster H1 were up-regulated when starch was used as a main carbon source.

Abundance of lignin degrading and other enzymes

Enzymes involved in lignin degradation such as laccase, isoamyl alcohol oxidase, alcohol dehydrogenase, bifunctional catalase-peroxidase and enzymes belonging to oxidoreductase family were identified in this study (Table S2, supplementary). Amylases that hydrolyze starch and pectate lyases that carried out eliminative cleavage of pectate yielding oligosaccharides with 4-deoxy-α-D-mann-4-enuronosyl groups at their non-

reducing ends were also iTRAQ quantified and regulated. The iTRAQ quantified amylases (Afu3g00900, Afu2g00710) were up-regulated only in starch containing medium and not in other substrate conditions, and their up-regulation suggested that amylases of *A. fumigatus* could have potential to degrade raw starch (Table S3, supplementary). The cellulolytic proteins of cluster C1 were up regulated in starch containing culture condition. Enzymes like GH5 endoglucanase (Afu2g09520) and dienelactone hydrolase (Afu6g12740) were significantly up-regulated when starch was main carbon source. This indicates that starch can stimulate expression of some specific cellulolytic enzymes.

Peptidases and protease like carboxypeptidase S1, aspartyl aminopeptidase, dipeptidyl peptidase, alkaline serine protease and aspartyl aminopeptidase were up-regulated when cellulose and xylan were used as a substrate. While enzymes like aminopeptidase, metallopeptidase, inorganic diphosphatase, aminopeptidase B, dipeptidyl peptidase III, dipeptidyl peptidase, aminopeptidase P, aspartyl aminopeptidase etc. were up-regulated when starch was used as a substrate. The enzymes like tripeptidyl peptidase A, aspartic endopeptidase, carboxypeptidase S1, tripeptidyl peptidase A, serine carboxypeptidase were down- regulated when starch were used as a substrate.

Zymograph and Enzyme Activities

As seen from zymogram (Fig 6B), some intense bands of proteins with cellulolytic activity were noted when SDS-PAGE containing CMC was used for cellulase activity. The prominent bands such as B3 were excised, tryptic digested and further analyzed by LC-MS/MS. The LC-MS/MS analysis confirmed abundance of endoglucanase (Afu7g06150), cell wall glucanase (Afu1g16190) endo-1,4-beta-xylanase (Afu3g00320) and several hypothetical proteins (Table S4, Supplementary). Enzyme

activities under different substrates suggested high endoglucanase and exoglucanase activity in cellulosic conditions, followed by xylan and then starch, while xylanse activity was higher in xylan culture condition (Fig 6A).

Post translational modifications

This study presents the deamidation of lignocellulolytic enzymes of thermophilic fungal strain *A. fumigatus* LF9. Based on the functional groups, 15% of identified cellulases and glycosyl hydrolases, 6% hemicellulases, 22% lignin degrading enzymes, 3% peptidases and proteases, and 2% of proteins with unknown function was deamidated. Based on the number of available deamidation sites in native protein and identified deamidation sites, the degree of deamidation varies from protein to protein. The list of modified enzymes, peptide sequences and sites of the modifications were listed in Table 2. The MS and MS/MS spectra, peptide sequence and modification sites are provided as supplementary information (MS and MS/MS spectra, and fragment ions assignment). The enzymes like endoglucanase (Afu7g06150|), glucanase (Afu7g05610|), exo-beta-1,3-glucanase (Afu6g13270|), endo-1,3-beta-glucanase (Afu1g04260|), glycosyl hydrolase (Afu2g00920|), cellobiohydrolase D (Afu6g07070|), endo-1,4-beta-xylanase (Afu3g00320|), alpha-mannosidase (Afu1g14560|), glucan 1,4-alpha-glucosidase (Afu2g00690|), beta-xylosidase (Afu3g02090|), isoamyl alcohol oxidase (Afu6g03620|), oxidoreductase (Afu2g14480|) etc. were detected with deamidation (Table 2). To elucidate the detailed biochemical functions of these PTMs and their impact on the fungal physiology and synthesis of these lignocellulolytic enzymes, a more detailed study with utilizing a high-throughput approach integrating proteomic data, metabolic

network analysis, protein structure analysis, and targeted genome engineering is required.

Discussion

Rapid and efficient enzymatic conversion of lignocellulosic biomass into fermentable sugars is a major challenge for the sustainable production of bio-chemicals and biofuels. The thermal pretreatment of biomass, followed by enzymatic treatment using thermotolerant enzymes could speed up the biomass hydrolysis rate and improve process efficiency. Hence, several researchers attempted to improve the thermotolerance of cellulases and hemicellulases by adopting techniques such as introduction of disulfide bridges, ionic pairs, tightly bound water molecules, protein packing and oligomerization [27]. Despite the improvement in the thermoactivity of these engineered cellulases and hemicellulases, their instability remains a major issue. These issues can be resolved by searching for naturally occurring thermostable plant biomass degrading fungi. The main advantages of thermotolerant enzymes from thermostable fungi for bioprocesses are their prolonged stability at room temperature, a reduced risk of process contamination, and an increased tolerance to organic solvents [40]. Our previous study [33] isolated *A. fumigatus* LF9 from compost and profiled its secretome at different temperatures between 20 and 70 ºC using saw dust as a substrate, and adopting solid state fermentation technique. The enzyme activities and protein abundances as determined by exponentially modified protein abundance index (emPAI) indicated the maximum activities at the range of temperature 40−60°C, demonstrating the thermophilic nature of this strain.

Characterization of the thermostability of the secretome suggested the thermotolerant nature of biomass degrading enzymes [33].

As of now, little work has been done on the proteomic analysis of the secretome of *A. fumigatus* and their applications in recycling of lignocellulosic biomass. *A. fumigatus* is pathogenic species but its potential biomass hydrolyzing enzymes could be useful if these enzymes are heterologously expressed in a GRAS (generally recognized as safe) organism and then used. Several strains of *Aspergillus* species are extremely useful in biotechnological industry and biomass recycling. Hence, the genomes of *A. clavatus* [41], *A. flavus* [42], *A.fumigatus* [43], *A. nidulans* [44], *A. niger* [45], *A. oryzae* [46], *A. terreus* etc. have been sequenced. Some *Aspergillus* species have been exploited for extracellular glucohydrolases secretion, which includes enzymes such as cellulases, hemicellulases, pectinases, and β-glucosidase [33, 47-51]. In this study, we profiled global view of the secretome of *A. fumigatus* LF9 in cellulose, xylan and starch containing culture media using iTRAQ-based quantitative proteomics. The efficient hydrolysis of lignocellulosic biomass requires several types of glycohydrolases, including different cellulases and hemicellulases. iTRAQ quantitative proteomics quantified 65 cellulolytic proteins, comparable to 65 proteins expressed in *T. reesei* but were higher than 52 in *P. chrysosporium* when cultivated in cellulosic media [12, 35]. The comparisons of cellulolytic enzymes encoded in the genome of *T. reesei* and *A. fumigatus* support a higher cellulolytic activity in *A. fumigatus* with 19 cellulases genes as compared to 10 in *T. ressei* [19]. Although the *P. chrysosporium* genome encodes 27 genes for cellulolytic enzymes [19] but, gene expression depends on carbon source and several other factors [9, 13]. The significant up-regulation of endoglucanases (GH12,

GH5 and GH45), cellobiohydrolase (GH7), beta-glucosidase (GH3) suggested potential candidates for lignocellulosic bioenergy. Two-dimensional gel and MALDI-TOF-MS analysis of the secretome of *A. oryzae* with wheat bran as a major carbon source demonstrated the abundance of 43 and 37 cellulolytic proteins in solid states and submerged cultures [52]. Further classification of these iTRAQ quantified 65 cellulolytic proteins revealed twenty endo-1-3(4)-beta-glucanase, six exo-glucanase, sixteen different glycosyl hydrolases, three alpha-glucosidases, six beta glucosidases, one beta-1-6-glucanase, one cellobiose dehydrogenase, and seven glucanosyltransferases.

Numerous processes during substrate utilization are governed not only by the relative abundance of proteins but also by their regulation, localization, protein-protein interactions and post translational modifications (PTMs). Glycosylation of secreted enzymes is one of the most frequent and abundant enzyme modifications and is well studied in *Aspergillus* sp., *T. reesei* and *Phanerochaete chrysosporium* [29, 32, 53]. According to Robinson [54, 55], protein deamidation serve as molecular clocks and plays a major role in protein turnover, organism development and aging. However, no literature exists on the presence and extent of fungal protein deamidation.

The impact of protein deamidation on their function and biomass hydrolysis potential requires more research that can integrate proteomic data, metabolic network analysis, protein structure analysis, and targeted genome engineering. It is essential to know which enzymes are deamidated and this study reports the deamidated enzymes. The three-dimensional structure of cellobiohydrolase I resolved by Divne et al [56] reveals a 40 angstrom long active site tunnel that plays a major role in its interaction with solid cellulose. Using this three dimensional structure, we found that deamidation sites

are located away from the enzyme active sites. Recently, it has been documented that low molecular weight monosaccharides or disaccharides produced by hydrolysis of cellulose and hemicellulose triggers enzyme production [57, 58]. However, in some cases, these compounds need to be further modified by glycosylation prior to functioning as physiological inducers [58]. Thus, PTMs play important role in biomass hydrolysing enzyme production and more focused research needs to be done. Also, the impact of deamidation and its role in secreted enzymes has not been documented.

Xylan belongs to a group of hemicelluloses and are abundant in hardwood and agricultural residues [59]. Xylan is almost as ubiquitous as cellulose in plant cell wall, so its fermentation is essential for the economic conversion of lignocellulose to ethanol [60, 61]. Interestingly, 60% (39 of 65) of iTRAQ quantified cellulolytic enzymes were up-regulated when xylan was used as a substrate. The enzymes belonging to GH families including GH7, GH62, GH43, GH5, GH31, GH71, GH1, GH61, GH5 etc. were up regulated and their iTRAQ ratios ranged between 6.12±0.74 and 2.68±1.76, when xylan was used as a substrate. Surprisingly, these cellulolytic enzymes were not up regulated when cellulose was used as a substrate. This indicates that cellulose and xylan induces the expression of some specific cellulolytic enzymes. The enhanced production of cellulases in xylan culture condition could be explained by the currently documented role of transcriptional activator (XlnR) in coordinating and controlling expression of both xylanolytic and cellulolytic enzymes [62, 63]. Current literature on cellulase and hemicellulases [57, 58] indicated that monosaccharides or disaccharides produced by hydrolysis of cellulose and hemicellulose triggers enzyme production. The saccharides derived from cellulosic biomass act as an inducer for cellulase and hemicellulases

production, e.g. D-xylose in *A. niger* [64], cellobiose in *A. oryzae* [65] and sophorose in *A. terreus* and *T. Reesei* [66]. When xylan was used as a substrate, 70.58% of iTRAQ quantified hemicellulases were up regulated. This significant up-regulation of hemicellulases could also be due to the thermotolerant nature of the enzymes secreted by this thermostable fungal strain because *Aspergilli* endoxylanases demonstrates the maximal activities at a range of 42°C–60°C, and a pH range of 4.0–7.0 [33, 67]. Xylanolytic enzymes have great biotechnological potential and are exploited for various industrial processes, including feed, food, pulp, and others [68-70]. Interestingly, enzymes like endo-1,4-beta-xylanase, beta-xylosidase, glucuronidase, arabinofuranosidase and acetylxylan esterases that act on different heretopolymers were expressed and significantly up-regulated when xylan was used as a substrate, indicating their potential use in lignocellulosic biorefineries. The most significantly up-regulated enzymes likes GH10 endo-1,4-beta-xylanase, GH11 endo-1,4-beta-xylanase, GH43 xylosidase, acetyl xylan esterase, GH43 xylosidase/arabinosidase, alpha-L-arabinofuranosidase, alpha-galactosidase, GH62 arabinofuranosidase could be potential enzymes for biomass degradation and hence more detailed and focused research is required on these enzymes. Beta-mannosidase and alpha-1, 2-mannosidase, which plays major roles in glucomannan degradation were also up-regulated when xylan was used as a substrate. Thus, taken together, xylanolytic enzymes from *A. fumugatus* LF9 could be exploited for several industrial processes including biomass hydrolysis.

Although GH10 and GH11 xylanases differ in produced xylo-oligosaccharides [67, 71], both were quantified with iTRAQ ratios of 7.58 and 6.9, showing a significantly higher abundance of these enzymes produced by *A. fumigatus* LF9. With 36

hemicellulase genes, *A fumigatus* has largest set of hemicellulases , as compared to *T. reesei* (16 genes), *Neurospora crassa* (19 genes) and *P. chrysosporium* (19 genes). Interestingly, when starch was used as a substrate, hemicellulolytic enzymes belonging to GH families GH2, GH92, GH43, GH47, GH43 etc. were up-regulated. Enzymes such as GH11 endo-1,4-beta-xylanase (Afu3g00320|), GH11 endo-1,4-beta-xylanase (Afu3g00470|), GH3 beta-xylosidase (Afu3g02090|), alpha-L-arabinofuranosidase (Afu6g14620|) and alpha-galactosidase (Afu4g03580|) that were quantified with iTRAQ ratios of 6.90±0.51, 4.49±0.98, 5.03±0.25, 6.18±0.54 and 6.17±0.72 respectively, were identified to be deamidated. The role of deamidation in secretory enzymes and their impact on biomass hydrolysis requires further detailed research.

In addition to cellulose and hemicellulose degrading enzymes, this study also identified and quantified lignin degrading enzymes like laccase, isoamyl alcohol oxidase, aryl-alcohol dehydrogenase, glutathione reductase, Cu-Zn superoxide dismutase and proteins of oxidoreductase family. Although laccase was iTRAQ quantified, but it was found to be down-regulated, indicating that the substrates like cellulose, xylan or starch could not induce its production. Further analysis of PTMs revealed the deamidation of laccase (Afu1g15670|), isoamyl alcohol oxidase (Afu6g03620|) and oxidoreductase (Afu2g14480|). Peptidases such as aspartyl aminopeptidase (Afu5g03990) and secreted dipeptidyl peptidase (Afu2g09030) were up-regulated in all studied substrate conditions. In xylan containing substrate conditions, 66.6% of identified peptidases and proteases were up-regulated while aspartic endopeptidase (Afu5g13300), serine carboxypeptidase (Afu6g00310) and tripeptidyl-peptidase (Afu4g03490) were down-regulated. Of these iTRAQ quantified peptidases, aminopeptidase (Afu4g09030|) and secreted dipeptidyl

peptidase (Afu2g09030|) were identified and found to be deamidated. In starch containing culture conditions, peptidases such as aminopeptidase (Afu4g09030), metallopeptidase (Afu7g05930), dipeptidyl peptidase III (Afu4g06140), aspartyl aminopeptidase (Afu5g03990) etc. were up-regulated. Of the 50 iTRAQ quantified proteins with unknown functions, 11, 26 and 23 proteins were up-regulated when cellulose, xylan and starch respectively were used as substrates, suggesting these enzymes contribute in biomass degradation. This study showed a significant up-regulation of cellulases, hemicellulases and lignin degrading enzymes of *A. fumugatus* LF9 in cellulose and xylan culture conditions. A previous study[33] of this strain (*A. fumugatus* LF9) in presence of saw dust as a substrate identified significant up-regulation of lignocellulolytic enzymes. Thus, *A. fumugatus* LF9 has the potential to degrade lignocellulosic biomass and can be exploited in lignocellulosic biorefinery.

Conclusions

Thermostable enzymes from thermophilic microbes have potential applications in the biotechnological industries and biomass hydrolysis. Proteomic characterization of the secretome of themophilic *A. fumigatus* LF9 in cellulose, xylan and starch culture conditions suggested the up-regulation of a large number of cellulases, hemicellulases, and lignin degrading proteins, demonstrating biomass degradation potential of this stain. The comparison of quantitative iTRAQ ratios suggests that cellulose and xylan causes expression and up-regulation of specific cellulases and hemicellulases. Post translational modifications analysis of the secretome of *A. fumigatus* revealed deamidation of key cellulases, hemicellulases and lignin degrading enzymes. The knowledge of deamidated

enzymes along with the specific sites of modifications could be crucial information for further functional studies of these enzymes in *A. fumigatus*.

Conflict of interest

None for this study.

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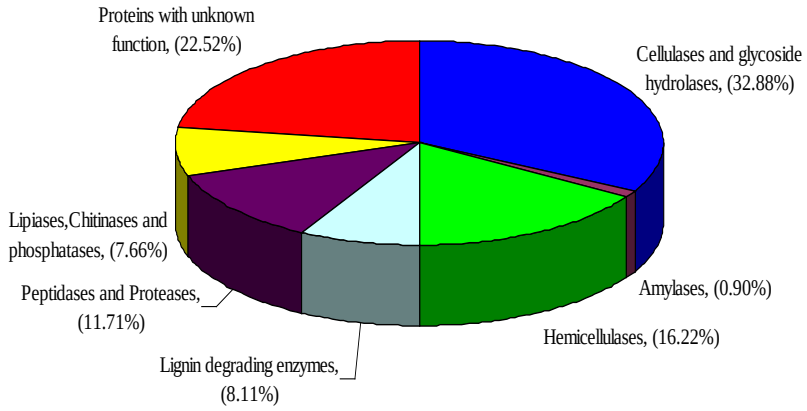

Figure 1. Functional classification of iTRAQ quantified proteins. The secretome of *A. fumigatus* LF9 in glucose, cellulose, xylan and starch containing culture media were characterized for protein composition.

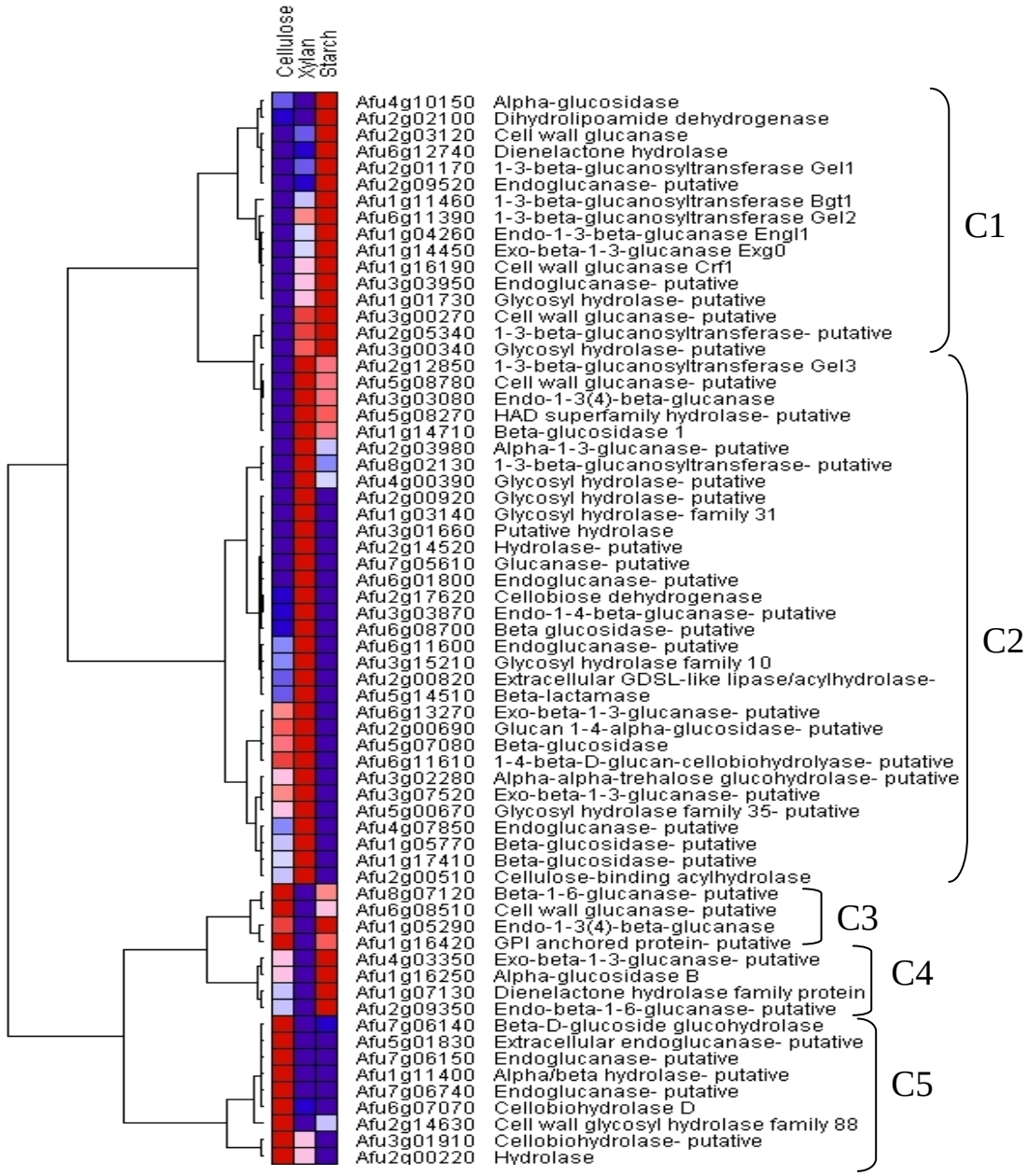

Fig 2. Hierarchical clustering of cellulolytic protein by *A. fumigatus* LF9, exhibiting their expressions when grown on cellulose, xylan and starch as the major carbon source. Up-regulated protein expression values are displayed in red, while the down-regulation values are in blue, and the intermediate values are in shades of red and blue.

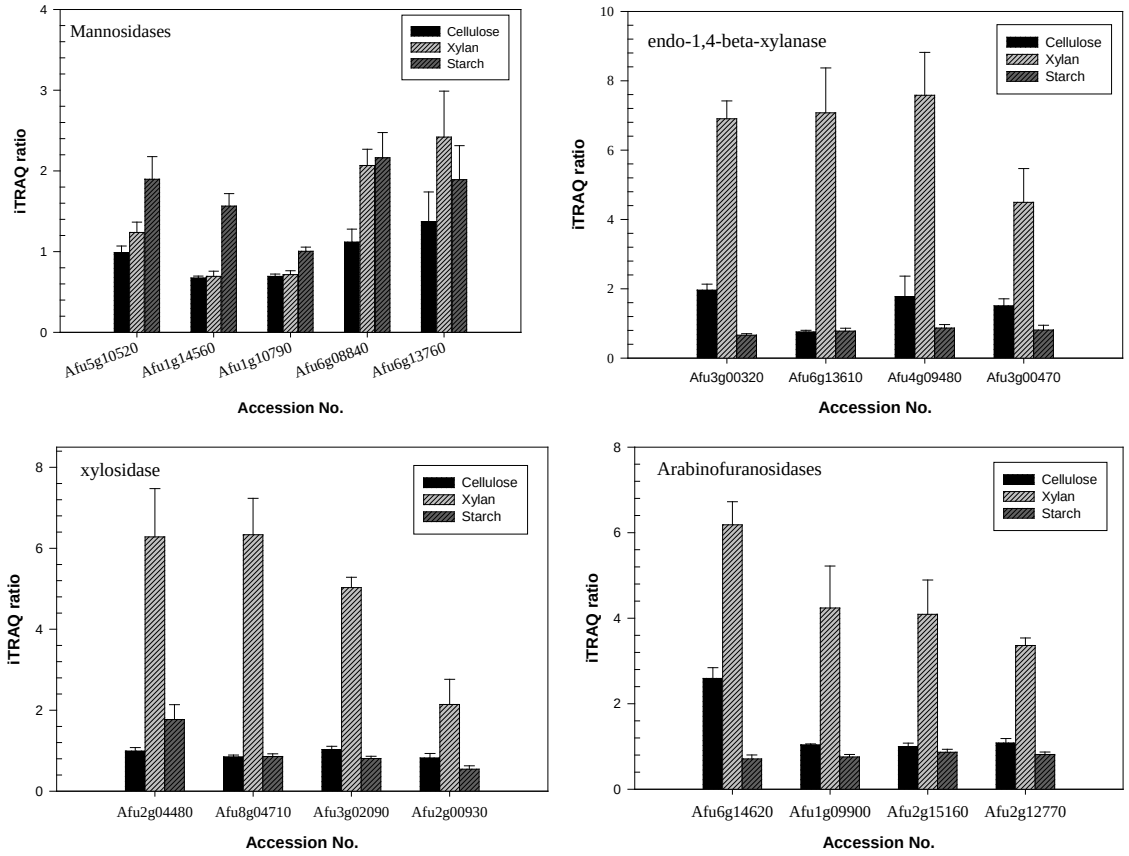

Figure3. Comparative abundance of different hemicellulose degrading enzymes of *A. fumigatus* LF9 in different carbon sources (Afu5g10520: alpha-1,2-mannosidase, Afu1g14560: alpha-mannosidase, Afu1g10790: alpha-1,2-mannosidase, Afu6g08840: beta-mannosidase, Afu6g13760: alpha-1,2-mannosidase, Afu3g00320: endo-1,4-beta-xylanase, Afu6g13610: endo-1,4-beta-xylanase, Afu4g09480: extracellular endo-1,4-beta-xylanase, Afu3g00470: endo-1,4-beta-xylanase, Afu2g04480: xylosidase, Afu8g04710: xylosidase, Afu3g02090: beta-xylosidase, Afu2g00930: beta-xylosidase, Afu6g14620: alpha-L-arabinofuranosidase, Afu1g09900: alpha-L-arabinofuranosidase, Afu2g15160: alpha-L-arabinofuranosidase A, Afu2g12770: arabinofuranosidase)

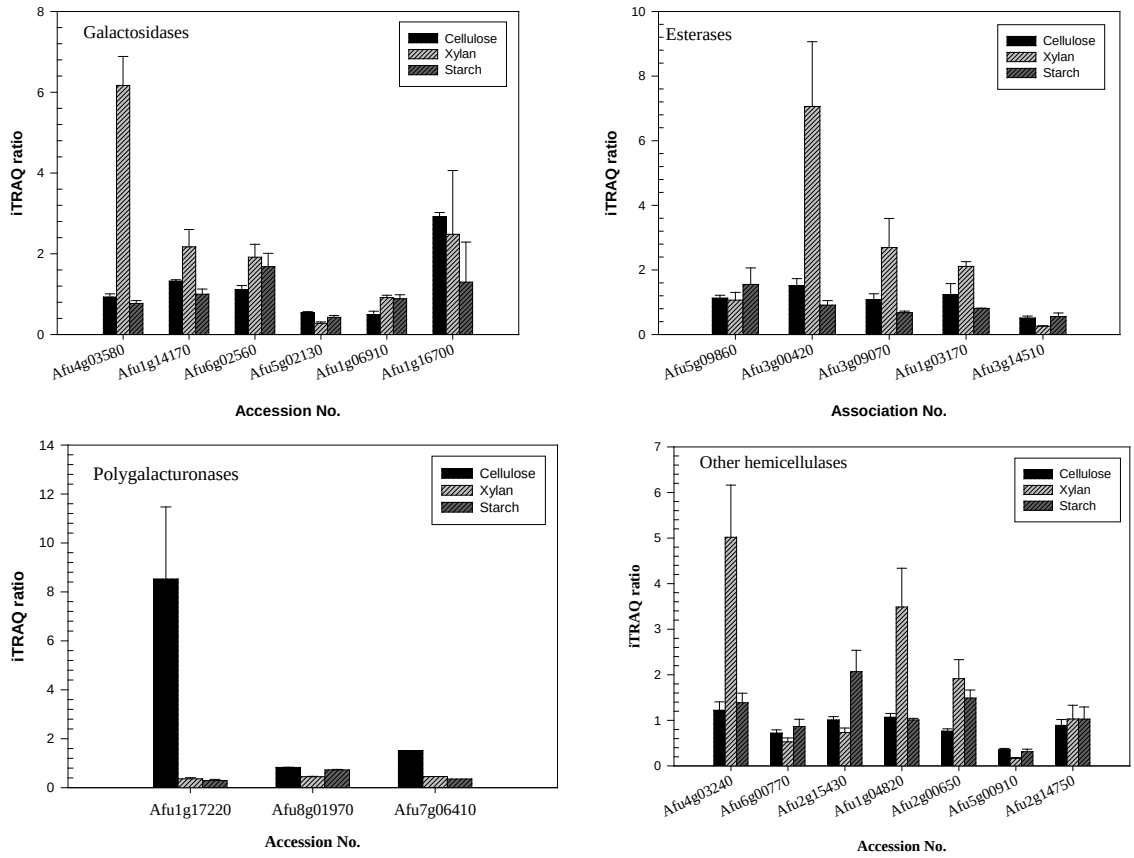

Figure 4. Comparative abundance of different hemicellulose degrading enzymes of *A. fumigatus* LF9 in different carbon sources (Afu4g03580: alpha-galactosidase, Afu1g14170: beta-galactosidase, Afu6g02560: alpha-galactosidase, Afu5g02130: alpha-galactosidase, Afu1g06910: arabinogalactan endo-1,4-beta-galactosidase, Afu1g16700: beta galactosidase, Afu5g09860: esterase,Afu3g00420: acetyl xylan esterase, Afu3g09070: carboxylesterase, Afu1g03170: esterase, Afu3g14510: rhamnogalacturonan acetylesterase, Afu1g17220: extracellular polygalacturonase, Afu8g01970: extracellular endo-polygalacturonase, Afu7g06410: exo-polygalacturonase, Afu4g03240: cell wall galactomannoprotein Mp1, Afu6g00770: extracellular arabinanase, Afu2g15430: L-xylulose reductase, Afu1g04820: D-xylose reductase (Xyl1), Afu2g00650: arabinosidase, Afu5g00910: mannose binding protein, Afu2g14750: endo-arabinase)

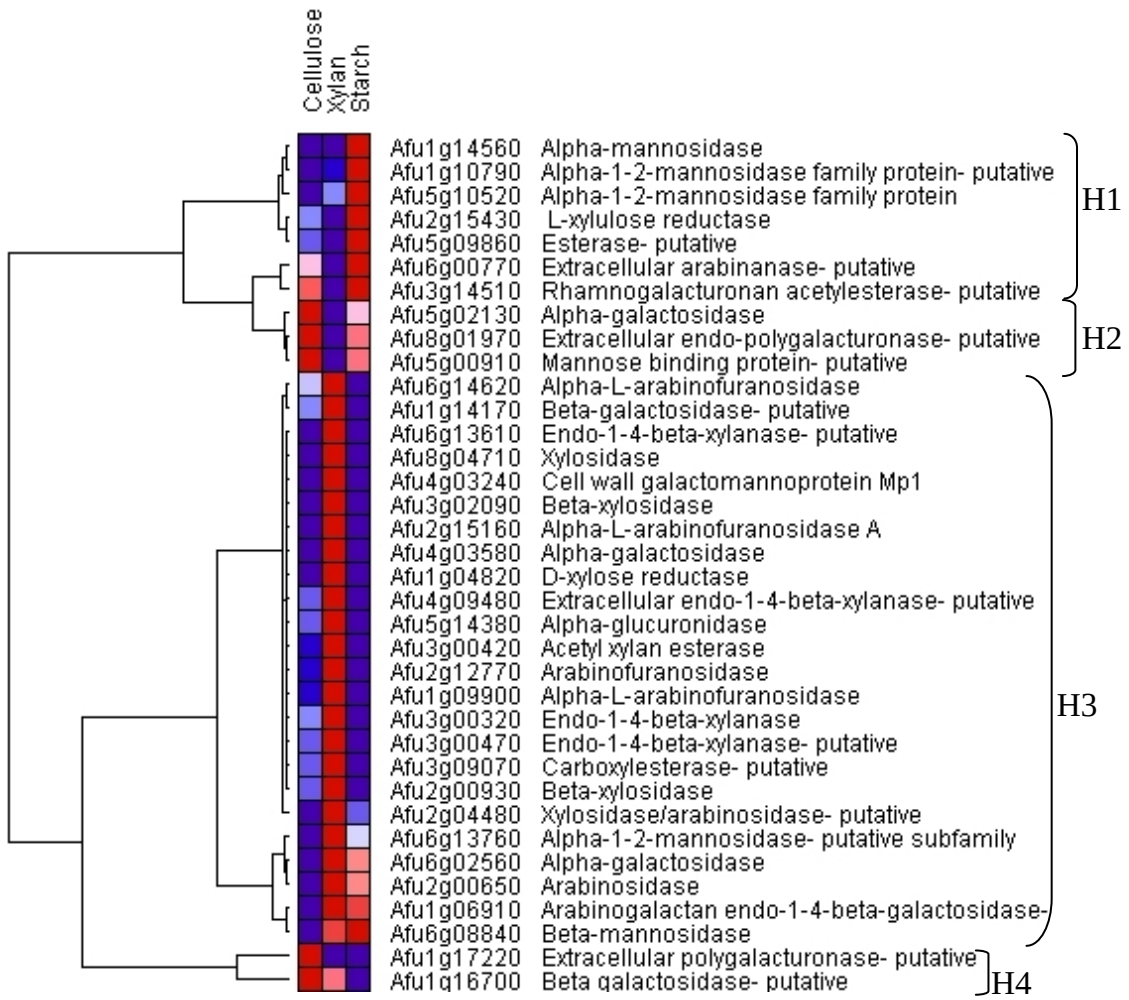

Fig 5. Hierarchical clustering of hemicellulolytic proteins by *A. fumigatus* LF9 exhibiting their expressions when grown on cellulose, xylan and starch as the major carbon source. Up-regulated protein expression values are displayed in red, while the down-regulation values are in blue, and the intermediate values are in shades of red and blue.

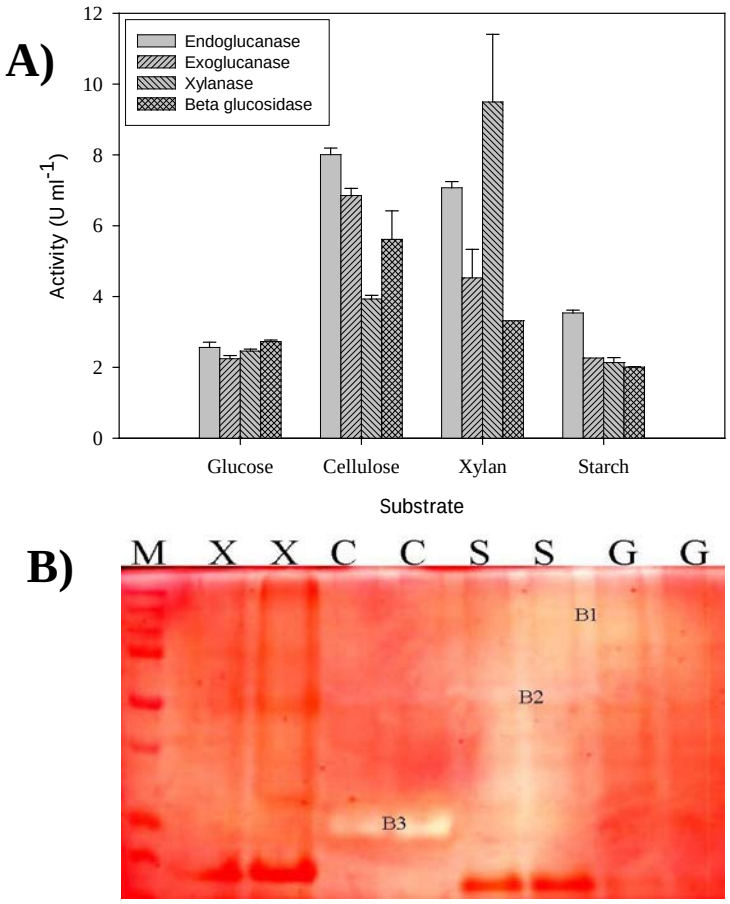

Fig 6. Actvities of enzymes in the secretome of *A. fumigatus* LF9 when grown in glucose, cellulose, xylan and starch culture media. A) Activities were determined by spectrometric methods; B) Zymogram showing cellulolytic activity in the secretome of A. fumigatus. SDS zymogram was performed using carboxymethyl cellulose as a substrate, stained with Congo red and destained by 1 M NaCl. The cellulase activity can be seen as a white band in zymogram analysis. The bands were further analyzed by LC-MS/MS. (M: Protein Marker, X: xylan, C: cellulose, S: starch and G: glucose)

Table 1. Comparative quantitative expression of cellulases, glycosyl hydrolases and amylases by *Aspergillus fumigatus* LF9 in different carbon sources

Unused Prot-Score	Total	% Cov	Accession No.	Name	Family	Peptide (95%)	Cellulose	Xylan	Starch
68.95±14.02	68.95±14.02	72.51±4.60	Afu2g00690	glucan 1,4-alpha-glucosidase, putative	GH15	123±45	1.84±0.03	1.95±0.15	1.39±0.11
65.34±8.92	7.15±2.28	33.21±18.28	Afu6g12740	dienelactone hydrolase family protein	NA	4±2	0.73±0.02	1.53±1.54	12.26±5.65
45.17±6.20	45.17±6.20	61.21±5.99	Afu3g00270	cell wall glucanase, putative	NA	77±25	0.52±0.03	0.95±0.11	0.98±0.07
43.24±6.92	43.24±6.92	54.88±4.44	Afu2g05340	1,3-beta-glucanosyltransferase, putative	GH72	48±14	0.82±0.06	2.02±0.58	2.14±0.64
42.74±6.79	42.74±6.79	64.97±4.24	Afu2g03980	alpha-1,3-glucanase, putative	GH71	59±18	0.97±0.05	4.18±0.41	1.97±0.22
39.27±7.95	39.27±7.95	49.06±6.31	Afu4g10150	alpha-glucosidase	GH31	33±10	1.34±0.22	1.06±0.23	3.14±0.70
37.28±6.38	37.28±6.38	40.86±3.81	Afu6g13270	exo-beta-1,3-glucanase, putative	PL3	49±15	1.21±0.03	1.37±0.15	0.83±0.09
35.43±3.57	35.43±3.57	81.84±2.93	Afu7g06150	endoglucanase, putative	GH12	80±26	7.05±1.00	0.53±0.05	0.50±0.04
34.88±7.94	34.88±7.94	63.98±7.70	Afu6g06770	enolase	NA	37±13	1.06±0.08	0.59±0.06	2.21±0.28
33.20±9.34	33.20±9.34	60.84±5.67	Afu2g01170	1,3-beta-glucanosyltransferase Gel1	GH72	34±11	0.59±0.01	0.68±0.07	1.31±0.12
32.96±6.95	32.96±6.95	56.80±8.47	Afu7g06140	beta-D-glucoside glucohydrolase	GH3	27±7	3.34±0.17	0.31±0.05	0.61±0.05
31.56±6.89	31.56±6.89	55.80±9.55	Afu7g05610	glucanase, putative	GH5	20±6	0.89±0.04	4.89±0.38	0.73±0.08
30.92±4.85	30.92±4.85	37.96±2.86	Afu1g04260	endo-1,3-beta-glucanase Engl1	GH81	35±13	1.31±0.11	1.91±0.32	2.58±0.23
24.03±3.79	24.03±3.79	55.44±2.04	Afu1g16190	cell wall glucanase Crf1	GH16	27±9	1.07±0.04	1.81±0.20	2.39±0.30
22.43±2.35	22.43±2.35	61.64±4.95	Afu1g11460	1,3-beta-glucanosyltransferase Bgt1	NA	23±6	0.56±0.02	0.86±0.10	1.44±0.10
22.20±6.11	22.20±6.11	57.38±9.21	Afu8g07120	beta-1,6-glucanase, putative	GH30	27±8	1.85±0.33	0.85±0.20	1.49±0.11
21.83±5.72	21.83±5.72	37.83±5.08	Afu6g11610	1,4-beta-D-glucan-cellobiohydrolyase, putative	GH7	24±10	3.29±0.06	3.60±0.55	0.54±0.05
21.32±3.08	21.32±3.08	48.66±8.98	Afu3g00900	alpha-amylase AmyA	NA	17±4	0.76±0.04	1.10±0.16	1.32±0.21
21.04±3.91	21.04±3.91	62.89±13.36	Afu5g09230	transaldolase	NA	17±4	0.96±0.03	1.70±0.21	4.02±0.64
20.32±4.13	20.32±4.13	37.59±6.08	Afu5g14380	alpha-glucuronidase	GH67	11±3	1.31±0.08	5.16±0.56	0.75±0.06
20.26±4.16	20.26±4.16	41.49±4.26	Afu2g17620	cellobiose dehydrogenase	CDH	16±5	1.03±0.08	3.34±0.32	0.84±0.08
19.10±2.19	19.10±2.19	35.35±2.20	Afu3g02280	alpha,alpha-trehalose glucohydrolase, putative	GH65	13±3	1.50±0.04	1.76±0.18	1.13±0.10
18.87±4.26	18.88±4.26	31.86±3.83	Afu2g00710	alpha-amylase, putative	NA	15±5	1.06±0.08	0.57±0.05	1.25±0.23

16.88±6.66	16.88±6.66	31.39±5.29	Afu1g14450	exo-beta-1,3-glucanase Exg0	PL3	10±3	0.77±0.13	0.98±0.10	1.24±0.15
16.29±3.14	16.29±3.14	45.99±7.16	Afu8g06880	pectin methylesterase	NA	24±7	0.31±0.02	0.27±0.04	0.16±0.03
15.13±2.52	15.13±2.52	50.95±7.37	Afu2g00920	glycosyl hydrolase, putative	GH62	14±4	0.86±0.17	5.85±0.71	0.89±0.11
15.01±3.19	15.01±3.19	44.78±5.06	Afu5g01830	extracellular endoglucanase, putative	GH5	10±3	6.57±0.25	0.64±0.07	0.49±0.04
13.75±2.26	14.12±2.27	42.65±7.79	Afu2g12850	1,3-beta-glucanosyltransferase Gel3	GH72	10±1	0.72±0.07	0.94±0.15	0.88±0.11
12.76±2.08	12.76±2.08	25.34±3.13	Afu2g03120	cell wall glucanase	NA	21±7	0.66±0.05	1.14±0.20	3.18±0.95
12.68±5.49	12.68±5.49	36.09±11.81	Afu3g00310	extracellular phytase, putative	NA	9±4	0.52±0.02	0.24±0.05	0.79±0.10
12.62±3.06	12.62±3.06	39.69±9.30	Afu2g00820	extracellular GDSL-like lipase/acylhydrolase,	NA	7±2	1.20±0.06	3.94±1.17	0.71±0.11
12.58±3.13	12.58±3.13	37.20±10.89	Afu5g07080	beta-glucosidase	GH3	8±2	1.92±0.34	2.22±0.61	0.81±0.13
12.47±3.77	12.47±3.77	33.70±3.85	Afu4g03350	exo-beta-1,3-glucanase, putative	PL3	8±3	0.52±0.04	0.40±0.04	0.61±0.04
12.29±1.13	13.29±2.08	28.84±3.97	Afu7g01240	phytase, putative	NA	16±4	0.97±0.04	0.60±0.09	2.37±0.51
11.97±3.09	11.97±3.09	57.31±8.74	Afu2g02100	dihydrolipoamide dehydrogenase	NA	5±2	1.00±0.08	0.78±0.06	3.01±0.76
11.64±1.41	11.64±1.41	31.80±2.38	Afu3g00340	glycosyl hydrolase, putative	GH76	9±1	0.74±0.04	2.07±0.28	2.27±0.25
11.44±2.89	11.44±2.89	38.28±3.55	Afu6g11600	endoglucanase, putative	GH5	6±2	1.31±0.09	3.11±0.51	0.82±0.14
10.05±1.34	10.05±1.34	50.78±5.43	Afu2g00760	pectate lyase A	NA	6±1	0.88±0.03	0.99±0.14	1.40±0.18
9.01±1.74	9.01±1.74	29.97±6.23	Afu1g03140	glycosyl hydrolase, family 31	GH31	5±1	0.89±0.10	4.61±1.18	0.86±0.11
8.96±2.75	8.96±2.75	38.07±2.89	Afu3g01660	putative hydrolase	GH43	9±3	0.89±0.10	5.33±0.80	1.01±0.21
8.66±2.65	8.66±2.65	29.37±14.92	Afu6g11390	1,3-beta-glucanosyltransferase Gel2	GH72	7±2	0.84±0.08	1.68±0.20	2.08±0.22
8.31±3.51	8.32±3.52	32.44±6.73	Afu6g08510	cell wall glucanase, putative	GH16	5±2	0.58±0.11	0.33±0.08	0.48±0.02
8.06±1.22	8.06±1.22	34.06±3.10	Afu1g05290	endo-1,3(4)-beta-glucanase	GH16	7±2	0.33±0.01	0.19±0.02	0.34±0.03
8.05±3.80	8.05±3.80	33.87±11.43	Afu5g08780	cell wall glucanase, putative	NA	5±2	0.94±0.13	1.42±0.22	1.32±0.25
8.02±1.43	8.02±1.43	38.30±8.20	Afu3g15210	Glycosyl hydrolase family 10	GH10	5±1	1.26±0.06	3.53±2.45	0.66±0.11
8.02±2.47	8.02±2.47	22.72±4.31	Afu3g03080	endo-1,3(4)-beta-glucanase	GH16	5±2	1.15±0.12	1.68±0.20	1.57±0.21
7.79±2.23	7.79±2.23	31.50±8.42	Afu3g01910	cellobiohydrolase, putative	GH6	5±2	1.01±0.36	0.76±0.43	0.43±0.20
7.71±2.04	5.39±2.03	22.91±11.56	Afu1g16700	beta galactosidase, putative	GH35	2±1	2.92±0.11	2.48±1.58	1.30±0.99
7.70±1.53	7.70±1.53	19.00±4.00	Afu8g02130	1,3-beta-glucanosyltransferase, putative	GH72	5±1	0.65±0.04	1.70±0.33	0.94±0.10
7.58±2.85	5.98±2.21	22.37±10.56	Afu4g07850	endoglucanase, putative	GH61	3±2	1.07±0.13	2.69±0.18	0.45±0.01
7.52±1.55	7.52±1.55	40.48±7.94	Afu6g08700	beta glucosidase, putative	GH3	4±1	1.30±0.13	4.10±0.47	1.10±0.12

7.42±3.13	7.42±3.13	24.67±5.11	Afu6g01800	endoglucanase, putative	GH7	5±2	1.05±0.06	6.12±0.74	0.78±0.16
7.30±2.20	7.30±2.20	31.50±7.51	Afu1g16420	GPI anchored protein, putative	NA	4±1	1.53±0.01	0.73±0.11	1.41±0.14
7.05±1.05	7.05±1.05	33.05±4.93	Afu1g07130	dienelactone hydrolase family protein	NA	5±2	0.78±0.03	0.57±0.15	1.13±0.12
6.43±1.42	6.43±1.42	29.06±3.11	Afu1g17410	beta-glucosidase, putative	GH3	3±1	1.81±0.19	2.88±0.47	0.97±0.08
6.35±1.60	6.35±1.60	26.61±8.92	Afu2g14520	hydrolase, putative	GH2	3±1	1.04±0.17	2.76±0.53	1.13±0.17
6.02±0.02	6.02±0.02	32.76±3.92	Afu5g14510	beta-lactamase	NA	3±0	0.62±0.05	1.43±0.24	0.50±0.04
6.00±0.84	7.51±1.41	24.01±3.58	Afu6g07070	cellobiohydrolase D	GH7	4±1	3.97±1.15	0.83±0.21	0.42±0.03
5.52±1.69	5.52±1.69	29.20±8.72	Afu5g08270	HAD superfamily hydrolase, putative	NA	3±1	1.58±0.68	1.68±0.39	1.66±0.41
4.27±0.86	4.27±0.86	38.39±4.65	Afu2g09350	endo-beta-1,6-glucanase, putative	GH5	2±0	0.82±0.06	0.68±0.11	1.08±0.14
4.27±1.10	4.27±1.10	27.09±4.95	Afu1g05770	beta-glucosidase, putative	GH3	2±1	1.36±0.21	2.99±1.23	0.61±0.15
4.09±1.54	4.09±1.54	24.72±5.62	Afu1g16250	alpha-glucosidase B	GH31	2±1	0.94±0.07	0.52±0.07	1.34±0.22
3.79±2.37	3.79±2.37	22.28±8.03	Afu4g00390	glycosyl hydrolase, putative	GH2	2±1	0.99±0.08	1.85±0.23	1.34±0.22
3.55±1.23	3.55±1.23	27.77±4.61	Afu2g00220	hydrolase	NA	2±1	1.32±0.22	1.05±0.15	0.71±0.15
2.80±1.45	3.60±1.40	11.03±4.45	Afu1g14710	beta-glucosidase 1	GH1	2±0	0.86±0.00	3.97±2.86	3.29±2.01
2.72±1.77	3.19±0.75	7.83±5.83	Afu3g03870	endo-1,4-beta-glucanase, putative	GH61	2±0	1.21±0.00	6.85±0.00	0.74±0.00
2.69±0.76	2.84±0.23	5.73±4.56	Afu2g00510	cellulose-binding GDSL lipase/acylhydrolase,	NA	2±0	0.91±0.08	1.68±1.11	0.45±0.00
2.67±1.57	3.34±0.95	13.29±3.13	Afu1g11400	alpha/beta hydrolase, putative	GH4	2±0	1.33±0.00	0.46±0.00	0.47±0.00
2.67±0.97	2.00±0.00	12.64±7.54	Afu5g00670	glycosyl hydrolase family 35, putative	GH35	2±0	1.10±0.21	1.53±0.11	0.66±0.13
2.6±1.27	2.69±0.95	4.89±2.75	Afu2g09520	endoglucanase, putative	GH5	2±0	0.77±0.00	2.68±1.76	16.64±6.12
2.49±1.23	3.38±1.63	12.23±4.72	Afu3g07520	exo-beta-1,3-glucanase, putative	PL3	2±1	0.89±0.00	1.12±0.00	0.52±0.00
2.27±0.16	2.09±0.00	11.46±3.25	Afu1g01730	glycosyl hydrolase, putative	GH76	3±0		0.92±0.00	1.54±0.00
2.00±0.00	2.28±1.00	12.17±4.16	Afu2g14630	cell wall glycosyl hydrolase family 88	NA	3±0	0.37±0.00	0.17±0.00	0.23±0.00
2.00±0.00	2.00±0.00	17.19±0.96	Afu7g06740	endoglucanase, putative	GH45	2±0	3.96±0.36	0.88±0.09	1.00±0.21
2.00±0.00	2.51±0.50	16.76±4.76	Afu3g03950	endoglucanase, putative	GH61	2±0	1.06±0.00	1.29±0.00	1.49±0.00

NA: Data not available

PL: Pectate lyase

Table 2. Deamidated enzymes and their modification with peptide sequence showing modification site (MS spectrum showing the precursor ion mass, and MS/MS spectrum showing peptide fragmentation and site of modification are provided in supplementary information)

Accession No.	Name	Peptide sequence and modification site
Afu7g06150|	Endoglucanase, putative (235 aa)	LVSQISSIPTTVQWSYDNTN*TR
Afu7g06150|	Endoglucanase, putative (235 aa)	N*HGYPASSQYLINMQFGTEPFTGGPATLR
Afu7g06150|	Endoglucanase, putative (235 aa)	VSQWTASVN
Afu7g05610|	Glucanase, putative (471 aa)	YSLLGEQ*LR
Afu1g05290|	Endo-1,3(4)-beta-glucanase, putative (357 aa)	NTAQDTGLIFAN*GAVYMGVDHTNVAGSSGR
Afu6g07070|	Cellobiohydrolase D (453 aa)	GSVVIDAN*WR
Afu6g13270|	Exo-beta-1,3-glucanase, putative (805 aa)	ATPGFTGLGLIDGDQ*YQGDGNQGWISTNVFFR
Afu1g04260|	Endo-1,3-beta-glucanase Engl1 (975 aa)	GN*LMLGILR
Afu8g07120|	Beta-1,6-glucanase, putative (489 aa)	FMPPGAIVLNGSGSYTYSGGGGIQSVASLNPDGTR
Afu8g07120|	Beta-1,6-glucanase, putative (489 aa)	WSGNAPSQ*SVTTWVLPSA
Afu3g00270|	Cell wall glucanase, putative (447 aa)	ANAGIGTN*PDEIVSYIN*EVR
Afu2g03980|	Alpha-1,3-glucanase, putative (497 aa)	WQEILN*LGPR
Afu2g00690|	Glucan 1,4-alpha-glucosidase, putative (632 aa)	ATALIDFGNWLIDNGYSSYAVN*NIWPIVR
Afu2g00690|	Glucan 1,4-alpha-glucosidase, putative (632 aa)	DLTWSYAAFLTAN*MR
Afu2g00690|	Glucan 1,4-alpha-glucosidase, putative (632 aa)	SVYAIN*SGIPQGAAVSAGR
Afu2g00920|	Glycosyl hydrolase, putative (397 aa)	SQTTLSNFPNGMGN*TVIALSDSNPNNLFEASNVYR
Afu3g00320|	Endo-1,4-beta-xylanase (XlnA), putative (229 aa)	GTVN*TDGGTYNIYTAVR
Afu3g00320|	Endo-1,4-beta-xylanase (XlnA), putative (229 aa)	LGMNLGTHNYQIVATEGYQSSGSASITVY
Afu3g00320|	Endo-1,4-beta-xylanase (XlnA), putative (229 aa)	TFTQ*YWSVR
Afu3g00470|	Endo-1,4-beta-xylanase, putative (314 aa)	GTVTSDGSTYDIYEHQQ*VNQPSIVGTATFN*QYWSIR
Afu3g02090|	Beta-xylosidase (772 aa)	HFAAYDLEDWN*GVVR
Afu1g10790|	Alpha-1,2-mannosidase (840 aa)	SIN*GYPLPGGAFVR
Afu1g14560|	Alpha-mannosidase (494 aa)	N*ATIVSQILDHIAK
Afu1g14560|	Alpha-mannosidase (494 aa)	N*GWGASAVDALSTAIVMR
Afu1g14560|	Alpha-mannosidase (494 aa)	AGFYVQ*SGAYILRPEVIESFYYAYR
Afu6g14620|	Alpha-L-arabinofuranosidase (507 aa)	GANAASGSLSTFYN*GAR
Afu4g03580|	Alpha-galactosidase (892 aa)	IGN*DIIPAWR
Afu1g15670|	Laccase (597 aa)	MILTN*GQFPAPPLYVR
Afu6g03620|	Isoamyl alcohol oxidase (572 aa)	HGLAADQVLEWEVIDGQ*GNFLVANR
Afu6g03620|	Isoamyl alcohol oxidase (572 aa)	SVVQTN*NAELTAAYR

Afu2g14480|	Oxidoreductase, FAD-binding, putative (474 aa)	GGANN*FGIVTNFIFR
Afu4g09030|	Aminopeptidase (954 aa)	LTFSGILN*DNMAGFYR
Afu4g09030|	Aminopeptidase (954 aa)	QGIDEN*TMLTER
Afu2g09030|	Secreted dipeptidyl peptidase (722 aa)	LPVAEGLSLFN*VLQER
Afu4g11800|	Alkaline serine protease Alp1 (404 aa)	ASFSNYGSVVDIFAPGQDILSAWIGSTTATN*TISGTSMATPH
Afu6g03150|	Hypothetical protein (431 aa)	DQTQYLQ*MWGR
Afu6g0315|	Hypothetical protein (431 aa)	DQTQ*YLQMWGR
Afu6g03150|	Hypothetical protein (431 aa)	DQ*TQYLQMWGR
Afu3g02600|	Hypothetical protein (281 aa)	AQAALGSN*ALVSSTVVAASR

Table S1.iTRAQ quantified hemicellulases from *A. fumigatus* LF9 when different substrates were used as a main carbon sources.

Unused	Total	%Cov	Accession No	Name	Family	Peptide (95%)	Cellulose	Xylan	Starch
81.23±14.68	81.23±14.68	85.24±6.27	Afu1g14560	alpha-mannosidase	GH47	170±49	0.67±0.02	0.69±0.06	1.56±0.15
68.00±12.26	68.00±12.26	80.81±5.88	Afu3g00320	endo-1,4-beta-xylanase	GH11	135±44	1.96±0.17	6.90±0.51	0.66±0.05
44.21±8.10	44.21±8.10	67.13±5.85	Afu6g13610	endo-1,4-beta-xylanase, putative	GH10	84±28	0.75±0.05	7.07±1.30	0.78±0.08
38.48±5.65	38.48±5.65	70.20±6.46	Afu3g02090	beta-xylosidase	GH3	39±11	1.03±0.08	5.03±0.25	0.80±0.06
30.92±7.41	30.92±7.41	78.16±8.06	Afu4g09480	extracellular endo-1,4-beta-xylanase, putative	GH10	29±10	1.77±0.59	7.58±1.24	0.87±0.10
28.76±7.42	28.76±7.42	48.12±8.38	Afu1g10790	alpha-1,2-mannosidase family protein, putative	GH92	19±7	0.69±0.03	0.72±0.05	1.00±0.05
25.71±5.00	25.71±5.00	49.95±5.47	Afu6g14620	alpha-L-arabinofuranosidase	NA	28±10	2.59±0.25	6.18±0.54	0.71±0.09
24.93±7.90	24.93±7.90	62.80±8.13	Afu6g00770	extracellular arabinanase, putative	GH43	41±16	0.72±0.07	0.53±0.09	0.86±0.16
20.32±2.51	20.32±2.51	40.24±8.27	Afu4g03580	alpha-galactosidase	NA	20±5	0.93±0.08	6.17±0.72	0.77±0.07
19.27±4.90	19.27±4.90	51.25±5.68	Afu5g10520	alpha-1,2-mannosidase family protein	GH92	12±4	0.99±0.08	1.24±0.13	1.90±0.28
18.81±4.50	18.81±4.50	35.45±6.98	Afu1g14170	beta-galactosidase, putative	GH35	12±3	1.32±0.03	2.17±0.43	1.00±0.13
18.58±4.39	18.58±4.39	53.82±11.16	Afu8g04710	xylosidase	GH43	14±4	0.85±0.04	6.33±0.90	0.86±0.07
18.41±1.71	18.41±1.71	68.05±6.85	Afu4g03240	cell wall galactomannoprotein Mp1	NA	22±5	1.22±0.18	5.02±1.14	1.39±0.21
15.10±2.38	15.11±2.38	51.56±8.20	Afu6g02560	alpha-galactosidase	NA	8±1	1.11±0.11	1.92±0.32	1.68±0.33
12.72±2.29	12.72±2.29	63.91±11.09	Afu2g15430	L-xylulose reductase	NA	15±4	1.01±0.07	0.73±0.10	2.07±0.47
12.31±2.21	12.31±2.21	36.62±5.69	Afu6g13760	alpha-1,2-mannosidase, putative subfamily	GH92	8±2	1.37±0.37	2.42±0.57	1.89±0.42
11.38±1.91	11.38±1.91	37.44±5.95	Afu5g02130	alpha-galactosidase	NA	8±3	0.54±0.02	0.28±0.03	0.43±0.05
11.18±4.43	11.18±4.43	51.75±11.91	Afu5g09860	esterase, putative	NA	6±3	1.13±0.09	1.06±0.24	1.55±0.51
10.40±2.90	10.40±2.90	40.15±7.09	Afu3g00420	acetyl xylan esterase	NA	8±2	1.51±0.22	7.06±2.01	0.91±0.14

10.02±3.16	10.42±3.54	44.43±9.34	Afu2g12770	arabinofuranosidase	GH62	6±2	1.08±0.10	3.36±0.18	0.81±0.06
9.18±2.66	11.33±2.71	36.74±5.92	Afu3g00470	endo-1,4-beta-xylanase, putative	GH11	10±4	1.51±0.20	4.49±0.98	0.81±0.14
8.77±3.15	8.77±3.15	36.06±9.26	Afu1g06910	arabinogalactan endo-1,4-beta-galactosidase,	GH53	5±2	0.50±0.08	0.91±0.06	0.88±0.10
8.70±3.25	8.70±3.25	29.35±3.63	Afu1g09900	alpha-L-arabinofuranosidase	NA	5±2	1.04±0.02	4.24±0.98	0.76±0.06
7.66±1.86	7.66±1.86	36.59±10.54	Afu2g15160	alpha-L-arabinofuranosidase A	NA	5±1	1.00±0.08	4.09±0.80	0.86±0.07
7.00±3.31	7.00±3.31	58.48±12.31	Afu3g14510	rhamnogalacturonan acetylesterase, putative	NA	4±2	0.51±0.06	0.26±0.02	0.56±0.11
6.60±2.24	6.60±2.24	27.08±2.94	Afu6g08840	beta-mannosidase	GH2	4±2	1.12±0.16	2.07±0.20	2.16±0.31
5.98±2.68	5.98±2.68	20.23±4.42	Afu2g04480	xylosidase/arabinosidase, putative	GH43	5±2	0.99±0.08	6.28±1.19	1.77±0.37
5.02±1.01	5.02±1.01	53.33±16.36	Afu1g04820	D-xylose reductase	NA	3±1	1.07±0.09	3.49±0.85	1.01±0.03
4.23±1.41	4.23±1.41	24.14±8.65	Afu2g00930	Possible beta-xylosidase, family 43 of glycosyl	GH43	2±1	0.82±0.11	2.14±0.62	0.54±0.08
3.88±1.33	3.88±1.33	28.47±5.03	Afu2g00650	arabinosidase	GH43, GH32, GH62, GH68	2±1	0.76±0.05	1.91±0.42	1.49±0.18
3.51±1.66	3.51±1.66	27.58±6.78	Afu1g17220	extracellular polygalacturonase, putative	GH28	2±1	8.53±2.94	0.36±0.04	0.29±0.05
3.51±1.67	3.51±1.67	28.23±10.04	Afu8g01970	extracellular endo-polygalacturonase, putative	GH28	2±1	0.83±0.01	0.44±0.02	0.72±0.02
2.63±1.51	2.63±1.51	27.38±6.56	Afu3g09070	carboxylesterase, putative	NA	2±1	1.08±0.18	2.69±0.90	0.68±0.05

NA: data not available

PL: Pectase lyase

Table S2. Lignin degrading enzymes, peptidases and proteases by *A. fumigatus* LF9 when different substrates were used as a main carbon sources.

Unused	Total	%Cov	Accession No.	Name	Peptide (95%)	Cellulose	Xylan	Starch
Lignin degrading enzymes								
13.95±3.54	13.95±3.54	32.25±5.81	Afu1g01180	isoamyl alcohol oxidase	9±3	1.17±0.08	1.24±0.23	1.45±0.21
20.69±4.39	20.69±4.39	50.48±7.37	Afu6g03620	isoamyl alcohol oxidase	31±10	0.80±0.01	1.09±0.10	1.29±0.11
42.64±5.93	42.64±5.93	73.38±7.29	Afu8g01670	bifunctional catalase-peroxidase Cat2	34±7	1.15±0.06	1.21±0.10	3.00±0.42
15.02±2.71	15.02±2.71	89.08±8.53	Afu5g09240	Cu,Zn superoxide dismutase	14±4	0.86±0.04	4.08±0.42	0.74±0.18
14.22±2.29	14.22±2.29	53.17±12.29	Afu2g14480	oxidoreductase, FAD-binding, putative	14±4	0.95±0.01	1.03±0.16	0.91±0.10
11.79±0.84	11.79±0.84	72.50±4.60	Afu1g14550	Mn superoxide dismutase MnSOD	9±2	1.08±0.10	0.75±0.08	2.17±0.24
10.00±0.73	10.00±0.73	57.17±8.98	Afu4g09110	cytochrome c peroxidase, putative	7±1	1.03±0.02	4.38±0.63	2.56±0.37
29.63±3.42	29.63±3.42	65.29±10.53	Afu3g00840	FAD-dependent oxygenase, putative	44±10	0.63±0.05	0.97±0.13	0.81±0.10
11.10±2.09	11.10±2.09	60.37±4.97	Afu4g11580	Mn superoxide dismutase	6±1	1.02±0.05	0.89±0.17	2.91±0.91
3.77±1.77	3.77±1.77	29.57±8.05	Afu1g15670	laccase	2±1	0.43±0.08	0.52±0.04	0.33±0.02
233.67±64.97	2.87±1.61	10.17±11.20	Afu7g05070	FAD dependent oxidoreductase, putative	2±0	1.09±0.00	2.67±1.52	7.27±5.87
10.53±4.52	10.53±4.52	39.87±8.66	Afu3g02270	mycelial catalase Cat1	5±2	1.83±0.05	2.83±0.39	0.74±0.11
2.01±0.00	227.51±25.50	18.48±6.47	Afu4g00610	aryl-alcohol dehydrogenase, putative	2±0	1.04±0.00	0.82±0.00	1.42±0.00
243.25±76.43	2.27±0.34	16.18±20.02	Afu1g15960	glutathione reductase	3±0	0.75±0.00	0.55±0.00	0.88±0.00
3.14±0.90	3.14±0.90	37.64±8.99	Afu1g15610	oxidoreductase,	2±1	1.54±0.12	1.00±0.19	1.05±0.19
2.98±0.73	2.98±1.73	34.70±17.35	Afu7g01010	alcohol dehydrogenase, putative	2±1	1.29±0.10	1.10±0.04	1.14±0.02

2.02±0.02	2.02±0.02	20.05±4.31	Afu2g04710	cytochrome b5, putative	2±0	2.13±0.64	4.18±1.44	1.06±0.29
Peptidases and Proteases								
58.93±12.65	58.93±12.65	62.59±8.26	Afu4g09030	aminopeptidase	48±14	1.01±0.04	1.47±0.16	3.31±0.31
36.78±11.98	36.78±11.98	60.05±5.90	Afu7g05930	metallopeptidase MepB	23±9	1.03±0.06	1.44±0.18	2.64±0.19
30.41±6.90	30.41±6.90	54.61±7.48	Afu4g06140	dipeptidyl peptidase III	22±6	1.05±0.06	1.35±0.15	2.27±0.25
29.95±5.32	29.95±5.32	71.09±4.54	Afu4g11800	alkaline serine protease Alp1	92±36	0.75±0.02	2.21±0.37	1.28±0.21
25.83±6.31	25.83±6.31	59.86±8.32	Afu5g08050	aminopeptidase P, putative	16±7	0.97±0.06	1.70±0.17	2.08±0.33
20.45±3.67	20.45±3.67	44.43±5.79	Afu5g03990	aspartyl aminopeptidase	14±4	1.63±0.32	2.07±0.21	1.74±0.32
26.25±6.38	26.26±6.38	50.00±4.73	Afu5g04330	aminopeptidase B	15±4	0.96±0.04	1.74±0.18	2.42±0.21
18.67±4.24	18.67±4.24	45.51±5.96	Afu5g13300	aspartic endopeptidase Pep1	12±4	1.13±0.06	0.52±0.03	0.64±0.05
21.43±5.53	21.43±5.53	42.09±6.52	Afu2g09030	secreted dipeptidyl peptidase	15±5	1.49±0.11	2.14±0.33	2.25±0.48
12.78±2.27	12.78±2.27	34.99±8.53	Afu5g01200	carboxypeptidase S1, putative	9±3	0.67±0.05	1.12±0.09	0.59±0.06
11.21±2.27	11.21±2.27	37.50±4.89	Afu4g03490	tripeptidyl-peptidase	7±2	0.32±0.00	0.09±0.01	0.09±0.01
19.49±1.57	19.49±1.57	38.71±2.39	Afu3g07030	glutaminase A	15±3	2.18±0.08	2.93±0.26	0.73±0.06
14.58±3.91	14.58±3.91	68.81±7.17	Afu3g08380	inorganic diphosphatase, putative	9±3	1.40±0.23	0.99±0.18	2.57±0.79
13.03±3.69	13.03±3.69	50.56±5.14	Afu7g04760	gamma-glutamyltranspeptidase	8±3	0.97±0.04	1.95±0.18	1.30±0.17
8.28±2.53	8.28±2.53	43.38±8.56	Afu3g08930	tripeptidyl peptidase A	4±1	0.62±0.06	0.61±0.02	0.57±0.03
11.75±2.83	11.75±2.83	40.30±7.49	Afu8g07080	elastinolytic metalloproteinase Mep	10±4	0.93±0.32	1.62±0.41	0.81±0.05
5.04±1.76	5.04±1.76	35.10±8.17	Afu2g01250	serine peptidase, family S28, putative	4±2	0.83±0.08	2.04±0.50	1.10±0.20
93.84±5.98	4.99±0.70	25.84±15.28	Afu6g06800	peptidase, putative	3±1	1.49±0.00	0.70±0.09	0.64±0.05
4.65±0.95	4.65±0.95	29.32±3.40	Afu6g00310	serine carboxypeptidase	3±1	1.15±0.09	0.51±0.05	0.38±0.09

3.43±0.80	3.43±0.80	23.88±5.62	Afu3g02970	aspergillopepsin, putative	5±2	5.89±0.06	0.27±0.03	0.23±0.01
7.09±4.24	7.09±4.24	36.11±8.78	Afu3g08290	aspartyl aminopeptidase	5±3	1.37±0.13	2.94±1.78	0.82±0.22
4.70±1.65	4.70±1.65	25.79±3.82	Afu8g04730	oligopeptidase family protein	2±1	0.82±0.24	4.83±1.71	1.07±0.38
2.19±1.70	2.38±0.27	10.82±12.06	Afu5g09210	alkaline serine protease Alp2	2±0	1.44±0.00	1.44±0.00	0.93±0.00
2.02±0.00	2.21±1.99	3.79±1.76	Afu4g14000	tripeptidyl peptidase A	2±0	1.29±0.00	0.79±0.00	0.66±0.00
2.70±1.65	4.06±1.67	24.29±12.92	Afu6g10250	alkaline serine protease AorO, putative	2±1	1.09±0.03	1.68±0.58	1.18±0.66
2.00±0.00	2.00±0.00	20.90±3.92	Afu8g04120	carboxypeptidase S1, putative	2±1	4.51±0.29	1.74±0.18	0.49±0.04

Table S3. iTRAQ quantified amylases, proteins involved in cell morphogenesis and hypothetical protein in different substrate conditions.

Unused	Total	%Cov	Accession No.	Name	Peptide (95%)	Cellulose	Xylan	Starch
Amylases								
18.87±4.26	18.88±4.26	31.86±3.83	Afu2g00710	alpha-amylase, putative	15±5	1.06±0.08	0.57±0.05	1.25±0.23
21.32±3.08	21.32±3.08	48.66±8.98	Afu3g00900	alpha-amylase AmyA	17±4	0.76±0.04	1.10±0.16	1.32±0.21
Lipiases, Chitinases and phosphatases								
4.26±1.70	4.26±1.70	37.72±5.90	Afu2g03110	alkaline phosphatase	2±1	1.06±0.24	5.89±0.79	1.11±0.21
7.51±2.61	7.51±2.61	45.18±12.80	Afu1g17250	conidial hydrophobin RodB	4±1	0.52±0.09	0.27±0.04	0.31±0.05
5.20±0.88	5.21±0.88	31.61±5.88	Afu8g00930	chitosanase, putative	3±1	0.38±0.01	0.35±0.06	0.50±0.04
8.66±3.66	8.66±3.66	36.87±6.02	Afu5g02040	extracellular lipase, putative	14±6	0.72±0.11	4.10±1.80	0.60±0.07
7.22±1.40	7.22±1.40	29.41±5.98	Afu1g03570	acid phosphatase PHOa	5±1	0.67±0.04	1.13±0.19	1.16±0.13
11.15±2.04	11.15±2.04	45.06±5.65	Afu4g01290	endo-chitosanase, pseudogene	13±4	1.15±0.04	0.23±0.04	0.60±0.06
10.57±3.17	11.45±3.23	50.81±14.57	Afu3g11280	class V chitinase, putative	9±4	1.13±0.19	2.61±0.73	3.19±0.46
7.97±1.50	7.97±1.50	44.88±8.22	Afu1g16480	acid phosphatase, putative	4±1	1.09±0.08	1.56±0.98	1.65±0.20
14.15±1.92	14.15±1.92	43.12±4.98	Afu3g07160	class V chitinase, putative	12±4	0.74±0.05	2.19±0.58	1.53±0.33
9.05±2.56	9.17±2.50	40.63±7.73	Afu1g07440	Hsp70 chaperone	5±1	0.96±0.02	1.35±0.18	3.16±0.34
15.18±4.17	15.18±4.17	52.04±9.63	Afu3g01530	phosphatidylglycerol specific phospholipase C,	20±6	1.28±0.06	1.84±0.30	2.47±0.46
17.55±3.60	17.55±3.60	47.34±3.18	Afu4g03660	acid phosphatase, putative	25±7	0.81±0.03	0.58±0.09	1.63±0.24
39.47±5.47	39.47±5.47	72.40±1.89	Afu8g01410	class V chitinase ChiB1	83±25	1.78±0.46	2.63±0.62	2.64±0.57
29.09±3.50	29.09±3.50	80.11±11.16	Afu6g07940	lactoylglutathione lyase	23±4	1.02±0.05	0.80±0.08	2.88±0.18
7.53±3.43	7.53±3.43	32.94±6.59	Afu3g14680	lysophospholipase Plb3	4±2	0.70±0.05	1.67±0.12	1.83±0.14

2.80±0.66	2.80±0.66	9.46±0.81	Afu5g03760	class III chitinase ChiA1	3±1	0.96±0.13	5.23±1.10	0.76±0.07
Hypothetical proteins								
3.19±1.17	3.19±1.17	15.03±5.07	Afu8g02510	hypothetical protein	2±1	2.68±0.20	5.34±0.88	0.79±0.09
6.24±3.08	6.24±3.08	55.56±9.87	Afu2g05720	conserved hypothetical protein	6±3	0.82±0.13	0.70±0.04	2.18±0.77
40.90±6.62	40.90±6.62	68.56±3.40	Afu2g05240	conserved hypothetical protein	64±20	0.65±0.06	0.29±0.02	0.51±0.05
36.89±8.46	36.89±8.46	46.39±3.20	Afu6g03150	hypothetical protein	44±15	0.98±0.05	5.66±1.07	0.78±0.06
24.40±4.32	24.40±4.32	81.67±5.49	Afu3g01130	hypothetical protein	17±5	0.84±0.02	3.16±0.24	1.29±0.07
24.65±2.01	24.65±2.01	59.55±4.11	Afu3g02600	hypothetical protein	35±9	0.49±0.05	1.59±0.14	0.38±0.04
20.79±5.77	20.79±5.77	48.58±7.94	Afu7g04680	conserved hypothetical protein	11±3	1.14±0.04	3.65±0.58	0.80±0.08
13.75±5.95	13.75±5.95	45.61±7.20	Afu5g01190	conserved hypothetical protein	8±3	1.84±0.35	3.98±1.60	0.61±0.07
15.01±0.96	15.01±0.96	64.34±9.28	Afu5g01120	conserved hypothetical protein	10±1	0.55±0.05	0.43±0.03	0.67±0.06
13.52±2.90	13.52±2.90	32.18±8.18	Afu3g07870	conserved hypothetical protein	9±2	1.47±0.16	2.28±0.34	0.93±0.08
11.88±2.27	11.88±2.27	42.38±10.45	Afu5g10930	hypothetical protein	12±4	0.53±0.05	0.53±0.05	1.08±0.13
9.29±3.09	9.29±3.09	34.26±9.01	Afu3g12690	conserved hypothetical protein	6±3	1.02±0.06	1.15±0.10	1.78±0.50
11.04±1.54	11.04±1.54	59.75±11.75	Afu5g14560	hypothetical protein	7±2	1.41±0.05	2.15±0.27	1.06±0.09
9.30±2.48	9.30±2.48	32.11±4.55	Afu6g06560	conserved hypothetical protein	6±1	0.97±0.08	1.95±0.45	0.81±0.11
11.36±4.17	11.36±4.17	26.77±4.14	Afu4g13990	hypothetical protein	21±6	0.40±0.06	0.83±0.07	0.73±0.06
5.96±1.93	5.96±1.93	18.83±3.60	Afu7g05340	conserved hypothetical protein	3±1	0.68±0.06	0.84±0.11	0.95±0.31
8.53±0.87	8.53±0.87	56.57±6.12	Afu2g17630	hypothetical protein	7±2	0.92±0.06	4.88±0.91	0.87±0.08
6.51±1.65	6.51±1.65	46.59±4.10	Afu5g01420	conserved hypothetical protein	4±1	1.02±0.33	0.75±0.13	0.91±0.13
5.80±1.81	5.80±1.81	47.76±11.07	Afu5g03520	hypothetical protein	3±1	0.94±0.05	1.14±0.10	1.05±0.21
5.32±0.77	5.32±0.77	57.11±20.77	Afu6g00180	hypothetical protein	16±6	0.66±0.04	1.21±0.13	1.82±0.18

9.03±1.92	9.03±1.92	31.32±3.36	Afu5g02100	hypothetical protein	6±1	1.05±0.07	3.21±0.53	2.96±0.50
6.48±2.16	6.48±2.16	31.56±4.72	Afu1g16430	hypothetical protein	4±1	1.34±0.24	1.42±0.42	2.32±0.57
6.09±1.44	6.09±1.44	57.80±13.78	Afu8g00430	hypothetical protein	5±1	1.01±0.33	0.93±0.33	1.66±0.56
5.75±0.74	5.75±0.74	31.41±6.26	Afu6g14470	hypothetical protein	3±0	1.71±0.17	7.23±2.00	1.18±0.16
5.00±1.00	5.00±1.00	27.40±3.51	Afu1g09960	conserved hypothetical protein	4±2	1.21±0.37	0.85±0.06	2.50±0.28
7.38±2.20	6.07±1.62	18.82±9.49	Afu3g03610	hypothetical protein	3±1	0.94±0.04	1.06±0.27	1.14±0.28
4.15±1.26	4.15±1.26	50.30±9.53	Afu4g08450	conserved hypothetical protein	4±1	0.51±0.07	0.54±0.10	0.47±0.05
4.00±0.00	4.00±0.00	18.73±3.65	Afu6g02010	hypothetical protein	4±0	0.98±0.13	5.24±0.60	1.39±0.11
4.04±0.04	4.04±0.04	35.44±3.10	Afu3g00960	conserved hypothetical protein	3±1	0.96±0.09	2.07±0.34	2.30±0.45
4.04±1.39	4.04±1.39	57.18±12.84	Afu4g08310	conserved hypothetical protein	3±1	0.66±0.05	0.74±0.07	1.32±0.18
3.74±1.99	3.74±1.99	23.28±13.73	Afu3g10390	conserved hypothetical protein	2±1	1.13±0.22	1.05±0.15	1.58±0.55
5.00±1.73	5.00±1.73	18.99±3.91	Afu6g14400	conserved hypothetical protein	3±1	1.47±0.01	1.25±0.12	0.87±0.10
3.44±0.60	3.44±0.60	39.15±6.72	Afu1g02290	conserved hypothetical protein	4±2	0.98±0.10	1.19±0.23	1.52±0.26
5.00±1.00	5.00±1.00	62.05±11.48	Afu3g14940	hypothetical protein	3±1	0.94±0.14	1.05±0.10	0.90±0.09
9.28±3.30	9.28±3.30	50.87±12.89	Afu2g15770	conserved hypothetical protein	5±2	1.07±0.12	1.90±0.38	2.48±0.56
3.62±0.56	3.62±0.56	24.72±3.98	Afu2g15420	hypothetical protein	3±1	1.20±0.12	1.03±0.23	0.56±0.08
6.24±2.33	6.24±2.33	50.36±2.57	Afu4g02840	conserved hypothetical protein	3±2	1.02±0.15	0.71±0.14	1.57±0.31
8.50±34.70	3.67±0.85	12.16±5.44	Afu8g00630	conserved hypothetical protein, putative	2±1	1.57±0.64	4.12±2.52	3.07±2.72
3.10±1.02	3.10±1.02	32.34±8.54	Afu2g15710	hypothetical protein	2±1	1.02±0.10	1.79±0.50	1.91±0.64
2.76±0.44	2.00±0.00	11.30±13.15	Afu2g09140	conserved hypothetical protein	2±0	1.02±0.00	1.65±0.00	1.63±0.00
2.06±0.05	2.10±0.09	29.09±8.16	Afu2g01840	conserved hypothetical protein	2±0	1.62±0.50	1.40±0.36	3.10±0.87
3.50±0.87	3.50±0.87	28.33±2.92	Afu6g11720	hypothetical protein	2±0	1.03±0.25	1.20±0.15	1.37±0.21

2.12±0.41	2.12±0.41	14.00±6.15	Afu5g14420	hypothetical protein	2±0	0.49±0.08	0.65±0.08	1.51±0.13
3.41±1.41	3.41±1.41	20.87±5.92	Afu1g05270	conserved hypothetical protein	4±1	1.05±0.09	2.46±0.31	1.17±0.13
2.00±0.00	2.00±0.00	31.39±12.63	Afu6g10840	hypothetical protein	2±0	0.88±0.09	1.90±0.33	5.26±1.22
2.00±0.00	2.00±0.00	22.38±14.72	Afu2g12000	conserved hypothetical protein	2±1	0.51±0.01	0.31±0.04	0.55±0.07

Table S4. The band showing cellulolytic activity was cut, digested and analyzed by LC-MS/MS. The proteins identified in the band are listed below along with their mass, protein score, emPAI and Peptides detected.

Accession	GS	Name	Pro_Mass	Prot_score	emPAI	Peptides
Afu3g00320	Afu3g00320	endo-1,4-beta-xylanase (XlnA), putative (229 aa)	24536	1111	2.16	42
Afu7g06150	Afu7g06150	endoglucanase, putative (235 aa)	25883	742	2.37	33
Afu1g16190	Afu1g16190	cell wall glucanase Crf1 (396 aa)	40487	132	0.27	9
Afu3g02970	Afu3g02970	aspergillopepsin, putative (270 aa)	28225	52	0.25	3
Afu1g07300	Afu1g07300	tetracycline transporter-like protein, putative (461 aa)	49371	45	0.07	1
Afu1g09060	Afu1g09060	conserved hypothetical protein (513 aa)	57133	43	0.06	2
Afu2g03620	Afu2g03620	oxidoreductase, short-chain	53366	42	0.06	2
Afu3g12510	Afu3g12510	vesicular fusion ATPase, putative (771 aa)	84710	41	0.04	4
Afu4g02960	Afu4g02960	UDP-sugar hydrolase, putative (397 aa)	43996	39	0.08	1

Afu3g09300	Afu3g09300	conserved hypothetical protein (168 aa)	17248	29	0.2	3
Afu5g12560	Afu5g12560	hypothetical protein (733 aa)	83681	26	0.04	2
Afu1g15080	Afu1g15080	hypothetical protein (620 aa)	69179	26	0.05	1
Afu7g06780	Afu7g06780	hypothetical protein (235 aa)	26238	26	0.13	1
Afu4g03730	Afu4g03730	dienelactone hydrolase (493 aa)	54642	25	0.06	3
Afu5g00230	Afu5g00230	hypothetical protein (583 aa)	64230	25	0.05	2
Afu1g10610	Afu1g10610	hypothetical protein (175 aa)	19842	24	0.17	2
Afu6g12310	Afu6g12310	conserved hypothetical protein (300 aa)	33701	24	0.1	1
Afu3g12340	Afu3g12340	conserved hypothetical protein (693 aa)	75736	23	0.04	3
Afu1g06060	Afu1g06060	hypothetical protein (1503 aa)	164339	23	0.02	2
Afu2g04140	Afu2g04140	conserved hypothetical protein (1560 aa)	171043	23	0.02	4
Afu1g12200	Afu1g12200	conserved hypothetical protein (313 aa)	35110	23	0.09	1

Afu4g13050	Afu4g13050	hypothetical protein (485 aa)	54219	21	0.06	2
Afu4g11600	Afu4g11600	hypothetical protein (845 aa)	94168	21	0.03	2
Afu4g08030	Afu4g08030	conserved hypothetical protein (625 aa)	69539	19	0.05	2
Afu2g04920	Afu2g04920	conserved hypothetical protein (1467 aa)	154207	18	0.02	1
Afu2g05940	Afu2g05940	conserved hypothetical protein (370 aa)	40602	18	0.08	1
Afu6g07380	Afu6g07380	conserved hypothetical protein (634 aa)	65757	18	0.05	1
Afu2g10690	Afu2g10690	MFS phosphate transporter, putative (667 aa)	75128	17	0.04	1
Afu2g09270	Afu2g09270	hypothetical protein (409 aa)	45569	16	0.07	1
Afu2g14260	Afu2g14260	conserved hypothetical protein (960 aa)	102846	16	0.03	1