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Quantitative proteomic analysis of lignocellulolytic enzymes by *Phanerochaete chrysosporium* on different lignocellulosic biomass

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ABSTRACT

Lignocellulosic biomass from agricultural crop residues and forest waste represents an abundant renewable resource for bioenergy and future biofuel. The current bottleneck of lignocellulosic biofuel production is the hydrolysis of biomass to sugar. To understand the enzymatic hydrolysis of complex biomasses, in this report, lignocellulolytic enzymes secretion by *Phanerochaete chrysosporium* cultivated in different natural lignocellulosic biomass such as corn stover, hay, sawdust, sugarcane baggase, wheat bran and wood chips were quantitatively analyzed with the iTRAQ technique using LC-MS/MS. A diverse groups of enzymes, including cellulases, glycoside hydrolases, hemicellulases, lignin degrading enzymes, peroxidases, esterases, lipases, chitinases, peptidases, protein translocating transporter and hypothetical proteins were quantified, of which several were novel lignocellulosic biomass hydrolyzing enzymes. The quantitative expression and regulation of lignocellulolytic enzymes by *P. chrysosporium* were dependent on the nature and complexity of lignocellulosic biomass as well as physical size of the biomass. The iTRAQ data revealed oxidative and hydrolytic lignin degrading mechanism of *P. chrysosporium*. Numerous proteins presumed to be involved in natural lignocellulosic biomass transformation and degradation were expressed and produced in variable quantities in response to different agricultural and forest wastes.

1. Introduction

Lignocellulose, a complex polymer constituting cellulose, hemicellulose and lignin is abundant, renewable and attractive low cost feedstock that could be hydrolyzed into sugars for subsequently fermenting into biohydrogen or bioethanol. The present bioethanol production technology utilizes maize starch, sugarcane juice or molasses as substrates, however, the use of food sources increases the production cost [1]; and affects the food supply and socioeconomics. A sustainable alternative is the conversion of low cost forest and agricultural wastes into biofuels to replace petroleum fuel for mitigating global warming and minimizing environmental pollution. According to Sanchez and Cardona [2], the prospective

Abbreviations: ACN, acetonitrile; ATCC, American Type Culture Collection; CBQ, cellobiose-quinone oxidoreductase; CDH, cellobiose dehydrogenase; CMC, carboxymethyl cellulose; DAN, 1,8-diaminonaphthalene; FDR, false discovery rate; GLX, glyoxal oxidase; GST, glutathione S-transferase; iTRAQ, isobaric tag for relative and absolute quantification; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LiP, lignin peroxidase; MMTS, methyl methanethiosulfonate; MnP, manganese-peroxidase; PAGE, polyacrylamide gel electrophoresis; TCEP, tris 2-carboxyethyl phosphine hydrochloride.

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lignocellulosic materials that can be hydrolyzed into sugars include crop residues, hardwood, softwood, cellulosic waste, herbaceous biomass and municipal solid wastes. Several researchers used different feedstock with pretreatments for the ethanol production and their work has been summarized in several reviews [2–5].

Numerous microbial strains from fungal and bacterial domains have the potential to degrade cellulose, hemicellulose and pectin [6–9]; while lignin is degraded predominantly by fungi [9]. According to Martinez et al. [9], *P. chrysosporium* genome encodes more than 240 carbohydrate active enzymes that include 166 glycoside hydrolases, 14 carbohydrate esterases and 57 glycosyltransferases; while its lignin depolymerizing enzymes includes lignin peroxidase encoded by ten *lip* genes, manganese peroxidase (five *mnp* genes) and multicopper oxidases [10]. Its several hydrolytic and oxidative enzymes have been individually isolated and purified [11,12]; while expressions of lignocellulolytic proteins on commercial cellulose and lignin were also explored [13]. The comparative gene expressions on Aspen and *Pinus strobus* by *P. chrysosporium* and *P. placenta* have been documented [14]. The enzyme production depends upon the carbon source and little is known about comprehensive enzyme secretion and their expression profile on natural lignocellulosic biomass including different agricultural wastes.

The structural complexity and proportion content of lignin, cellulose, and hemicellulose vary significantly with type of agricultural/forest residues. The red oak wood chips [15], glucose, cellulose and wood chips [16], softwood chips [17], benzoic acid [18] as a carbon source have been tested and *P. chrysosporium* secreted proteins were separated by using 2D gel electrophoresis, however, only a few proteins (between 45 and 79) were identified which could be due to the technical limitations of 2D gel electrophoresis [19–22]. The advances in genome sequencing and proteomics technology provided comprehensive identification and assessment of proteins that contribute in systematic understanding at the molecular level. However, most of the previous studies on *P. chrysosporium* enzyme secretion used traditional colorimetric methods, hence comparative secretome that may eventually lead to the discovery of novel enzymes involved in lignocellulosic biomass hydrolysis is still lacking. The secretory protein profile of *P. chrysosporium* varies with different crop residues due to structural complexity, degree of lignification and variable contents of cellulose, hemicellulose and lignin. In an iTRAQ technique, iTRAQ reagents are designed as an isobaric tags consisting of a charged reporter group that is unique to each of the four or eight reagents; a peptide reactive group; and a neutral balance portion to maintain an overall mass of 145. It can be used for multiplexing of up to four or eight different samples [19–21]. These unique reagents, upon MS/MS fragmentation give rise to four or eight unique reporter ions (m/z=114–117 or m/z=113–121) that are used to quantify their respective samples. Therefore, the objective of this study was to identify, quantify and compare the secretory proteins of *P. chrysosporium* involved in natural lignocellulosic biomass hydrolysis by high throughput quantitative iTRAQ-based LC-MS/MS proteomics approach under corn stover, hay, sawdust, sugarcane baggase, wheat bran and wood chips as the carbon source respectively.

2. Experimental procedures

2.1. Microorganism cultivation conditions and secretome extraction

The *P. chrysosporium* strain (ATCC 20696) procured from the American Type Culture Collection was grown in malt extract (250 ml) by inoculating the pellet (approximately 0.2 g) received from supplier (ATCC). The cell mass was collected by centrifugation, washed with sterilized MilliQ water and inoculated into medium having composition of 2.6 g/L $(NH_4)_2SO_4$; 2.2 g/L 2,2 dimethylsuccinic acid; 1.1 g/L KH_2PO_4; and micronutrients 0.500 g/L $MgSO_4.7H_2O$; 0.010 g/L $FeSO_4.7H_2O$; 0.005 g/L $MnSO_4.H_2O$; 0.074 g/L $CaCl_2.2H_2O$ 0.001 g/L $COCl_2$; 0.1 g/L thiamin. The cell mass was collected and used to inoculate six separate test flasks (500 ml) containing basic media with 10 g/L agricultural waste/crop residues (corn stover, sugarcane baggase and wheat bran), forest wastes (hay, wood chips) and sawdust, respectively. The basic media containing glucose with strain served as a control. The cultures were incubated at 37 °C, 80 rpm and culture supernatants from each test flask were collected at exponential phase (120 h) by centrifugation at 10,000 ×*g* at 4 °C (Beckman Coulter, USA). The experiment was performed for three times and mean with standard deviation were reported. The secretome was further clarified by filtration through 0.2 μm filter. The proteins in the supernatants were concentrated by lyophilization technique, suspended in 100 mM acetate buffer and protein contents were quantified using the 2D Quant kit (Amersham Biosciences, USA) according to the manufacturer's protocol.

2.2. Protein digestion and iTRAQ labeling

The protein digestion, peptide extraction and spectrometric analysis were performed as described previously [23–25]. In brief, 150 μg proteins from each sample was loaded on SDS-PAGE (12% polyacrylamide) and run for 30 min at 100 V. Each lane was sliced separately, cut into small pieces (approximately 1 mm^2), and processed separately. After washing with 50% acetonitrile containing 25 mM TEAB (Sigma, USA) gel pieces were reduced with TCEP (5 mM) and then alkylated by MMTS (10 mM). The dehydrated gel pieces were subjected to sequencing grade modified trypsin (Promega, Madison, WI) digestion in 50 mM TEAB buffer at 37 °C for overnight. The peptides were extracted using 50% ACN/5% formic acid, and then dried with vacuum centrifuge for subsequent iTRAQ reagent labeling.

The iTRAQ labeling of peptides obtained from test samples with different agricultural waste as a carbon source were performed using 8-plex iTRAQ reagent Multiplex kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. The iTRAQ labeled peptides were fractionated using a WAX column (200×4.6 mm, 5 μm particle size, 300 Å pore size) by a HPLC system (Shimadzu, Japan) at flow rate 1.0 ml/min as described previously [23–25].

2.3. LC-MS/MS analysis

The quantitative proteomics analysis was performed using a LC-MS/MS system as reported earlier [23–25]. In brief, labeled

peptides were separated on a home-packed nanobored C18 column with a picofrit nanospray tip (75 μm ID × 15 cm, 5 μm particles) (New Objectives, Wubrun, MA) with a TempoTM nano-MDLC system coupled with a QSTAR® Elite Hybrid LC-MS/MS system (Applied Biosystems, USA) at a constant flow rate of 300 nl/min. Each sample fraction was divided into two equal aliquots and independently analyzed by the LC-MS/MS using 90 min gradient. The 90 min gradient consisted of 10–40% buffer B (80% ACN, 0.1% FA) for 65 min, 40–80% buffer B for 10 min, 80% buffer B for 5 min, followed by 90% buffer A (0.1% FA) for 10 min. The QStar Elite mass spectrometer was set in positive ion mode using Analyst® QS 2.0 software (Applied Biosystems, USA) and data was acquired with a selected mass range of 300–16000 m/z. The three most abundant peptides with +2 to +4 charges and above a five-count threshold were selected for MS/MS, and each selected target ion was dynamically excluded for 30 s with ±30 mDa mass tolerance. Smart information-dependent acquisition 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.

2.4. Mass spectrometric data search and analysis

The data acquisition was performed using the Analyst QS 2.0 software (AB/MDS SCIEX). Protein identification and quantification was performed using ProteinPilot Software 2.0.1, Revision Number: 67476 (Applied Biosystems) and further processed by a Pro Group algorithm where isoform-specific quantification was adopted to trace the differences between expression of various isoforms. User defined parameters were as follows: (i) Sample Type — iTRAQ 8-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 [26]; Database-A concatenated target — decoy database (20096 sequences and 9138356 residues) of *P. chrysosporium* proteins obtained from organism's genome project (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html database version 2.1); (x) Search effort-thorough. For iTRAQ quantitation, the peptides were automatically selected by Pro Group algorithm to calculate the reporter peak area, error factor (EF) and *p*-value. 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. The existence of signal peptide sequence was checked using the signal peptide prediction program SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/).

2.5. Zymogram analysis and enzyme assay

The activities of the enzyme were determined by zymogram analysis on SDS-PAGE by loading 10 μg of protein. The polyacrylamide gels were prepared with 1% CMC and xylan for cellulase and hemicellulase activities respectively. The SDS-PAGE was run in Mini Protean (Bio-Rad) gel running system for about 120–180 min at 100 V; gel was washed with phosphate buffer (pH 7.2 ± 0.2) containing 40% isopropanol. The enzyme reaction was performed at 42 ± 2 °C after overnight renaturation at 4 °C and cellulase and xylanase bands were developed with 1% Congo red staining.

While, peroxidase and phenoloxidases activity was analyzed as described by Hoopes and Dean [27]. In brief, following electrophoresis, gels were incubated in 50 mM sodium acetate (pH 5.0) containing 1% dimethyl sulfoxide and 2 mM DAN. Hydrogen peroxide was added to the final concentration of 5 mM and gel was incubation at 20 °C. The reaction was stopped by immersing gel in 50% (v/v) methanol containing 10% (v/v) acetic acid at 50 °C. Gel was immersed in 20% trichloroacetic acid to darken the DAN oxidation product. The enzyme activities were determined on the samples collected from the test culture flask. The endo-1,4-β-glucanase and exo-1,4-β-glucanase were assayed by measuring the reducing sugars [24].

3. Results

3.1. Functional classification of the iTRAQ quantified proteins of P. chrysosporium

This study quantified 329 proteins with ≤1% FDR and unused protein score ≥2.0 as a cutoff value. These iTRAQ quantified proteins were functionally classified according to their biological role. As depicted in Fig. 1, proteins were grouped under cellulases (16.6%), glycoside hydrolases (19.9%), hemicellulases (15.9%), lignin depolymerizing enzymes (10.0%), peptidases and proteases (19.9%), lipases (1.4%), chitinases (2.4%, transport (7.1%) and hypothetical proteins (7.1%). Some intracellular proteins were also identified that may suggest some cell lysis or cell death. An iTRAQ ratio of ≤0.80 were set as a down regulation while ≥1.2 as up-regulation criterion. Figs. S1 and S2 (supplementary) illustrates in a heat map showing altered lignocellulolytic enzyme expression of *P. chrysosporium* due to different lignocellulosic biomass. The heat map readily shows changes in the secreted quantity of the lignocellulose degrading proteins.

3.2. Proteins involved in cellulose hydrolysis

The hydrolysis of cellulose catalyzed by cellulases produced by both fungi and bacteria were grouped into endocellulase, exocellulase, cellobiase and cellulose depolymerizing oxidative cellulase and cellulose phosphorylase [28]. Although this work identified and iTRAQ quantified numerous cellulose hydrolyzing enzymes and glycoside hydrolases, only sixty eight proteins that qualified the applied cut-off criterion were considered during classification. The comparative expressions of the cellulolytic proteins were presented in Fig. 2. The cellulose degrading GH6 exocellobiohydrolase (jgi|Phchr1|133052|) and GH61 endoglucanase B (jgi|Phchr1|41123|) were significantly upregulated in all tested lignocellulosic substrates. The GH7 cellulase (jgi|Phchr1|137372|) was abundantly produced demonstrating highest unused protein score (189.88 ± 12.6) and peptide number (293 ± 24) and was significantly upregulated when strain was cultivated with corn stover, hay, sawdust, sugarcane baggase, and wheat bran (Fig. 2). When the tested agricultural wastes were used as a carbon source, GH7 cellulase was significantly upregulated with unused protein score, percentage coverage and peptides (confidence 95%) 189.88, 73.95 and 293 respectively (Table S1). While when

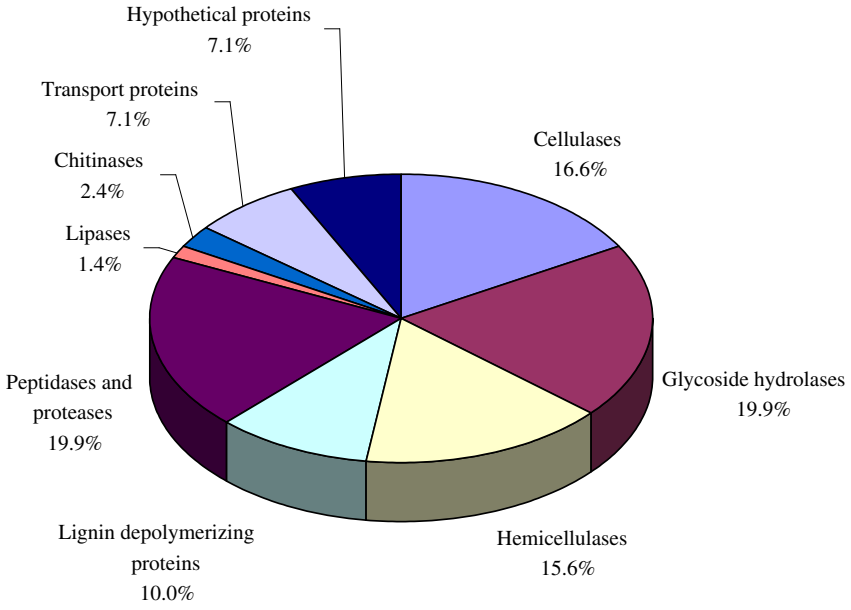

Fig. 1 – Functional classification of iTRAQ quantified secretory proteins of *P. chrysosporium* under different agricultural and forest wastes as a major carbon source. The proteins identified and iTRAQ quantified in all test substrate and having ≥2.0 unused protein score were considered for classification.

corn stover, hay, sawdust, sugarcane baggase, wheat bran and woodchips were used as a major carbon source; iTRAQ ratios of GH6 exocellobiohydrolase (jgi|Phchr1|133052|) were 1.81, 2.84, 4.57, 1.26, 1.62 and 1.39 respectively. In this study, when different type of lignocellulosic biomass was used as carbon source, six exo-cellobiohydrolases were expressed and iTRAQ quantified. The cellobiose hydrolyzing GH1 beta-glucosidases (jgi|Phchr1|131484|) was upregulated in all tested lignocellulosic substrates except sugarcane baggase. While GH3 beta-glucosidase (jgi|Phchr1|128442|) was upregulated when corn stover, wheat bran and wood chips were used as a major carbon source. We iTRAQ quantified two laminarinase that have endoglucosidase activity, of which laminarinase (jgi|Phchr1|123909|) was upregulated when corn stover, wheat bran and wood chips were used as a carbon source. In addition to cellulases, this study iTRAQ quantified thirty eight glycoside hydrolases and their comparative quantitations were presented in Fig. 3. *P. chrysosporium* encodes extensive set of cellulolytic proteins, but their expression depends upon carbon source. The agricultural waste contains significant amount of starch [29], which explains iTRAQ quantification and significant upregulation of different alpha amylases.

3.3. *Proteins involved in hemicellulose and pectin hydrolysis*

This study identified hemicellulose hydrolyzing xylanases; xylan esterases; rhamnogalacturonases; alpha-, beta-, mannosidases; arabinases; mannanases; carboxyesterases; alpha-L-arabinofuranosidases; galactosidases; polysaccharide lyases, novel phytases etc. (Table S1, supplementary information). The comparative expressions of iTRAQ quantified hemicellulose and pectin degrading proteins were presented in Fig. 4. The mannan gets hydrolyzed into monomer sugars by mannanases and mannosidases through random cleavage within 1,4-beta-D-mannan. [28,30]. This study noted the expression of mannan hydrolyzing CEL4a mannanase (jgi|Phchr1|140501|), mannanase Man5C (jgi|Phchr1|5115|), endo-1,4-beta-mannosidase Man5D (jgi|Phchr1|6579|) and beta-1,3-mannanase (jgi|Phchr1|137167|). The expression of endo-1,4-beta-xylanase B, GH27 alpha-galactosidase, acetyl xylan esterase, GH5 exo-beta-1,3-glucanase etc. varied with substrate type and were discussed in the Discussion section.

3.4. *Lignin depolymerization and other proteins*

When *P. chrysosporium* was cultivated with different agricultural or forest wastes as a major carbon source, copper radical oxidases, CDH, glucose oxidase, isoamyl alcohol oxidase, glutathione reductase, glutathione S-transferase, peroxiredoxins, pyranose 2-oxidase, quinone oxidoreductase, iron-containing alcohol dehydrogenase, cytochrome *c*, were identified and iTRAQ quantified (Table 1). The variable iTRAQ ratios of cellobiose dehydrogenase, copper radical oxidase, glutathione S-transferase, peroxiredoxins and quinone oxidoreductase could be due to difference in nature of substrates and degree of lignifications.

The glutathione S-transferase enzyme that precisely cleaves beta-aryl ether linkage of the major intramolecular bond in lignin [26] was found to be upregulated in all test conditions except sugarcane baggase while its iTRAQ ratios were highest in wheat bran followed by corn stover. In addition to the hydrolytic lignin degrading enzymes, this study iTRAQ quantified five copper radical oxidases, of which copper radical oxidase (jgi|Phchr1|8882|) was significantly upregulated in all tested lignocellulosic biomass. The glucose oxidase and pyranose 2-oxidase that generate H_2O_2 were also identified and iTRAQ quantified.

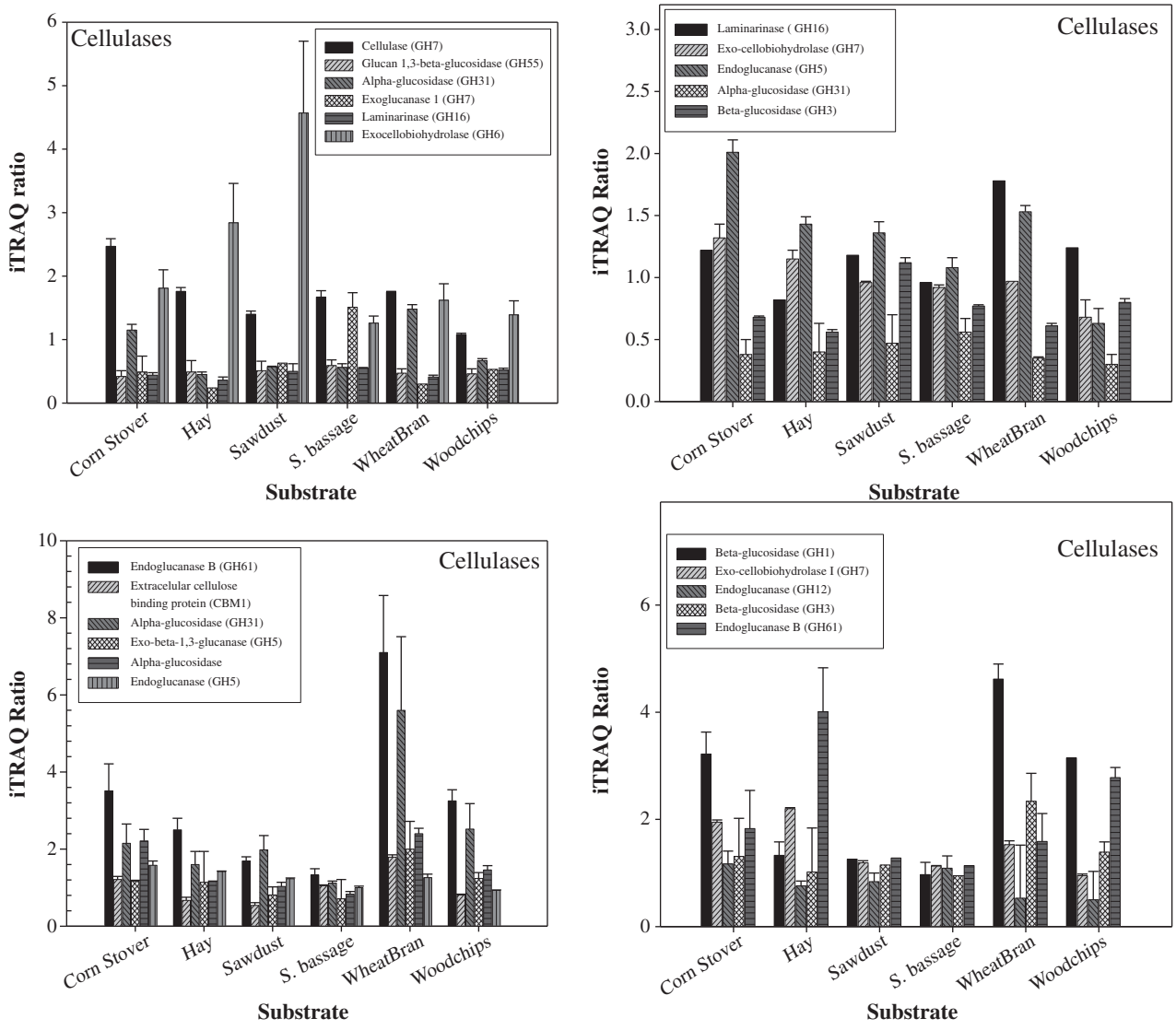

Fig. 2 – Comparative iTRAQ ratios of cellulases by *P. chrysosporium* on different lignocellulosic biomass [Cellulase GH7 (jgi|Phchr1|137372|); Glucan 1,3-beta-glucosidase GH55 (jgi|Phchr1|8072|); Alpha-glucosidase GH31 (jgi|Phchr1|125462|); Exoglucanase 1 GH7 (jgi|Phchr1|137216|); Laminarinase GH16 (jgi|Phchr1|10833|); Exocellobiohydrolase GH6 (jgi|Phchr1|133052|); Laminarinase GH16 (jgi|Phchr1|123909|); Exo-cellobiohydrolase GH7 (jgi|Phchr1|127029|); Endoglucanase GH5 (jgi|Phchr1|6458|); Alpha-glucosidase GH31 (jgi|Phchr1|968|); Beta-glucosidase GH3 (jgi|Phchr1|129849|); Endoglucanase B GH61 (jgi|Phchr1|41123|); Extracelular cellulose binding protein CBM1 (jgi|Phchr1|6482|); Alpha-glucosidase GH31 (jgi|Phchr1|135833|); Exo-beta-1,3-glucanase GH5 (jgi|Phchr1|135724|); Alpha-glucosidase (jgi|Phchr1|35408|); Endoglucanase GH5 (jgi|Phchr1|4361|); Beta-glucosidase GH1 (jgi|Phchr1|131484|); Exo-cellobiohydrolase I GH7 (jgi|Phchr1|129310|); Endoglucanase GH12 (jgi|Phchr1|8466|); Beta-glucosidase GH3 (jgi|Phchr1|128442|); Endoglucanase B GH61 (jgi|Phchr1|122129|)].

The iTRAQ data demonstrated secretion of several proteases and peptidases among which proteinase (jgi|Phchr1|6069|), peptidase (jgi|Phchr1|5061|), aspartic peptidase A1 (jgi|Phchr1|128676|, (jgi|Phchr1|126189|), glutamate carboxypeptidase (jgi|Phchr1|131104|) and proteinase (jgi|Phchr1|6070|) (Table S2, supplementary information) were significantly induced when *P. chrysosporium* was cultivated with different agricultural and forest wastes. The various transport proteins and hypothetical proteins were detected in the secretome of *P. chrysosporium*. Although the functions of hypothetical proteins were unknown but the upregulation of several hypothetical proteins reasonably support their possible role in lignocellulose degradation.

3.5. *Enzyme assay and zymography*

Fig. 5 presents the zymograms showing cellulase, xyalanase and reactive radical generating lignin depolymerising enzymes. As seen from Fig. 5A, more than nine intense bands of cellulases were observed when SDS-PAGE containing CMC was used for cellulase activity suggesting diversity of cellulases. Band 9 was noted in secretome of all test samples, while band 7 and 8 were observed only in corn stover, hay, sawdust and sugarcane baggase. The variable band intensities (bands 7 and 8) suggest cellulase production as a function of substrate. Similarly, xylanase expressions were also dependent on substrate type

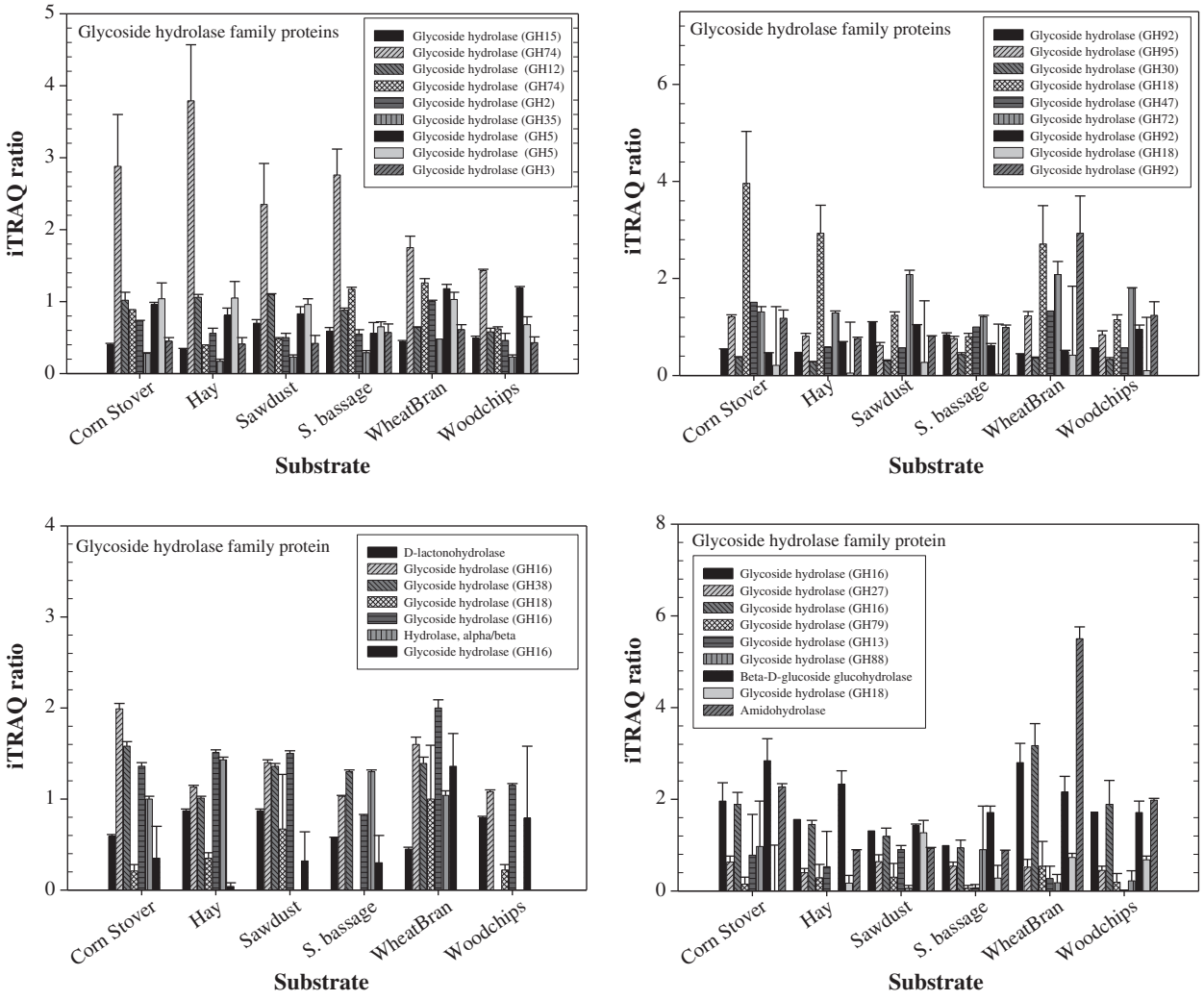

Fig. 3 – Comparative iTRAQ ratios of glycoside hydrolases by *P. chrysosporium* on different lignocellulosic biomass. [Glycoside hydrolase GH15 (jgi|Phchr1|138813|); Glycoside hydrolase GH74 (jgi|Phchr1|134556|); Glycoside hydrolase GH12 (jgi|Phchr1|7048|); Glycoside hydrolase GH74 (jgi|Phchr1|138266|); Glycoside hydrolase GH2 (jgi|Phchr1|135385|); Glycoside hydrolase GH35 (jgi|Phchr1|9466|); Glycoside hydrolase GH5 (jgi|Phchr1|122884|); Glycoside hydrolase GH5 (jgi|Phchr1|5607|); Glycoside hydrolase GH3 (jgi|Phchr1|139063|); Glycoside hydrolase GH92 (jgi|Phchr1|133585|); Glycoside hydrolase GH95 (jgi|Phchr1|6997|); Glycoside hydrolase GH30 (jgi|Phchr1|9011|); Glycoside hydrolase GH18 (jgi|Phchr1|40899|); Glycoside hydrolase GH47 (jgi|Phchr1|4550|); Glycoside hydrolase GH72 (jgi|Phchr1|6433|); Glycoside hydrolase GH92 (jgi|Phchr1|1930|); Glycoside hydrolase GH18 (jgi|Phchr1|9211|); Glycoside hydrolase GH92 (jgi|Phchr1|35714|); D-lactonohydrolase-like protein (jgi|Phchr1|7809|); Glycoside hydrolase GH16 (jgi|Phchr1|2630|); Glycoside hydrolase GH38 (jgi|Phchr1|35305|); Glycoside hydrolase GH18 (jgi|Phchr1|2991|); Glycoside hydrolase GH16 (jgi|Phchr1|3846|); Hydrolase, alpha/beta (jgi|Phchr1|8062|); Glycoside hydrolase GH16 (jgi|Phchr1|126622|); Glycoside hydrolase GH16 (jgi|Phchr1|138982|); Glycoside hydrolase GH27 (jgi|Phchr1|4422|); Glycoside hydrolase GH16 (jgi|Phchr1|7122|); Glycoside hydrolase GH79 (jgi|Phchr1|1503|); Glycoside hydrolase GH13 (jgi|Phchr1|122628|); Glycoside hydrolase GH88 (jgi|Phchr1|121728|); Beta-D-glucoside glucohydrolase (jgi|Phchr1|131440|); Glycoside hydrolase GH18 (jgi|Phchr1|6412|); Amidohydrolase (jgi|Phchr1|894|)].

(Fig. 5b). The reactive radical generating lignin depolymerizing enzymes and peroxidases were detected in the secreted enzymes (Fig. 5c). Both endoglucanase and exoglucanase activities were detected in the secretome of *P. chrysosporium* when corn stover, hay, sawdust, sugarcane baggase, wheat bran and wood chips were used as a major carbon source (Fig. 6). However, endoglucanase was higher when corn stover, hay and wheat straw were used as a major carbon source; while exoglucanase were higher when sawdust was used as a substrate.

4. Discussion

In this research work, we report for the first time, comprehensive iTRAQ based proteomic analysis of *P. chrysosporium* secretome under lignocellulosic biomass derived from different agricultural/forest wastes. Since lignocellulosic bioethanol technologies are hindered due to structural complexity of lignin, genetic engineering of crop plants to reduce plant cell

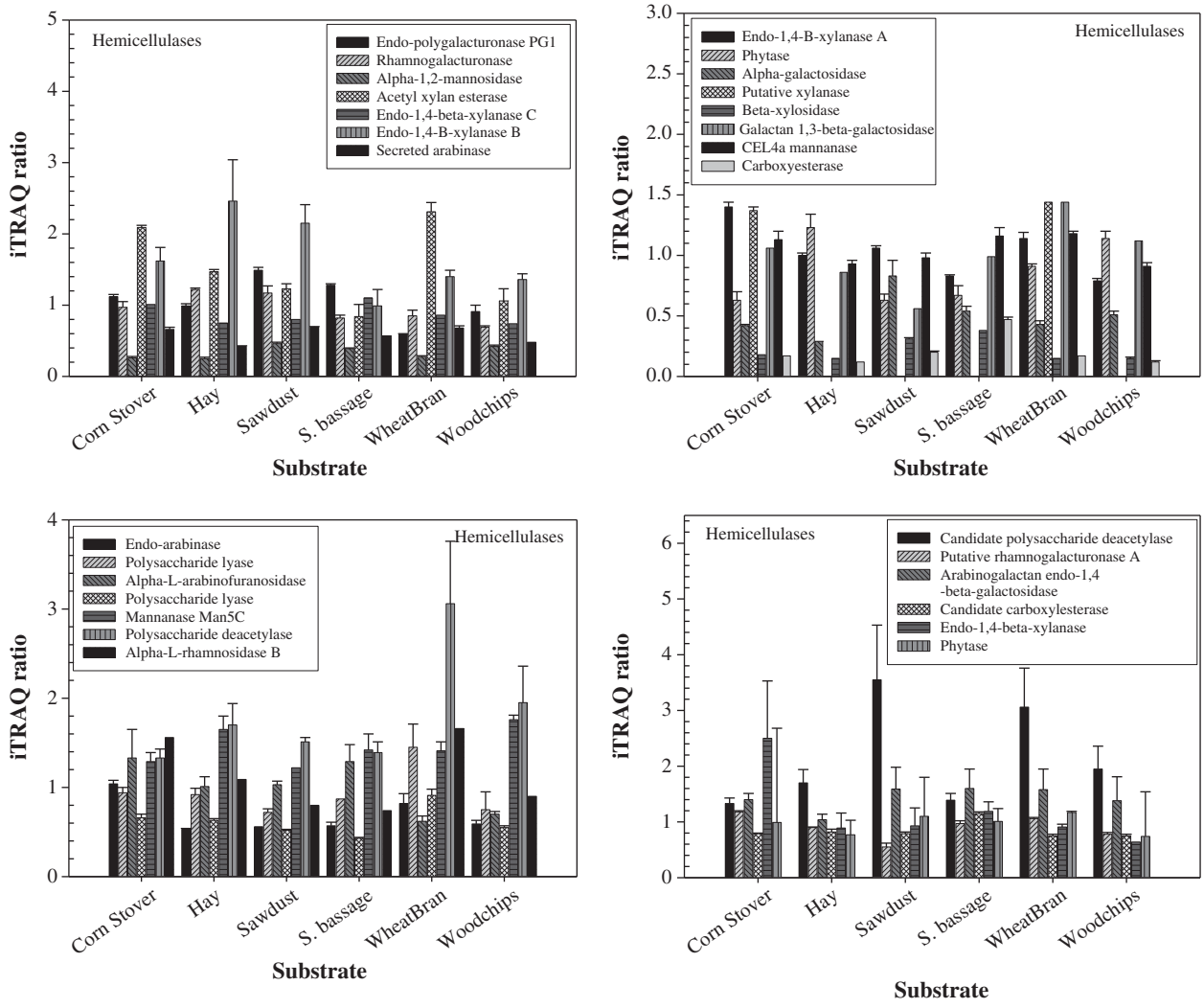

Fig. 4 – Comparative iTRAQ ratios of hemicellulases and pectinases by *P. chrysosporium* on different lignocellulosic biomass. [Endo-polygalacturonase PG1(jgi|Phchr1|3805|); Rhamnogalacturonase (jgi|Phchr1|29397|); Alpha-1,2-mannosidase (jgi|Phchr1|3431|); Acetyl xylan esterase (jgi|Phchr1|126075|); Endo-1,4-beta-xylanase C (jgi|Phchr1|138715|); Endo-1,4-beta-xylanase B (jgi|Phchr1|133788|); Secreted arabinase (jgi|Phchr1|4822|) Endo-1,4-beta-xylanase A (jgi|Phchr1|138345|); Phytase (jgi|Phchr1|137138|); Alpha-galactosidase (jgi|Phchr1|125033|); Putative xylanase (jgi|Phchr1|7045|); Beta-xylosidase (jgi|Phchr1|9257|); Galactan 1,3-beta-galactosidase (jgi|Phchr1|297|); CEL4a mannanase (jgi|Phchr1|140501|); Carboxyesterase (jgi|Phchr1|3761|); Endo-arabinase (jgi|Phchr1|133070|); Polysaccharide lyase (jgi|Phchr1|6736|); Alpha-L-arabinofuranosidase (jgi|Phchr1|3651|); Polysaccharide lyase (jgi|Phchr1|964|); Mannanase Man5C (jgi|Phchr1|5115|); Alpha-L-rhamnosidase B (jgi|Phchr1|122292|); Candidate polysaccharide deacetylase (jgi|Phchr1|29074|); Putative rhamnogalacturonase A (jgi|Phchr1|3795|); Arabinogalactan endo-1,4-beta-galactosidase (jgi|Phchr1|138710|); Candidate carboxylesterase (jgi|Phchr1|126191|); Endo-1,4-beta-xylanase (jgi|Phchr1|125669|) and Phytase (jgi|Phchr1|121720|).

wall lignin remain under consideration. However, decreasing lignin content may increase the susceptibility of crop plants to insect, fungal, and pest attack affecting their sustainability. The best approach is to develop technologies for hydrolyzing agricultural wastes including crop residues and forest wastes. Therefore, this study profiled the secretory proteins of *P. chrysosporium* using agricultural and forest wastes. In total, 329 high confident secreted proteins were quantified which were significantly higher than 79 or 45 protein reported earlier [15,31].

4.1. *Expressions of cellulases and hemicellulases*

Some lignocellulolytic enzymes of *P. chrysosporium* were isolated, purified and characterized by several researchers [6–12,32], but comprehensive quantitative lignocellulolytic protein expression on lignocellulosic biomass was rarely documented. This study demonstrated quantitative expressions of 68 cellulose hydrolyzing proteins and 32 hemicellulases that constitutes >41% of *P. chrysosporium* carbohydrate active enzymes encoded in its genome. The iTRAQ quantification indicated

Table 1 – Lignin degrading proteins identified in the secretome of *Phanerochaete chrysosporium* when grown on different lignocellulosic biomass derived from agricultural or forest wastes.

Unused	%Cov	%Cov (95)	Accession	Name	Peptides (95%)	cornstover : control	Hay: control	Sawdust : control	Sugarcane baggase : control	Wheatbran: control	Woodchips: control	SignalP
Lignin depolymerizing proteins												
46.19 ±10.75	39.71 ±2.98	20.50 ±3.04	jgi|Phchr1| 11098|	Cellobiose dehydrogenase	36±10	2.12±0.10	1.68±0.06	2.10±0.81	1.64±0.05	2.26±0.16	1.46±0.01	Y
21.41 ±4.33	32.56 ±5.17	13.11 ±3.17	jgi|Phchr1| 134241|	Copper radical oxidase variant A	17±7	0.94±0.05	0.71±0.14	0.98±0.34	0.51±0.18	1.01±0.55	1.01±0.65	Y
17.85 ±2.90	43.65 ±5.41	15.28 ±2.27	jgi|Phchr1| 6270|	Glucose oxidase, putative	13±3	0.90±0.05	0.43±0.01	0.51±0.01	0.64±0.05	1.61±0.13	0.71±0.01	Y
14.53 ±1.08	35.16 ±4.66	15.25 ±2.08	jgi|Phchr1| 3896|	Isoamyl alcohol oxidase, putative	11±2	1.02±0.05	0.88±0.10	0.71±0.07	0.65±0.02	1.01±0.07	0.83±0.08	Y
19.64 ±2.00	52.86 ±3.28	16.95 ±1.91	jgi|Phchr1| 135167|	Glutathione reductase	16±2	2.29±0.40	1.23±0.18	1.23±0.13	1.29±0.08	5.31±1.43	2.20±0.56	N
8.59 ±4.58	19.79 ±5.73	4.99 ±2.96	jgi|Phchr1| 8882|	Copper radical oxidase	5±3	1.57±0.05	1.76±0.03	1.49±0.17	1.31±0.32	2.84±0.02	1.83±0.08	Y
9.18 ±1.72	42.27 ±0.82	19.90 ±2.14	jgi|Phchr1| 6766|	Glutathione S-transferase, epsilon class (AGAP0	6±1	2.28±0.14	1.19±0.00	1.40±0.01	0.99±0.04	3.44±0.01	1.44±0.20	N
5.31 ±3.06	21.51 ±4.39	4.28 ±2.71	jgi|Phchr1| 121730|	Copper radical oxidase	3±2	0.96±0.22	1.10±0.39	1.03±0.11	1.00±0.18	1.89±0.75	1.17±0.41	N
5.85 ±1.49	32.17 ±8.87	5.48 ±1.00	jgi|Phchr1| 124009|	Copper radical oxidase	3±1	1.62±0.10	1.10±0.07	1.19±0.08	0.78±0.25	1.95±0.62	1.39±0.27	Y
2.52 ±3.52	16.29 ±17.29	2.34 ±3.34	jgi|Phchr1| 2685|	Putative oxidoreductase	2±1	1.84±0.02	0.98±0.07	1.12±0.03	1.21±0.19	3.20±0.22	1.46±0.05	N
1.41 ±2.41	24.38 ±25.38	4.95 ±5.95	jgi|Phchr1| 8807|	Peroxiredoxins	2±1	0.31±0.03	1.17±0.17	0.19±0.01	0.04±0.00	0.52±0.05	0.43±0.03	N
3.23 ±0.00	13.02 ±0.58	2.32 ±0.00	jgi|Phchr1| 26890|	Multicopper oxidase; Fet3 ferroxidase protein	1±0*	0.89±0.03	1.17±0.02	0.77±0.02	0.96±0.01	1.09±0.05	0.93±0.02	Y
3.19 ±0.00	33.26 ±3.95	2.74 ±0.00	jgi|Phchr1| 137275|	Pyranose 2-oxidase	1±0*	1.00±0.03	1.16±0.02	1.32±0.03	–	1.99±0.09	–	—
2.37 ±1.32	28.82 ±8.95	1.15 ±1.15	jgi|Phchr1| 3442|	Putative quinone oxidoreductase	1±1*	1.52±0.00	1.33±0.00	1.66±0.00	1.17±0.00	1.56±0.00	1.02±0.00	N
2.01 ±0.00	41.12 ±1.87	10.28 ±0.00	jgi|Phchr1| 2835|	Cytochrome *c* C1	1±0*	1.07±0.03	0.97±0.02	0.68±0.01	1.34±0.02	0.84±0.04	0.85±0.02	–
2.00 ±0.00	8.47 ±0.80	2.56 ±0.00	jgi|Phchr1| 5263|	Copper radical oxidase	1±0*	0.50±0.01	1.24±0.03	–	0.62±0.01	0.52±0.02	–	–
2.33 ±0.00	36.13 ±2.25	2.80 ±0.00	jgi|Phchr1| 140211|	NAD-dependent formate dehydrogenase	1±0*	2.43±0.18	1.43±0.18	1.66±0.11	1.43±0.05	4.43±0.17	2.11±0.07	–

*Peptide sequence listed in Table S4 (Supplementary information), –: not detected; — not available.

*Peptide sequence listed in Table S4 (Supplementary information), –: not detected; — not available.

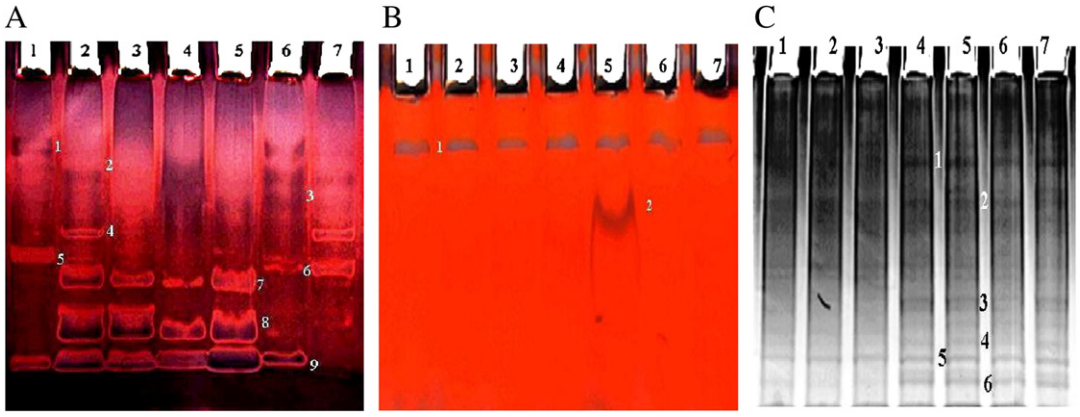

Fig. 5 – Proteins and Zymogram showing cellulase (A), xyalanase (B) and reactive radical generation lignin depolymerising enzymes (C). SDS-PAGE was used with 0.2% carboxymethyl cellulose and xylan as a substrate for cellulases and xylanses respectively. (Lane 1: control; 2: corn stover; 3: hay; 4: sawdust; 5: sugarcane baggase; 6: wheat bran; 7: wood chips).

significant upregulation of GH6, GH7 exoglucanases and GH5, GH12, GH16, GH61 endoglucanases. The induced expression of GH7 exoglucanase was further profiled by zymogram with protein score of 219 (Table S3, supplementary information). The novel GH1, GH3, GH31, GH55 beta-, alpha-glucosidases were expressed and significantly upregulated in tested substrates. The cellobiose inhibits cellobiohydrolases [33] and hence external supplementation of beta-glucosidase was thought to be an alternative to overcome inhibitory effects. However, this study noted expressions of GH1, GH3 beta-glucosidase (jgi|Phchr1|129849|, jgi|Phchr1|131484|, jgi|Phchr1|128442|). The quantitative expression suggested significant upregulation of GH1 beta-glucosidase (jgi|Phchr1|131484|) and GH3 beta-glucosidase (jgi|Phchr1|128442|). Again, GH16 laminarinase (jgi|Phchr1|10833|, jgi|Phchr1|123909|) that acts as an endoglucosidases [15] were detected in the secretome of *P. chrysosporium*. In addition to these glucosidases, GH31 alpha-glucosidase jgi|Phchr1|135833|, jgi|Phchr1|125462|, jgi|Phchr1|968|, jgi|Phchr1|35408|) and GH55 glucan 1,3-beta-glucosidase (jgi|Phchr1|8072|) were iTRAQ quantified. Based on expression and regulation profile of different beta-glucosidases, laminarinases, cellobiose reducing CDH, it can be concluded that *P. chrysosporium* have the potential to overcome cellobiohydrolases inhibitory effect. According to Soham et al. [34], hemicellulose constituents contains alpha-1,4, -1,3; -1,2 bonds that supports the expressions of different alpha-glucosidases. Taken together the expressions of exo-, endo-glucanases, beta-, alpha-glucosidases and CDH; *P. chrysosporium* is a potential cellulose degrading fungus and their enzymes have potential application in lignocellulosic bioenergy.

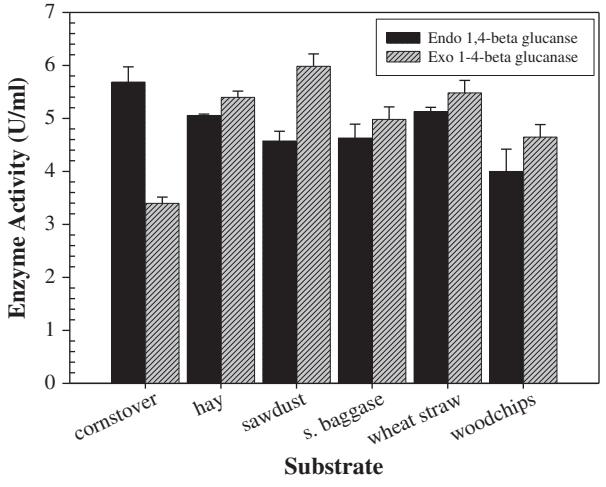

Fig. 6 – The endoglucanase and exoglucanase activities when *Phanerochaete chrysosporium* was grown in different lignocellulosic biomass as a major carbon source.

Hemicellulose, the second most abundant renewable biomass represents about 15–35% of plant biomass. The hemicellulose degrading xylanase, endo-polygalacturonase PG1, rhamnogalacturonase, beta-xylosidase, alpha-glucuronidase, ferulic and *p*-coumaric esterases, alpha-L-arabinofuranosidase, endo-arabinase, carboxyesterase, acetylxylan esterase etc. were iTRAQ quantified. These enzymes act synergistically and degrade heteropolymers of hemicellulosic biomass [28,35]. The iTRAQ quantification of mannanase Man5C, beta-1,3-mannanase and CEL4a mannanase in the secretome of *P. chrysosporium* suggested its mannan hydrolysis potential. The hemicellulolytic enzyme expression profile revealed softwood and hardwood hydrolyzing potential of *P. chrysosporium*. According to Moreira and Filho [30], complete depolymerization of mannans requires synergetic and cooperative action between enzymes acting on the main chain and side chain substituent. An iTRAQ ratio of GH11 endo-1,4-beta-xylanase B ranged between 1.21 and 1.80 with corn stover, sugarcane baggase, wheat bran and woodchips as a carbon source while the corresponding values were 3.04–4.42 when hay and sawdust were used as a carbon source.

4.2. *Expressions of lignin degrading proteins*

The lignin peroxidase (LiP), manganese-peroxidase (MnP) and laccase were generally considered as lignin degrading enzymes.

However, according to Blanchette et al. [36] they are too big to penetrate plant cell wall. Hence, to get accesses microbes initially activate easily diffusing several oxidases, reactive radical generating enzymes, and quinine reducing enzymes [37,38]. The expression and iTRAQ quantification of copper radical oxidase, cellobiose dehydrogenase, glucose oxidase, isoamyl alcohol oxidase, peroxiredoxins, pyranose 2-oxidase, quinone oxidoreductase, iron-containing alcohol dehydrogenase etc. indicated *P. chrysosporium* degrade lignin through oxidases. This study found upregulation of CDH which is an extracellular redox enzyme of ping-pong type that generates hydroxyl radicals by reducing Fe^{3+} to Fe^{2+} and O_2 to H_2O_2 and play important role in lignin degradation [39]. According to Henriksson et al. [37], CDH play a major role in lignin degradation by (i) breaking beta-ethers [18], demethoxylating aromatic structures in lignin, and (iii) introducing hydroxyl groups in non-phenolic lignin. As reported by Henriksson et al. [37], CDH is involved in both lignin and cellulose hydrolysis; and can be converted into lignin degrading CBQ using proteolytic cleavage [40] or by proteases secreted by *P. chrysosporium* [41,42]. The detailed research on CHD is required to tailor its exact role in lignin degradation and elucidate lignin degradation mechanism.

The LiP- and MnP- requires H_2O_2 for their catalytic activities and the major source is copper radical oxidases and glyoxal oxidase. The present study identified five copper radical oxidases, of which jgi|Phchr1|8882| and jgi|Phchr1|124009 were significantly upregulated (Table 1). Wymelenberg et al. [31] identified six copper radical oxidase encoding genes (*cro*1–*cro*6), while based on their position Martinez et al. [9] emphasized the strong relationship between peroxidases and copper radical oxidases. This study noted the expression of GLX that generates H_2O_2 for LiP- and MnP-mediated reactions and also regulates peroxidase activity. Manganese peroxidase was identified and iTRAQ quantified, however, its confidence level was lower than set cut-off values. In addition, this study iTRAQ quantified expressions of beta-aryl ether linkage cleaving glutathione S-transferase. According to Otsuka et al. [43] and Masai et al. [44] glutathione S-transferase catalyzes the specific hydrolytic cleavage of lignin guaiacylglycerol beta-aryl ether linkages. Masai et al. [44] characterized three GST genes, *ligF*, *ligE*, and *ligG* from lignin degrading *Sphingomonas paucimobilis* SYK-6, and described their role in beta-aryl ether cleavage. The iTRAQ quantification and substrate specific regulation of glutathione S-transferase in tested lignocellulosic biomass revealed hydrolytic lignin degradation mechanism by *P. chrysosporium*.

4.3. *Effect of structural component content and particle size on lignocellulolytic protein expressions*

The efficiency of lignocellulosic biomass hydrolysis depends upon the content of rigidity imparting lignin and degree of lignifications [45]. Biomass in softwood comprises with 45–50% cellulose, 25–35% hemicellulose, and 25–35% lignin [46]; while the corresponding values in hardwood were 37–40, 23–29 and 21–23% respectively [47,48]. An iTRAQ ratio 4.57 of GH6 exocellobiohydrolase demonstrated highest expression of this protein when sawdust was used for the cultivation of *P. chrysosporium*, while its iTRAQ ratios ranged between 1.26 and 2.84 when corn stover, hay, sugarcane baggase, wheat bran and wood chips were used as a major carbon source. The cellulose, hemicellulose and lignin content in sugarcane bagasse and corn stover ranges between 19–24, 16–21, 15 and 32–44, 29–35, 45% respectively [49]. The significant upregulation of 34 cellulose degrading proteins of *P. chrysosporium* in wheat bran culture condition could be due to higher cellulosic content and lower lignifications. According to Ye et al. [50] wheat bran contains glucan (27–40%), xylan (14–28%), arabinan (1–5%) and lignin (11–25%); while protein (17.7%) and starch (8.8%) were also reported in wheat bran [51]. The induced production of hemicellulases including acetyl xylan esterase, endo-1,4-beta-xylanase B; xylanase; galactan 1,3-beta-galactosidase; mannanase Man5C; alpha-L-rhamnosidase B; polysaccharide deacetylase; arabinogalactan endo-1,4-beta-galactosidase etc. could be due to significant quantities of arabinoxylans, beta-glucans, arabinose , xylose, mannose, galactose in wheat bran. Taken together, variable iTRAQ ratios of lignocellulolytic enzymes were due to variable content of structural constituents and structural complexity among cellulose, hemicellulose and lignin. The gene expressions of *P. placenta* and *P. chrysosporium* were influenced by the wood substrate [14].

To make structural polysaccharides accessible to hydrolytic enzymes, Grabber et al. [45] manipulated lignin structure by altering the normal guaiacyl, syringyl, and p-hydroxy-phenyl; however degradability was not influenced. The wood becomes highly rigid on physiological maturity due to high lignin content and degree of lignifications but yet GH6 exocellobiohydrolase was quantified with an iTRAQ ratio of 1.39 when wood chips were used as a carbon source. This iTRAQ ratio further increased to 4.57 when wood were crushed to smaller particles (sawdust) demonstrating effect of particle size. Similarly, GH7 cellulase (jgi|Phchr1|137372|), GH5 endoglucanase (jgi|Phchr1|6458|) and GH3 beta-glucosidase, showed 32, 73 and 32% higher iTRAQ ratios in sawdust when compared to wood chips. In addition to these enzymes, iTRAQ ratios of proteins belonging to family GH15 (jgi|Phchr1|138813|), GH74 (jgi|Phchr1|134556|), GH18 (jgi|Phchr1|40899|), GH72 (jgi|Phchr1|6433|) etc. were higher in sawdust than wood chips. Thus, small particle size enhanced cellulose accessibility to the cellulases thereby increasing their expressions.

4.4. *Peptidases, lipase, chitinase, transport and hypothetical proteins*

The iTRAQ data demonstrated secretion of 43 proteases and peptidases. According to Van Soest [52], nutrient nitrogen limitation triggers the synthesis of the lignin degrading system while Sato et al. [16] proposed protease production in *P. chrysosporium* as a lignolytic or wood degrading response. The role of proteases in the activation of cellulases and in functional domains of CDH has been reported [41,42]. This study quantified numerous novel proteases and presence of proteins in plant cell wall validates its expressions and upregulation. Cassab et al. [53] reviewed the major content, composition and functional role of proteins in plant cell wall. In addition to peptidases, lipases and chitinases that contribute to remolding of cell wall during cell budding, growth and morphogenesis were also secreted by *P. chrysosporium* when agricultural or forest waste was used for its cultivation. The *P. chrysosporium* genome analysis predicts 359 hypothetical proteins that include some secreted proteins

5. Conclusion

The comprehensive quantitative proteomic analysis of *P. chrysosporium* secretome will highlight potential novel enzymes for developing optimized enzyme cocktail for efficient lignocellulose degradation. *P. chrysosporium* precultivated with different natural lignocellulosic materials (corn stover, hay, sawdust, sugarcane baggase, wheat bran and wood chips) highlighted differential expression of lignocellulolytic enzyme including numerous cellulases, glycoside hydrolases, hemicellulases, lignin degrading enzymes, esterases, lipases, chitinases, peptidases, protein translocating transporter and hypothetical proteins. Although *P. chrysosporium* considered as a lignin degrader, the iTRAQ quantified secretome data revealed cellulose hydrolyzing exo, endo glucanases, beta glucosidases, and glycoside hydrolases; hemicellulose degrading xylanases, mannosidases, mannanases, polygalacturonases, rhamnogalacturonases, esterases, arabinases suggesting that it degrades major constituent of plant cell wall including cellulose and hemicellulose. This study emphasized the importance of particle size, nature and complexity of lignocellulosic biomass during protein expressions by *P. chrysosporium*.

Supplementary materials related to this article can be found online at doi:10.1016/j.jprot.2011.11.020.

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