# Quantitative iTRAQ Secretome Analysis of Cellulolytic *Thermobifida fusca*

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## Abstract

*Thermobifida fusca*, a thermophilic bacterium belonging to Actinobacteria is a major degrader of plant cell walls. The protein profiles of the secretome produced by *T. fusca* grown in cellulose, lignin, and mixture of cellulose and lignin containing culture media, promoting production of respective substrate hydrolyzing enzymes was explored using proteomics approach with high throughput isobaric tag for relative and absolute quantification (iTRAQ) technique using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The iTRAQ quantification of the secretome revealed unique extracellular enzyme system, including discrete multi-enzyme complexes of cellulases, hemicellulases, glycoside hydrolases, proteases, peroxidases and protein translocating transporter proteins. When the strain was grown in these substrate conditions, proteins corresponding to cellulases, hemicellulases and transport proteins were highly up-regulated, while lignin degrading DyP-type peroxidase, novel non-heme peroxidases, catalase, cytochrome-c oxidase and superoxide dismutase were also identified. Numerous proteins presumed to be involved in lignocellulose hydrolysis were expressed in response to these different culture conditions and among these were several secreted hypothetical proteins that were not previously observed.

## Introduction

Lignocellulosic biomass composed of cellulose, lignin and hemicelluloses is one of the largest sink for fixed global carbon and hence considered as a potential feedstock in biofuel. In near future, the most expected and widely used biofuel around the globe would be cellulosic ethanol, produced from abundant and inexhaustible supplies of lignocellulosic biomass from plants and plant-derived materials such as forest waste, agricultural waste, and herbaceous crop residues. However, developing new technologies for efficient bioconversion of lignocellulosic biomass into biofuel would be prerequisite to substitute for petroleum-based energy. Several microorganisms are capable of hydrolyzing cellulose[1] and those efficiently hydrolyze hemicelluloses and convert lignin to monomeric components have been isolated and studied.[2] The efficient conversion of lignocellulosic biomass involves the release of long chain polysaccharides, specifically cellulose and hemicelluloses and subsequent breakdown of these polysaccharides into sugars. Due to complex nature of lignocellulose biomass, efficient hydrolysis requires the concerted action of multiple enzymes such as cellulase, glycanaeses, laccases and peroxidases and others.

White-rot fungi are the inhabitant of forest litter and fallen trees having the potential to degrade all components of plant cell walls including cellulose, hemicelluloses, and lignin; [3] and characterized for lignolytic enzymes such as lignin peroxidase, manganese peroxidase, and laccases. [1, 4] Although its genome sequence encodes lignin degrading secretory oxidases, peroxidases and hydrolytic enzymes, they cannot grow on lignin alone; but have the unique potential to degrade major proportion of it completely to

$CO_2$ and $H_2O$. [3] The cellulolytic, *Trichoderma reesei* was other mostly studied fungus and most of the commercial cellulases are produced industrially using genetically modified strain by random mutagenesis or by targeted genetic modifications such as introducing strong inducible promoters, increasing the gene copy numbers or removing undesired secreted proteins. [5] Earlier researches on lignocellulose degradation were focused on fungi since they are potential lignocellulose degraders. Recently, genome analysis of *Thermobifida fusca* revealed existence of several glycoside hydrolases in addition to the previously identified cellulases and xylanases. Though considerable research literature is available on the analysis of human pathogenic bacteria and yeasts, only limited number of studies exists on bacteria hydrolyzing renewable biomass.

*T. fusca*, an aerobic, thermophilic soil bacterium belonging to Actinobacteria*;* secretes extracellular glycoside hydrolases and is primary degrader of plant biomass in heated organic matter, soil, compost heaps, manure piles and rotting hay. [6, 7] Using traditional biochemical methods, Irwin et al.[8] identified six extracellulases (four endoglucanases and two exoglucanases) in *T. Fusca*. Further, extracellular xyloglucanase; [9] an intracellular β-glucosidase [10] and β-1, 3-glucanase [11-13] have been identified and characterized. In addition, the protein CelR, which regulates the induction of cellulases and related enzymes have been reported. [8, 14] Genome analysis of *T. fusca* encodes 45 hydrolytic enzymes including 36 glycoside hydrolases distributed in 22 GH-families, 9 carbohydrate esterases, and 2 polysaccharide lyases. In addition, genome analysis revealed 28 more putative glycoside hydrolases and enzymes potentially involved in plant cell wall degradation. Among the predicted genes, 68% have been assigned a function; 26% (830 genes) without predicted functions [6] and 3.3% (106 genes)

as a unique in *T. fusca*. The researchers [8-14] focused either one enzyme or one class of enzyme, and despite the cellulase secretion in response to cellobiose [15]; the comprehensive and systematic whole secretome analysis of *T. fusca* in response to different culture conditions containing cellulose, lignin or both is still lacking. This has created big gap between understanding the secreted/released lignocellulosic enzymes and their biotechnological application in lignocellulose biomass hydrolysis. To fill this gap, whole secretome analysis and identification of secreted enzymes under cellulose and lignin containing culture conditions is very much essential. Further, comparison of the *T. fusca* secretome under different culture conditions with cellulose and lignin might highlight different essential secreted proteins and also explore their response to these plant cell wall constituents. Therefore, the objective of this study was to identify and quantify the predominant secretary proteins involved in cellulose and lignin hydrolysis by high throughput quantitative iTRAQ-based LC-MS/MS proteomics approach under different culture conditions.

## Materials and Methods

### Microorganism cultivation conditions and secretome extraction

The previously grown *T. fusca* was inoculated into three test flasks containing media supplemented with 0.1% cellulose fibrous, long (Sigma, Cat No. C6663), 0.1% lignin alkali (Aldrich, Cat No. 471003) and 0.1% each of cellulose and lignin respectively. The control served only basic media without any supplement of cellulose or lignin. The media composed of 3.1 g $L^{-1}(NH_4)_2SO_4$; 2.0 g $L^{-1}$ glucose; 1.5 g $L^{-1}$ NaCl; 0.9 g $L^{-1}$ $KH_2PO_4$; 0.91 g $L^{-1}$ $K_2HPO_4$, and micronutrients 0.200 g $L^{-1}$ $MgSO_4.7H_2O$; 0.008 g $L^{-1}$

$ZnSO_4.7H_2O$; 0.02 g $L^{-1}$ $FeSO_4.7H_2O$; 0.015 g $L^{-1}$ $MnSO_4.H_2O$; 0.026 g $L^{-1}$ $CaCl_2.2H_2O$. After incubation at 55 ºC, 200 rpm for 72 h, culture supernatants were harvested by centrifugation at 12, 000 x g at 4 º C (Beckman Coulter, USA). The experimental design contained at least three independent experiments for each culture conditions. The supernatant from three independent biological replicates were pooled to minimize biological variation, complete protease inhibitor cocktail (50mM, Roche, Mannheim, Germany) was added and the pooled supernatants were further clarified using 0.2µm filter assembly. The proteins in the supernatant were precipitated with TCA (10% w/v) for overnight at 4 ºC and washed with ice cold acetone at 20,000 x g at 4 ºC. Finally the pellets were resuspended in 0.1% SDS and proteins were quantified using the 2D Quant kit (Amersham Biosciences, USA) according to the manufacturer's protocol.

**Protein digestion, peptide extraction, and mass spectrometric analysis**

Protein from each sample (200 µg) was separated by SDS-PAGE using 10% polyacrylamide at 70V for 60 min. The gel was stained with Coomassie brilliant blue and washed with buffer containing 40% methanol and 10% acetic acid. Each sample lane was sliced separately, washed with 75% acetonitrile containing Triethylammonium bicarbonate buffer (TEAB) (Sigma, USA) and subjected for destaining. After destaining, gel pieces were reduced with Tris 2-carboxyethyl phosphine hydrochloride (TCEP) (5 mM) and then alkylated by methyl methanethiosulfonate (MMTS) (10 mM). After dehydration with 100% acetonitrile, gel pieces were subjected to sequencing grade modified trypsin (Promega, Madison, WI) digestion at 37 º C for 20 h. The peptides were

extracted using 50% ACN/5% acetic acid, dried with vacuum centrifuge for subsequently iTRAQ reagent labeling.

**iTRAQ labeling and LC-MS/MS analysis**

The iTRAQ labeling of peptide samples derived from four different substrate conditions were performed using iTRAQ reagent Multiplex kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. Two iTRAQ labeling experiments were conducted for each pooled sample to minimize variations due to non-uniform labeling. The peptides labeled with respective isobaric tags, incubated for 2 h and vacuum centrifuged to dryness. The two labeled samples were pooled and reconstituted in Buffer A (10mM $KH_2PO_4$, 25% acetonitrile, pH 2.85), iTRAQ labeled peptides were fractionated using PolySULFOETHYL A$^{TM}$ SCX column (200 x 4.6mm, 5μm particle size, 200 A$^o$ pore size) by HPLC system (Shimadzu, Japan) at flow rate 1.0 ml min$^{-1}$. The 50 min HPLC gradient consisted of 100% buffer A (10 mM $KH_2PO_4$, 25% acetonitrile, pH 2.85) for 5 min; 0-20% buffer B (10 mM $KH_2PO_4$, 25% ACN, 500 mM KCL, pH 3.0) for 15 min; 20-40% buffer B for 10 min; 40-100% buffer B for 5 min followed by 100% buffer A for 10 min. The chromatograms were recorded at 218nm. The collected fractions were desalted with Sep-Pak® Vac C18 cartridges (Waters, Milford, Massachusetts), concentrated to dryness using vacuum centrifuge and reconstituted in 0.1% formic acid for LC-MS/MS analysis.

The mass spectroscopy analysis in triplicate was performed using a Q-Star Elite mass spectrometer (Applied Biosystems; MDS-Sciex, USA), coupled with online micro flow HPLC system (Shimadzu, JAPAN). Technical replicate with respect to Q-Star Elite

mass spectrometer was set at three as multiple injections give better coverage of the target secretome with superior statistical consistency. This is especially true for single peptide proteins as more MS/MS spectral evidence was obtained from multiple injections leading to a higher confidence of peptide identification and quantification. The peptides were separated using nanobored C18 column with a picofrit nanospray tip (75 µm ID x 15 cm, 5 µm particles) (New Objectives, Wubrun, MA). The separation was performed at a constant flow rate of 20 µl $min^{-1}$, with a splitter to get an effective flow rate of 0.2 µl $min^{-1}$. The mass spectrometer data acquired in the positive ion mode, with a selected mass range of 300-2000 *m/z*. Peptides with +2 to +4 charge states were selected for MS/MS. The three most abundantly charged peptides above a 5 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.

**Mass spectrometric data analysis**

The data acquisition was performed with Analyst QS 2.0 software (Applied Biosystems/ MDS SCIEX). Protein identification and quantification were performed using ProteinPilot Software 2.0.1, Revision Number: 67476 (Applied Biosystems, Foster City, CA). The Paragon algorithm in the ProteinPilot software was used for the peptide identification which was further processed by Pro Group algorithm where isoform-specific quantification was adopted to trace the differences between expressions of

various isoforms. User 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 *T. fusca* DB from NCBI RefSeq (target: 3110 sequences; 1030353 residues; downloaded from http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome&cmd=Retrieve&dopt=Protein+Table&list_uids=18653) and the corresponding reverse sequence (decoy: for false discovery rate (FDR=2.0*decoy_hit/total_hit) estimation); (x) Search effort, thorough. For iTRAQ quantitation, the peptide for quantification was 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. For the prediction of signal peptides, the whole secretome of *T. fusca* was analyzed using the SignalP 3.0 software (http://www.cbs.dtu.dk/services/SignalP). Metabolic pathways were assigned according to KEGG Pathway Database. For hypothetical proteins, function prediction was assigned by performing batch BLASTp (KoriBLAST 2.5) against NCBI non-redundant protein databases using BLOSUM62 matrix.

**Cellulose hydrolysis and enzyme assay**

Samples were withdrawn at regular intervals from the culture gown as stated above for the determination of residual cellulose, enzyme activities and cell growth. The residual cellulose was determined as stated by Huang and Forsberg [16] after digesting with

67% sulphuric acid (35ºC, 30 min) and quantifying released monosaccharides using anthrone method. [17] Carboxymethylcellulase (endo-1,4- β -glucanase, EC 3.2.1.4) and Avicelase (exo-1,4-β-glucanase, EC 3.2.1.91) were assayed by measuring the reducing sugars. The reaction mixture in CMCase contained 1 ml of crude enzyme solution, 1 ml of 1% carboxymethylcellulose (Sigma-Aldrich, St. Louis, MO) in 0.05 M potassium phosphate buffer (pH 6.0) while for Avicelase, the carboxymethylcellulose was substituted by 1 ml of 1% Avicel (Sigma-Aldrich). After incubation at 55ºC for 30 min, the reducing sugars were assayed by the anthrone method. One international unit (IU) of enzyme activity was defined as the amount of enzyme releasing 1 μmol reducing sugar per minute.

# Results

**Cellulose degradation and proteins secreted by *T. fusca* under different culture conditions**

Fig 1 presents the batch test cellulose hydrolysis results of *T. fusca* with initial cellulose concentration of 1.0-2.0 g $L^{-1}$ at 55 ºC. The cellulose was completely hydrolyzed in 96 h and 118 h at initial concentration of 1.0 and 2.0 g $L^{-1}$ respectively. The endoglucanse and exoglucanse activities were consistent with the iTRAQ results (Fig 2). All proteins identified in the secretome of *T. fusca* that were common in all culture conditions including control, cellulose, lignin and both cellulose and lignin were listed in table S1 (supplementary). Considering the complexity of secreted proteins, we used complete protease inhibitor cocktail during secretome collection and isolation of the secreted proteins to minimize degradation of secreted proteins by proteases. To minimize

the false positive identification, a cut-off with the unused protScore ≥ 2 was used as identification criteria, corresponding to a protein confidence level of 99%. The corresponding FDR is < 0.63%. Table 1 lists iTRAQ quantified major enzymes secreted in different culture conditions.

**Cellulases:** The cellulases, three β-1,4-endoglucanases (Cel5A, Cel6A and Cel9B); two β-1,4-exoglucanases (Cel6B and Cel48A); one processive endoglucanase (Cel9A) and one β-glucosidase were quantified in all three culture conditions. Of these secretory proteins only Cel9A (EC=3.2.1.4; EC=3.2.1.91) has no secretion signal. The enzyme β-1,4-exoglucanase (Tfu_1959) was significantly induced ($p<0.001$, ANOVA) in cellulose condition when compared to other culture conditions (Fig. 3). The complete cellulose hydrolysis requires endoglucanases, exoglucanases and β-glucosidases. Based on the iTRAQ analysis, cellulases Cel9A belonging to family GH-9; Cel9B, family GH-9; Cel6B, family GH-6; Cel48A, family GH-48 and β-glucosidase, family GH-1 were up-regulated. The proteins including Cel9A, Cel9B, Cel6B and Cel5A were induced when culture medium contained both cellulose and lignin.

**Hemicellulases:** The hemicellulases including endo-1,4-β-xylanase (family GH-10, Tfu_2791), α-L-arabinofuranosidase (family GH-43, Tfu_1616) were significantly expressed in cellulosic condition relative to control without cellulose (Fig3). When compared with cellulose and lignin containing culture conditions, the contents of α-L-arabinofyranosidase (Tfu_1616) and acetyl xylan esterase/chitin deacetylase (Tfu_2788) were high when both cellulose and lignin were used for strain culture. The xylanase and acetyl xylan esterase were also induced in lignin culture condition. Of these identified

hemicellulases, α-L-arabinofuranosidase (EC=3.2.1.55) has no secretion signal (Table 1).

**Other glycoside hydrolases and proteolytic enzymes:** In addition to earlier reported cellulases (Cel5A, Cel6A, Cel9B, Cel6B, Cel48A, Cel9A) and β-glucosidase, this study further reports cellulose-binding family II protein, trehalase/maltose phosphorylase, putative secreted cellulose-binding protein, pectate lyase and hypothetical protein Tfu_2130 proteins secreted by *T. fusca* with variable response to different culture conditions. The comparative relative iTRAQ quantification of the secreted proteins in cellulose, lignin and mixture of both cellulose and lignin containing culture conditions were presented in Fig. 3. The proteins cellulose-binding family II protein, pectate lyase, and putative secreted cellulose-binding protein were significantly up-regulated in all three culture conditions; while trehalase/maltose phosphorylase and hypothetical protein Tfu_2130 were induced only in mixture of cellulose and lignin containing culture medium. The putative secreted cellulose-binding proteins (Tfu_1268) were significantly induced ($p<0.001$, ANOVA) in cellulose condition when compared to other culture conditions (Fig. 3). The proteolytic enzymes such as NLP/P60 family secreted protein (Tfu_1030) streptogrisin C. Serine peptidase (Tfu_0484) and protease inhibitor precursor (Tfu_2085) were up-regulated in cellulose culture condition (Table1). The protein Tfu_0484 was induced in lignin while Tfu_2085 in cellulose culture conditions.

**Transport and hypothetical proteins:** Among the proteins classified under membrane transport, ABC-type dipeptide/oligopeptide (Tfu_0910), putative ABC transporter permease protein (Tfu_0932), putative solute binding lipoprotein (Tfu_0934), putative α-glucosides-binding ABC transporter (Tfu_2337) and ATPase (Tfu_0588) were

significantly up-regulated in all tested culture conditions (Fig 4). The protein Tfu_0910 was significantly induced ($p<0.001$, ANOVA) in lignin, and cellulose and lignin culture conditions when compared with cellulosic culture condition.

The major hypothetical proteins with their secretory signal were listed in table 1. The proteins Tfu_0517, Tfu_2236, Tfu_0458, Tfu_0448, Tfu_2403, Tfu_0814, Tfu_0581, Tfu_1213 and Tfu_2791 were up-regulated in the cellulosic culture condition (Fig 5). While Tfu_0607, Tfu_2403, Tfu_1018 and Tfu_0144 were induced in both lignin, and cellulose and lignin containing culture medium.

## 4. Discussion

The most of the earth's renewable carbon exits in the form of lignocellulose that mainly composed of cellulose (insoluble fibers of β-1,4-glucan), hemicelluloses (xylans, mannans and glucans) and lignin (polyphenolic polymer); and its hydrolysis into fermentable sugars has the potential of biofuel generation. The lignocellulose hydrolysis into sugars essentially need cocktail of lignocellulolytic enzymes. Microbial cellulases and hemicellulases production is dependent on the carbon source; [18] hence we used cellulase and lignocellulase inducing cellulose and lignin separately and in combination to culture *T. fusca*. In our study, iTRAQ quantified secreted proteins in these growth conditions were assigned to cellulases (Cel9A, Cel9B, Cel6B, Cel48A, Cel5A, Cel6A and β-glucosidase) and hemicellulases including endo-1,4-β-xylanase, xylanase/ α-L-arabinofuranosidase, acetyl xylan esterase /chitin deacetylase, pectate lyase, trehalase/maltose phosphorylase and several novel hypothetical proteins. Although considerable research literature exits on cellulose hydrolysis and cellulases production by

*T. fusca,* a major plant cell wall degrader; but with our best knowledge, this is first study that report identification and up-regulation of acetyl xylan esterase /chitin deacetylase, pectate lyase, trehalase/maltose phosphorylase, hypothetical protein Tfu_2130, cellulose-binding family II protein, putative secreted cellulose-binding protein in the tested culture conditions. The prominently secreted iTRAQ quantified cellulose hydrolyzing protein cocktail constitutes three β-1,4-endoglucanases (Cel5A, Cel6A and Cel9B); two β-1,4-exoglucanases (Cel6B and Cel48A); one processive endoglucanase (Cel9A) and one β-glucosidase and were consistent with literature reports.[8-15]

Among the cellulases β-1,4-exoglucanase (Cel48A) that attack the chain ends of cellulose, releasing cellobiose showed higher iTRAQ ratio (4.30) followed by β-1,4-exoglucanase (Cel6B) with iTRAQ ratio of 2.56 relative to control. The efficient degradation of lignocellulosic biomass depends upon the appropriate levels of endoglucanase, exoglucanase and β –glucosidase secretion.[19] The identified and regulated enzymes that involve in lignocellulose biomass hydrolysis were β-1,4-endo/exoglucanase; β-1,4-endoglucanase; β-1,4-exoglucanase; β-1,4-exoglucanase; β-glucosidase; endo-1,4-β-xylanase and xylanase/ α-L-arabinofuranosidase. Besides *T. fusca,* several bacterial strains such as *Rhodospirillum rubrum, Cellulomonas fimi, Clostridium stercorarium, Bacillus polymyxa, Pyrococcus furiosus, Acidothermus cellulolyticus,* and *Saccharophagus degradans* produces cellulases and hydrolyse cellulose. [20-22] However, they are not effective like *T. fusca* on the crystalline/fibrous cellulose as complete hydrolysis of crystalline/ insoluble fibrous cellulose requires synergetic action of cellulases.[19] Although *Trichoderma reesei,* has the potential to produces exoglucanases/cellobiohydrolases (CBH1 and CBH2), endoglucanases (EG1,

EG2, EG3, EG5), and β-glucosidase [18] essential for cellulose degradation; the cellulose hydrolysis efficiency is limited due to lower content of β-glucosidase. On the contrary, *Aspergillus niger* fungi produces cellulases having high β-glucosidase activity but lower endoglucanase levels.[23, 24] Further, the major advantages of *T. fusca* cellulase over fungal cellulases were its thermostable nature and broad pH range.

In addition to cellulases and hemicellulases, enzymes such as DyP-type peroxidase, novel non-heme peroxidases cytochrome-c oxidase, catalase and superoxide dismutase were also identified in the secretome of *T. fusca.* Thus, the present study highlights complete secretome of *T. fusca* that includes bacterial cellulases existing as a discrete multi-enzyme complex, xylan hydrolyzing xylaneses, pectin hydrolyzing pectate lyase and lignin depolymerizing peroxidases and oxidase.

**Actively Expressed proteins**

**Cellulases**: Cellulose is a homopolysaccharide composed of β-D-glucopyranose units that linked by β-1,4 glycosidic bonds. All six cellualses (Cel5A, Cel6A, Cel9B, Cel6B, Cel48A, Cel9A) and β-glucosidase that were identified in the *T. fusca* secretome showed significant up-regulation in cellulose containing culture conditions. The catalytic domains of these cellulase enzymes belong to four different families: 5, 6, 9 and 48. Interestingly, family 6 contains two cellulases but they are very different as one is an endocellulase (Cel6A) while the other is an exocellulase (Cel6B). Similarly, for family 9, one enzyme is an endocellulase (Cel9B), the other is a novel type of cellulase, a processive endoglucanase (Cel9A). Thus, all six cellulases identified in the secretome of *T. fusca* that have potential to degrade cellulose are different from each other.

The efficient cellulose hydrolysis potential of *T. fusca* was due to synergetic action of these up-regulated endoglucanases and exoglucanases, as endoglucanases catalyses random cleavage of internal bonds of the cellulose chain, exoglucanases attack the chain ends, releasing cellobiose; while β-glucosidases converts cellobiose into glucose. [19] The hydrolyzed cellulose further induces cellulases synthesis [25] and repressor CelR regulates the expression of these cellulase genes. [6, 10, 26] The relative iTRAQ ratio exhibited that *T. fusca* secretes required enough level of β-glucosidases that hydrolyse cello-oligo-saccharides preventing accumulation of cellobiose, which otherwise inhibits the synthesis of endo- and exoglucanases due to negative feedback. [27] Spiridonov and Wilson [26] characterized β-glucosidase using different substrates and demonstrated cleavage of β-1,4 and β-1,2 glycosidic bonds.

The putative secreted cellulose-binding proteins showed significantly higher iTRAQ ratio of 9.26 in presence of cellulose. According to gene ontology this proteins involve in hydrolyzing O-glycosyl compounds. Thus, their higher expressions in cellulosic condition and being as hydrolyzing enzyme it might be involved in cellulose hydrolysis and further detailed research needed to characterize this novel protein.

**Hemicellulases**: Hemicelluloses composed of pentoses and hexoses are second most abundant renewable biomass accounting 25–35% of lignocellulosic biomass [28] and also an important source of fermentable sugars for bio-refining applications. Hemicelluloses in hardwood contained mainly xylans, while in softwood glucomannans are most common. [19] In the secretome of *T. fusca*, four hemicellulases endo1,4-β –xylanase, α-L-arabinofuranosidase, xylanase and acetyl xylan esterase were identified in all three culture conditions. Since these enzymes have hemicelluloses hydrolysis potentials they

may play important role in converting hemicelluloses into sugars. The endo 1,4-β–xylanase, xylosidase α-L-arabinofuranosidase, glucoronidase, xylanase and acetyl xylan esterase act on different heteropolymers of plant hardwood. The glucomannan degrading mannan endo-1,4-beta mannosidase that act on the polymer backbone was also identified in this study.

**Glycoside hydrolases and other enzymes**: In addition to cellulases and hemicellulases, this study identified several hypothetic proteins. The hypothetical protein (Tfu_2130) belonging to family GH81 identified in the *T. fusca* secretome was recently characterized for its endohydrolytic action on callose. [12] Callose, a plant polysaccharide composed of glucose residues linked through β-1,3-linkages and termed as β-glucan. This hypothetical protein having β-1,3-glucanases activity showed significant up-regulation in cellulose and lignin culture condition supporting its involvement in β-glucan hydrolysis as earlier reported [12]. The extracellular proteases activate several isozymes by specific cleavages. [29] The extracellular proteases such as the most recognized thermostable serine protease and a putative proteolytic enzyme containing NLP/P60 domain proteins showed up-regulation in cellulose condition. TfpA, the most recognized *T. fusca* thermostable serine protease identified in the secretome activates cellulase isozymes by specific cleaves. [29-30] The trehalase/maltose phosphorylase enzyme identified in the *T. fusca* secretome could depolymerize polysaccharides using phosphates as catalytic nucleophile. Other identified glycoside hydrolases significantly up-regulated in the used culture conditions were hypothetical protein Tfu-2130 belonging to glycoside hydrolase family 81, putative secreted cellulose-binding protein and cellulose-binding family II protein containing carbohydrate binding domains whose catalytic functions have not yet been revealed. The

non-catalytic domains of putative secreted cellulose-binding protein and cellulose-binding family II protein participate in complex formation, substrate binding, or cell surface attachment facilitating the cellulose hydrolysis. [31] The surface protein also called collagen adhesion protein having unused score 127.46 was significantly abundant and was up-regulated when lignin and mixture of lignin and cellulose were used for culturing the strain. (Table S1). The LC-MS/MS identified isoamylase-type glycogen debranching enzyme (GlgX) also possesses high specificity for hydrolysis of carbohydrate chains with multiple glucose residues. [32] While, xylan hydrolyzing β-xylosidase identified in the secretome of *T. fusca* emphasizes its use for lignocellulose biomass hydrolysis.

**High abundant non- hydrolase protein**

In addition to functionally classified proteins, FAD binding protein dihydrolipoamide dehydrogenase that play major role in redox homeostasis regulation was abundant but not up-regulated. The unclassified multifunctional putative intracellular protease/amidase protein belonging to PfpI family was also high abundant (unused protein score =107.35) (Table S1, Supplementary) and based on ontology it has both hydrolase and protease activity. While according to gene ontology this protein has peptidase activity. In addition, several putative proteins having multiple functions including putative aminopeptidase that has hydrolase, protease and aminopeptidase activity were abundant in the *T. fusca* secretome. Restated, in addition to cellulases and hemicellulases *T. fusca* also produces proteases that partially degrade their cellulases into multiple active isozymes. [15 29] Several cytoplasmic proteins were also detected in the secretome of *T. fusca* suggesting possible cell damage during cell division or harvesting.

**Proteins involved in lignin degradation and their expression in *T. fusca* secretome**

Presently, it has been widely accepted that *T. fusca* could partially hydrolyze lignin components in lignocellulosic complex to get access to utilizable cellulose and hemicelluloses. [33] According to Crawford [34] *T. fusca* strain degrades lignin partially when incubated for 14 days with lignocellulosic pulps. Under lignin culture condition, we identified cytochrome-c oxidase, DyP-type peroxidase, catalase, novel non-heme peroxidases and superoxide dismutase in the secretome of *T. fusca*. Although, these proteins were not significantly up-regulated but their existence in the secretome were in consistent with the genome analysis of *T. fusca* that showed genes encoding for Mn-dependent peroxidases, heme catalases and Mn-dependent superoxide dismutase. [6] These novel non-heme peroxidases and superoxide dismutase produced by *T. fusca*, were shown to degrade lignin-containing substrates. [35, 36] The DyP-type peroxidases comprise a novel superfamily of heme-containing peroxidases which is unrelated to the superfamilies of known peroxidases and all members have not yet been characterized in detail. Recently, Van Bloois et al [37] biochemically characterized DyP-type peroxidase (TfuDyP) as a monomeric, heme-containing, thermostable, and Tat-dependently exported peroxidase. Further, the identified pectate lyase (Tfu_2168) showed up-regulation in lignin condition but not in cellulose culture condition. *T. fusca* grow well on pectin de-esterified derivatives but not on lignin alone. [6]

**Transport proteins secreted by *T. fusca***

The bacterium *T. fusca* has a *sec* system for general protein secretion and *sec*-independent twin-arginine protein translocation (TAT) system [6] to translocate fully

folded proteins; a unique protein transport system might have significant implications for the secretion of functional enzymes. The carbohydrate transport proteins: putative α-glucosides-binding ABC transporter and putative solute binding lipoprotein; amino acid transport proteins: ABC-type dipeptide/oligopeptide; solute transport proteins: ABC transporter and ATPase were regulated when *T. fusca* was cultured in tested culture conditions. The *T. fusca* genome analysis revealed extensive set of transporters including eight ABC disaccharide transporter cassettes and majority of them were localized on the chromosome next to glycoside hydrolases. [6] In lignin containing culture medium transporter systems including carbohydrate and amino acid transporter were up-regulated. These regulated transport proteins quantified by iTRAQ were in consistent with the *T. fusca* genome analysis as it identified the presence of cellobiose/cellotriose, maltose and xylobiose ABC transport systems, a permease of the major facilitator superfamily, four putative amino acid ABC transport systems, ion transport and heavy metal transporter. [6] The genome analysis study has unraveled that *T. fusca* employs sec secretion system as one of the ways for protein secretion. [6]

**Biotechnology for biofuels**

The lignocellulosic biomass such as agricultural waste and forest residues is abundant in the world and could be hydrolyzed into glucose using hydrolytic enzymes secreted by microorganisms. Bacterial cellulases exist as discrete multi-enzyme complexes, called cellulosomes that consist of multiple subunits. [18] The six cellualses and one β-glucosidase were identified in the secretome of *T. fusca* that have potential to catalyse the hydrolysis of lignocellulose biomass into its constituent sugars for downstream fermentation into

commercial products such as alternative fuels, novel commodities, and specialty chemicals. The *T. fusca* is a major plant cell wall degrader in soil, [6] therefore detailed studies with practical agriculture/forest waste would explore the useful enzymes for biotechnological application in biofuel.

## Conclusion

The *T. fusca* grown in cellulose and lignin culture medium produced extracellular enzymes including multi-enzyme complexes of cellulases, hemicellulases and glycoside hydrolases. In addition to cellulases and hemicellulases, *T. fusca* produced different extracellular proteases that activate multiple cellulase isozymes. The lignin de-polymerizing enzymes such as DyP-type peroxidase, novel non-heme peroxidases cytochrome-c oxidase, catalase and superoxide dismutase were also quantified using iTRAQ based proteomics technique. Thus, this study reports the comprehensive systematic quantification and regulation of *T. fusca* secretome constituent including cellulases, hemicellulases, other glycoside hydrolases, proteases, peroxidases and protein translocating transport proteins; and support the lignocellulosic biomass hydrolyzing enzyme secretion and their biotechnological application for biofuel and biorefinery. In addition, numerous hypothetical proteins produced by *T. fusca* having lignocellulose biomass hydrolysis potential were identified and their response to different culture conditions was explored.

## Acknowledgments

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**Figure legends**

Fig 1 Cellulose concentrations versus time curves showing cellulose hydrolysis by *T. fusca* at 55 ºC.

Fig 2 Time course of endoglucanse and exoglucanse production by *T. fusca*

Fig 3. iTRAQ ratios of regulated functional cellulase, hemicellulases, and glycoside hydrolase and protease proteins

Fig 4. iTRAQ ratios of regulated carbohydrate, amino acid, multiple sugar, ion, solute transporting and protein translocating proteins

Fig 5. iTRAQ ratios of regulated unmapped hypothetical proteins

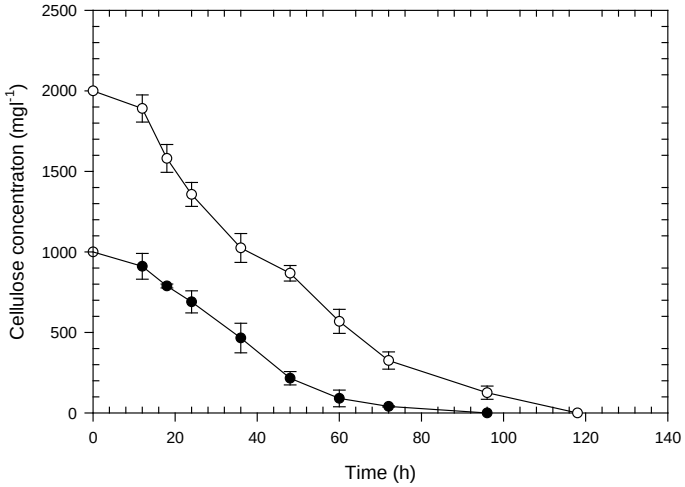



Fig 1 Cellulose concentrations versus time curves showing cellulose hydrolysis by *T. fusca* at 55 ºC.

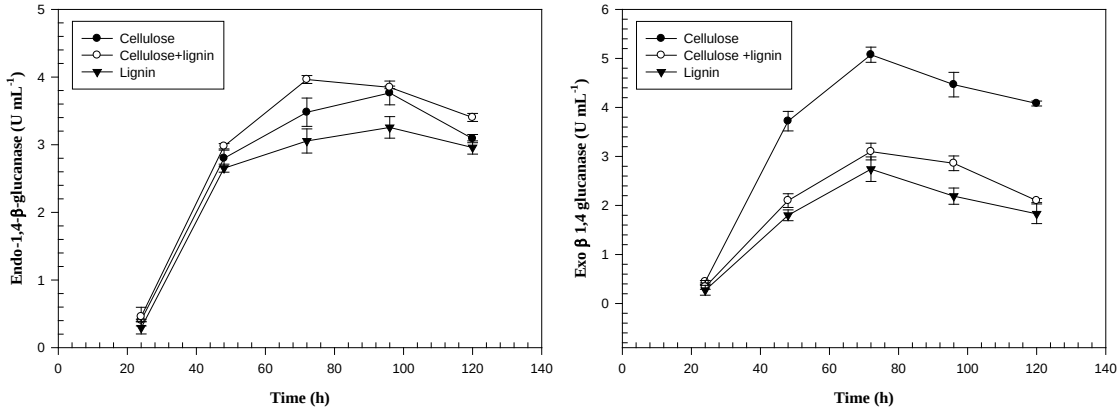



Fig 2 Time course of endoglucanse and exoglucanse production by *T. fusca*

Table 1. The functional classification of *T. fusca* secretary regulated proteins in different culture conditions.

| Gene locus[a] | Protein name | Accession number | Protein score (Unused) | No. of peptides * | Cellulose : control | Cellulose +lignin: control | lignin: control | SignalP (EC number) |
|---|---|---|---|---|---|---|---|---|
| **A) Cellulases** | | | | | | | | |
| Tfu_1959 | 1,4-beta-exoglucanase (E6, Cel48A) | gi\|72162358 | 30.04 | 20 | 4.30 | 2.36 | 1.68 | Y (EC=3.2.1.91) |
| Tfu_0620 | 1,4 beta-exoglucanase (E3, Cel6B) | gi\|72161024 | 16.72 | 16 | 2.56 | 1.76 | 1.56 | Y (EC=3.2.1.91) |
| Tfu_1627 | 1,4 beta-endo/exoglucanase (E1, Cel9A) | gi\|72162028 | 16.18 | 9 | 1.63 | 1.66 | 1.66 | N (EC=3.2.1.4; EC=3.2.1.91) |
| Tfu_0901 | 1, 4 beta-endoglucanase (E5, Cel5A) | gi\|72161305 | 8.06 | 4 | 1.42 | 1.30 | 2.54 | Y (EC=3.2.1.4) |
| Tfu_2176 | 1,4 beta-endoglucanase (E4, Cel9B) | gi\|72162575 | 20.28 | 11 | 1.77 | 1.85 | 1.22 | Y (EC=3.2.1.4) |
| Tfu_1074 | β-1,4-Endoglucanase (E2; Cel6A) | gi\|72161478 | 12.86 | 10 | 1.24 | 2.31 | 3.66 | Y (EC=3.2.1.4) |
| Tfu_0937 | β-glucosidase; galactosidase | gi\|72161341 | 2.41 | 6 | 1.60 | 0.89 | 0.79 | N (EC=3.2.1.21) |
| Tfu_2712 | glycoside hydrolase family protein | gi\|72163111 | 8.03 | 5 | 0.61 | 0.98 | 1.60 | Y (EC=3.2.1.4) |
| **B) Hemicellulases** | | | | | | | | |
| Tfu_2791 | endo-1,4-beta-xylanase | gi\|72163190 | 20.20 | 13 | 1.64 | 1.31 | 0.75 | Y (EC=3.2.1.8) |
| Tfu_1616 | α-L-arabinofuranosidase | gi\|72162017 | 12.29 | 5 | 1.85 | 2.56 | 1.17 | N (EC=3.2.1.55) |
| Tfu_2923 | Xylanase; endo-1,4-β-xylanase | gi\|72163322 | 21.30 | 14 | 0.86 | 0.72 | 1.46 | Y (EC=3.2.1.8) |
| Tfu_2788 | Acetyl xylan esterase | gi\|72163187 | 6.04 | 4 | 1.16 | 2.30 | 1.39 | Y |
| **C) Other glycoside hydrolase** | | | | | | | | |
| Tfu_3044 | Trehalase/maltose phosphorylase | gi\|72163443 | 6.71 | 6 | 1.18 | 1.82 | 1.21 | N (EC=2.4.1.216) |
| Tfu_1665 | cellulose-binding family II protein | gi\|72162066 | 8.06 | 6 | 1.96 | 2.11 | 1.38 | Y |
| Tfu_2168 | pectate lyase | gi\|72162567 | 36.36 | 22 | 1.42 | 1.66 | 2.93 | Y |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Tfu_1268 | Putative secreted cellulose-binding protein | gi\|72161672 | 2.00 | 2 | 9.26 | 1.74 | 1.79 | Y |
| Tfu_0900 | mannan endo-1,4-beta-mannosidase | gi\|72161304 | 6.00 | 24 | 0.77 | 0.67 | 1.17 | Y (EC=3.2.1.78) |
| Tfu_2130 | hypothetical protein Tfu_2130 | gi\|72162529 | 16.01 | 9 | 0.77 | 2.16 | 0.89 | Y |
| **D) Proteolytic enzymes** | | | | | | | | |
| Tfu_1030 | NLP/P60 family secreted protein | gi\|72161434 | 2.00 | 1 | 2.57 | 0.74 | 0.63 | Y |
| Tfu_0484 | streptogrisin C. Serine peptidase. | gi\|72160888 | 90.12 | 252 | 1.39 | 0.93 | 1.99 | Y |
| Tfu_2085 | protease inhibitor precursor | gi\|72162484 | 18.59 | 15 | 2.41 | 1.60 | 1.22 | Y |
| **E) Membrane Transport** | | | | | | | | |
| Tfu_0910 | ABC-type dipeptide/oligopeptide /nickel transport systems, | gi\|72161314 | | 6 | 1.76 | 5.84 | 4.32 | Y |
| Tfu_0932 | putative ABC transporter permease protein | gi\|72161336 | | 2 | 2.19 | 1.46 | 1.34 | N |
| Tfu_0934 | putative solute binding lipoprotein | gi\|72161338 | | 21 | 1.43 | 1.25 | 2.13 | Y |
| Tfu_0809 | extracellular solute-binding protein | gi\|72161213 | | 9 | 0.65 | 1.95 | 1.95 | Y |
| Tfu_1636 | periplasmic phosphate binding protein | gi\|72162037 | | 1 | 0.63 | 1.23 | 2.13 | Y |
| Tfu_2337 | Putative α-glucosides-binding ABC transporter | gi\|72162736 | | 2 | 1.27 | 1.45 | 1.95 | Y |
| Tfu_0808 | amino acid ABC transporter | gi\|72161211 | | 12 | 1.71 | 1.11 | 0.89 | N |
| Tfu_0588 | ATPase; putative ABC transporter | gi\|72160992 | | 1 | 1.55 | 1.62 | 1.84 | N |
| Tfu_0933 | ABC transport system substrate-binding proteinNLPA lipoprotein; | gi\|72161337 | | 2 | 0.49 | 0.77 | 0.56 | Y |
| **F) Unmapped proteins** | | | | | | | | |
| Tfu_0517 | hypothetical protein Tfu_0517 | gi\|72160921 | 4.00 | 2 | 1.95 | 1.11 | 1.39 | N |
| Tfu_0607 | surface protein | gi\|72161011 | 127.46 | 209 | 1.12 | 1.39 | 1.36 | Y |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Tfu_2236 | hypothetical protein Tfu_2236 | gi\|72162635 | 8.21 | 4 | 1.50 | 0.94 | 1.25 | Y |
| Tfu_0458 | secreted protein | gi\|72160862 | 11.50 | 7 | 1.30 | 0.47 | 0.45 | Y |
| Tfu_0448 | hypothetical protein Tfu_0448 | gi\|72160852 | 10.02 | 5 | 1.39 | 2.17 | 0.69 | N |
| Tfu_0957 | superoxide dismutase | gi\|72161361 | 48.76 | 102 | 1.23 | 0.26 | 0.25 | N(EC=1.15.1.1) |
| Tfu_2403 | aminopeptidase Y | gi\|72162802 | 61.86 | 68 | 1.27 | 1.52 | 1.53 | Y (EC=3.4.11.15) |
| Tfu_0814 | bacteriocin, lactococcin 972 | gi\|72161218 | 10.11 | 27 | 1.30 | 0.39 | 0.25 | Y |
| Tfu_1018 | hypothetical protein Tfu_1018 | gi\|72161422 | 2.14 | 1 | 0.90 | 1.52 | 1.53 | Y |
| Tfu_0985 | α-amylase domain-containing protein | gi\|72161389 | 60.78 | 88 | 0.68 | 0.39 | 0.47 | Y |
| Tfu_2874 | hypothetical protein Tfu_2874 | gi\|72163273 | 12.71 | 10 | 0.67 | 0.43 | 0.52 | Y |
| Tfu_2677 | hypothetical protein Tfu_2677 | gi\|72163076 | 2.98 | 3 | 0.58 | 0.49 | 0.31 | N |
| Tfu_1414 | FenI protein | gi\|72161818 | 18.74 | 10 | 0.57 | 0.58 | 0.62 | N |
| Tfu_2387 | hypothetical protein Tfu_2387 | gi\|72162786 | 16.61 | 14 | 0.67 | 0.84 | 0.89 | Y |
| Tfu_1922 | solute-binding protein | gi\|72162321 | 58.30 | 91 | 0.23 | 1.16 | 2.94 | Y |
| Tfu_0581 | hypothetical protein Tfu_0581 | gi\|72160985 | 32.86 | 38 | 1.32 | 0.55 | 0.64 | Y |
| Tfu_2964 | hypothetical protein Tfu_2964 | gi\|72163363 | 14.71 | 8 | 0.68 | 6.29 | 8.34 | Y |
| Tfu_1213 | Xylanase | gi\|72161617 | 10.09 | 8 | 1.51 | 1.16 | 1.31 | Y (EC=3.2.1.8) |
| Tfu_0144 | hypothetical protein Tfu_0144 | gi\|72160548 | 6.00 | 3 | 0.71 | 1.52 | 1.76 | N |
| Tfu_1643 | parallel beta-helix repeat-containing ricin B lectin | gi\|72162044 | 9.46 | 5 | 1.19 | 0.65 | 0.72 | N |
| Tfu_1582 | CRISPR-associated RAMP Cmr6 family protein | gi\|72161984 | 4.03 | 2 | 1.24 | 0.81 | 1.61 | N |
| Tfu_1864 | iron(III) dicitrate transport permease | gi\|72162263 | 8.03 | 3 | 0.99 | 0.68 | 0.69 | N |
| Tfu_2130 | hypothetical protein Tfu_2130 | gi\|72162529 | 16.01 | 9 | 0.76 | 2.16 | 0.89 | Y |
| Tfu_2315 | putative secreted protein | gi\|72162714 | 11.81 | 6 | 0.44 | 0.79 | 0.70 | N |
| Tfu_2791 | xylanase | gi\|72163190 | 20.20 | 13 | 1.63 | 1.31 | 0.75 | Y (EC=3.2.1.8) |

[a]From: http://genome.jgi-psf.org

*No. of unique peptides matched to each protein

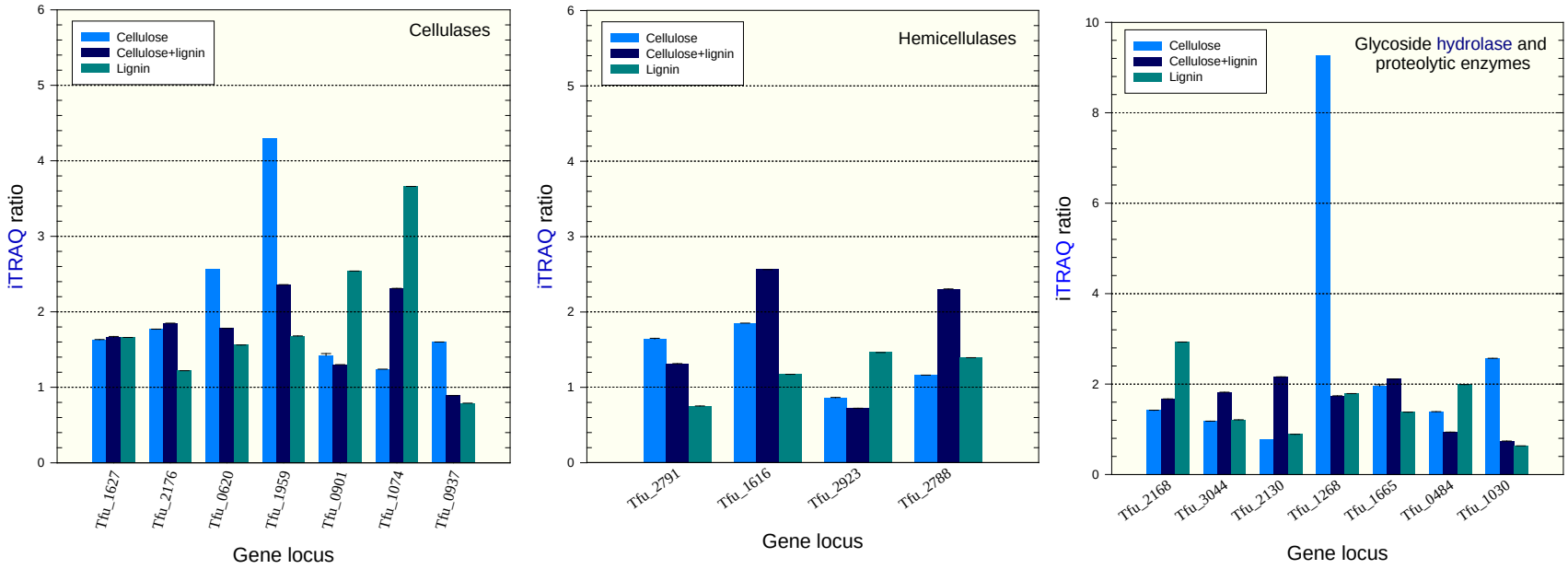



Fig 3. iTRAQ ratios of cellulases, hemicellulases, and glycoside hydrolase and protease proteins

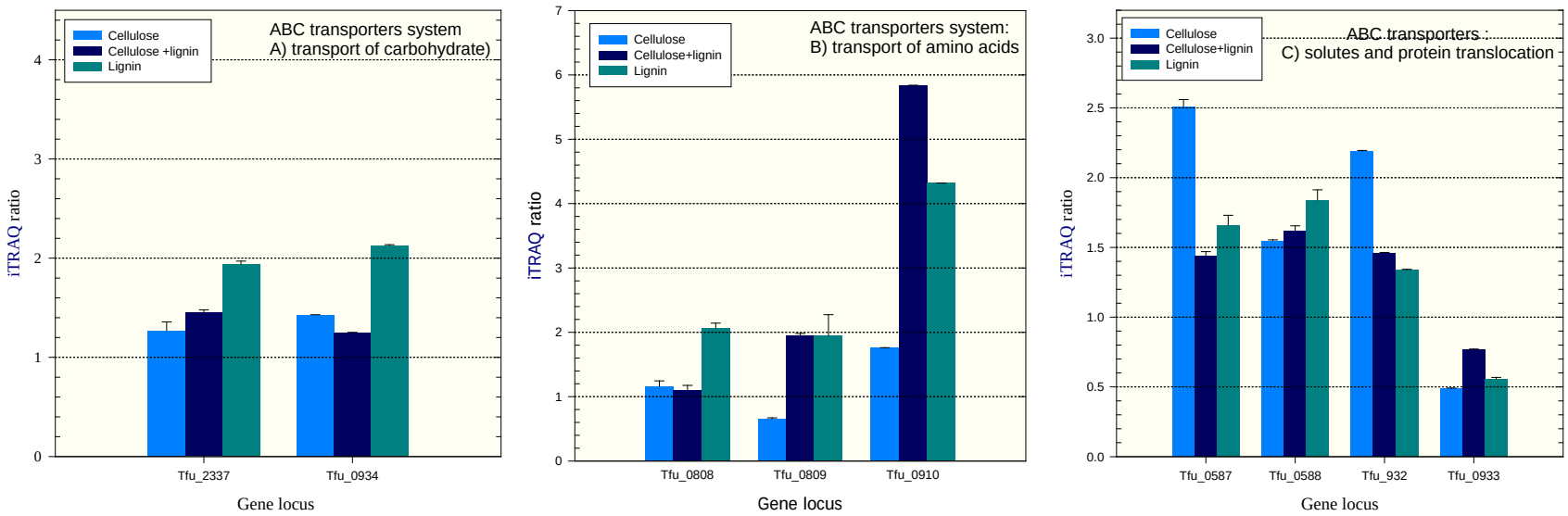



Fig 4. iTRAQ ratios of regulated carbohydrate, amino acid, solute transporting and protein translocating proteins

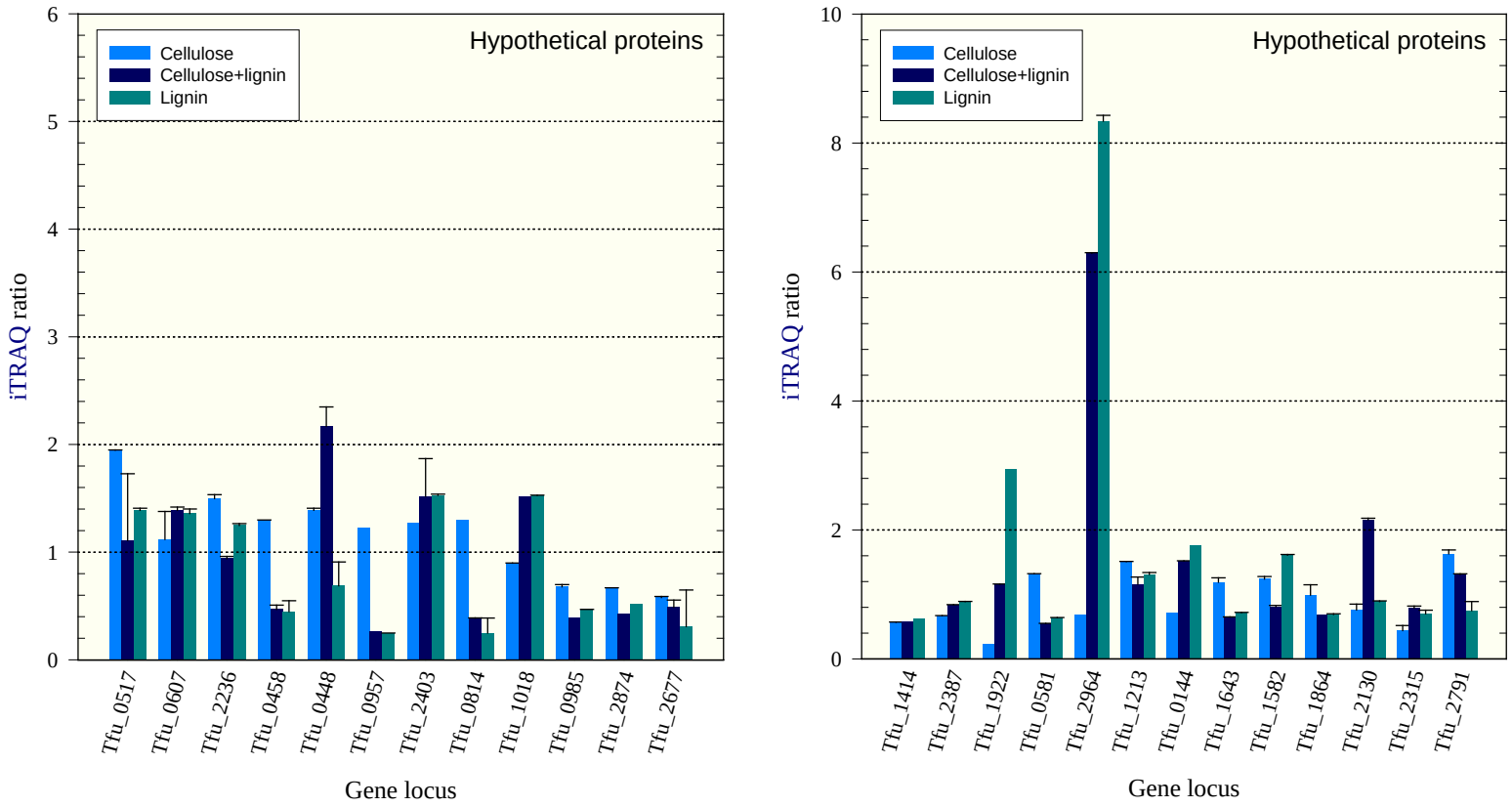
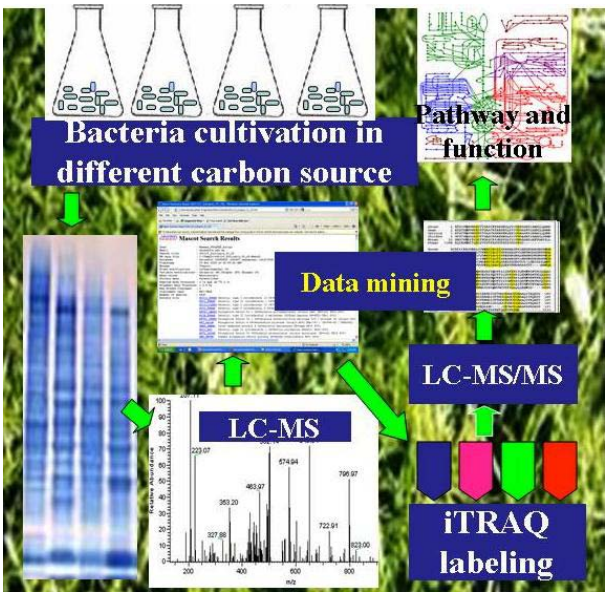



Fig 5. iTRAQ ratios of regulated unmapped hypothetical proteins

# Synopsis




Lignocellulose hydrolysis by bacteria is accomplished by secreted extracellular proteins. Regulation of extracellular secreted proteins of *T. fusca* under different carbon source including cellulose, lignocellulose and lignin were studied. The iTRAQ based proteomics approach comprehensively quantified regulated cellulases, hemicellulases, glycoside hydrolases, proteases and peroxidases. Numerous hypothetical proteins were also expressed in response to different carbon sources. The comparative up-regulation of cellulases, hemicellulases and other glycoside hydrolases exhibited lignocellulose hydrolysis efficiency of *T. fusca* secreted enzymes and its possible biotechnological application for biofuel and biorefinery.