

Transcriptome Based Analysis of a *Lentinus squarrosulus* Strain for the Lignolytic Versatile Peroxidases

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ABSTRACT

Versatile peroxidase, an extracellular heme protein of the lignolytic system is endowed with polyvalent catalytic sites that render this protein a very high redox potential. Versatile peroxidase is regarded as a hybrid of lignin peroxidase and manganese peroxidase as this enzyme possesses the catalytic features of both these enzymes in oxidation of aromatic compounds and is a potential biocatalyst with relevance in multitude of biotechnological applications. Transcriptomic analysis of *Lentinus squarrosulus* demonstrated the expression of versatile peroxidase besides a plethora of biomass degrading enzymes. Bioinformatic analysis identified ten putative versatile peroxidase transcripts with significant protein sequence similarity to the versatile peroxidase isoforms of other white-rot basidiomycetes. The enzyme was purified and visualized on Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with a molecular weight of 49 KDa. Further, application of this purified enzyme on lignocellulosic crop residues showed remarkable decrease in lignin content with corresponding increase of 18-20% *in vitro* dry matter digestibility. Transcriptome analysis of *L.squarrosulus* revealed significant facts about the biodegradative ability of this fungus potentially paving the way for efficient biotechnological applications utilizing its potency.

Keywords: *Lentinus squarrosulus*; Biodegradation; Transcriptome; Versatile peroxidase

INTRODUCTION

Basidiomycetes have been the focus of intense research by mycologists for their antioxidant, antiparasitic, immune modulating effects and considerably on biomass degradation [1]. Biodegradation of plant biomass especially cellulose and hemicellulose have been examined with different groups of basidiomycetes however, the distinctive ability of depolymerization of lignin is an attribute of relatively few species. This group of basidiomycetous fungi commonly referred to as “white-rot” are saprophytic on plant organic matter and they acquire energy from plant polysaccharides cellulose and hemicellulose by disrupting the lignin complex, a refractory polymer cementing the carbohydrates [2]. These white-rot fungi enhance delignification there by increasing the nutrient availability of agricultural crop residues for use in livestock feed systems. Delignification in white-rot is attributed to an extensive set of extracellular enzymes encompassing major oxidative

enzymes of Laccase, Lignin Peroxidase, Manganese Peroxidase and Versatile Peroxidase besides auxiliary Carbohydrate-Active enzymes (CAZymes) [3].

Lentinus squarrosulus is a tropical white-rot fungus characterized by rapid growth on diverse lignocellulosic substrates. Studies on biodegradation of crop residues by *L. squarrosulus* has shown it to be particularly promising in enhancing delignification however, fungal conversion of crop residues for animal feed demands longer incubation times besides severe reduction in organic matter [4]. Characterization and application of enzymes involved in bio-delignification is said to improve the efficiency of the former process thereby augmenting the potential for utilization of these abundant lignocellulosic biomass in downstream applications especially in the field of animal nutrition [5].

Versatile Peroxidase (reactive-black-5: hydrogen-peroxide oxidoreductase EC 1.11.1.16), a lignolytic enzyme secreted by

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Received: 10-Jul-2023, Manuscript No. EEG-23-25505; **Editor assigned:** 12-Jul-2023, Pre QC No. EEG-23-25505 (PQ); **Reviewed:** 26-Jul-2023, QC No. EEG-23-25505; **Revised:** 02-Aug-2023, Manuscript No. EEG-23-25505 (R); **Published:** 10-Aug-2023, DOI: 10.35248/2329-6674.23.12.222

Citation: Ravichandran A, Sridhar M, Kolte AP, Dhali A, Gopinath SM (2023) Transcriptome Based Analysis of a *Lentinus squarrosulus* Strain for the Lignolytic Versatile Peroxidases. *Enz Eng.* 12:222.

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white-rots, possess high redox potential for oxidation of diverse recalcitrant substrates without requirement of mediators. Also, this enzyme bears hybrid architecture combining structural elements of lignin peroxidase and manganese peroxidase, the other high potential peroxidases implicated in lignin degradation [3]. These features project this enzyme as an attractive choice for industrial applications. Nevertheless, narrow substrate specificity of lignolytic enzymes in general and hybrid nature of this enzyme in particular, limit apparent identification of this enzyme from the mixture of lignolytic enzymes secreted by the fungi through biochemical methods thereby restricting research on this enzyme to a relatively few species [6,7]. Accordingly, this study focuses on demonstrating the lignolytic enzymes of *L.squarrosulus* through biochemical assays complemented with transcriptomic study of total mRNA of the fungi cultured in highly oxidative medium. Transcriptomic studies assisted in comprehensive bioinformatic analysis of the biodegradative ability of the fungus, study of its lignolytic enzymes specifically versatile peroxidase, purification and consequently appreciation of this versatile peroxidase in biodegradation of low cost agro-wastes in the context of enriching ruminant nutrition.

MATERIALS AND METHODS

Fungal strain and culture conditions

The fungus used in the present study, *L.squarrosulus* is a wild isolate from Western Ghats, India determined through morphological analysis and ITS sequencing in our earlier studies (MTCC accession no 12922) [8]. Homogenized mycelium from freshly grown seed culture of the fungus was inoculated to potato dextrose broth with 0.01% reactive black 5 (RB5). The flasks were continuously agitated at 100 rpm and incubated at $28 \pm 2^\circ\text{C}$ for seven days. Fungus grown in culture medium without the azo dye served as control. Cell-free culture supernatant was used as crude enzyme for assessing enzymatic activities.

Enzyme activity measurements

Cellulase activity was deduced with carboxymethyl cellulase as substrate. The assay mixture consisted of substrate 0.25% and culture supernatant 0.2 ml in 50 mM phosphate buffer pH 6.8 [9]. The procedure for xylanase estimation was similar to cellulase with the substrate being 0.06% oat spelt xylan. Polygalacturonase assay reaction mixture comprised polygalacturonic acid 0.45%, culture supernatant 0.1 ml in 50 mM acetate buffer pH 5 [10]. Mannanase activity was inferred with 0.5% locust bean gum as substrate. 0.1 ml of culture supernatant was reacted with 0.9 ml substrate solution in phosphate buffer pH 6.8 [11]. Reducing sugars produced through the enzyme reactions was measured by Dinitro Salicylic acid (DNS) method at 575 nm [12]. D-glucose, D-xylose, D-galacturonic acid and D-mannose were used as standards for estimation of cellulase, xylanase, polygalacturonase and mannanase activities respectively.

α -glucosidase, β -glucosidase and xylan esterase activities were interpreted using 0.1% p-nitrophenol α -D glucopyranoside, 0.1% p-nitrophenol β -D glucopyranoside and 0.02% p-nitrophenol acetate as substrates correspondingly with p-nitrophenol as standard [13]. The substrates were accordingly dissolved in 0.1M phosphate buffer pH 6.8 prior to use and reacted with 0.1 ml of culture supernatant as crude enzyme sample. Enzyme activity was determined by quantifying the amount p-nitrophenol released from the reaction at 400 nm. Lignolytic enzymes in the culture supernatant were determined through the methods described

earlier [4,7].

Ribonucleic acid isolation and sequencing

Fungal cells from three culture replicates were frozen at -196°C during the late log phase and macerated to fine powder. Total Ribonucleic Acid (RNA) was isolated using Genetix RNA sure Plant mini kit according to the manufacturer's protocol. Concentration of RNA was measured on nanodrop spectrophotometer and integrity, quality assessed by denaturing agarose gel electrophoresis. Sequencing libraries were prepared using TruSeq stranded library preparation kit and quality, fragment length distribution was assessed through Agilent 4200 Tape station. The library was then subjected to paired end sequencing in Illumina NextSeq 500 platform.

Reads mapping and annotation

Raw reads were processed in Trimmomatic v 0.35 for removal of adapter sequences, ambiguous and low-quality sequences. The quality reads were then assembled in CLC assembly cell. Annotation was performed using BLAST2GO 5.2 software for identification of Gene Ontology (GO) and exhaustive functional annotations of the transcript sequences were performed through Function annotator [14,15]. Function Annotator assisted in annotation of enzymes, best hits to National Center for Biotechnology Information (NCBI) non-redundant database, GO terms, conserved domain, transmembrane protein, lipoprotein peptide, signal peptide and also subcellular localization. Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology designation was obtained based on homology searches in the highlighted text has to be replaced with KEGG Automatic Annotation Server (KAAS) [16].

Carbohydrate active enzymes annotation

The catalytic and other functional domains of enzymes involved in metabolism and transport of carbohydrates were elucidated based on signature domains of each Carbohydrate Active Enzyme (CAZy) families through dbCAN2 meta server [17]. Short sequence reads were submitted to dbCAN2 meta server for automated CAZyme annotation using tools HMMER, diamond and Hotpep. In addition, Conserved Unique Peptide Patterns (CUPP) in the CAZymes placed them in different functionally related protein groups. Finer level of classification based on the similarity of protein sequence and peptide signature of CUPP group was used to assign transcript sequences to protein families [18].

Gene prediction

Gene prediction softwares AUGUSTUS and FGENESH were used for identification of genes expressed in *L.squarrosulus* [19,20]. AUGUSTUS analysis was based on *Phanerochaete chrysosporium*, another White-rot genome as reference whereas FGENESH prediction was based on *Trametes cinnabarina* as reference. Parameters were set to detect genes from both strands and also through alternate splicing.

Enzyme purification

Proteins from cell free culture supernatant was precipitated with 65% ammonium sulphate at 4°C and dissolved in 20mM sodium acetate buffer pH 4.5. The protein solution was then applied to Q Sepharose fast flow anion exchange column equilibrated with acetate buffer (pH 4.5) post dialysis against 10 mM sodium acetate

buffer pH 4.5. Proteins were eluted with acetate buffer through a linear gradient of 0.2-1 M NaCl at a flow rate of 0.1 ml/min. The collected fractions with versatile peroxidase activity were pooled and added to gel filtration Sephadex G-75 column equilibrated with acetate buffer pH 4.5. Fractions of 2 ml were collected at a flow rate of 1 ml/min using sodium acetate buffer (pH 4.5). Enzyme activities of the fractions were deduced through RB5 and manganese oxidation assays whereas protein concentration was recognized by monitoring the absorbance of the sample at 280 nm. The resultant fractions with versatile peroxidase activity were concentrated on Amicon PM 10 membrane and stored at -20°C until further use.

Lignocellulose biodegradation

Agro residues finger millet straw and paddy straw chaffed to 1 mm × 1 mm were used for biodegradation analysis. Purified enzyme was applied to the straws at 25 U/g of straw and incubated for 24 hours at room temperature. The straw samples were subsequently dried at a constant temperature of 60°C and subjected to detergent fiber analysis of acid detergent fiber, neutral detergent fiber and acid detergent lignin through the methodology of Van Soest, et al. [21]. Digestibility analyses of the straw samples were determined through *in vitro* dry matter digestibility procedure of Tilley and Terry [22].

Statistical analysis of the data on proximate analysis of straw samples and degradation characteristics were determined through ANOVA and Tukey's HSD means comparison. Differences were considered significant for $p \leq 0.05$.

RESULTS

Transcriptome of *L.squarrosulus*, a white-rot belonging to *polyporaceae* family grown in simple culture medium with azo dye Reactive Black

5 (RB5) was analyzed. The study was focused towards the expression of degradative enzymes in the induced medium and not intended for differential expression analysis.

Enzyme activity measurements

L.squarrosulus grown in simple synthetic medium with Reactive Black 5 (RB5), an azo dye as inducer for the production of lignolytic enzymes demonstrated the presence of a repertoire of degradative enzyme activities in control and medium supplemented with chromogenic substrate RB5 (Figure 1). Lignin degrading enzymes of this fungus particularly versatile peroxidase, manganese peroxidase and laccase were strongly induced in presence of the azo dye. Activities of versatile peroxidases were higher in RB5 supplemented medium than control ($p < 0.01$). Manganese oxidizing peroxidase activity increased to a maximum of 10 U/ml in induced medium compared to the uninduced medium besides laccase activity. Only trivial α -glucosidase activity was observed in induced and uninduced media. The interference of the mannanase substrate, locust bean gum with glucose in the culture supernatant impeded the detection of this enzyme.

Rise in cellulase activity was observed on day-7 with activity higher in uninduced than induced medium. The same trend was observed with xylanase, acetyl esterase and polygalacturonase whereas β -glucosidase activity was higher in induced medium. Though the instance of maximum activity and the duration varied for each enzyme, the performance of uninduced medium was optimal for production of biomass degrading enzymes other than lignolytic enzymes. Lignolytic enzymes activity was higher in induced medium attributed to complex aromatic nature of the azo dye. This evidently signified that the specific activity of lignolytic enzymes were higher in induced medium.

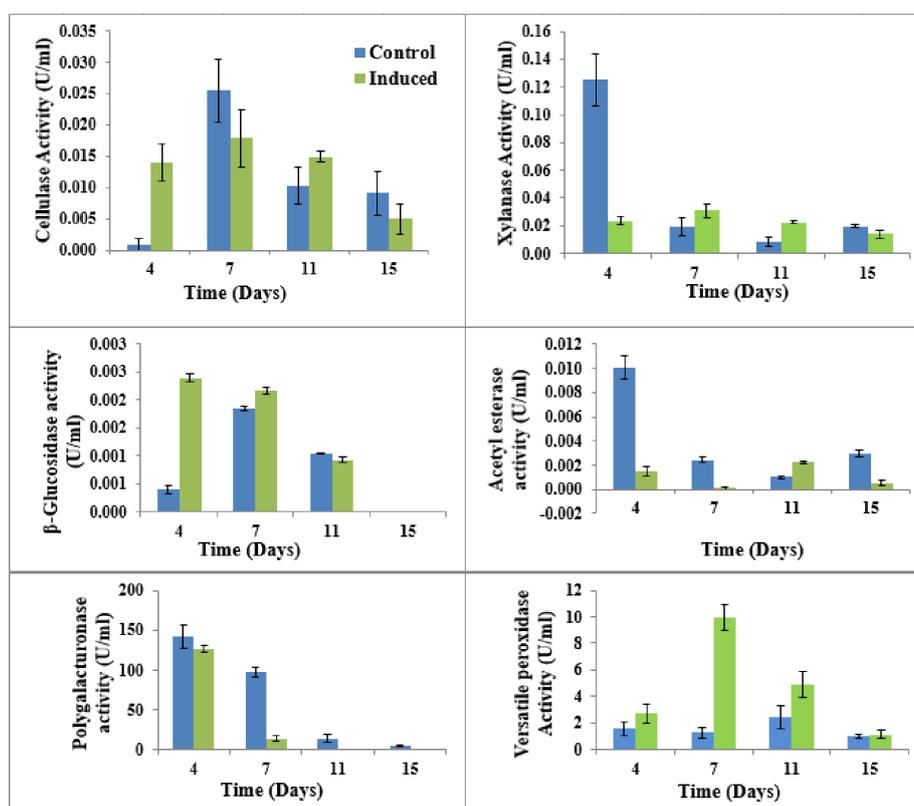


Figure 1: Biochemical analysis of few biomass degrading enzymes in the culture supernatant of *L.squarrosulus*.

L.squarrosulus transcriptome

L. squarrosulus transcriptome sequencing yielded 6,679,162 high quality paired end reads. After trimming of adaptors and low-quality bases, the raw reads were assembled de novo through CLC assembly cell resulting in 25,244 contigs with an N50 of 1340 bp (Table 1). The raw sequencing data referred in this project was submitted to the NCBI sequence read archive under the accession number PRJNA640439.

Table 1: Summary of *L.squarrosulus* transcriptome assembly.

Raw reads	Yield obtained
Number of contigs	25, 244
Total size of contigs (bp)	2,31,81,074
Average Length (bp)	918
Length SD (bp)	856
GC content (%)	57.93
N50 contig length (bp)	1,340
N50 contig count	5,091

Functional annotation of *L.squarrosulus* transcriptome

Following the assembly, the contigs were annotated through Function annotator and BLAST2GO. The taxonomic information furnished based on the proportion of similarity sequences placed *L.squarrosulus* close to *Dichomitus squalens* at the species level (Figure 2). The subsequent best hits were *Trametes versicolor* and *Trametes cinnabarina* with all these white-rots belonging to the core polyporoid clade, one of the major clade with rich catabolic ability under Polyporales [23].

Overall, 3,365 GO terms were assigned to 10,494 genes. The most abundant GO term predicted by Function annotator was GO:0055114, specifying the oxidation-reduction process (biological process) with gene products marking to manganese peroxidase 3 precursor of *Phlebia radiata* (PEM3_PHLRA), laccase 1A of *Trametes pubescens* (AF414808.1, AF491761, AF414807.1), ligninase H2 of *Phanerochaete chrysosporium* (LIG4_PHACH), Mannitol Dehydrogenase (MTLD_BACP2), NADPH dependent D-xylose reductase (XYL1_CANBO), Arabinitol Dehydrogenase (ARD1_UROFA), Arabinan Endo-1,5-alpha-L-arabinosidase A (ABNA_EMENI), Pyranose Dehydrogenase (PDH3_LEUMG), α -Fucosidase A (AFCA_ASPNC). This substantiates the potential of this fungus in biomass degradation through production of diverse hydrolytic enzymes. Subsequently rich GO terms were GO: 0005524 (Molecular function: ATP binding), GO: 0008152 (Cellular function: Metabolic process) and GO: 0016021 (Cellular component: integral membrane components).

Best matching hits of the putative proteins encoded by the transcripts against NCBI non-redundant protein database were available for 16,779 transcripts that expressed similarity to chiefly *Dichomitus* and *Trametes* protein sequences. 3,217 probable enzymes were determined through PRIAM based on ENZYME database. Though top abundant hits were the proteins involved in genome integrity and regulation like RNA dependent RNA polymerase, RNA helicase, protein kinases, there were significant representation of biomass degrading enzymes like endo-1,3(4)-beta-glucanase, glucose oxidase, choline oxidase and a range of lignocellulose active enzymes as depicted in Figure 3. This demonstrates that expressions of these enzymes by the fungus are not dependent on the presence of lignocellulose substrate in the culture medium.

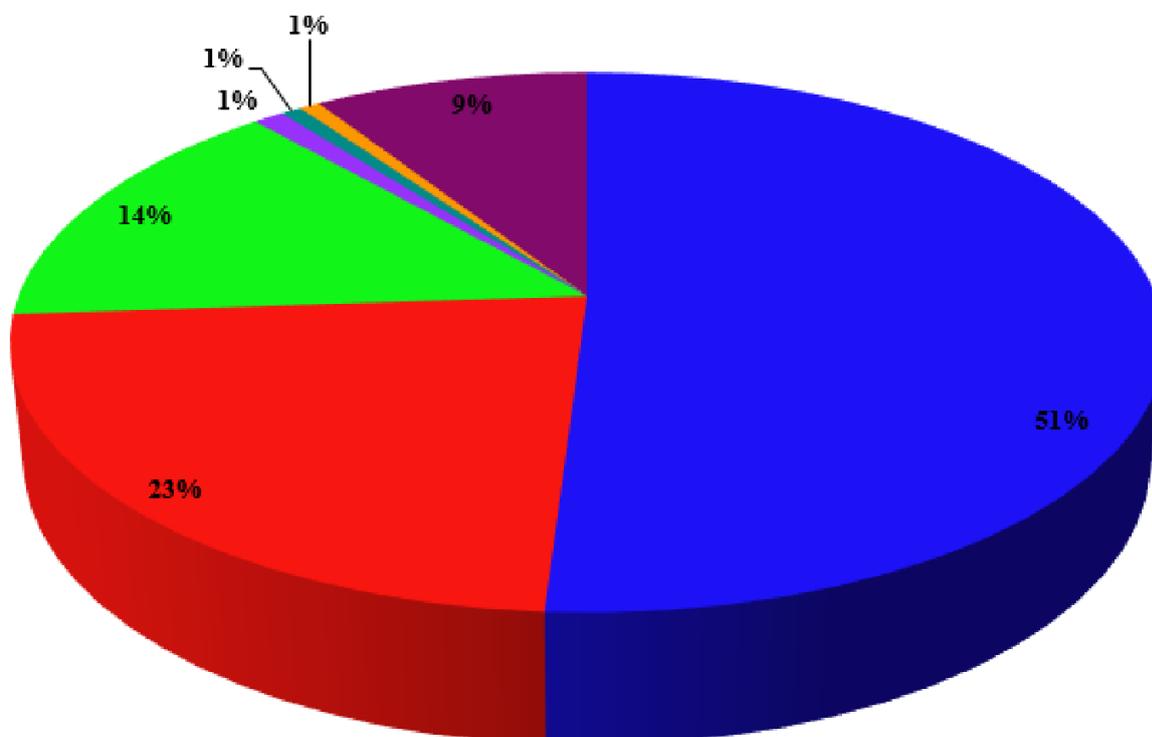


Figure 2: Taxonomic distribution of *L.squarrosulus* nucleotide sequences at species level. Note: (■) *Dichomitus squalens* LYAD-421 SS1, (■) *Trametes cinnabarina*, (■) *Fibroporia radiculosa*, (■) others, (■) *Trametes Versicolor* FP-101664 SS1, (■) *Gelatoporia subvermispora* B, (■) *Fomitopsis pinicola* FP-58527 SS1.

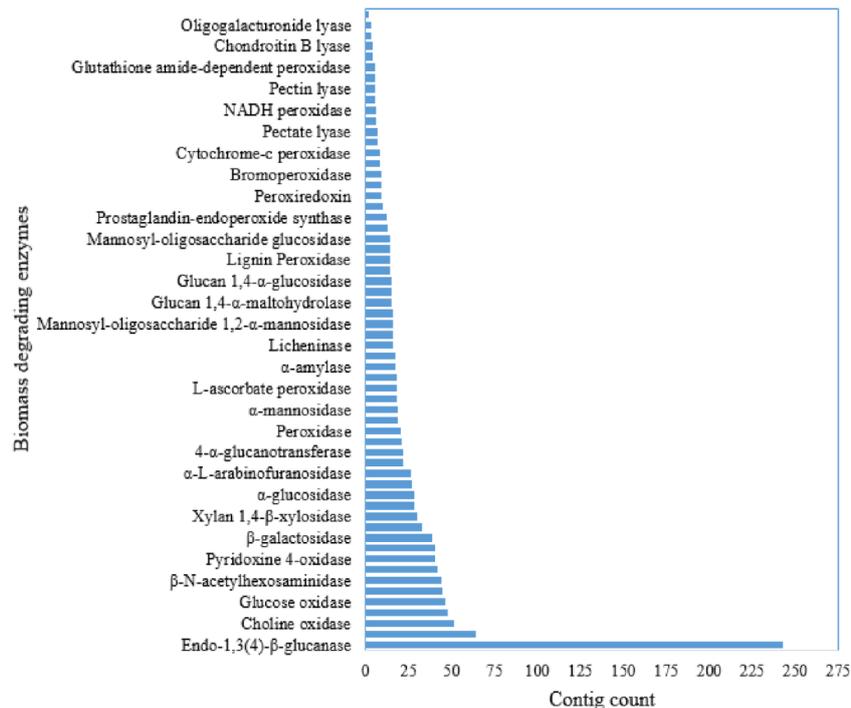


Figure 3: Putative biomass degrading enzymes of *Lentinus squarrosulus*.

Putative domain hits illustrated by Function annotator were based on PFAM database. 4952 unique conserved domains were identified against 11,585 transcripts. The most abundant domain hit was Major Facilitator Superfamily of secondary transporters (pfam07690) followed by Tymo_45kd_70kd (pfam03251), a kind of transposable element detected in Basidiomycetes. Similar transposable elements were also reported in *Pleurotus ostreatus* [24].

KEGG orthology designations were obtained for 3,327 transcripts. Transcripts encoding 20 putative cytochrome P450 polypeptides were present in *L.squarrosulus* transcriptome. Cytochrome P450 monooxygenases and oxidoreductases revealed enhanced oxidative environment in the culture medium. Associated with cytochrome P450 transcripts were the glutathione reductase that functions in regeneration of co-oxidants for aromatics metabolism and flavin containing monooxygenases and dioxygenases that function in aromatic ring cleavage [25,26]. The probable pathway depiction for cytochrome P450 was metabolism of aromatic compound RB5.

A considerable depiction of H_2O_2 producing oxidases were made by Glucose-Methanol-Choline (GMC) superfamily encoding transcripts which supplies the peroxide requisite for lignolytic peroxidases. Glyoxal oxidase of this fungus exhibited 66% similarity in protein sequence to that of *P.chrysosporium*.

Phylogenetic analysis of versatile peroxidase

The transcriptome analysis identified six transcripts of laccase with 100% sequence congruence to laccases of *Trametes cinnabarina*, *Polyporus*, *Lentinus tigrinus* and ten transcript sequences to encode versatile peroxidase with significant protein similarity to versatile peroxidase protein isoforms of *Pleurotus eryngii* and of *Trametes versicolor*. Two isoforms of manganese peroxidase were identified based on the conserved domains and sequence similarity. However, there were no lignin peroxidase transcripts observed with this

species as confirmed through the biochemical analysis.

Protein sequences of putative versatile peroxidases were subjected to multiple alignments through CLUSTALW with experimentally determined protein sequences of lignolytic peroxidases in Protein Data Bank (PDB). Rooted Phylogenetic tree through Unweighted Pair Group Method with arithmetic mean (UPGMA) method of the alignment is presented in (Figure 4). Notably, transcript sequences 4933 and 6622 are equivalent to MnP4, a versatile peroxidase isozyme of *P.ostreatus*.

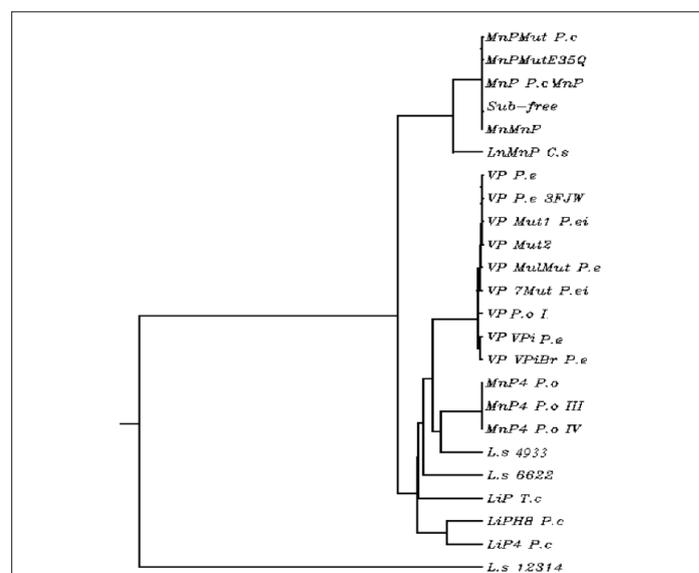


Figure 4: Multiple sequence comparison of lignolytic peroxidases with *L.squarrosulus* putative versatile peroxidase sequences. Note: (P.c - *Phanerochaete chrysosporium*; C.s - *Ceriporiopsis subvermispora*; P.e-*Pleurotus eryngii*; P.o- *Pleurotus ostreatus*; T.c-*Trametes cervina*; L.s-*Lentinus squarrosulus*).

CAZyme annotation

Prediction of CAZy families on peptide signatures demonstrated preponderance of glycoside hydrolases followed by auxiliary activities 3 family. The transcriptome was found enriched with transcripts of carbohydrate esterase's 10 families of esterase. Carbohydrate esterase's deacetylate the conjugates of glucans and are binding components of polysaccharide degrading machinery. Glycoside hydrolases of family 16 showed significant depiction in the transcriptome of *L.squarrosulus* (Figure 5). Lignolytic enzymes belonging to AA2 family that act in synchronization with the glycoside hydrolases were also prominent in the transcriptome as revealed by CAZyme annotation. The other affluent CAZymes reported were GMC oxidoreductase (AA3), lytic polysaccharide

monoxygenases cleaving cellulose chains (AA9) and lytic polysaccharide monoxygenases cleaving xylans (AA14).

CUPP assigned 508 transcripts to 6 families of polysaccharide lyases, 51 families of glycoside hydrolases, 23 families of glycoside transferases, 7 families of carbohydrate esterases and 10 families of auxiliary activities family (Figures 6 and 7). Of the enzymes characterized through CUPP based on the peptide signatures, chitinase (3.2.1.14) and chitin synthase (2.4.1.16) was predominant followed by laccase (1.10.3.2). Ten transcript sequences were assigned to laccase family and seven transcripts encoded peptide signatures typical of versatile peroxidase (1.11.1.16). The top hits also included glyoxal oxidase (1.2.3.15) and glucan endo-1,3-beta-D-glucosidase (3.2.1.39).

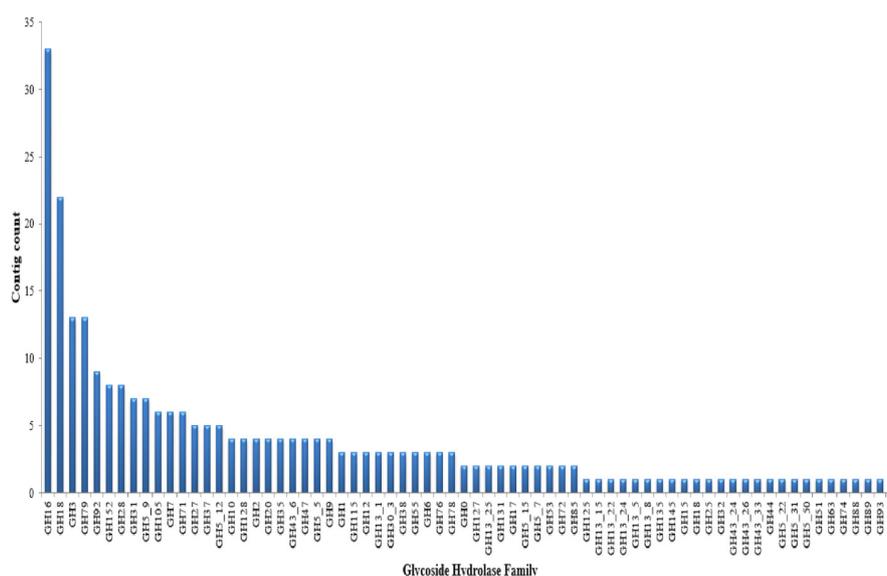


Figure 5: Distribution of Glycoside hydrolases (GH) in the transcriptome predicted based on CAZyme database.

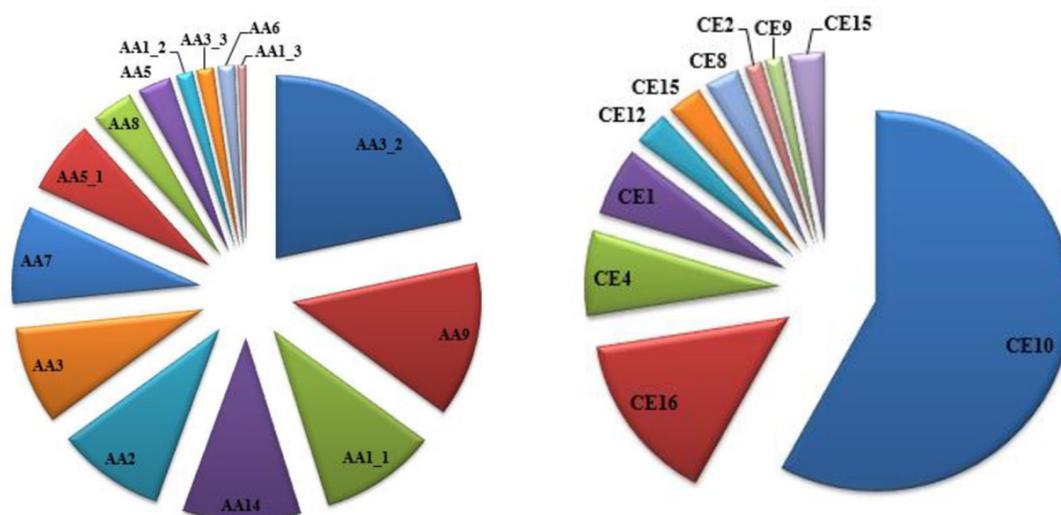
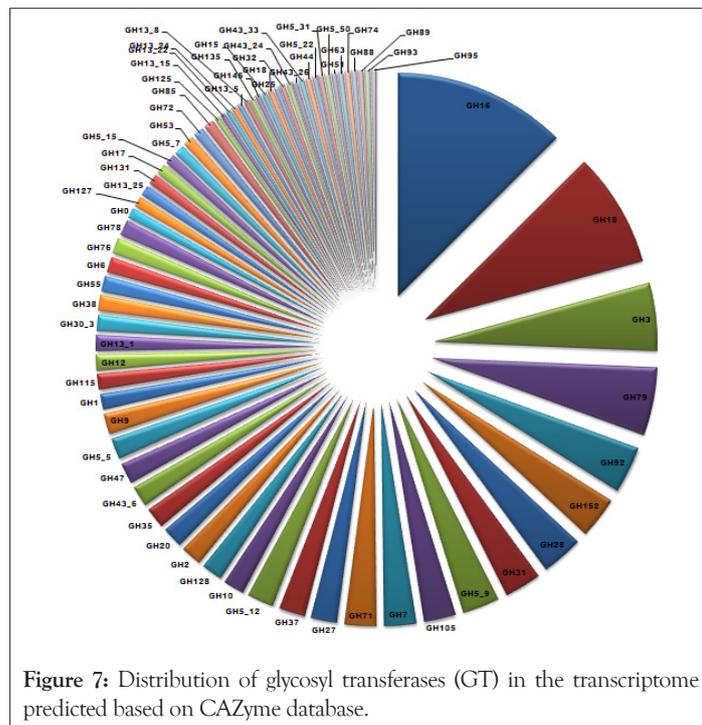


Figure 6: Distribution of Auxiliary activities (AA) enzymes and carbohydrate esterases (CE) in the transcriptome predicted based on CAZyme database.



Gene prediction

The protein sequences predicted by AUGUSTUS and FGENESH were similar except for slight length variations for a few transcripts. Laccase expression was visualized in the medium devoid of external supplementation of copper in contrast to cultures requiring Cu^{2+} addition for laccase induction [27, 28]. Though two of the sequences were only partial, three sequences encoded proteins with length >399 aa. The predicted proteins exhibited more than 90% similarity to laccases of *Lentinus tigrinus* and *Polyporus brumalis*. The sequences exhibited conserved L1-L4 signatures typical of laccases involved in copper ion binding [29]. Ten transcripts were identified to encode product of versatile peroxidase as assessed through amino acid sequence alignment. The sequences exhibited significant homology with manganese dependent and repressed peroxidase isoforms of *Lentinus tigrinus* and *Trametes versicolor*. A complete versatile peroxidase coding sequence was also predicted with 92% protein identity to manganese peroxidase 1 isoform of *Lentinus tigrinus*. The predicted proteins did include fatty acid dehydrogenases involved in peroxidation of lipids which generates lipid radicals as lignolytic oxidants for non-phenolic lignin degradation by manganese peroxidases. Protein sequence comparison of predicted lignolytic enzymes is illustrated in Table 2.

Table 2: Putative lignolytic enzymes of *L.squarrosulus*.

Transcript ID	Putative function	BLAST best hit	Sequence length	e-value	Amino acid identity
Lsqua4933	Versatile peroxidase	manganese peroxidase 1 (<i>Lentinus tigrinus</i>)	369aa	0	92%
Lsqua6622	Versatile peroxidase	manganese-repressed peroxidase (<i>Trametes versicolor</i>)	280aa	$4e^{-139}$	72%
Lsqua18051	Versatile peroxidase	manganese peroxidase isozyme precursor (<i>Polyporus brumalis</i>)	179aa	$8e^{-117}$	94%
Lsqua10154	Versatile peroxidase	Mn peroxidase MNP6 (<i>Lentinus tigrinus</i>)	168aa	$1e^{-109}$	96%
Lsqua13135	Versatile peroxidase	manganese peroxidase isozyme precursor (<i>Lentinus tigrinus</i>)	72aa	$7e^{-35}$	86%
Lsqua15894	Versatile peroxidase	manganese peroxidase isozyme precursor (<i>Lentinus tigrinus</i>)	65aa	$1e^{-31}$	92%
Lsqua15340	Versatile peroxidase	manganese peroxidase isozyme precursor (<i>Lentinus tigrinus</i>)	135aa	$2e^{-84}$	93%
Lsqua10666	Versatile peroxidase	Mn peroxidase MNP6 (<i>Lentinus tigrinus</i>)	76aa	$3e^{-38}$	87%
Lsqua14505	Versatile peroxidase	Mn peroxidase MNP6 (<i>Lentinus tigrinus</i>)	76aa	$3e^{-38}$	87%
Lsqua12314	Versatile peroxidase	heme peroxidase (<i>Lentinus tigrinus</i>)	406aa	0	80%
Lsqua20933	Laccase	laccase (<i>Lentinus tigrinus</i>)	68aa	$9e^{-24}$	72%
Lsqua6164	Laccase	laccase 1 (<i>Polyporus brumalis</i>)	516aa	0	88%
Lsqua18153	Laccase	laccase (<i>Polyporus brumalis</i>)	474aa	0	92%
Lsqua18367	Laccase	laccase LCC3-3 (<i>Polyporus ciliatus</i>)	399aa	0	93%
Lsqua21132	Laccase	Cu-oxidase-domain-containing protein (<i>Lentinus tigrinus</i>)	133aa	$3e^{-84}$	96%
Lsqua21539	Laccase	laccase (<i>Lentinus tigrinus</i>)	244aa	$1e^{-149}$	91%

Protein purification

Proteins possessing versatile peroxidase activity were eluted from Q Sepharose column at a gradient of 0.6 M NaCl. Pooled fractions with enzyme activity were applied to Sephadex G-75 column and collected at an elution volume of 70 ml. The SDS-PAGE analysis of the purified versatile peroxidase stained with silver nitrate showed two isoenzymes as bands of around 49 KDa (Figure 8). The purified enzyme exhibited specific activity towards RB5 and manganese in distinct reactions however reaction rate of the enzyme towards RB5 decreased in presence of manganese elucidating the presence of versatile peroxidase.

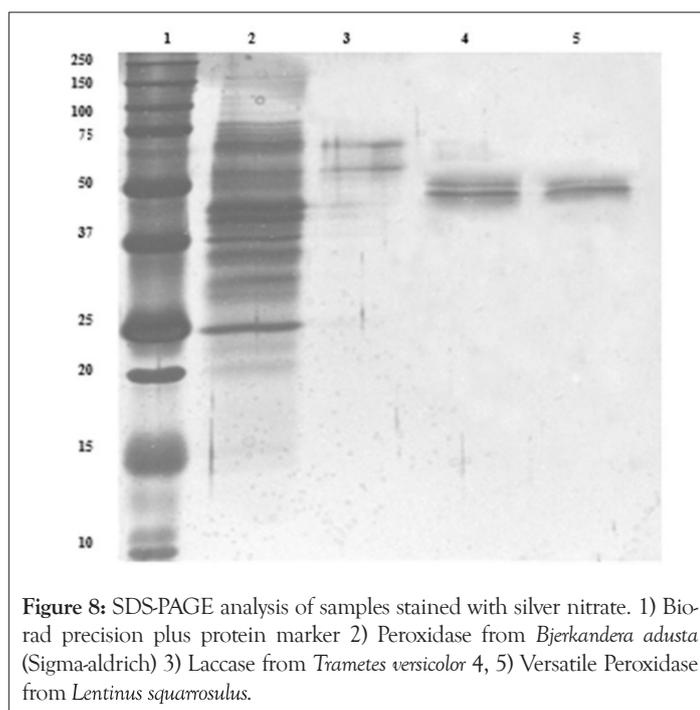


Figure 8: SDS-PAGE analysis of samples stained with silver nitrate. 1) Bio-rad precision plus protein marker 2) Peroxidase from *Bjerkandera adusta* (Sigma-aldrich) 3) Laccase from *Trametes versicolor* 4, 5) Versatile Peroxidase from *Lentinus squarrosulus*.

Lignocellulose biodegradation

Purified versatile peroxidase at 25 U/g of straw caused reduction of 6.8% NDF and 4.8% ADF in finger millet and paddy straws compared to untreated control. The concentration of acid detergent lignin decreased by 17% and 11% in finger millet straw and paddy straw respectively. The decrease in refractory cell wall components caused a corresponding increase in digestibility as estimated through *In vitro* Dry Matter Digestibility (IVDMD). The DMD of finger millet straw and paddy straw increased by 18% and 20% respectively (Table 3).

Table 3: Evaluation of IVDMD of control and purified versatile peroxidase treated straw samples.

Straw	Sample	IVDMD [*]
Finger millet	Control	51.06 ± 0.30
	Purified versatile peroxidase treated	60.35 ± 0.11
Paddy	Control	65.54 ± 0.27
	Purified versatile peroxidase treated	78.58 ± 1.12

Note: * indicates statistically significant (P<0.05).

DISCUSSION

Versatile peroxidase from tropical fungus *L.squarrosulus* discussed

in the study was first reported from our initial screening studies for the enzyme among the selected wood rotting basidiomycetes. Biochemical analysis coupled with enzyme specific assays indicated the secretion of versatile peroxidase from this fungus and our earlier studies on application of this crude enzyme to finger millet and paddy straw revealed significant reduction in lignin content with concomitant increase in *in vitro* digestibility. During this study, we corroborated the secretion of versatile peroxidase and its associated CAZymes by the fungus *L.squarrosulus*.

Lignolytic enzymes like versatile peroxidase are of economic and industrial importance owing to their high oxidative ability in degradation of recalcitrant lignin and other xenobiotics. Here, white rot fungi occupy a unique niche for their high redox potential lignolytic enzymes. Hence, researchers worldwide have worked on characterization of diverse white rot fungi for their extensive degradation abilities. Despite this, there is dearth of studies on the application prospects of these lignolytic enzymes in areas of biodelignification and bioremediation. This could be attributed to the complexity in interpreting the lignolytic enzymes in the fungal culture medium through biochemical analysis.

Transcriptomic analysis of the fungus from the medium exhibiting high levels of versatile peroxidase revealed significant expression of CAZymes especially lignolytic enzymes in support of the fact that lignolytic enzymes are mainly expressed in nutrient limited conditions rather than presence of lignocellulosic substrates. Multiomics studies on *Phanerochaete chrysosporium* lignocellulolytic networks also substantiated that nutrient limitation were major drivers of lignolytic and cellulolytic gene expression rather than the presence of lignocellulosic substrates [29-32]. Cellulase, xylanase, polygalacturonase, β -glucosidase, laccase and versatile peroxidase were shown to be produced in considerable amounts despite the lack of lignocellulosic substrate. Though the cellulase, xylanase activities were comparable between the control and RB5 supplemented medium, titer of lignolytic enzymes were remarkably higher in the RB5 supplemented medium.

Putative cellulases (EC 3.2.1.4) and xylanases (EC 3.2.1.8) were identified in the transcriptome of the fungus in spite of presence of glucose in the medium which is presumed to cause catabolite repression. However, glucose concentration in the medium during the period of RNA isolation was comparatively low that might have plausibly caused expression of cellulose degrading enzymes. In addition, transcripts encoding putative hemicellulose degrading enzymes of arabinanase, xylosidase, mannanase were also seen expressed in the transcriptome of *L.squarrosulus*. These results are in contrast to the transcriptomic analysis of *Pycnoporus sanguineus* also grown in the absence of lignocellulosic substrate wherein cellulases were not present [33]. However, in our present study, cellulases and hemicellulases were perceived to be expressed even in the absence of lignocellulosic substrates. In addition, Lytic Polysaccharide Mono Oxygenases (LPMO), pectinases and a range of other polysaccharide degrading enzymes were detected elucidating the superior biodegradative ability of the fungus. Similar oxidative enzymes acting on polysaccharides were seen expressed in *Ceriporiopsis subvermiformis* and *Phanerochaete chrysosporium* cultures containing lignocellulosic substrates [34,35]. Ligninolysis is substantially connected to free radical and peroxide production. *L.squarrosulus* transcriptome revealed diverse peroxide generating enzymes like cellobiose dehydrogenases, aryl alcohol oxidases and other members of glucose-methanol-choline oxidoreductases. In addition, multiple SKN7 transcription factor sequences and RTA1

domain containing sequences that activate the genes in response to oxidative stress were observed in the transcriptome.

Molecular mass of versatile peroxidase protein observed on SDS-PAGE closely matches with the predicted enzyme based on the mRNA sequence in the transcriptome. The versatile peroxidase protein was purified with a specific activity of 62 U/mg. Reactivity of the purified versatile peroxidase on the natural lignocellulosic substrates of finger millet and paddy straw showed 17% and 11% reduction in lignin content per 25 U of enzyme. This resulted in increase in digestibility of 18-20%. The results on pre-treatment of crop residues with versatile peroxidase was thus encouraging to advance the research on the application of this enzyme in enhancing the nutritive value of abundant crop residues in support of animal nutrition.

In a nutshell, the fungus undertaken in our study, *L.squarrosulus* is evidently competent to efficiently degrade lignocelluloses through its extensive machinery of hydrolytic and oxidative enzymes targeting multiple components of the substrate. Additionally, these enzymes working on lignocellulose bioconversion also react on diverse aromatic compounds like dyes, pesticides, endocrine disruptors and other environmental contaminants which convey their importance. This study on the transcriptome analysis of *L.squarrosulus* revealed significant facts on this front and will definitely enhance the knowledge about the biodegradative ability of this fungus potentially paving the way for efficient biotechnological applications utilizing its potency.

CONCLUSION

Lentinus squarrosulus evidently is competent to efficiently degrade lignocelluloses through its extensive machinery of hydrolytic and oxidative enzymes targeting multiple components of the substrate. Additionally, these enzymes working on lignocellulose bioconversion also react on diverse aromatic compounds like dyes, pesticides, endocrine disruptors and other environmental contaminants which convey their importance. Though multiple White-rot Basidiomycetes were studied for lignin degradation, each species is unique in its ability to oxidize the aromatic macromolecule. Though there are multiple studies on biochemical characterization of lignocellulolytic enzymes of this fungus, research on expression pattern of the lignocellulose degrading enzymes is not attempted. Transcriptome analysis of *L.squarrosulus* revealed significant facts on this front and will definitely enhance the knowledge about the biodegradative ability of this fungus paving the way for efficient biotechnological applications utilizing its maximum potential.

ACKNOWLEDGEMENT

The financial assistance by Department of Science and Technology (DST), Ministry of Science and Technology, Govt. of India, under the WOSA scheme (SR/WOS-A/LS-32/2016) is gratefully acknowledged by the first author. The authors thank the Director, ICAR - National Institute of Animal Nutrition and Physiology, Bangalore (Karnataka) India, for providing the necessary facilities to carry out the research work.

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