

A Versatile Peroxidase from *Lentinus squarrosulus* towards Enhanced Delignification and *in vitro* Digestibility of Crop Residues

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The scarcity of quality feed is a major constraint to livestock productivity, and it is attributed to recalcitrant lignin, which hinders the utilization of crop residues. White-rot lignicolous fungi have been used for enrichment of crop residues through fungal pre-treatment, but dry matter loss is inevitable. Versatile peroxidase oxidizes a diverse class of aromatics without mediators and plays a key role in ligninolysis. In this study, the efficacy of a novel versatile peroxidase isolated from a wild species was evaluated for its effect in the delignification of crop residues. The strain identified as *Lentinus squarrosulus* (TAMI004, BankIt2098576 MH172167) demonstrated predominant versatile peroxidase activity amongst screened isolates in solid-state and submerged fermentations; it displayed hybrid characteristic of manganese oxidation and manganese independent reactions on aromatic compounds. The manganese oxidizing peroxidase activity was 12 U/mL in submerged and 131 U/mL in solid-state fermentation. Treatment of crop residues with *L. squarrosulus* extract rich in versatile peroxidase showed a decrease in neutral detergent fiber, acid detergent fiber, and acid detergent lignin contents, prompting delignification. Thus, the use of versatile peroxidase in enhancing the digestibility of straws is substantiated through proximate and *in vitro* digestibility analysis. Thus, the potential use of versatile peroxidase in increasing the *in vitro* degradation of straws for enhancing feed utilization in ruminants was substantiated.

Keywords: White rot fungi; Wild isolate; Delignification; Versatile peroxidase; Ruminants; Feed utilization

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INTRODUCTION

Sustainable energy and waste management are the pillars of socio-economic development. Environmental health is equally important in promoting socio-economic prosperity. Thus, there is an increasing global demand for food and fuel, prompting the search for alternate sources of feedstock for fuel without compromising food security (Eisler *et al.* 2014). Sustainable agricultural systems are drivers of food security and environmental viability. Livestock plays an inexorable role in supporting sustainable agriculture. However, the quest for quality feed for the expanding livestock community is still a concern impacting the efficiency of livestock production. One of the keys to these challenges lies in the lignocellulosic biomass generated through cropping practices and as by-products of agro-industries worldwide (Owen and Jayasuriya 1989). Interestingly, the utilization of lignocellulosic biomass also addresses the issue of residue disposal, which in

current times is a major contributor to greenhouse gas emissions (Lal 2005; Bhattacharyya and Barman 2018). Lignocellulosic residues are pertinent in diverse applications of biofuel production, paper pulp manufacture, animal feed, *etc.* The exploitation of these lignocelluloses relies heavily on cost-effective destruction of the biomass. Biomass deconstruction is necessary for efficient exploitation of the polysaccharides for biotechnological applications because of the recalcitrance of lignocellulosic complexes, which is due to crystalline cellulose and the rigidity and hydrophobicity of lignin (Grabber 2005). Although diverse pretreatment methods have their own advantages, the biological method of lignin degradation stands out for economic reasons. The white-rot fungi dominate the biological lignin degraders due to their rich network of ligninolytic enzymes. White-rot fungi are superior to other lignin degrading bacteria and fungal groups on account of this selective lignin degrading ability (Kirk 1987). Hence, this group of fungi is widely used for the treatment of crop residues to improve their nutritive value (Sharma and Arora 2010a, b). However, the loss of polysaccharides during lignin degradation limits fungal pretreatment application at large scale (Martínez *et al.* 2005; Sridhar *et al.* 2015). There has been recent research to characterize these ligninolytic enzymes for their ability to disintegrate lignocellulosic complex in a way that is amenable to downstream biotechnological applications (Kumar *et al.* 2015). The ligninolytic system of these white-rot fungi is comprised of laccase, manganese peroxidase, lignin peroxidase, versatile peroxidase, and other enzymes involved in peroxide generation (Knop *et al.* 2015). Laccase, phenol oxidase, and heme peroxidases such as manganese peroxidase and lignin peroxidase oxidize the non-phenolic units of lignin through mediators, while the phenolic units are attacked directly (Christian *et al.* 2005; Ruiz Duenas and Martinez 2009a; Falade *et al.* 2016). Extensive research has established the role of these enzymes in lignin degradation (Ruiz Duenas *et al.* 2009a; Falade *et al.* 2016). Versatile peroxidase is unique in the system on account of its ability to oxidize the phenolic and non-phenolic compounds free of redox mediators (Ruiz Duenas *et al.* 2009b; Knop *et al.* 2016). The hybrid architecture of lignin peroxidase and manganese peroxidase present in versatile peroxidase and the adaptation of its amino acid residues in the catalytic environment make it favorable for high potential substrate oxidation (Ruiz-Duenas *et al.* 2009b). Versatile peroxidase oxidizes high molecular weight substrates through catalytic tryptophan at the surface resembling lignin peroxidase and Mn^{2+} to Mn^{3+} as in manganese peroxidase (Revankar and Lele 2006). Nevertheless, there is a lack of research on versatile peroxidase, as this enzyme exhibits considerable complexity for identification among the other ligninolytic peroxidases. While exogenous fibrolytic enzymes are currently exploited in ruminant nutrition, the role of ligninolytic enzymes, particularly versatile peroxidase, is still emerging. This study focused on the screening of wild isolates for a novel versatile peroxidase having exceptional efficiency for aromatics and manganese oxidation. This enzyme was targeted for its *in vitro* delignification potential of crop residues for better utilization in downstream applications.

EXPERIMENTAL

Chemicals

Chemicals used in the study were of analytical grade unless otherwise stated. Reactive Black 5 (RB5), 2, 2'-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) and 2, 6 dimethoxyphenol (DMP) used for assay were procured from Sigma Aldrich (St.

Louis, MO, USA).

Fungal Culture Propagation and Screening

Twenty isolates of wood-rotting fungi from humid, tropical regions of Western Ghats (12.9763° N, 77.5929° E, 8.9339° N, 77.2780° E) were brought to pure cultures by aseptic propagation of inner tissue of pileus/stipe of basidiocarp on potato dextrose agar slants at 28 °C (Jorgensen and Knud 1953). All isolates were appraised for their ligninolytic ability through the Bavendamm test of oxidation with 30 mM gallic acid and 3 mM tannic acid in discrete reactions (Christian *et al.* 2003). The enzymatic reactivity of the isolates towards guaiacol (O-methoxyphenol) at a concentration of 8 mM on solid media confirmed their phenolic oxidation ability. The strains with positive reactions to the above analysis were selected for the screening of versatile peroxidase production.

Subsequently, agar plates supplemented with 100 µM Reactive Black 5, an azo dye, were observed for oxidative decoloration. The plates were inoculated with 4 mm mycelial plugs from freshly grown culture and observed at regular intervals for a halo of decolorization. Reactive Black 5 was chosen because it is a heavily recalcitrant double azo dye possessing high redox potential. Decolorization of the sulfonphthaleine dyes bromophenol blue, bromothymol blue, and bromocresol green were studied by supplementing liquid culture medium with 0.01% of the corresponding dye (Krishna Prasad *et al.* 2005).

Culture Conditions

The basal medium for cultivation comprised the following per liter: glucose, 10 g; NH₄H₂PO₄, 2 g; yeast extract, 2 g; and trace element solution, 1 mL. Trace elements included: MgSO₄, 3 g/L; CuSO₄, 0.005 g/L; ZnSO₄, 0.1 g/L; FeSO₄, 0.1 g/L; and CaCl₂, 0.05 g/L. Fungal cultures pre-grown in glucose-ammonium medium were used as inocula. Prior to inoculation, the cells were homogenized and added to the production medium. Flasks were maintained under continuous agitation of 120 rpm. Solid-state fermentation was implemented using pre-treated poplar wood chips moistened with basal medium (Kumar *et al.* 2015). A 250-mL Erlenmeyer flask containing 5 g of pre-treated wood chips layered with 25 mL basal medium was inoculated with homogenized mycelia from freshly grown culture. Cultures were incubated at 28 °C in the dark. Enzyme analysis was performed using extracellular fluids from submerged and solid-state fermentation. The effect of supplementation of different concentrations of manganese (0 to 500 µM) on Versatile Peroxidase production was analyzed by adding manganese as MnSO₄ to the basal medium. All experiments were performed in triplicate.

Decolorization Studies

To ascertain the rate of degradation of the azo dye Reactive Black 5, liquid medium supplemented with 100 µM dye was inoculated with a 4-mm mycelial plug from freshly grown culture and monitored for decolorization spectrophotometrically at the absorbance maxima of the dye once in 24 h. Flasks were incubated at 28 °C under continuous agitation at 120 rpm. A non-inoculated flask supplemented with dye served as a control. Decolorization efficiency was assessed as % decolorization using Eq. 1,

$$\% \text{ decolourization} = \frac{C_0 - C_t}{C_0} \times 100 \quad (1)$$

where C_0 is the initial concentration of the dye and C_t is concentration of the dye at t h. All

experiments were performed in triplicate.

Analytical Measurements

Laccase activity was estimated by the oxidation of 1.6 mM ABTS in 100 mM sodium acetate buffer (pH 4.5) at 420 nm (ϵ_{420} 36000 M⁻¹ cm⁻¹) (Ghose 1987). The oxidation of ABTS by peroxidases was corrected by subtracting the activity in the presence of 0.5 µg/mL catalase. Lignin peroxidase activity was measured by monitoring the oxidation of 2 mM veratryl alcohol in 100 mM sodium tartrate buffer pH 3. The formation of veratrylaldehyde was measured at 310 nm (ϵ_{310} 9300 M⁻¹ cm⁻¹). Manganese peroxidase activity was deduced from the formation of Mn³⁺ malonate at 270 nm. The assay mixture consisted 0.5 mM MnSO₄ and 100 mM malonate buffer pH 4.5, and the reaction was initiated with 0.1 mM H₂O₂. Oxidation of RB5 was determined in 100 mM sodium tartrate buffer pH 3 with 10 µM RB5. The reaction was initiated by addition of 0.1 mM H₂O₂ and monitored through the decrease in absorbance at 598 nm (ϵ_{598} 24000 M⁻¹ cm⁻¹). Versatile peroxidase activity of the selected wild isolate TAMI004 (TC04) was compared with *Pleurotus sajor-caju* and *Trametes versicolor*, rich producers of the enzyme. Enzyme activity was assessed through oxidation of 2,6 dimethoxyphenol after correcting for laccases. The oxidation of 2,6 dimethoxyphenol (DMP) was determined at 468 nm in a reaction mixture containing 0.1 mM DMP, 100 mM sodium tartrate buffer pH 3, and 0.1 mM H₂O₂. One unit of enzyme is defined as the amount of enzyme that transforms 1 µmol of substrate or that forms 1 µmol of product per minute. Absorbance measurements were carried out in Shimadzu UV 1800 spectrophotometer (Kyoto, Japan). Reducing sugars were estimated by the dinitrosalicylic acid (DNS) method with D-glucose as a standard (Rao *et al.* 2018). Microbial biomass was deduced by filtering the media contents through pre-weighed dried Whatman no 1 filter paper followed by overnight drying at 70 °C.

Culture Identification

This efficient strain exhibiting ligninolytic activity was identified in a discrete study undertaken by Rao *et al.* (2018). Genomic DNA from freshly grown fungal culture was isolated by microwave method and served as a template for PCR with ITS 1 (5'-TCC GTA GGT GAA CCT GCG G- 3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT G-3') as primers. The amplified product spanning 18s rDNA, 28s rDNA, and 5.8s rDNA along with ITS1 and ITS4 regions was sequenced, and the ITS sequence obtained was then subjected to sequence comparison through NCBI BLAST nucleotide search as documented in (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). A low complexity filter was set, and the sequences with lowest expect value and maximal identity were selected.

Proximate Analysis and Estimation of *in vitro* Dry Matter Digestibility (IVDMD)

Five commonly available crop residues such as paddy straw, finger millet straw, foxtail millet straw, little millet straw, and barnyard millet straw were milled to 1 to 2 cm length and dried at a constant temperature of 70 ± 2 °C for use in biodegradation studies. Crude enzyme produced by the fungus through solid-state and submerged fermentation modes was harvested on the peak of enzyme production and exploited for treating the crop residues. The crop residues were treated with the crude enzyme at 40% (v/w) and incubated for 24 h followed by drying at 70 ± 2 °C. The samples were analyzed for proximate principles of neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent

lignin (ADL) (Van Soest *et al.* 1991). Subsequent to proximate analysis, finger millet straw and little millet straw were analyzed for their *in vitro* dry matter digestibility through a two-stage method (Minson and McLeod 1972). Though *in vitro* digestibility does not directly determine ruminant digestibility, this method is preferred as it is time saving and cost effective as compared to *in vivo* trials for primary investigations on the potential of feed materials. However in most of the trials, *in vitro* results were found to be correlative to the *in vivo* digestibility results (Minson and McLeod 1972; Geisert *et al.* 2007). Prior to digestion of the samples, fermentation medium was prepared by adding 2.5 g/L casein hydrolysate and 0.15% Resazurin solution to McDougall's buffer. McDougall's buffer was composed of the following per liter: NaHCO₃, 9.80 g; Na₂HPO₄, 7 g; KCl, 0.57 g; NaCl, 0.47 g; MgSO₄·2H₂O, 0.12 g; and CaCl₂, 0.04 g. Reducing agent (625 mg cysteine hydrochloride dissolved in 95 mL distilled water, 4 mL of 1 N NaOH added followed by addition of 625 mg sodium sulphide flakes) was introduced to the fermentation medium and flushed with CO₂ to remove the air from the medium. Rumen contents were obtained from a male cow through a permanent fistula. The contents were maintained at 39 °C and carefully filtered through two layers of cheese cloth. Next, 40 mL of fermentation medium with 10 mL of rumen liquor were then added to 0.5 g of finely ground sample. The sample flasks were flushed with CO₂ and incubated at 39 °C for 48 h with continuous shaking. After incubation, the samples were extracted using neutral detergent solution to assess their digestibility on dry matter basis.

Statistical Analysis

All treatments were performed in three replicates. Proximate analysis was evaluated using PROC GLM in SAS 9.3 software (Cary, NC, USA). Significant differences between treatments were determined using t-test at α value of 0.05.

RESULTS AND DISCUSSION

Screening of Wild Isolates for Ligninolytic Enzymes

The oxidation of phenolics was indicated by the formation of brown to reddish brown radiance on agar plates (Fig. 2(1,3)). Strains were considered positive for ligninolytic activity only on oxidation of all the phenolic indicator compounds subjected in the study. Accordingly, 60% of the screened wood-rotting strains were positive for ligninolytic activity. Subsequently, 30% of strains were found to decolorize the recalcitrant azo dye RB5 and were considered producers of versatile peroxidase. Though sulfonphthaleine dyes decolorization was observed in most isolates, the azo dye decolorization was exhibited by markedly few isolates. The typical decolorization paces of the isolates are depicted in Fig. 1. The efficient isolate designated as TAMI004 (TC04 in Fig. 1) as explored through these preliminary studies was further evaluated in liquid culture for production of ligninolytic enzymes. In addition, microscopic observation of the hyphae, septa, clamp connections, and spores confirmed these fungal strains as belonging to basidiomycetous family.

Identification of Wild Isolate

Genomic DNA extracted *via* microwave method was of highest purity as determined through nanodrop spectrophotometer. Amplification through ITS 1 and ITS 4 primers yielded a 700-bp sequence encompassing the ITS 1, ITS 2, and rDNA regions,

which were deposited to Genbank with accession numbers MH172167 and MH172168. Sequence comparison through NCBI BLAST identified the strain as the basidiomycete *Lentinus squarrosulus* (TAMI004, BankIt2098576 MH172167).

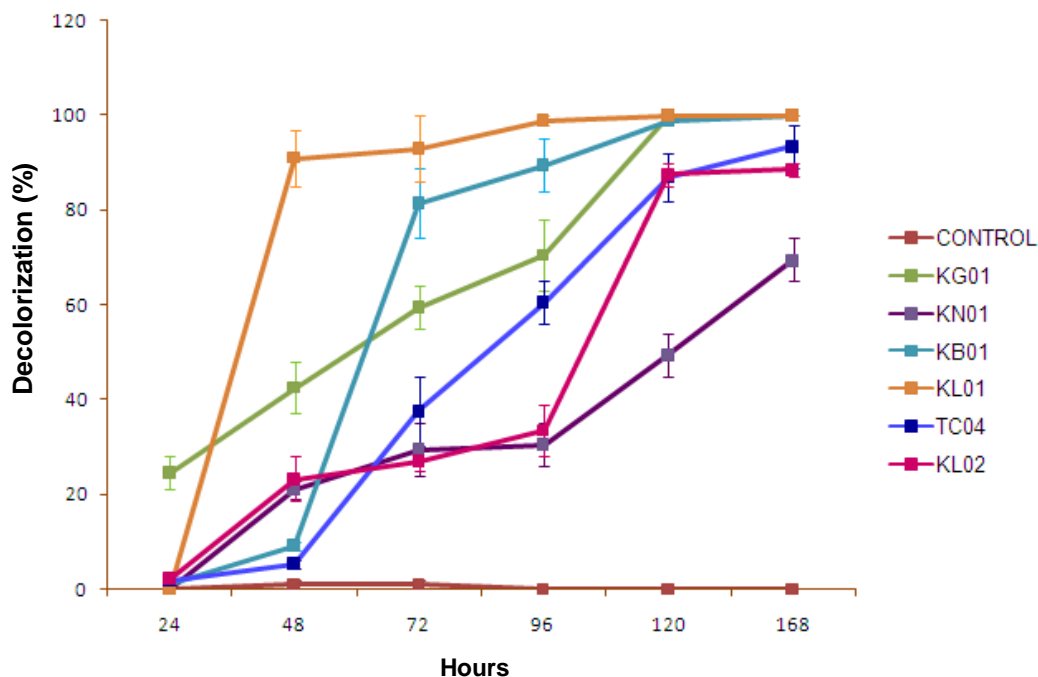


Fig. 1. RB5 Decolorization profile of the wild isolates in potato dextrose medium

Characterization of Ligninolytic Enzyme Production

The *Lentinus* species studied here is a rich producer of laccase as determined by ABTS oxidation ability alongside Versatile Peroxidase activity. The decolorization of RB5 and RBBR were observed in manganese-independent reactions (Fig. 2). The decolorization of the sulfonphthaleine dye bromophenol blue is illustrated in Fig. 3. In liquid culture, manganese-oxidizing peroxidase activity reached its maximum after 7 days, at the very end of active tropophase (log phase), after which the activity declined.

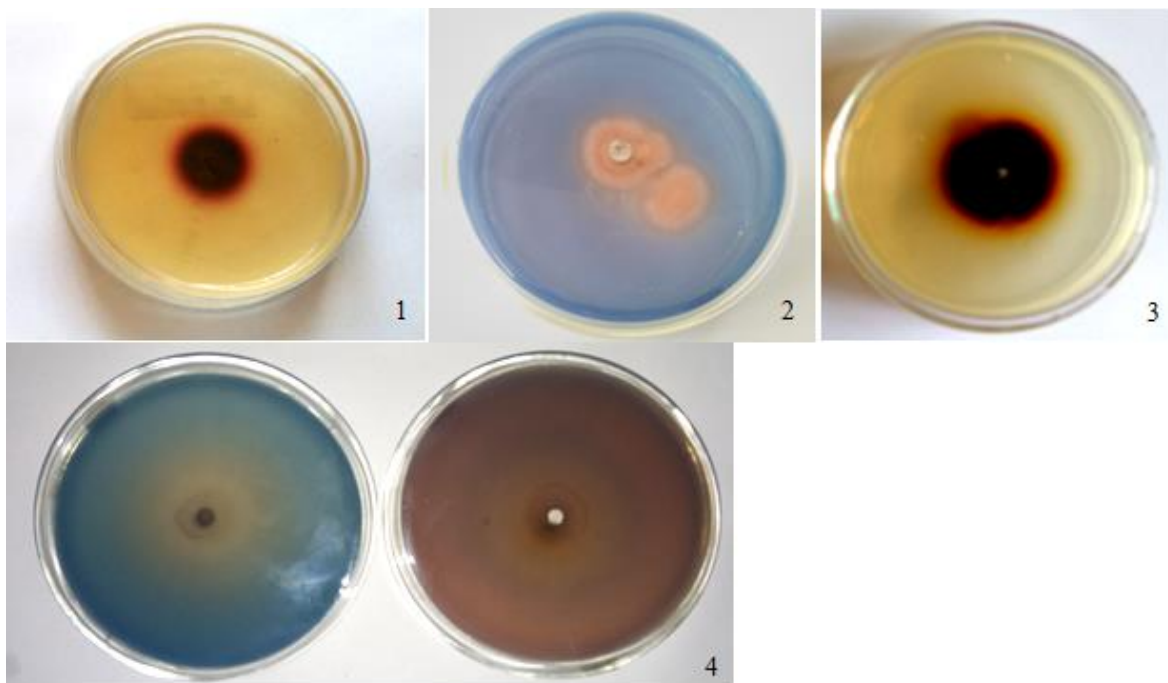


Fig. 2. Phenolics oxidation by TAMI004. (1) Oxidation of guaiacol, (2) decolorization of RB5, (3) oxidation of gallic acid, and (4) decolorization of RBBR after 3 and 7 days of culture

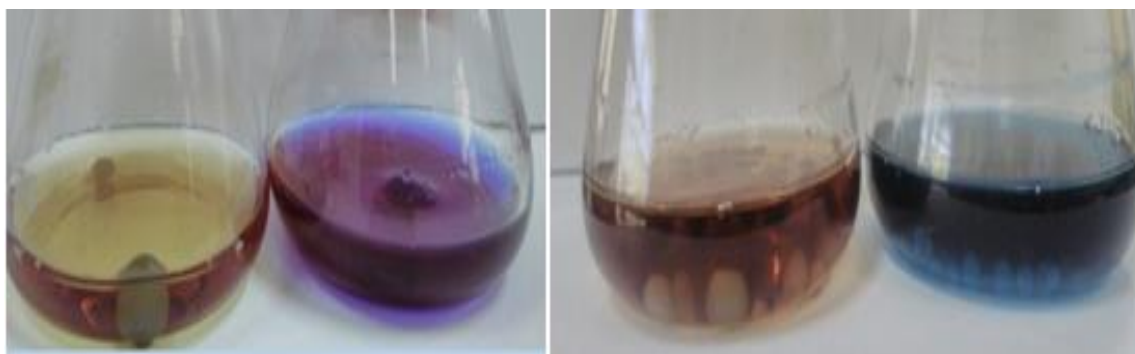


Fig. 3. Decolorization of (A) sulfonphthaleine dye bromophenol blue and (B) RB5 in liquid culture in comparison to negative controls

Residual reducing sugar was nearly negligible at this phase (Fig. 4). To evaluate the activity of the manganese-oxidizing peroxidase enzymes in manganese-containing medium, the above isolate was cultivated in basal medium with varying concentrations of manganese from 0 to 500 μM . The RB5-oxidizing peroxidase activity was predominant in the medium devoid of manganese, though manganese-oxidizing peroxidase activity was significant in media with 0 and 500 μM manganese (Fig. 5). In contrast, only trivial veratryl alcohol oxidation was observed with this isolate. Manganese dependent and independent activities on 2,6 dimethoxy phenol were also exhibited by this species. Manganese independent activity on 2,6 dimethoxy phenol of the species relatively with *Pleurotus sajor-caju* and *Trametes versicolor* is depicted in Fig. 6.

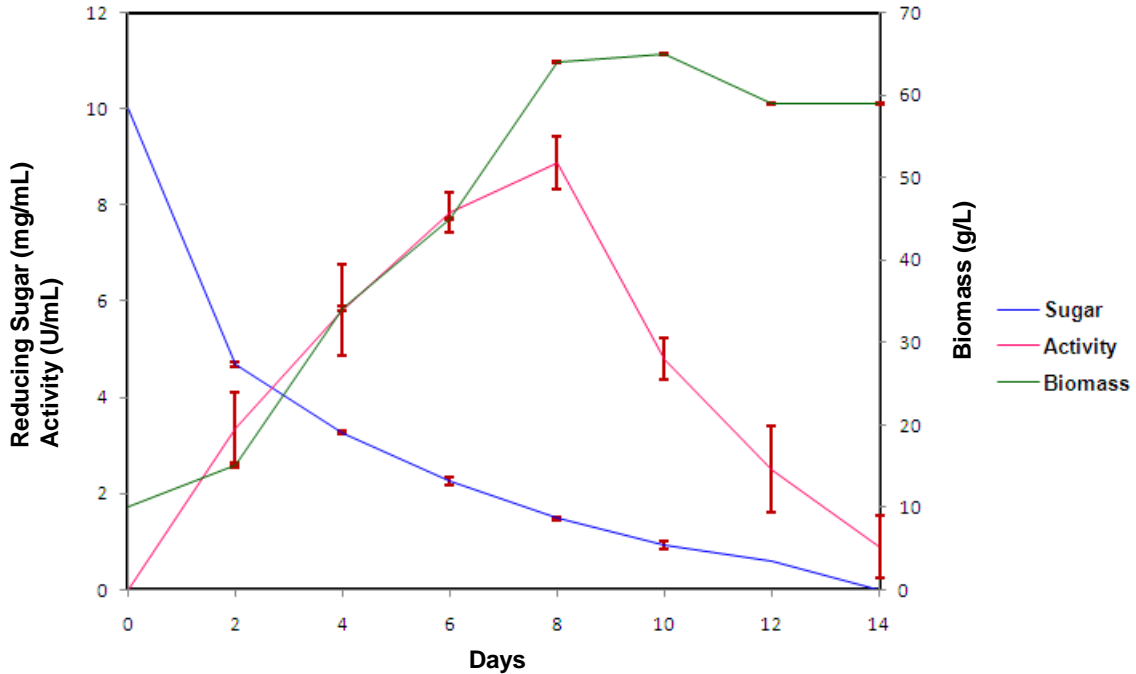


Fig. 4. Versatile peroxidase activity, biomass growth, and reducing sugar profile of TAMI004 during submerged fermentation

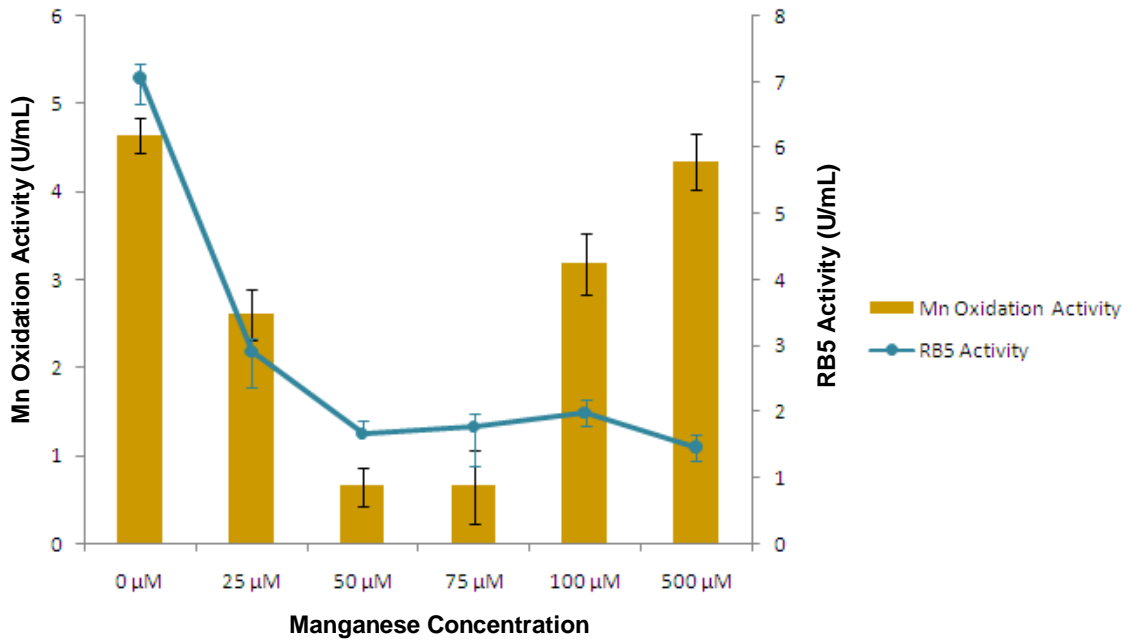


Fig. 5. Manganese oxidizing peroxidase activity and RB5 decolorizing peroxidase activity at varying concentrations of manganese (0 to 500 µM)

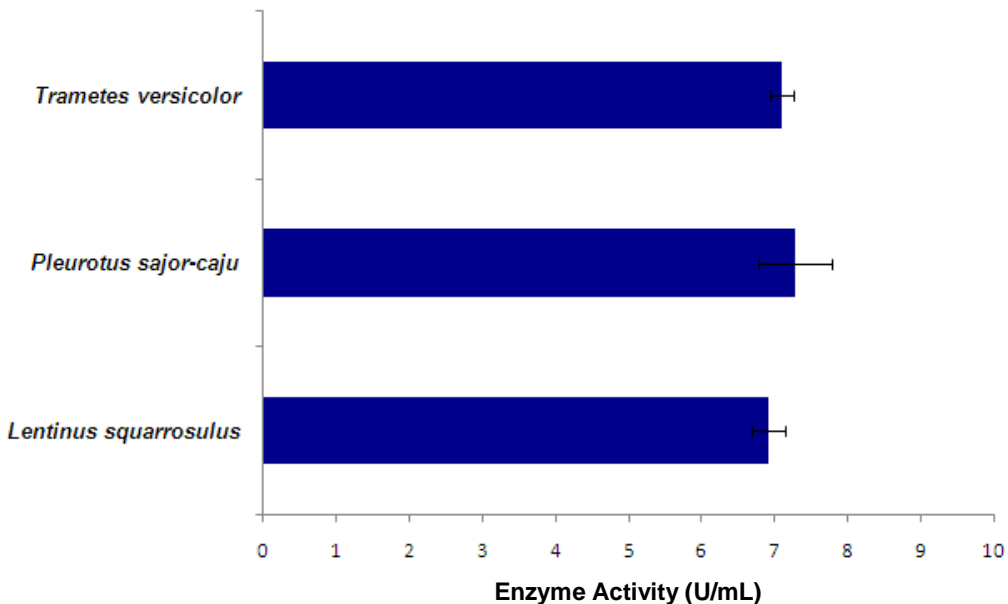


Fig. 6. Comparison of manganese-independent oxidation of 2,6 dimethoxy phenol (DMP) by different versatile peroxidase producing strains

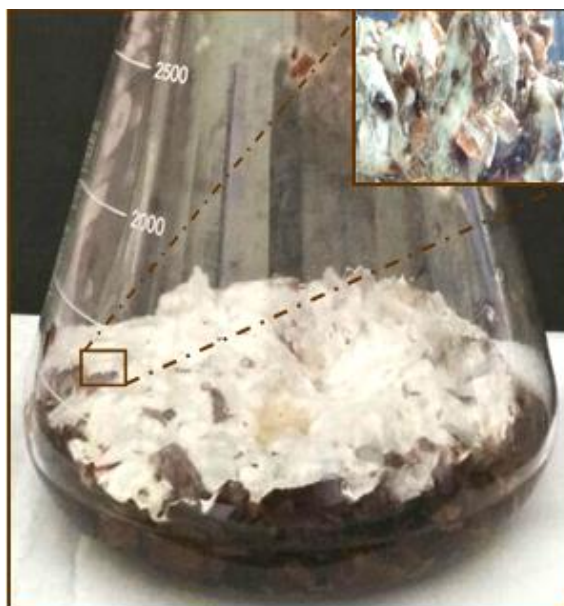


Fig. 7. Solid-state fermentation of TAMI004 on wood chips with a close up view of colonization of the fungus on wood chips (inset).

The pH optima for RB5 oxidizing activity and manganese oxidizing activity were observed as 3 and 4.5, respectively. Solid-state fermentation demonstrated superior ligninolytic ability in comparison to submerged fermentation. The manganese-oxidizing peroxidase activity evidenced in submerged fermentation was 12 U/mL, whereas in solid-state fermentation it was 131 U/mL. The activity values obtained in the current study were comparable to those produced by *Bjerkandera* sp. in glucose ammonium medium (Heinfling *et al.* 1998a). This *Lentinus* strain also grew profusely in solid-state fermentation on wood chips, as wood is the preferred substrate of this fungus (Fig. 7).

Estimation of Proximate Principles and *in vitro* Dry Matter Digestibility (IVDMD)

A majority of the straws exhibited reductions in the NDF, ADF, and ADL contents of enzyme-treated samples irrespective of the mode of fermentation, as shown in Table 1 and Figs. 8, 9, and 10. NDF recorded 3.6% and 4.2% reduction in solid-state (SSF) and submerged (SmF) fermentation enzyme treatments, respectively. There was a 5.2% and 2.9% decrease in acid detergent lignin in SSF and SmF enzyme treatments, respectively. Little millet, foxtail millet, and paddy straw exhibited statistically promising results, while finger millet straw and barnyard millet showed the slightest response. Thus, solid-state fermentation yielded significantly superior results compared to submerged fermentation.

Table 1. NDF, ADF, and ADL in Control and Enzyme-Treated Straws

Straws	Method	NDF	ADF	ADL
		Means \pm SD	Means \pm SD	Means \pm SD
Barnyard Millet	Control	73.32 \pm 0.26	48.83 \pm 0.16	6.6 \pm 0.03
	SSF	71.79 \pm 0.43*	36.36 \pm 0.28***	6.44 \pm 0.02
	SMF	72.09 \pm 0.64	46.59 \pm 0.31**	6.41 \pm 0.1
Foxtail Millet	Control	74.16 \pm 0.38	44.13 \pm 0.18	5.89 \pm 0.08
	SSF	66.38 \pm 1.46**	38.58 \pm 0.79***	5.48 \pm 0.16*
	SMF	68.7 \pm 0.42**	43.73 \pm 0.42	5.9 \pm 0.09
Finger Millet	Control	76.75 \pm 0.22	46.66 \pm 0.48	4.13 \pm 0.07
	SSF	76.45 \pm 1.06	46.51 \pm 0.02	3.98 \pm 0
	SMF	74.82 \pm 0.82*	45.77 \pm 0.01*	4.2 \pm 0.01
Paddy	Control	81.48 \pm 0.4	62.06 \pm 0.23	4.34 \pm 0.04
	SSF	79.99 \pm 0.51	51.37 \pm 0.12***	4.14 \pm 0.16
	SMF	80.14 \pm 0.94	57.79 \pm 0.3**	4.28 \pm 0.31
Little Millet	Control	67.38 \pm 0.47	42.87 \pm 0.01	5.94 \pm 0.04
	SSF	65.41 \pm 0.43*	38.14 \pm 0.1**	5.43 \pm 0.08*
	SMF	61.72 \pm 0.41**	39.11 \pm 0.03**	5.3 \pm 0.26*

NDF- Neutral Detergent Fiber, ADF – Acid Detergent Fiber, ADL – Acid Detergent Lignin
(*P \leq 0.05, **P \leq 0.01, ***P \leq 0.001)

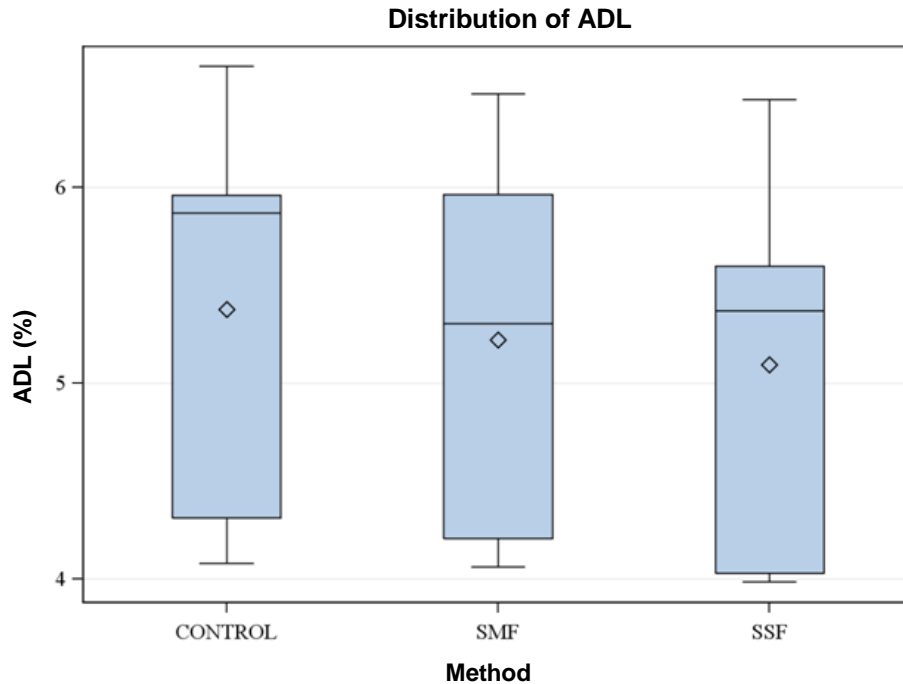


Fig. 8. Distribution of ADL in control, solid-state (SSF), and submerged (SmF) enzyme treatments of crop residues

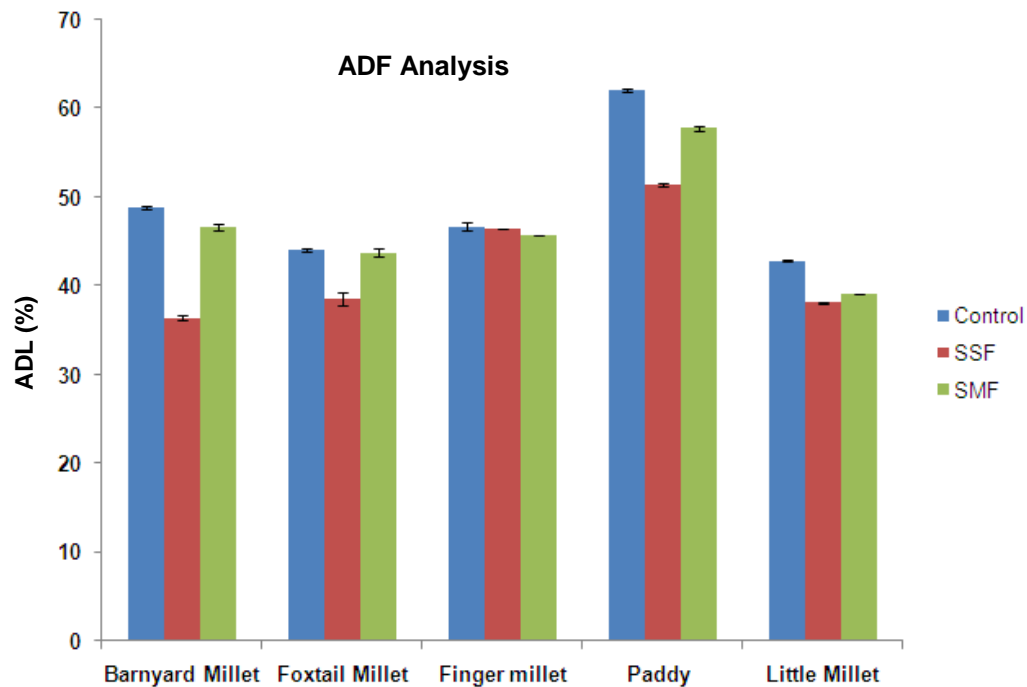


Fig. 9. Distribution of ADF control, solid-state (SSF), and submerged (SmF) enzyme treatments of crop residues

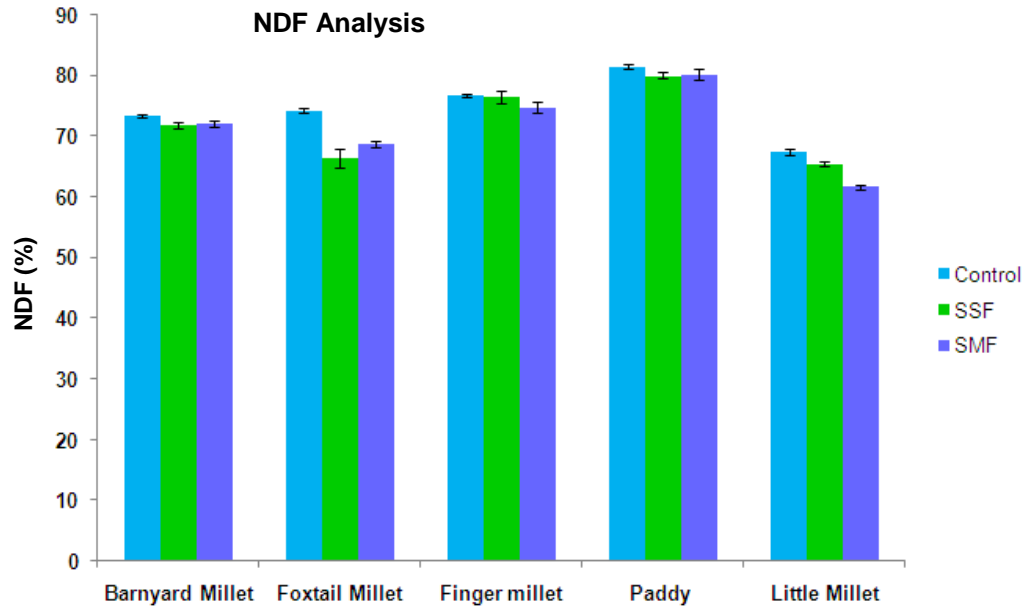


Fig. 10. Distribution of NDF control, solid-state (SSF), and submerged (SmF) enzyme treatments of crop residues

Enhanced digestibility was observed with both little millet and finger millet straws (Table 2). A maximum of 32% increase in digestibility compared with the control was recorded with little millet straw treated with enzyme harvested through solid-state fermentation and 29% for finger millet straw treated with the same enzyme. Enzyme produced through submerged fermentation provided 14% and 16% increase in digestibility, respectively, for these straws (Table 2). The negative correlation between lignin content and digestibility was evident from the correlation coefficients -0.8 for finger millet straw and -0.46 for little millet straw. The augmentation of digestibility through delignification in the enzyme-treated straws is illustrated in Fig. 11.

Table 2. *In vitro* Dry Matter Digestibility (IVDMD) Statistical Means of Control and Enzyme Treated Straws (**P ≤ 0.01)

Straws	Method	IVDMD (Means ± SD)
Little Millet	Control	43.02 ± 2.2
	SSF	56.71 ± 0.28**
	SMF	49.02 ± 1.03
Finger Millet	Control	51.06 ± 2.5
	SSF	66.05 ± 0.68**
	SMF	59.4 ± 1.94

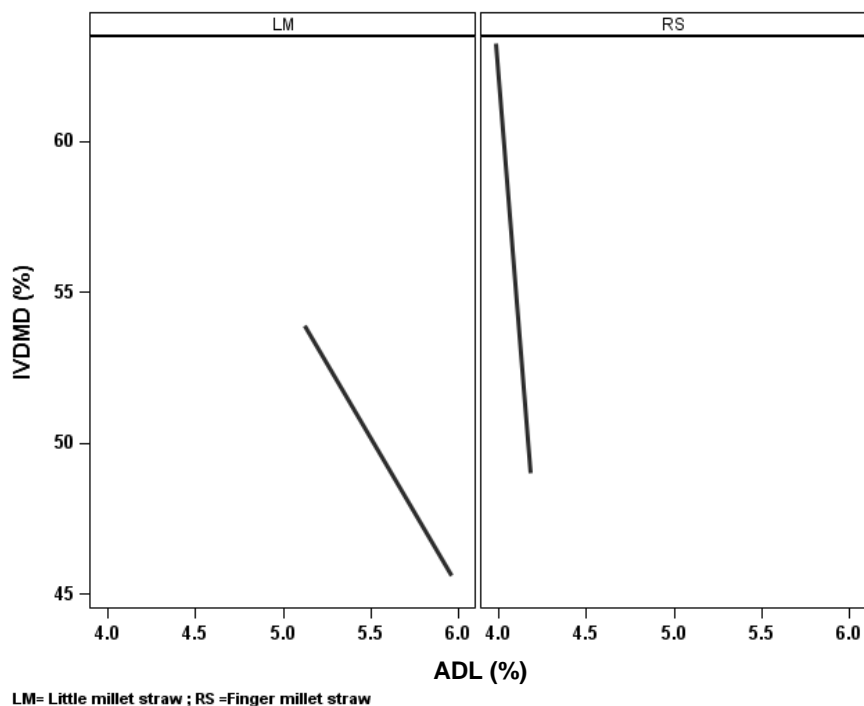


Fig. 11. Correlation plot of ADL vs. IVDMD of solid-state (SSF) and submerged (SmF) enzyme treatments of little millet (LM) and finger millet (RS) straw

Tannic acid and gallic acid are traditional substrates of the Bavendamm reaction, while guaiacol and syringaldehyde are phenolic lignin substructures produced upon lignin oxidation. These indicator compounds provided direct evidence of ligninolytic activity; nevertheless, diverse aromatic indicator compounds were used for substantiation of the lignin degrading ability of the isolates. In nature, wood decay by white-rot fungi requires two or more of the ligninolytic enzymes. Secondary compounds of this lignin macromolecule are readily oxidized by the ligninolytic machinery of white-rot fungi. Though these substrates are readily oxidized by the ligninolytic enzyme array, the mode of action and their potential differ. This is the rationale behind selective oxidation of certain substrates by the ligninolytic system. For this reason, Reactive Black 5 (RB5) is reportedly oxidized by versatile peroxidase and dye decolorizing peroxidases (Martínez *et al.* 2005; Salvachua *et al.* 2013). RB5 decolorization proves the secretion of high redox potential peroxidase, which was displayed by only few isolates screened in this study. The best isolate with this high potential for oxidation of RB5 was studied to decolorize azo dye RB5 and anthraquinone dye RBBR autonomously devoid of manganese. The TAMI004 strain was relatively rapid and competent in its action on phenolics and dyes. This prompted further characterization for its ligninolytic potential. The decolorization of Reactive Black started from day 3 and reached its maxima after 5 days; complete decolorization was observed after 7 days. A similar pattern of decolorization was also perceived with Remazol brilliant blue R; both dyes were oxidized equally and efficiently in manganese independent reactions. The oxidation of diverse substrates makes this high redox potential enzyme similar to those from *Pleurotus* and *Bjerkandera* (Heinfling *et al.* 1998b,c; Ruiz Duenas *et al.* 2001).

White rot fungi typically oxidize natural lignocellulosic substrates through the collaborative effect of two or more of the ligninolytic enzymes. Growth conditions strongly

influence the production of these enzymes from diverse eco-physiological groups. Therefore, the enzyme activities of this isolate were studied in solid-state and submerged fermentation modes. Ligninolytic enzymes are effective in solid-state fermentation (Rodriguez and Sanroman 2005; Palma *et al.* 2016). Likewise, in this study, the activity of manganese oxidizing peroxidase was exponentially higher in solid-state fermentation of wood than in submerged fermentation. In submerged fermentation, the manganese-oxidizing peroxidase activity was higher in the medium without manganese. Though manganese oxidizing activity was predominant in media containing 0, 100, and 500 μM manganese, RB5 oxidizing peroxidase activity declined from 0 μM through 500 μM . This suggests the existence of two distinct peroxidases in media void of manganese and in media with more than 100 μM manganese (Martinez *et al.* 1996). Maximum activity peak in solid-state fermentation was observed on day 12, after which activity declined and maintained until day 20, whereas in submerged fermentation activity peaked on 7th day and dropped thereafter. Maximal activity in submerged fermentation was observed at the end of log phase when the residual sugar concentration was almost 30%. Reducing sugar consumption was rapid during the log phase and was almost negligible during stationary phase. Preliminary investigation of the ligninolytic enzymes secreted by this wild isolate established the presence of high redox potential versatile peroxidase. Microscopic observation of spores, hyphae, and septa confirmed this strain as affiliated with the basidiomycetes group. The internal transcribed sequence (ITS) region was acquired from the genomic DNA isolated from the fungus through the use of ITS 1 and ITS 4 primers. This strain was identified as *Lentinus squarrosulus* through comparison with BLAST of the fungal barcode sequence in a separate study (Rao *et al.* 2018). To the authors' knowledge, this is the first report describing production of versatile peroxidase from *Lentinus squarrosulus*. *Lentinus squarrosulus* is an edible white-rot mushroom of family Polyporaceae that grows extensively on decaying wood. Analysis of research conducted have established the antioxidant and nutritional properties of this white-rot fungus, but ligninolytic abilities of this fungus have not been reported (Omar *et al.* 2011). The ligninolytic potential of this fungus was realized through the crude enzyme treated straws, wherein there was a noteworthy improvement in the fiber fraction reduction, as illustrated through proximate principles. The reduction in the lignin component of the crude enzyme treated straws is likely to improve its digestibility and concomitantly animal productivity. This was confirmed through the digestibility studies carried out on little millet and finger millet straws treated with the versatile peroxidase rich enzyme extract. The significant increase in *in vitro* digestibility in the enzyme treated straws confirms the potential of this enzyme to enrich the vastly available crop residues for utilization by ruminants. These results are salient from the ruminant nutrition perspective, as a small increase in digestibility makes a great difference in livestock productivity (Reddy and Krishna 2009). Though much work is required towards enhancing the activity of this high potential peroxidase and a deeper insight on its role in enhancing ruminant digestibility, this work will be an impetus for further advancement in this direction. Production of high potential versatile peroxidase by this strain has been indicated through this work, which will be of use in biotechnological and industrial applications, especially in ruminant nutrition.

CONCLUSIONS

1. Wild isolates of wood rotting Basidiomycetes were screened and characterized for

identification of a potential versatile peroxidase producer.

2. The role of a novel versatile peroxidase from tropical white-rot *Lentinus squarrosulus* identified in this study in enhancing the nutritive value of crop residues as demonstrated through proximate and *in vitro* digestibility analysis was substantiated.
3. This enzyme holds immense potential for biotechnological applications in ruminant nutrition through enrichment of lignocellulosic crop residues.

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CONFLICT OF INTEREST

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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