Biological depolymerization of lignin using laccase harvested from the autochthonous fungus \textit{Schizophyllum commune} employing various production methods and its efficacy in augmenting in vitro digestibility in ruminants

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A laccase-producing hyper performer, \textit{Schizophyllum commune}, a white-rot fungus, was evaluated for its ability to selectively degrade lignin of diverse crop residues in vitro. Relative analysis of crop residue treatment using laccase obtained from immobilized cells demonstrated degradation of 30–40% in finger millet straw and sorghum stover, 27–32% in paddy straw, 21% in wheat straw, and 26% in maize straw, while 20% lignin degradation was observed when purified and recombinant laccase was used. Further investigations into in vitro dry matter digestibility studies gave promising results recording digestibility of 54–59% in finger millet straw 33–36% in paddy straw and wheat straw, 16% in maize straw for laccase obtained from cell immobilization method, whereas 14% digestibility was observed when purified and recombinant laccase was used. Sorghum stover recorded digestibility of 13–15% across all straws treated with laccase. The results obtained elucidated the positive influence of laccase treatment on lignin degradation and in vitro dry matter digestibility. The present research gave encouraging figures confirming the production of laccase using the cell immobilization method to be an efficient production method commensurate with purified and recombinant laccase under conditions of submerged cultivation, proclaiming a cost-effective, environmentally safe green technology for effectual lignin depolymerization.

Ruminant livestock occupies a unique niche on account of their inherent ability to use crop residues as feeds, the key contributors to livestock feed resources as roughages, especially in the developing and transition countries. The widespread global availability of these residues and their importance in crop-livestock systems assign them the position of important strategic natural resources\textsuperscript{1}. Considering the huge quantities of lignocellulosic biomass available and the high nutritive quality of their polymeric constituents, the hexose and pentose sugars, cereal straws normally contain at least 70% carbohydrates\textsuperscript{2} and are, therefore, no doubt a potential source of energy for livestock, which can be utilized through microbial fermentation in the rumen. Lignin has been reported as one of the most significant recalcitrance factors affecting biomass recalcitrance\textsuperscript{3,4} and the inability of rumen microbes to release adequate levels of energy from many crop residues has limited their value and utility for livestock production. Technologies that significantly enhance delignification and promote utilization of trapped energy from crop residues thus represent an unprecedented opportunity for enhanced productivity from existing agricultural systems, especially where crop residues are underutilized.

Among the various methods hitherto employed for improving nutrient availability from crop residues, biological treatment with white-rot fungi (Basidiomycetes) is known to be the safest green technology being endowed

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with the inherent capability to selectively degrade lignin on account of their major ligninolytic enzymes viz. Laccases, Lignin peroxidases, Manganese peroxidases, and Versatile peroxidases.

Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) is a blue-copper oxidoreductase that is the most widely studied of these enzymes having application in a vast number of biotechnological processes, including materials science, bioremediation, biofuels, fiber modified nanobiotechnology, biosensor, food chemistry, paper, and pulp industry, and biodegradation. Laccase catalyzes the oxidation of a wide range of substrates including phenolic compounds with the concomitant reduction of molecular oxygen to water. Traditionally, laccase has been produced by white-rot fungi using submerged fermentation batch culture a very convenient nevertheless expensive process, especially if the enzyme requires a high fold purification as well as solid-state fermentation (SSF) from agro-waste materials. The research to date about laccase production on agro-based residues was chiefly targeted toward biofuel and bioethanol production and laccase production at the laboratory level from fungi such as Agaricus bisporus, Coriolus versicolor, Pleurotus ostreatus, Trametes versicolor, and Rhus vernicifera has been widely reported. Schizophyllum commune, a white-rot basidiomycete was studied for ligninolytic enzymes (manganese peroxidase, lignin peroxidase, and laccase) production in solid-state fermentation (SSF) for delignification of various agro-industrial residues from the banana stalk, corn cobs, sugarcane bagasse, and wheat straw, respectively, and were also employed for ethanol production from wood chips by consolidated bioprocessing while Han et al. used S. commune mixed with other species and reported that the fungal co-culture and the mixed lignocellulosic wastes contributed to the improvement of laccase activities and enhanced laccase yields within a short period.

WRF such as Phanerochaete chrysosporium, Pleurotus sp., Lentinus edodes, Coriolus versicolor, Phlebia sp., and Ceriporiopsis subvermispora was used to ferment various crop residues like wheat straw, olive mill solid waste, madake bamboo, tanniniferous lеспедеza plants, oil palm fronds, etc. which were then used for feeding ruminants as such or as crude enzyme extracts. Microbial conversion though a practical and promising alternative for enhancing the nutritional value of crop residues results in severe losses in organic matter.

The amount of enzyme secreted by fungi in the native state is not sufficient to meet the current industry demands and created the dire necessity for methods of enhancing secretion or obtaining novel potent enzymes from wild isolates. Studies in this direction on the application of ligninolytic enzymes in general and laccase in particular to enhance the digestibility of crop residues for better ruminant productivity are scarce. Laccase obtained from a wild isolate of the white-rot fungi S. commune showed positive delignification and enhanced digestibility for ruminants in vitro. In the concerted efforts and in the light of this background the current study was conducted (a) to validate the potential of laccase enzyme harvested from NI-07 strain by various methods in terms of secretion or obtaining novel potent enzymes and (b) to authenticate the efficacy of enzyme production encompassing greater efficiency, economic viability, reduction in production complexities and harnessing enormous stability to pave the future direction for utilization of the most economically feasible technology for bulk production of laccase to improve digestibility in ruminants.

Materials and methods

Chemicals. Unless otherwise stated, all chemicals used for analysis were of analytical grade and were purchased from Sigma–Aldrich (USA). Restriction enzymes and the 2,2′-azino bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was procured from Hi-Media. The substrate 2,2′-anisidine and 1.77 mM catechol. Cultures were grown using similar conditions as mentioned previously.

Organisms. Schizophyllum commune NI-07, a potent laccase producer used in the present study, was isolated and deposited with MTCC, Chandigarh, and is available for procurement (MTCC 11893). Trametes versicolor (MTCC 138) was used as the reference standard. Both cultures were maintained on Malt Extract Agar (MEA) slants and plates.

Laccase production in liquid cultures. Laccase was produced under submerged cultivation conditions (SmF) using Malt extract broth as the growth medium and modified basal salt solution (BSS) as the production medium. Mycelial plugs (5 mm × 3/100 ml media) inoculated and cultured in MEB for 5 days (30 °C, 120 rpm) were used as inoculum for laccase production in BSS. One litre of BSS comprised of glucose (20 g), l-asparagine (2.5 g), l-phenylalanine (0.15 g), KH₂PO₄ (1 g), MgSO₄·7H₂O (0.5 g), CaCl₂ (0.01 g), FeSO₄·7H₂O (0.01 g), MnSO₄·4H₂O (0.001 g), ZnSO₄·7H₂O (0.001 g), CuSO₄·5H₂O (0.002 g), and thiamine hydrochloride (1 mg). Flasks containing the production medium were incubated at 30 °C for 7 days (120 rpm). Cell-free culture supernatant was used as a crude enzyme for further analysis after centrifugation (2000g, 15 min).

The medium used for laccase production under submerged conditions was optimized (SmF/Opt) using response surface methodology (RSM) and the Proc General Linear Model procedure of SAS (Version 9.3). This was adapted to maximize laccase production by altering the various culture conditions that affect the growth of fungal biomass by one factor at a time (OFAT) approach. Statistically optimized media obtained from RSM for enhancing laccase production contained 1.82% glucose, 1.35% fructose, 0.23% l-asparagine, 0.19% yeast extract, 2.45 mM p-anisidine and 1.77 mM catechol. Cultures were grown using similar conditions as mentioned above. Cell-free culture supernatant was used as a crude enzyme for further studies after centrifugation (2000g, 15 min).

Whole-cell immobilization (Icell/Lac) was performed using inert matrix polyurethane foam (PUF). PUF sheets were procured from Shriram Polymers (Mumbai, India), cut into 1 × 1 × 1 cm cubes, pre-treated by boiling in water for 20 min at 80°C, soaked overnight in methanol before washing with distilled water, dried, and autoclaved. Flasks containing 5 g/100 ml media of autoclaved cubes were inoculated with mycelial plugs (5 mm × 3/100 ml media) homogenized under aseptic conditions and incubated (30 °C) under continuous illumination for 7 days (120 rpm).
shaking (120 rpm) for five days. MEB used as growth media was decanted slowly from the flasks and replenished with BSS, retaining the cubes immobilized with fungus. Flasks were further incubated (30 °C, 120 rpm) for lac
case production. Aliquots from the media were regularly sampled for measuring laccase activity, and laccase-
rich media was harvested between the 6th and 7th days when there was maximum laccase production. Cell-free laccase-rich media was obtained by centrifugation (2000xg, 15 min) of the harvested media.

Purified laccase was obtained by subjecting the cell-free supernatant obtained using immobilization to 70%
(NH₄)₂SO₄ precipitation, kept overnight at 4 °C and centrifuged (5000xg, 30 min). The precipitate was dissolved
in 0.4 M Na-acetate buffer (pH 5.2). The solution was dialyzed overnight against 0.1 M Na-phosphate buffer
(pH 6.8) and loaded onto a Sephadex G-50 column (44×3 cm) equilibrated with the same buffer. Collected
fractions were assayed for both protein and laccase activity. Active fractions were pooled and concentrated on
an Amicon ultra cell-30 membrane, Millipore, USA (Pure/Lac) and reconstituted in sodium acetate buffer (pH
5.2) for further analysis.

The nucleotide sequence of the full-length cDNA from Schizophyllum commune containing a 1554 bp open
reading frame encoding a polypeptide of 518 amino acid residues was adopted from Hatamoto²⁶, and the gene
was codon-optimized and directionally subcloned into the pPIC9 vector using EcoR1 and NotI restriction
enzymes. The vector was linearized using BglII and transformed into the Pichia GS115 strain²⁵. Single colonies
from the transformed plate were inoculated into 10 ml of buffered minimal glycerol (BMG) medium (prepared
by using 100 ml of potassium phosphate buffer (1 M, pH 6.0), 100 ml 10× YNB, 2 ml 500× Biotin and 100 ml 10×
glycerol and brought up to 1000 ml with sterile distilled water) and grown at 30 °C (250 rpm) for 18 h. The cells
were then harvested by centrifugation (2000xg, 5 min). The supernatant was decanted, and the cell pellet was
resuspended in buffered minimal methanol (BMM) (prepared using 100 ml of 10% methanol instead of glycerol).
Other components are the same as BMG. Cultures were incubated at 30 °C (250 rpm), and 100% methanol was
added to a final concentration of 0.5% (v/v) every 24 h to maintain induction. Cell-free culture media (Rec/Lac)
was eventually used for analysis.

Cell-free supernatant obtained from all the production methods was stored at –20 °C until further use, and
all enzymatic procedures were performed at room temperature.

Enzyme assays. Laccase activity was determined by the oxidation of ABTS²⁷ at 28 ± 2 °C. The reaction
mixture contained 0.6 ml of 1.6 mM ABTS, 0.6 ml of sodium acetate buffer (pH 5.2) and 0.6 ml of culture filtrate. Distilled water (0.6 ml) without enzyme served as a control. ABTS oxidation was monitored by measuring the increase in absorbance at 420 nm (ε₄₂₀=36,000 M⁻¹ cm⁻¹). To correct for ABTS oxidation by peroxidases (in media), 0.5 µg/ml catalase was used. One unit of laccase activity was defined as µmoles of ABTS oxidized per minute per ml. Protein concentration was determined by the method of Lowry (1951)²⁸ using bovine serum albumin (BSA) as the standard.

Sectioning of PUF cubes. Polyurethane foam (PUF) cubes inoculated and immobilized with S. commune
for 20 days were subjected to 0.1 mm micro sectioning using a refrigerated microtome cryostat LEICA CM
1510S. Uninoculated PUF cubes soaked in distilled water served as control. The sections were then observed for
fungal colonization using phase-contrast microscopy.

Preparation of substrate. Paddy straw (PS), finger millet straw (FMS), wheat straw (WS), maize straw
(MS), and sorghum stover (SS) were procured from the local market and manually chaffed into lengths of 2 cm.
Known weights of the straw were treated by spraying laccase enzyme obtained from different production meth-
ods under hydrated conditions. For every 3 g of straw used, 5 ml of enzyme-rich broth was used at a ratio of 3:5
(w/v). Purified laccase was obtained by subjecting the cell-free supernatant obtained using immobilization to 70%
(NH₄)₂SO₄ precipitation, kept overnight at 4 °C and centrifuged (5000xg, 30 min). The precipitate was dissolved
in 0.4 M Na-acetate buffer (pH 5.2). The solution was dialyzed overnight against 0.1 M Na-phosphate buffer
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Statistical analysis. Statistical analysis was performed using R statistical software (version 4.1.0)³⁹. The data on various nutritional parameters are presented as the mean ± SD (standard deviation) and tabulated. Box
plots were designed using ggboxplots, and correlation graphs were plotted using the ggpubr R package. R package ggplot2 was used to create a line graph to see the variations in laccase titre levels across incubation days for different production methods employed. Each component of the graphs—axes, scales, colors, objects, etc. was built up sequentially one component at a time. Regression analysis of IVDMD against lignin content governed by each individual production method was performed using the linear regression method to measure the strength of the linear relationship between lignin degradation and in vitro dry matter digestibility. The Geom_smooth function of R with the formula \( y \sim x \) was used to analyze the correlation between ADL and IVDMD of five different straws treated with laccase-rich cell-free media.

The coefficients of determination \( (R^2) \) were calculated using the formula
Methods of laccase production using WRF. *S. commune* NI-07 strain. Photographs of (A) Pure culture of *S. commune* on Malt Extract agar plate (B) *S. commune* grown on Laccase Detection Agar (gl-1 media contained KH₂PO₄-1 g; C₂H₃N₂O₄-0.5 g; MgSO₄-0.5 g; CaCl₂-0.01 g; Yeast Extract-0.01 g; CuSO₄-0.001 g; FeSO₄-0.001 g; MnSO₄-0.001 g; 1.6% w/v agar) with 0.1% ABTS as substrate (C) Modified LBM with *S. commune* immobilized PUF cubes (D,F) Phase-contrast micrograph (×40 dry objective; wet mount) showing a section of the uninoculated polyurethane foam (PUF) cube (F) A section of PUF cube immobilized with *S. commune* (G) Elution profile of *S. commune* laccase from Sephadex G-50 gel filtration column (H) SDS PAGE of laccase obtained through immobilization: Lane 1: Protein standard marker (Biorad Precision Plus), Lane 2: Purified laccase (I) Transformed *E. coli* cells analyzed for the presence of pPIC9K/Scom-Lac expression cassette (a): Colony PCR of transformed *E. coli* DH5α cells with pPIC9K/Scom-Lac expression cassette. L1-Gene ruler 1 kb plus DNA Ladder, L2&3–5 µl PCR product from two different clones selected on LB/ampicillin plates and loaded on 1% agarose gel (J) Laccase expression in methanol induced minimal broth with 0.1 mM CuSO₄ and 0.5 mM ABTS A-Control GS115 strain without insert; B-Laccase induced strain (GS115/pPIC9K/Scom-lac); C-Sec HSA strain (GS115 Albumin Mut) (K) Laccase production using basal salts medium. Production (a) under Submerged fermentation (SmF), Submerged fermentation using optimized media (SmF/Opt), Fermentation using immobilized cell (Icell/Lac), Fermentation using recombinant laccase (Rec/Lac). One unit laccase is expressed as the activity of the enzyme that catalyzes the conversion of 1.0 µmole of ABTS min⁻¹.

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r = \frac{\sum (x - m_x)(y - m_y)}{\sum (x - m_x)^2 \sum (y - m_y)^2},
\]

where 'mx' and 'my' are the means of x and y variables. The p-value of the correlation was determined using a correlation coefficient table for degrees of freedom: df = n - 2, where n is the number of observations in the x and y variables. For all statistical analyses, significance was declared at P ≤ 0.05 for variables to remain in the model. ANOVA was used to compare the means of ADL and IVDMD of enzyme-treated straws. Further independent t-tests were performed to compare the effect of different methods on each type of straw.

Results and discussion

Rumen physiology demands the use of both roughages and concentrates in the diet in varying proportions influencing productivity and maintenance costs. The low quality of dry roughages necessitates the need for enhancing the nutritive value of the feed using economically favorable delignification methods. Recalcitrant lignin is made up of three monolignols: p-coumaryl, coniferyl, and sinapyl alcohols and digestion of cell wall fractions of forage in the rumen is incomplete due to the complex links which limit their degradation. White rot fungi are known to be active lignin degraders as they harness a plethora of ligninolytic enzymes and the depolymerization rate by fungi ranges from 20% to nearly 100%, depending on different lignin sources. Most of the studies using WRF were conducted to a large extent employing SSF. In this study, the native isolate *S. commune* NI-07 was initially grown under solid-state fermentation (SSF) conditions by inoculating the fungus directly onto the lignocellulosic substrate. It produced laccase effectively measuring laccase activity of 304.62 U ml⁻¹ on the 5th day, which was relatively higher than that observed for the reference culture *T. versicolor*. However, regardless of SSF appearing to be a viable option, there were dry matter losses as the culture started utilizing the carbohydrate components for carbon source, limiting induction of ligninolytic enzymes, a fact attributed to the fungus shifting its reproductive cycle from asexual to sexual mode. High laccase activity was nevertheless observed during the mycelial growth stage, which dropped down rapidly through fruiting body formation (data not shown). Therefore, the focus was shifted to submerged fermentation methods for laccase production to overcome the limitations of SSF.

Han et al. also reported the use of *S. commune* Han 881 to produce laccase in submerged fermentation in mixed cultures accelerating enzyme production. Liquid cultivation of the native isolate *S. commune* (Fig. 1A) produced laccase in the culture media extracellularly and a confirmatory test for laccase activity was made quantitatively by spectrophotometric enzymatic analysis. There was an appearance of a green halo around the colony using the ABTS plate test assay (Fig. 1B) confirming the conversion of ABTS to ABTS⁺ radicals in the presence of laccase. Laccase production by *S. commune* under SmF using modified liquid basal solution was reverberated by the overlay of blue color on the mycelium (Fig. 1C), as type I copper on interaction with laccase protein influenced the formation of blue color. Laccase titres were greatly influenced by the production methods employed. The production of laccase under SmF conditions (Fig. 1K) showed maximum laccase activity on the 6th and 7th days in modified BSS (SmF) and the 8th and 9th days in statistically optimized culture media (SmF/Opt).

Immobilization of the native isolate was performed following submerged cultivation as it provides a solution for more cost-effective and economical use of enzymes. Here, *S. commune* cells (Icell/Lac) immobilized
using polyurethane foam (PUF) (Fig. 1D) further enhanced laccase production. PUF was used as a supporting platform for adhesion because of its inert nature, excellent dimensional stability, and strength. In the present study, maximum laccase activities were observed on the 6th and 7th days of production. Media-rich in laccase could be recovered easily without compromising cell viability for 9–10 batches. Figure 1E,F show sections micrographs of PUF cubes after 20 days of fungal immobilization (Fig. 1F). Rapid colonization of foam was observed using a phase-contrast microscope when microsections of PUF inoculated with S. commune were observed at 40× magnification compared to the control (Fig. 1E), ascertaining adsorption of the fungal mycelia within the matrix. PUF has been used earlier as a support matrix to immobilize fungus. Luke and Burton⁴⁶ immobilized Neurospora crassa in a membrane bioreactor to witness the continuous production of enzymes without deactivation for several weeks. Struszczyn-Świta⁴⁹ used PUF-immobilized fungal chitosanase–lipase preparation that was found to have a half-life of 200 days when stored at room temperature. Krishna Prasad et al.⁵⁰ have immobilized Pleurotus ostreatus 1084 on PUF cubes and reported increased laccase yields. Reference⁵¹,⁵² immobilized Aspergillus flavus A5p1 on PUF and showed enhanced RB4 decolorization efficiency. All these reports are in line with our current study and suggest the use of PUF as a suitable matrix for cell immobilization.

The harvested media from immobilization was further used to purify the enzyme. Purification and characterization epitomized the isolate's superior pH and temperature tolerance ability. Figure 1G shows the elution profile after the Sephadex G-50 size exclusion chromatography. The specific activity of the pure enzyme was 360 U mg⁻¹. Gel electrophoresis after concentrating the active fractions from size exclusion chromatography resulted in an apparent molecular weight of about 75,000 Da (Fig. 1H). The results are in agreement with Vantamuri and Kailiwal⁴⁵, where a monomeric protein with a molecular mass of ~75 kDa as estimated by non-denaturing polyacrylamide gel electrophoresis was isolated from Marasmius species BBKAV79 with greater pH and thermal stability. Irshad and Asgher⁴⁴ purified laccase from Schizophyllum commune IBL-06 and obtained a 63 KDa band on SDS-PAGE. These variations observed could stem from the same organism or across different organisms are because of changes in the glycosylation patterns that occur due to structural and chemical changes and glycosylation improves the stability of the protein post immobilization. The higher stability observed in the obtained laccase is because immobilization stabilizes the structure of the enzyme and alters the spectral properties both physically and chemically and glycosylation improves the overall stability of the protein as well as increases its molecular weight.

The Laccase gene was expressed heterologously in the Pichia pastoris GS115 strain for recombinant protein production. The expression cassette containing the optimized laccase gene was transformed into E. coli DH5α first for propagation and then transformed into Pichia. The transformants from E. coli, containing the pPIC9K/Scom-Lac expression cassette with α-factor secretion signal and 3′AOX1 primers were subjected to colony PCR to confirm the presence of insert. Parent plasmid pPIC9K will produce a 195 bp fragment and with a laccase insert of 1520 bp, the amplification product is 1715 bp as evident from the gel photograph corresponding to the primer positions on the vector (Fig. 1I) confirming the successful cloning of pPIC9K with optimized laccase gene. Liquid cultivation of the positive Pichia clone using buffered minimal methanol (BMM) decolorized the broth to yellowish-green (Fig. 1J) suggesting proper processing of the signal sequence and extracellular expression of laccase protein was confirmed by ABTS assay. The highly expressing transformant gave a wet cell weight of 0.19 g/10 ml of broth with laccase activity of 344 U ml⁻¹ after 5 days of growth at 30 °C. Rec/Lac expressed in the medium contained very little native protein, laccase being the sole enzyme produced with maximum activity on the 5th day (Fig. 1K). Hirai et al.⁵³ had also expressed laccase cDNA from white-rot fungus S. commune in a transgenic tobacco plant by decreasing the CpG-dinucleotide motif content for phytoremediation of recalcitrant environmental pollutants.

The results obtained in the present study across various production methods, accentuated for 5–7 days of cultivation in liquid cultures for laccase production at 30 °C. It is partially in agreement with the works of Irshad and Asgher (2016) where maximum enzyme activity from S. commune was recorded after 192 h using banana stalk under SSF conditions. A pH range between 4 and 5.5 was ideal for laccase production, as it recorded maximum laccase activity in all the production methods employed in that pH range. Many studies on fungi have previously reported the optimal pH range for laccases to be between 3.0 and 6.0⁵⁵,⁵⁶ and are substrate-dependent. Differential substrate protonation patterns contribute to differences in optimal pH for activity. This is completely in agreement with the results of the current study as well as the study by Nagai et al.⁵⁷, who observed the optimal pH to be 3.0 and 4.0 when the substrates ABTS and o-toluidine were used. Each production step employed greatly increased laccase yields. This could be due to the inherent differential capacity of organisms to synthesize ligninolytic enzymes⁵⁸ under the reaction conditions used.

Crop residues are generally fed to ruminants and form an intricate part of their diets. Previous studies have revealed enhanced ruminant digestibility upon feeding pre-treated crop residues using ligninolytic enzymes of WRF⁵⁹. The present research explored the interaction of laccase enzymes with five different crop residues: paddy straw (PS), finger millet straw (FMS), wheat straw (WS), maize straw (MS), and sorghum stovers (SS). Herein, the influence of various production methods on laccase titre and crop residue oxidation was studied considering parameters such as loss in total dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL). Straws were cut into 2 cm lengths and steam sterilized first to provide enough hydration before enzyme treatment, as a hydrated environment creates natural conformation of the enzyme, allowing functional degradation of lignin. Varying concentrations of the enzyme with varying specific activities were obtained when different production methods were employed. Enzyme-rich broth was then sprayed directly onto the straws and mixed well to create a large surface area for enzyme action.

The proximate composition of each straw treated with laccase obtained from various methods was analyzed. The distribution of parameters in treated straws compared to the control is illustrated using box-and-whisker plots (Fig. 2). A decrease in dry matter (Fig. 2A,E,L,M,Q) was observed in all the straws (Control > SmF > SmF/ Opt > Icel/Lac > Pure/Lac > Rec/Lac), except for SS (Fig. 2Q), where enzyme treatment did not influence...
Pure/Lac, and Rec/Lac for PS, WS, and MS in terms of lignin content. Differences existed in the p-hydroxyphenyl SmF and SmF/Opt methods, no significant differences were observed between laccase produced from Icell/Lac, (Fig. 2B,F,J,N,R) content was observed from SmF to Rec/Lac, with Rec/Lac treatment showing higher averages, results obtained. Values of NDF (Fig. 2C,G,K,O,S), ADF (data not presented) and ADL (Fig. 2D,H,L,P,T) showed a steep decrease from control through various methods up until Rec/Lac with marginal difference in values of NDF and ADF for the straws treated with laccase obtained from Pure/Lac and Rec/Lac methods. Decreases in NDF, ADF, and ADL suggested that vegetal cell wall components of the straws were degraded due to laccase treatment, facilitating enhanced digestibility. The results are consistent with earlier reports where different types of dry pastures were treated with 60% enzyme.

There were significant differences in ADL between laccase production from SmF (M = 9.6; SD = 0.19), SmF/Opt (M = 8.32; SD = 0.22) and Rec/Lac (M = 6.35; SD = 0.12) in FMS and SmF/Opt (M = 6.39; SD = 0.07), SmF/Opt (M = 3.17; SD = 0.08) and Rec/Lac (M = 2.15; SD = 0.03) in SS when compared to control (M = 10.61; SD = 0.11 and M = 3.59; SD = 0.03 for FMS and SS simultaneously). While PS, WS, and MS showed significant differences in the SmF and SmF/Opt methods, no significant differences were observed between laccase produced from Icell/Lac, Pure/Lac, and Rec/Lac for PS, WS, and MS in terms of lignin content. Differences existed in the p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) subunits of lignin, and these differences influenced the rate of biodegradability of the straws. Wheat straw contains percent H, G, and S units in a ratio of 5:49:46 ratio of 6:4:30:45-66 with β-O-4′-ether linkages, whereas the H, G, and S units appear in a ratio of 15:45:40 in rice straw. However, an H:G ratio of 5:71:24 with higher levels of β-O-4′-alkyl-aryl lignin was observed by Rosado et al.63. Akin et al.64 correlated the degradation of guaiacyl monomers in lignin to improve digestibility, while Crestini et al.65 observed depolymerization of lignin on the cleavage of the β-O-4′ bonds.

ANOVA was conducted to compare the means of ADL and IVDMD of straws treated with laccase. An independent t-test was performed to determine the effect of different production methods on the ADL content and IVDMD of the treated straws (Table 1). Mean values concerning each method for ADL and IVDMD were found to be significantly different (p < 0.001). Treatment demonstrated a gradual decrease in lignin content, with a maximum degradation of 40.1% observed in FMS and SS, followed by PS (32%) and WS (21.7%), showing the least degradation. These observations suggest that crop residue lignin is guaiacyl rich, with β-O-4′-linkages dominating the structure. Additionally, cell wall cross-linking impacts biomass saccharification levels. McKinley et al.66 observed changes in the hemicellulosic-bound p-coumaric (pCA) and ferulate (FA) of sorghum stems, showing improved saccharification owing to the presence of a larger proportion of fermentable sugars for access, improving digestibility. The ash content in the straws measures degradation indirectly. Organisms capable of more lignocellulose degradation leave more residual ash. The increase in ash content in all the treated straws compared to the control further validated the results (data not shown). The increase in ash content was similar to the studies conducted by Yasar and Tosun67 where the nutritional quality of tomato pomace was improved by Pleurotus ostreatus and Phanerochaete chrysosporium fermentation. Substrate particle size also influenced treatment significantly, as it created a large surface-to-volume ratio, thereby providing access to carbohydrate components. In the present study, crop residues were cut into 2 cm lengths before treatment. This is in agreement with earlier studies from Salvachua et al.68, where the digestibility of Irepex latus-treated wheat straw cultures improved significantly with a reduction in particle size.

IVDMD (t-test) showed a significant difference in treated straws for the SmF, SmF/Opt, and Icell/Lac methods, while no significant difference was observed for Icell/Lac, Pure/Lac, and Rec/Lac in any of the straws used (Table 1). Maximum digestibility was observed in FMS, recording 59% enhancement, while PS and WS showed 36% enhancement in digestibility. Increases in IVDMD of 14.44% and 15.83% were recorded in MS and SS, respectively. Although SS showed maximum lignin degradation, IVDMD was very low compared to other straw samples and can be attributed to the genetic variability it carries. Si et al.69 isolated the laccase gene from Agrobacterium sp. S5-1 in soil humus and heterologously expressed in Escherichia coli. The authors observed that laccase produced significantly (p < 0.05) increased dry matter digestibility of maize straw from 23.44 to 27.96% and from 29.53 to 37.10% after 8 h and 24 h of digestion. Many researchers have reported similar observations for straw treatment under SSF conditions. Results obtained in the present study are in agreement with the experiments conducted by Datsymor et al.70 where pretreatment of rice straw using P. ostreatus showed a marked decrease in acid detergent lignin when compared to control. The IVDMD and the total VFA concerning P. ostreatus were markedly higher than both the control and the other treatments. Similar results were obtained by Sufyan et al.71 where the nutritional value and digestibility of wheat straw, rice straw, and corn cob using Pleurotus species improved.

Comparative analysis of the correlation between lignin content and dry matter digestibility on treatment with laccases (Fig. 3) showed a positive effect on delignification irrespective of the production method employed. A strong negative correlation was observed between enhancement in digestibility and lignin degradation in all the production methods used, with Icell/Lac, Pure/Lac, and Rec/Lac showing maximum degradation and increased digestibility.

Linear regression graphs used to generate ‘r’ values (Fig. 3, Insert) showed a very strong negative correlation for both FMS (Fig. 3A) and MS (Fig. 3D) (r = 0.9595558 and 0.9698371, respectively), while PS (Fig. 3B) (r = 0.9315393), WS (Fig. 3C) (r = 0.9356731) and SS (Fig. 3E) (r = 0.9348795) gave a strong negative correlation (p < 0.0001). Laccases from Icell/Lac, Pure/Lac, and Rec/Lac showed maximum lignin degradation with increased digestibility while having similar effects in all the straws studied with Pure/Lac and Rec/Lac. The linear increase obtained in the present study in IVDMD of five straws upon treatment with laccase...
Figure 2. Box and whisker plots showing the distribution of proximate composition of five different crop residues after treatment using laccase enzyme: (A–D) finger millet (FMS); (E–H) paddy (PS); (I–L) wheat (WS); (M–P) maize (MS) and (Q–T) sorghum (SS). Labels (A,E,I,M,Q) represents dry matter (DM)%, (B,F,J,N,R) represents Protein %, (C,G,K,O,S) represents Neutral Detergent Fiber (NDF)%, (D,H,L,P,T) represents Acid Detergent Lignin (ADL)% of FMS, PS, WS, MS and SS respectively. The representative color codes for each production method are as follows Control (pink); laccase from Submerged fermentation, SmF (amber); laccase produced using optimized media in submerged fermentation, SmF/Opt(green); laccase obtained from immobilized cells, Icell/Lac (cyan); purified laccase, Pure/Lac (sky blue) and recombinant laccase, Rec/Lac (Magenta). Bars represent the standard error of means from three replicates.
is in keeping with earlier reports. Ravichandran et al.\textsuperscript{63} used versatile peroxidase enzyme produced through submerged fermentation for treating finger millet straw and recorded a 14% and 16% increase in digestibility. A maximum improvement of 20% in IVDMD was obtained upon treatment of different straws with purified lignin peroxidase by Thammaiah et al.\textsuperscript{22}. In the current study, lignin degradation and digestibility in maize straw were more effective when Icell/Lac was used compared to Pure/Lac and Rec/Lac. Crop residue lignin of maize straw is known to be syringyl rich with beta aryl ether linkages, as confirmed by 2D NMR studies\textsuperscript{72}. Syringyl propane units predominantly are acid-soluble. However, the pattern of degradation and digestibility differed in all other straws, which are rich in guaiacyl units. This confirms the fact that the guaiacyl–syringyl ratio plays a major role in influencing the degradation pattern.

Extracellular enzymes are generally released in minute quantities and research favors the use of optimization strategies to improve enzyme production\textsuperscript{73}. Many workers have emphasized the need for developing cost-effective environmentally safe strategies to ameliorate the nutritive value of the feed for better productivity using WRF. Zhang et al.\textsuperscript{74} highlighted the potential of WRF to use enzymes for higher fiber digestion with low price and environmentally friendly properties. Zhu et al.\textsuperscript{43} pointed out the use of crude ligninolytic enzyme extracts to be a novel approach that can help develop a cost-effective and environmentally acceptable technology, as they present several additional advantages over the use of purified enzymes. The presence of proteins, mediators, or other factors in the medium may stabilize crude enzymes and mediate the action of these enzymes\textsuperscript{75}.

In the present study, employing immobilized, highly stable laccase enzyme-rich media, to enhance in vitro digestibility of the tested crop residues parallels the aforementioned research.

Digestibility values obtained upon the use of Icell/Lac, Pure/Lac, and Rec/Lac after treatment did not demonstrate remarkable variation proving that the former method was equally effective with the latter two. This would save the additional expenses and labor involved in the purification of laccases, as immobilized laccase can be repeatedly produced at a very low input cost along with the fact of remaining a relatively easy, safe, and environmentally friendly technology.

Table 1. Evaluation of ADL and IVDMD of crop residues treated with laccase obtained using different production methods. Variables with different superscripts within groups were found to be statistically significant. (p-value < 0.05 for t-test); ** statically significant if p-value < 0.05 (for ANOVA).

| Straw     | Method    | ADL       | P value** | IVDMD     | P value** |
|-----------|-----------|-----------|-----------|-----------|-----------|
| Finger millet | Control  | 10.61 ± 0.11* | < 0.0001 | 40.34 ± 0.3* | < 0.0001 |
|           | SmF      | 9.6 ± 0.19*  |          | 45.92 ± 0.75* |          |
|           | SmF/Opt  | 8.32 ± 0.22* |          | 49.84 ± 1.23* |          |
|           | Icell/Lac| 7.46 ± 0.05* |          | 62.37 ± 0.07* |          |
|           | Pure/Lac | 7.44 ± 0.11* |          | 62.4 ± 0.11*  |          |
|           | Rec/Lac  | 6.35 ± 0.12* |          | 64.16 ± 0.08* |          |
| Paddy     | Control  | 5.88 ± 0.05*  |          | 42.4 ± 0.21*  |          |
|           | SmF      | 5.19 ± 0.16*  |          | 44.76 ± 1.02* |          |
|           | SmF/Opt  | 4.6 ± 0.12*   |          | 49.5 ± 1.55*  |          |
|           | Icell/Lac| 4.25 ± 0.03*  |          | 56.51 ± 0.21* |          |
|           | Pure/Lac | 4.15 ± 0.03*  |          | 58.5 ± 0.27*  |          |
|           | Rec/Lac  | 3.99 ± 0.06*  |          | 57.84 ± 1.15* |          |
| Wheat     | Control  | 7.85 ± 0.16*  |          | 43.57 ± 0.09* |          |
|           | SmF      | 7.26 ± 0.08*  |          | 44.94 ± 0.78* |          |
|           | SmF/Opt  | 6.54 ± 0.21*  |          | 49.81 ± 0.82* |          |
|           | Icell/Lac| 6.19 ± 0.09*  |          | 58.12 ± 0.12* |          |
|           | Pure/Lac | 6.07 ± 0.04*  |          | 59 ± 0.44*    |          |
|           | Rec/Lac  | 6.14 ± 0.04*  |          | 59 ± 0.44*    |          |
| Maize     | Control  | 4.64 ± 0.13*  |          | 48.84 ± 0.04* |          |
|           | SmF      | 4.18 ± 0.05*  |          | 51.55 ± 1.39* |          |
|           | SmF/Opt  | 3.97 ± 0.05*  |          | 54.14 ± 0.58* |          |
|           | Icell/Lac| 3.42 ± 0.05*  |          | 56.73 ± 0.23* |          |
|           | Pure/Lac | 3.67 ± 0.05*  |          | 55.71 ± 0.17* |          |
|           | Rec/Lac  | 3.67 ± 0.12*  |          | 55.71 ± 0.17* |          |
| Sorghum   | Control  | 3.59 ± 0.03*  |          | 60.34 ± 0.08* |          |
|           | SmF      | 3.39 ± 0.07*  |          | 62.25 ± 1.3*  |          |
|           | SmF/Opt  | 3.17 ± 0.08*  |          | 65.18 ± 0.25* |          |
|           | Icell/Lac| 2.88 ± 0.03*  |          | 68.19 ± 0.21* |          |
|           | Pure/Lac | 2.22 ± 0.09*  |          | 69.63 ± 0.17* |          |
|           | Rec/Lac  | 2.15 ± 0.03*  |          | 69.63 ± 0.17* |          |
Conclusion
Ruminant livestock is endowed with the inherent ability to use crop residues as feeds. The enormous potential of laccase for several applications necessitated the need for newer hyper-producing strains to overcome the limitations of ruminant digestibility by acting on the lignin polymer selectively. Laccase produced employing solid-state fermentation (SSF) of crop residues results in severe losses in organic matter and is not feasible in the context of ruminant feeding. Laccase enzyme produced using submerged fermentation batch culture is a very convenient nevertheless expensive process. The results of the current study are extremely promising evincing that delignification of all tested crop residues employing immobilized laccase was the most effective and an extremely lucrative technology for potential use in enhancing ruminant digestibility. It also exhibits immense scope in biotechnological and industrial applications. Pilot-scale studies for large-scale production of laccase enzyme through immobilization corroborated with in vivo feeding trials in ruminants are however warranted and would add to confirming the finding of the current study.

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V.P.K. conceptualized, planned and executed the experiments and is a major contributor in writing the manuscript. M.S. designed, supervised, reviewed and edited the manuscript. R.G.R. performed statistical analysis. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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