Purification, Characterization, and Biodelignification Potential of Lignin Peroxidase from Immobilized *Phanerochaete chrysosporium*

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Lignin peroxidase (LiP), which has been studied extensively in white-rot Basidiomycetes with regard to bio-pulping and bio-bleaching, plays a role in the biodegradation of plant cell wall lignin. In the current study, LiP obtained from a wild isolate of *Phanerochaete chrysosporium* immobilized on polyurethane foam cubes was purified 21-fold using ammonium sulphate precipitation and size exclusion chromatography. The enzyme with a molecular mass of 55 kDa exhibited a considerably higher pH tolerance and thermostability compared with the native enzyme. It showed a strong affinity for the substrate veratryl alcohol and had kinetic constant values of 142.86 µmol and 65 µM. Cysteine, sodium azide, mercaptoethanol, and silver nitrate inhibited the activity, while ethanol, EDTA, Cu^{2+}, Mn^{+}, Na^{+}, and Fe^{2+} exhibited induction. Purified LiP completely decolorized (100%) bromo phenyl blue, bromothymol blue, and bromocresol green. The 96 and 72% degradation obtained with phenol and congo red was also higher compared to crude LiP. Treatment with LiP showed reduction in ADL as compared to untreated straws, with a maximum of 2.87 units obtained in JR followed by 2.66 units in PS. The digestibility of all straws increased, the response varying from a maximum of 21.27 units in PRM to a minimum of 12.32 units obtained in LM.

Keywords: Ligninolytic enzyme; Lignin peroxidase; Phanerochaete chrysosporium; Immobilization; Stability; Delignification; Ruminants

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INTRODUCTION

Lignin is considered to be the most abundant renewable raw material, other than cellulose, and it plays an important role in the carbon cycle of the biosphere (Schmidt 2006). It prevents microbial degradation of accessible carbohydrates within cell walls (Jeffries 1994). Because of their selective lignin degrading properties, the ligninolytic enzymes of white-rot fungi have immense potential in various biotechnological applications, including in the kraft pulping for the paper industry, wastewater treatment, bio leaching, ethanol production, denim stone washing, *etc*. The interest of the authors in ligninolytic enzymes focused on the deconstruction of lignin in lignocellulosic biomass for use as animal feed for ruminants (Rodrigues *et al*. 2008; Sharma and Arora 2010a,b; Sarnklong *et al*. 2010, 2011; Sridhar *et al*. 2015; van Kuijk *et al*. 2015). Ligninolytic enzyme systems consist mainly of manganese peroxidase, lignin peroxidase (LiP), and laccase. Some evidence has shown that many ligninolytic fungi use a combination of any two of these three enzymes (Kuwahara *et al*. 1984; Kantelinen *et al*. 1989).

Lignin peroxidases (EC 1.11.1.14) play a central role in the biodegradation of plant cell wall lignin (Piontek *et al*. 2001). They belong to the family of oxidoreductases, first described in *Phanerochaete chrysosporium* (Glenn *et al*. 1983). Lignin peroxidase is an extracellular hemeprotein that is H_2O_2-dependent, with an unusually high redox potential and low optimum pH. It is capable of oxidizing a variety of reducing substrates, including polymeric substrates (Schoemaker and Piontek 1996; Piontek *et al*. 2001). It has the distinction of being able to oxidize methoxylated aromatic rings without a free phenolic group, which generates cation radicals that can react further by a variety of pathways, including ring opening, demethylation, and phenol dimerization. In contrast with laccases, LiP does not require mediators to degrade high redox-potential compounds, but it needs H_2O_2 to initiate catalysis.

It has been observed that extracellular enzymes of white-rot fungi play an important role in the deconstruction of lignin in lignocellulosic biomass (Sridhar *et al*. 2015; Datta *et al*. 2017). Consequently, lignin degradation of crop residues by the laccases of *Schizophyllum commune* has been studied (Kumar *et al*. 2015).

Most studies on lignin biodegradation have been done with the white-rot fungus *P. chrysosporium*. Two ligninolytic peroxidases, LiP and manganese peroxidase, have been studied (Kirk and Farrell 1987). Though not well elucidated, LiP may also play a major role in the bio-delignification of lignocellulose in crop residues. Not all species of white-rot fungi secrete LiP. In the native state, white-rot fungi produce these enzymes in minute quantities that are sensitive to denaturing conditions typically found in the gut (anaerobic conditions, temperature, and pH), which should be considered when enhancing the digestibility of crop residues for ruminants. Their potential for use in enhancing livestock productivity has stimulated the need to produce these enzymes with better stabilities in sufficiently large quantities in an economically viable manner.

Within the frame of the development of a bioprocess using the competencies of LiP to enhance the digestibility of crop residues, the present study was aimed at enhancing the production of LiP by a wild isolate of *P. chrysosporium* immobilized on polyurethane foam cubes (PUF) by submerged fermentation. The produced enzyme was purified and characterized. This is the first report on the purification and characterization of LiP obtained after immobilization of whole fungal cells. The potential of partially purified LiP in the deconstruction of lignin in crop residues was also evaluated *in vitro*. To the knowledge of the authors, there are no studies on enhancing the digestibility of crop residues by way of delignification with LiP produced from immobilized *P. chrysosporium*.

EXPERIMENTAL

Materials and Methods

Chemicals, reagents, and dyes

Substrates and bovine serum albumin (BSA) were purchased from Sigma Chemicals (St. Louis, USA), while all of the other chemicals and dyes were purchased from HiMedia (Mumbai, India). Finger millet (FMS), little millet (LM), bajra (BA), barnyard millet (BRM), paddy (PS), maize stover (MS), jowar (JR), foxtail millet (FXM), and prosomillet (PRM) straws were procured from a local market.

Strain selection and enzyme production

White-rot fungi were isolated from decaying wood from Western Ghats of Coorg,

Karnataka, India. Basidiocarps (fruiting bodies) were inoculated into mycological agar slants and the tissue culture technique was employed for isolation (Revankar and Lele 2006). Two selected cultures were grown on 2% malt extract agar plates and maintained at 4 °C. The isolates were screened for LiP production on potato dextrose agar, malt extract, and mycological agar for 7 d at 30 °C. The isolate was morphologically identified as *P. chrysosporium* by the Microbial Type Culture Collection (Chandigarh, India) and was able to produce detectable levels of LiP enzyme. It was cultivated using submerged fermentation to immobilize the fungus on PUF cubes after pretreatment (Prasad *et al*. 2005). The medium contained 30 g/L malt extract, 5.0 g/L peptone, and 15.0 g/L agar, and was supplemented with penicillin and streptomycin to prevent bacterial contamination. The flasks were inoculated with 0.6 mL of the homogenized mycelia under aseptic conditions after sterilization and incubated at room temperature (28 °C ± 4 °C) under continuous shaking on an orbital shaker (120 rpm). The growth medium was removed and replenished with Kirk's basal medium, which contained 0.22 g/L ammonium tartrate, 0.2 g/L potassium di-hydrogen phosphate, 0.05 g/L magnesium sulphate heptahydrate, 0.01 g/L calcium chloride, 1 mg/L thiamine, 10 mL/L Tween (10%), 1.5 mM veratryl alcohol, 10 mL/L trace element solution, and 5 g/L glucose. The pH of the solution was adjusted to 3.5, and the cultures were incubated at room temperature (28 °C ± 4 °C) on a rotary shaker (120 rpm) for 25 d with five replicates. The LiP activity was monitored regularly.

LiP assay

The LiP activity was assayed spectrophotometrically (Shimadzu UV 1800 spectrophotometer, Kyoto, Japan) by measuring the rate of H_2O_2-dependent oxidation of veratryl alcohol to veratraldehyde (Tien and Kirk 1988), as well as by the Dye B Azure method (Arora and Gill 2001). The veratryl alcohol assay contained 0.8 mM veratryl alcohol in 0.1 M sodium tartrate buffer (pH = 3.0). To this, 1 mL of culture filtrate buffer was added in the presence of 150 mM H_2O_2. The linear absorbance was read at 310 nm for 1 min at 30 °C. One unit of LiP activity was defined as 1 µmol veratraldehyde formed/min and was expressed as U/mL (molar extinction coefficient, E_{max} = 9300 $M^{-1} \cdot cm^{-1}$).

Determination of the protein

The protein concentration was determined by the method of Lowry *et al*. (1951) using BSA as a standard. The protein of the fractions collected by column chromatography (Wipro GE Healthcare Pvt Ltd, Bangalore, India) was estimated by reading the absorbance at 280 nm.

LiP purification and electrophoretic analysis

All of the procedures were performed at 4 °C, unless otherwise stated. The culture media from the culture flasks were harvested on day 10. Solids were precipitated using 65% saturated $(NH_4)_2SO_4$ and centrifugation at 10600 g for 15 min at 4 °C. The precipitate was dissolved in 100 mL of distilled water and dialyzed overnight against 100 mL of 0.5 M sodium tartrate buffer with a pH of 3.5 at 4 °C using dialysis tubing with a molecular weight cut off of approximately 8000 Da. It was loaded onto a 44cm×3.0cm Sephadex G-75 column (Sigma Aldrich Chemicals Pvt Ltd Bangalore, India) pre-equilibrated with 0.5 M sodium tartrate buffer (pH = 3.5) and eluted with the same buffer. Five-milliliter fractions were collected at a flow rate of 1 mL/min. The collected fractions were assayed for both protein and LiP enzyme activity. The active fractions were pooled and

concentrated on an Amicon PM 10 membrane (Merck Millipore, Darmstadt, Germany) and stored at -20 °C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) was performed using 12.5% separating gel and 4% stacking gel at room temperature with a precision plus protein standard (Bio-Rad Laboratories India Pvt. Ltd., Gurugram, Haryana, India). The enzyme sample was boiled for 10 min and centrifuged at 2000 rpm for 10 min. The gel was run at 60/80 V until the dye reached the end of the separating gel. Staining of the protein bands was done using Coomassie Brilliant Blue R-250, which was followed by fixing and de-staining.

Biochemical characterization of the partially purified LiP

The properties and kinetics of the purified LiP obtained through immobilization of *P. chrysosporium* on PUF cubes were studied.

Effect of the pH

The LiP activity was assayed using 100 mM veratryl alcohol at different pH values ranging from 3 to 10 with an interval of 0.5 using appropriate buffers. The buffers included sodium phosphate citric acid buffer with a pH of 5 to 6.5, sodium phosphate buffer with a pH of 7 to 8.5, sodium tartrate buffer with a pH of 3 to 4.5, and carbonate buffer with a pH of 9 to 10, all of which were prepared at a concentration of 0.5 M. The pH stability of the purified LiP was determined by exposing the enzyme to pH values ranging from 3 to 10 with an interval of 0.5 and estimating the activity after 30 s and 15 min.

Effect of the temperature on the LiP activity

Lignin peroxidase was incubated with 100 mM veratryl alcohol in 0.5 M sodium tartrate buffer with a pH of 5.5. The temperature ranged from 25 °C to 40 °C with an interval of 0.5 °C, and the optimum temperature was determined. Furthermore, the enzyme was incubated for various time intervals ranging from 0 min to 10 min with 2-min intervals at temperatures ranging from 25 °C to 85 °C with an interval of 10 °C to examine the effect of the incubation temperature on the enzyme activity. The thermostability of the enzyme was determined by incubating the enzyme at 60 °C, 65 °C, 70 °C, 75 °C, and 80 °C for periods ranging from 20 min to 120 min with an interval of 20 min. The tubes were then immediately cooled in an ice bath and the activity was determined.

Effect of various activators and inhibitors on the LiP activity

The stimulatory/inhibitory effects of different organic compounds (EDTA, Cysteine, ethanol, sodium azide, mercaptoethanol, and silver nitrate) on the LiP activity were investigated. These compounds were added at concentrations of 10^{-3} M and 10^{-4} M, and the residual activity was determined after 10 min of incubation. The activity of the purified LiP in the presence of only veratryl alcohol was taken as 100%.

Different metal ions (Cu^{2+}, Fe^{2+}, Mn^{+}, Na^{+}, Zn^{+}, Mg^{2+}, Ca^{2+}, and Ag^{+}) were evaluated in a similar fashion by incubating the enzyme with a metal ion for 10 min at concentrations of 10^{-3} M and 10^{-4} M. The activity of the purified LiP in the presence of only veratryl alcohol was taken as 100%.

Effect of various substrates on the LiP

Several compounds were tested as potential LiP substrates at a concentration of 100 mM and the list of substrates (mediator compounds) tested is given in Table 1. The rate of

substrate oxidation was measured by determining the increase in the absorbance at their respective wavelengths.

Table 1. List of Compounds Tested as Potential Substrates for LiP

Substrate	E_{max} ($M^{-1} \cdot cm^{-1}$)	Wavelength (nm)
Veratryl alcohol	9300	310
Guaiacol	6400	436
Catechol	2211	392
Butyl alcohol	-	310
Vanillic acid	-	390
Amyl alcohol	-	310
Pyrogallol	2490	450

Determination of the kinetic constants

Various concentrations of veratryl alcohol (0 mM to 140 mM) as the substrate were used to determine the values of the kinetic constants K_m and V_{max} of the LiP from *P. chrysosporium* at a pH of 3.5 using sodium tartrate buffer (0.5 M). All of the reactions were performed at 30 °C. The E_{max} for veratryl alcohol was found to be 9300 $M^{-1} \cdot cm^{-1}$. A Lineweaver-Burk plot was plotted between the inverse of the substrate concentration and velocity of the reaction to determine the K_m and V_{max} of the enzyme ($1/v = K_m/V_{max} \times 1/S + 1/V_{max}$).

Decolorization extent assays

The decolorizing ability of the LiP was evaluated with five dyes, *e.g.*, Tetrabromophenol Blue or 3,3',5,5'-tetrabromophenolsulfonphthalein (bromophenol blue) (industrial dye); 3',3''-Dibromo thymol sulfonephthalein (bromothymol blue); Bromo cresol green (triphenylmethane dye); Congo red (diazo dye); and Phenol sulfonephthalein (phenol red or PSP). The reaction mixture consisted of 0.890 mL of 0.2 M citrate/phosphate buffer with a pH of 4.0, 0.1 mL of each dye at 1% final concentrations, and 0.01 mL of pure LiP (final concentration of 5 U/mL). Assays were performed at 25 °C and the reaction was initiated with the addition of the enzyme. After incubation for 30 min, decolorization of the dye was monitored at the maximum visible absorbance. The results were expressed as the percent of remaining color as a function of the incubation time, according to the following relationship,

$$\textit{Remaining Color}\ (\%) = (\textit{Abs final} / \textit{Abs initial}) \times 100\% \quad (1)$$

where *Abs final* is the absorbance value at the indicated incubation time and *Abs initial* is the initial absorbance value.

To exclude the possibility that decolorization of the dyes occurred because of non-biological oxidation, the dyes were incubated with 150 mM H_2O_2 in the absence of the enzyme. None of the dyes showed any change in absorption after 30 min of incubation.

Deconstruction of lignin in the crop residues and in vitro incubation

The LiPs obtained after immobilization to enhance production (T1) and after purification (T2) were used in the study. The FMS, LM, BA, BRM, PS, MS, JR, FXM, and PRM were procured from a local market, manually chaffed to a 2-cm length, and treated by spraying with LiP enzyme extracts of both T1 and T2 at an enzyme to straw ratio of 1:2.5 (Sridhar *et al*. 2015). Untreated straws served as controls. The straws were left

overnight for treatment, dried at 60 °C, ground to pass through a 1-mm screen, and then analyzed. The dry matter of the untreated and treated dried samples was determined at 100 °C ± 5 °C after 8 h. The nitrogen (N) content was determined by the standard Kjeldahl method (AOAC 2005), and the crude protein content was calculated (N × 6.25). The ash content was determined using a muffle furnace. The neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) contents were determined (Van Soest *et al*. 1991). The hemicellulose content of each sample was calculated as the difference between the NDF and ADF contents, while the cellulose content was calculated as the difference between the ADF and lignin contents. The *in vitro* dry matter digestibility (IVDMD) was determined according to Tilley and Terry (1963) and as described by Goering and Van Soest (1970). Each sample was run with five replicates.

Statistical analysis

All of the experiments were performed with three replicates. A proximate analysis was evaluated using PROC GLM in SAS 9.3 software (SAS Institute Inc., Cary, NC, USA). Significant differences between the treatments were determined using the t-test at an α value of 0.05.

RESULTS AND DISCUSSION

Strain Selection, Immobilization, and Enzyme Production

Phanerochaete chrysosporium is a wood-rotting basidiomycete and dominant LiP enzyme synthesizer. Differences in titers of enzyme yielded by organisms in different studies could have been because of the different inherent capacities of the organisms to synthesize ligninolytic enzymes, growth conditions, nutritional requirements, and inducers (Niku-Paavola *et al*. 1990; Rogalski *et al*. 1991; Schlosser *et al*. 1997).

Though LiPs are the most investigated ligninolytic enzymes, this study characterized the enzyme produced by immobilized *Phanerochaete* sp. The wild isolate of *P. chrysosporium* (LPS1) was confirmed to be strongly positive for LiP production in both the plate screening tests (Fig. 1) and submerged culture. Reports are available on enhancing the secondary metabolite production using the immobilized approach (Nakamura *et al*. 1997; de Ory *et al*. 2004). A variety of matrices, such as agar carrageenan, calcium alginate gels, polyacrylamide, *etc*., have been used, but the nature of the cell to be immobilized, nature of the substrates and products formed, and culture conditions are major factors to consider when choosing the matrix and immobilization procedure. Adsorption to surfaces and encapsulation within gels or porous materials (a particular type of physical entrapment) have been the most widely studied methods for the immobilization of microbes. These techniques represent a particular form of cellular adhesion based on the ability of certain microorganisms to fix themselves to solid surfaces by means of the secretion of polymucosaccharides (Moonmangmee *et al*. 2002). Of the various matrices tested for the immobilization of fungi for ligninolytic enzyme production, PUF proved to be the ideal synthetic matrix, according to the 10-fold increase in the activity, which was in agreement with Nakamura *et al*. (1997). The *P. chrysosporium* (LPS1) culture immobilized on PUF cubes secreted the highest LiP levels in the submerged culture between days 6 and 8, and thus was selected for further study (Fig. 2).

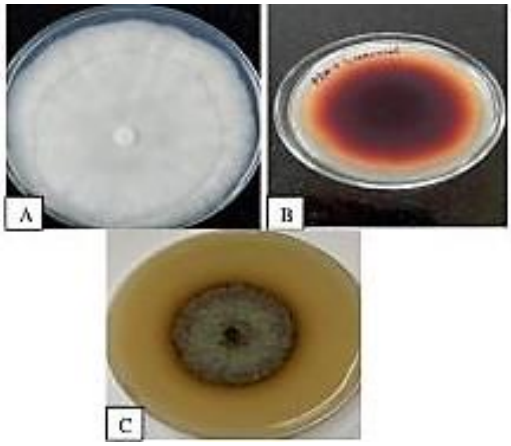

Fig. 1. (A) White-rot fungi; (B) a reddish-brown color formed around the colonies because of guaiacol; and (C) screening with 2,3-dimethoxybenzidine (0.1%) (confirmation test for LiP)

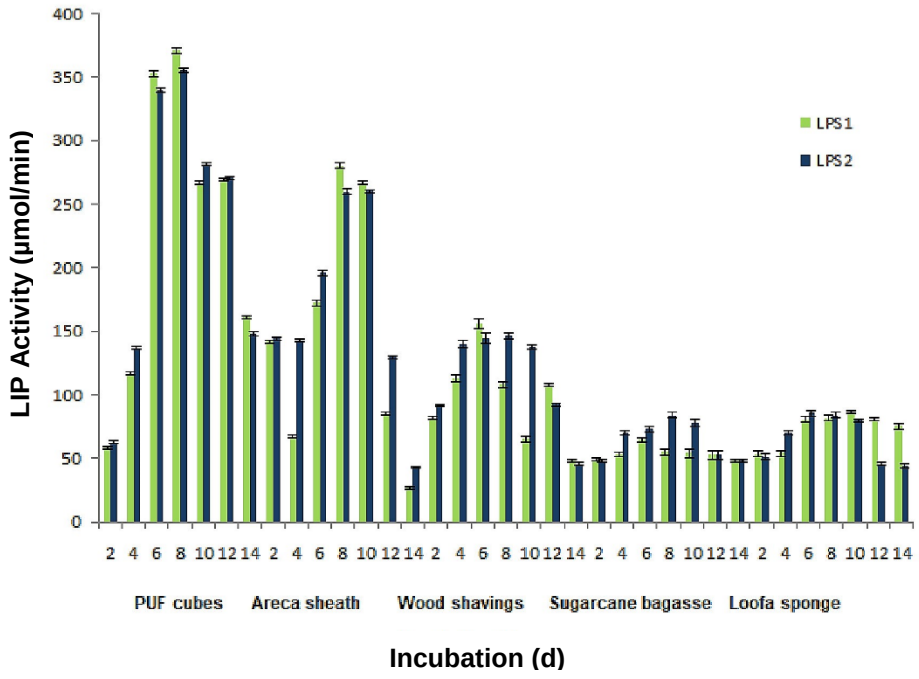

Fig. 2. Production of LiP in the submerged culture from *P. chrysosporium* immobilized on different matrices

LiP Purification and Biochemical Characterization

Sugiura *et al*. (2003) cultivated a novel LiP from the white-rot fungus *P. sordida* YK-624 in a Mn-deficient nitrogen-limited media for 7 d, and it was purified to homogeneity by anion-exchange chromatography and gel-permeation chromatography. It showed a 7.4-fold increase with a yield of 144%. Asgher *et al*. (2012) isolated LiP from an indigenous *Trametes versicolor* IBL-04 strain cultured in a solid-state fermentation medium of corn cobs, and a maximum LiP activity of 592 U/mL ± 6 U/mL was reported after 5 d of incubation under the optimum culture conditions. The crude LiP was purified 3.3-fold with a specific activity of 553 U/mg after passing through the chromatography columns. The purified LiP exhibited a relatively low molecular weight (30 kDa)

homogenous single band in native and SDS-PAGE. Yang *et al*. (2005) purified and characterized LiP from a liquid culture of *Penicillium decumbens* P6, a lignite-degrading fungus, using $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography on DEAE-cellulose and CM-cellulose, gel filtration, and non-denaturing discontinuous PAGE. The enzyme had a molecular weight of 46.3 kDa in SDS-PAGE.

Lignin peroxidases are heme-containing glycoproteins, and the subunit molecular weight of white-rot fungal LiP is reported to range between 38 kDa and 48 kDa (Fakoussa and Hofrichter 1999; Sugiura *et al*. 2003; Yang *et al*. 2005; Arora *et al*. 2011). Employing gel filtration, LiP was eluted as one major peak (Fig. 3) in the current study and was purified 21-fold with a yield of 69% (Table 2). The results of the SDS-PAGE showed that the LiP obtained from the immobilized *P. chrysosporium* was a homogenous monomeric protein, which was evident by the single homogenous band corresponding to 55 kDa (Fig. 4). This was higher than for the standard LiP, which showed a molecular weight of 48 kDa. The findings in the present study suggested that higher molecular mass enzymes are produced upon fungi immobilization compared with those produced in a submerged culture/solid-state culture and are in keeping with earlier studies with laccase (Kumar *et al*. 2015).

Table 2. Purification of the LiP obtained from the Immobilized *P. chrysosporium*

Purification Step	Total Volume (mL)	Total LiP Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification Fold
Crude extract	1400	322.58	856.8	0.376	100	1.0
Ammonium sulphate precipitation	100	280.65	72.1	3.892	87.00	10.35
Ultrafiltration	82 ± 4.99	268.81	61.91	4.341	83.31	11.54
Sephadex G-75	75 ± 5.0	221.50	60.9	7.896	68.66	21.0

One unit of LiP activity was defined as 1 µmol of veratraldehyde formed/min.

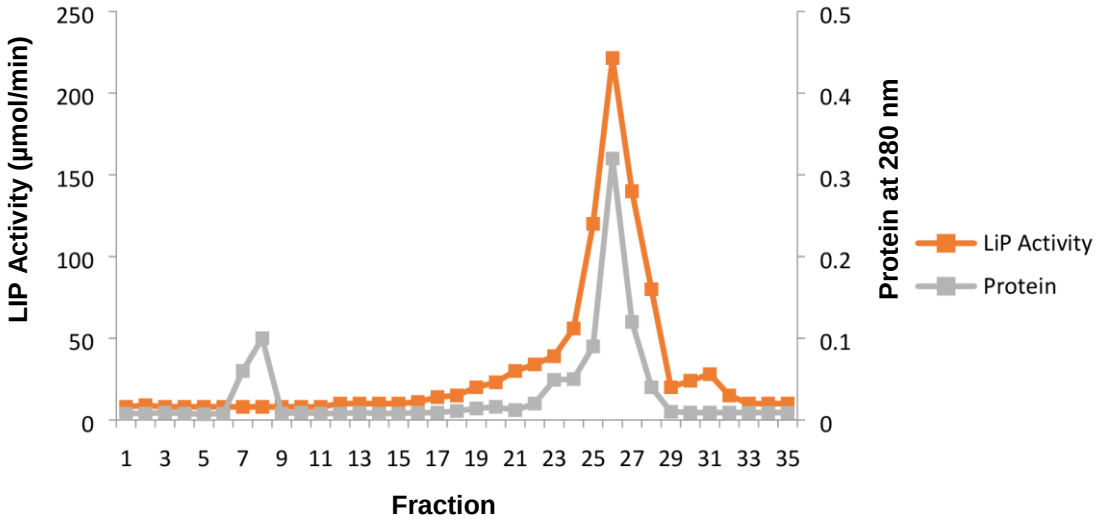

Fig. 3. Elution profile of the LiP upon gel filtration

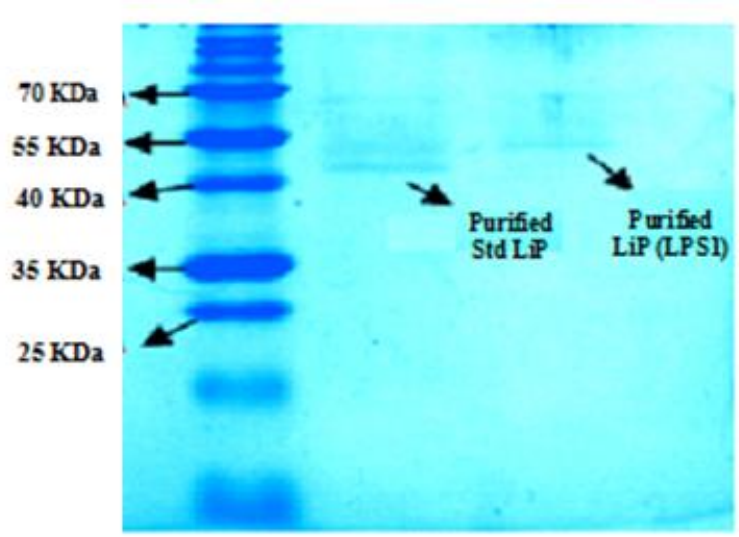

Fig. 4. SDS-PAGE results: lane 1 – molecular weight markers; lane 2 – purified standard LiP; and lane 3 – purified LiP obtained by immobilization of *P. chrysosporium* on PUF cubes

Effect of the pH on the LiP Activity

Phanerochaete chrysosporium enzymes show a higher stability at a pH of 3 to 3.5 and 30 °C, and are still stable in the pH range of 5 to 6. They modify kraft lignin and phenolic compounds containing hydroxy and methoxy groups. With veratryl alcohol as the substrate, LiP exhibits an optimum catalytic pH in the acidic range (Niku-Paavola *et al.* 1988). Asgher *et al*. (2012) showed LiP from *Schizophyllum commune* to be optimally active at a pH of 5 and 35 °C. Fakoussa and Hofrichter (1999) reported the pH range for LiP to be between 2.0 and 5.0, with an optimum between 2.5 and 3.0.

In this study, the purified LiP obtained through immobilization had an optimum pH of 5.5 with veratryl alcohol as the substrate (Fig. 5A). The enzyme exhibited an enhanced pH stability compared with the native enzyme obtained in the submerged cultures. It retained over 75% of the activity at a pH of 6.5 for over 15 min (Fig. 5B). This showed that it was superior to the purified LiP obtained from a liquid culture of *Penicillium decumbens* P6 (Yang *et al*. 2005), which exhibited an optimum pH of 4.0, with 70.6% of the relative activity remaining at a pH of 9.0.

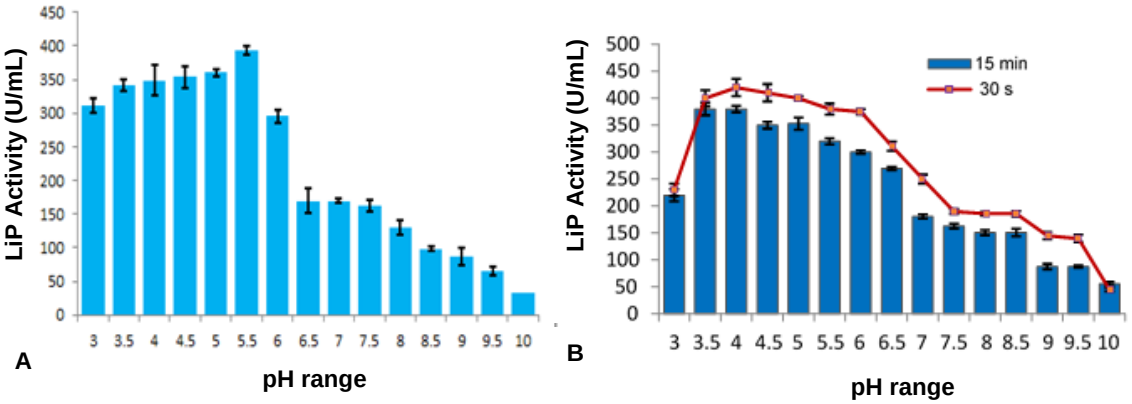

Fig. 5. (A) pH optima and stability of the purified LiP; and (B) pH stability of the purified laccase after 15 min (columns) and 30 s (curve) of incubation

Effect of the Temperature on the LiP Activity

Lignin peroxidase from different white-rot fungi show optimum activities in the temperature range of 35 °C to 60 °C. Lignin peroxidase from *Guanidium lucidium* shows thermal stability at 25 °C and the ligninolytic activity decreases as the temperature further increases (Hariharan and Nambison 2013). Lignin peroxidase from a liquid culture of *Penicillium decumbens* P6 has an optimum temperature of 45 °C (Yang *et al*. 2005), but is not stable at higher temperatures.

The LiP obtained in a submerged culture through immobilized *P. chrysosporium* had an optimum activity at a temperature of 30 °C (Fig. 6A). The purified enzyme exhibited a higher thermostability and retained over 50% of the activity, even after 120 min of incubation at both 60 °C and 65 °C. At 70 °C, there was a loss of 25% of the activitiy at the end of 120 min of incubation. Only 25% of the activity was maintained after 100 min of incubation at both 75 °C and 80 °C, and the activity was completely lost at the end of 120 min (Fig. 6B). Purified LiP exhibited superior thermostability (Fig. 6 C), evaluated by incubating the enzyme at temperatures ranging between 25°C to as high as 80 °C, with a maximum exposure of 10 min. at each temperature, and enzyme activity measured at every two min. intervals. Purified LiP failed to reflect loss in activity at temperatures of up to 60 °C even up to 10 min. after exposure. A drastic loss in activity of over 50% was evident upon exposure of the enzyme to temperatures of 70 °C and 80 °C, with only 50 units of residual activity being obtained after 8 and 10 min. at the latter temperature.

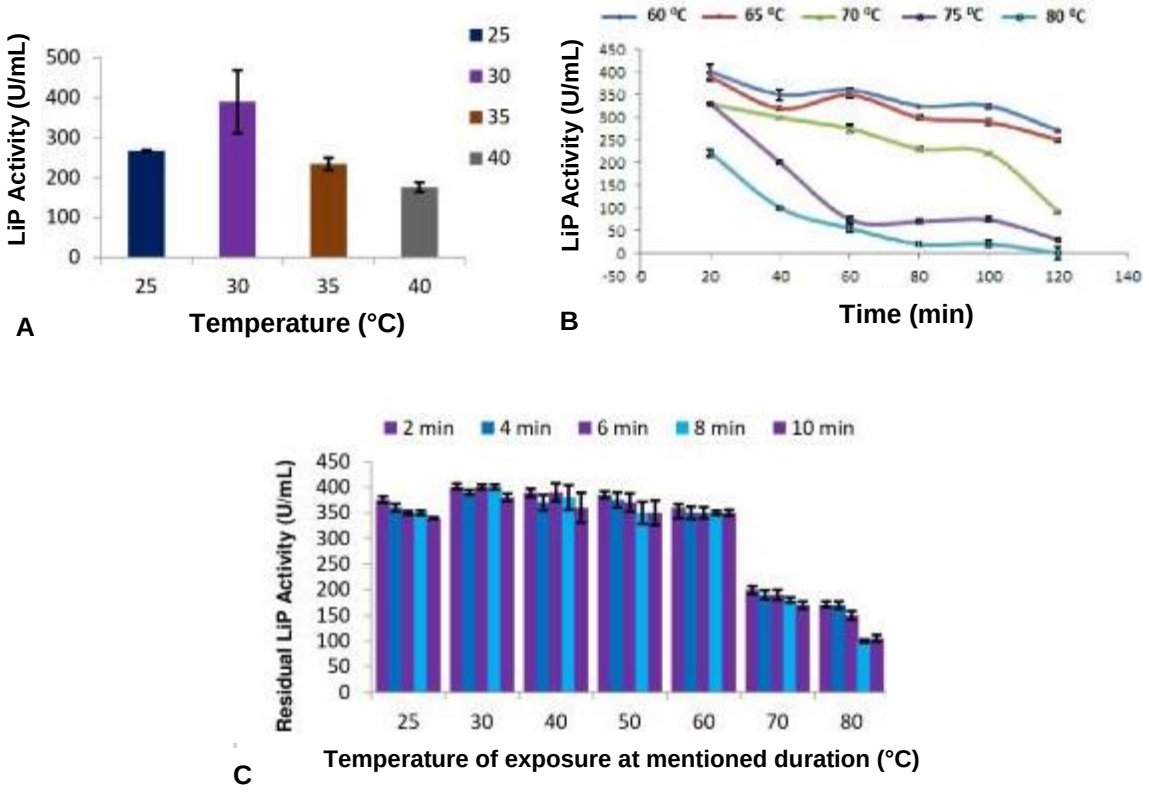

Fig. 6. (A) Temperature optima of the purified LiP; (B) temperature tolerance of the purified LiP; and (C) exposure at various temperatures for ten minutes showing thermostability of purified LiP

The results clearly highlighted that the LiP produced by the immobilized *P. chrysosporium* was thermostable compared with the native enzyme produced in the submerged culture. This finding was similar to that of Asgher *et al*. (2012), who observed optimum temperatures of 60 °C and 80 °C for free and immobilized LiPs, respectively. Asgher *et al*. (2012) concluded that immobilization enhanced the activity and thermostability potential of the LiP remarkably and immobilized LiP remained stable over broad pH and temperature ranges compared with the free enzyme.

Effect of the Activators and Inhibitors on the LiP Activity

The stimulatory/inhibitory effects of different organic compounds (EDTA, Cysteine, ethanol, sodium azide, mercaptoethanol, and silver nitrate) and metal ions (Cu^{2+}, Fe^{2+}, Mn^{2+}, Na^{+}, Zn^{2+}, Mg^{2+}, Hg^{2+}, Ca^{2+}, and Ag^{+}) on purified LiP were investigated. The results showed that the LiP was greatly inhibited by Cysteine, sodium azide, mercaptoethanol, and silver nitrate, and it was activated by ethanol and EDTA (Fig. 7A). Among the metal ions used, Ag^{+} and Zn^{+} caused LiP inhibition to various extents, whereas Cu^{2+}, Mn^{+}, Na^{+}, and Fe^{2+} were LiP activators at both concentrations (Fig. 7B). The ions Mg^{2+} and Ca^{2+} failed to have any effect on the LiP activity. Tuisel *et al*. (1991) also reported an inhibition of LiP activity by sodium azide. Asgher *et al*. (2012) reported the activity of a novel extra thermostable LiP was stimulated to various extents by Cu^{2+}, Mn^{2+}, and Fe^{2+}, whereas Cysteine, EDTA, and Ag^{+} had inhibitory effects.

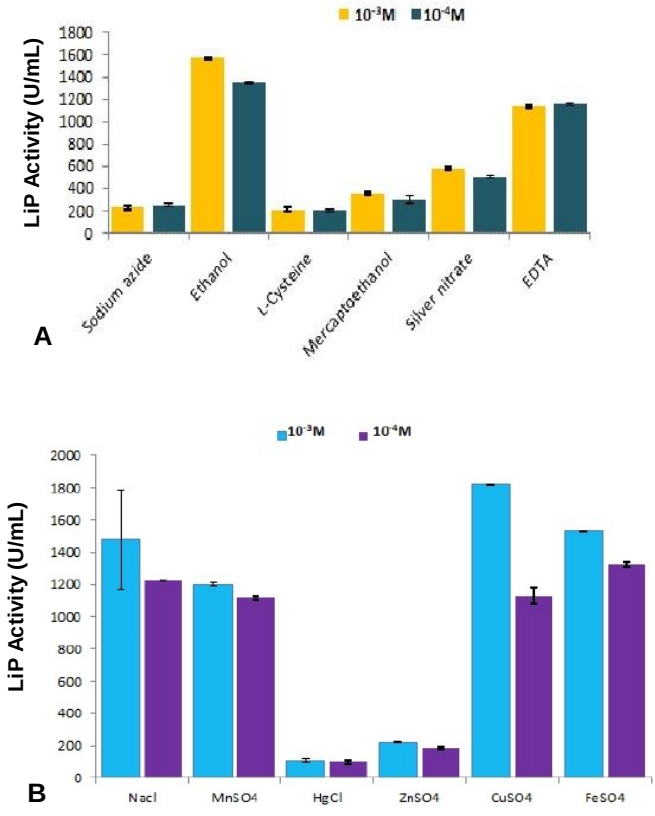

Fig. 7. Effect of the various (A) activators/inhibitors and (B) metal ions on the purified LiP

Effect of Various Substrates on the LiP Activity

Lignin peroxidases can oxidize a number of aromatic phenolic compounds in a wide variety of applications. Amongst all of the mediator compounds tested, veratryl alcohol, which is known to act as an inducer, charge transfer mediator, or stabilizer in most white-rot fungi, caused an increase in the activity (Fig. 8). All of the other compounds tested failed to influence the LiP activity.

Determination of the Kinetic Constants of the LiP

The LiP from a liquid culture of *Penicillium decumbens* P6 exhibited K_m and V_{max} values of 0.565 mmol/L and 0.088 mmol/mg protein·min, respectively, using veratryl alcohol as the substrate (Yang *et al*. 2005), while the kinetic constants K_m and V_{max} reported for free and immobilized LiPs were 70 μM and 56 μM, and 588 U/mg and 417 U/mg, respectively (Asgher *et al*. 2012).

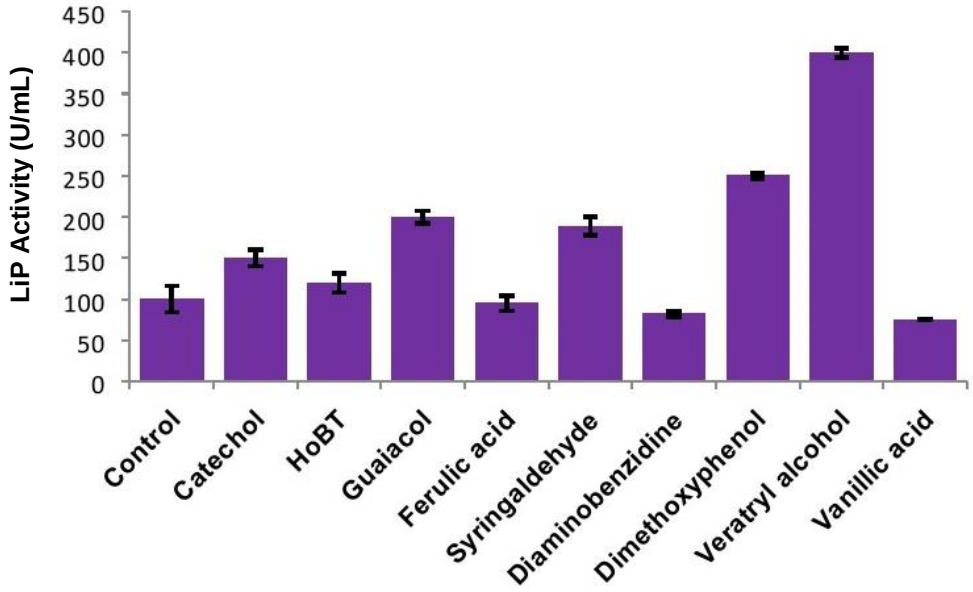

Fig. 8. Effect of the different substrates on the LiP activity

The highest LiP activity was obtained with veratryl alcohol, which exhibited a strong affinity for this substrate. Various concentrations of veratryl alcohol (μM) ranging from 10 mM to 140 mM were plotted against the respective initial specific activities (*V*) of the purified LiP obtained from immobilized *P. chrysosporium*. A Lineweaver-Burk reciprocal plot (Fig. 9A) was constructed. The Michalis-Menten kinetics yielded a hyperbolic curve (Fig. 9B). The K_m and V_{max} values of the purified LiP were 65 μM and 142.86 μmol, respectively, which indicated that the enhanced substrate affinity and catalytic efficiency of the LiP was obtained through immobilization on the PUF cubes.

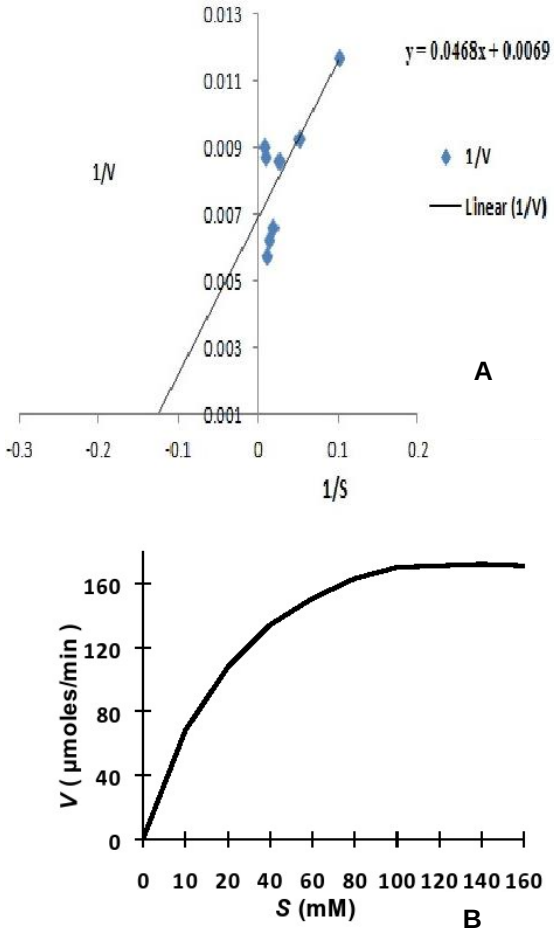

Fig. 9. (A) Lineweaver-Burk plot and (B) Michaelis Menton plot for the purified LiP obtained from the immobilized *P. chrysosporium*

Dye Decolorization Assays

Lignin peroxidase catalyzes the oxidation of phenolic, non-phenolic, and polycyclic aromatic hydrocarbon compounds, as well as a variety of recalcitrant aromatic compounds. Decolorization of synthetic dyes has also been reported. Alam *et al*. (2009) observed a 40% removal of methylene blue after the addition of approximately 0.5 U/mL LiP from *P. chrysosporium*. Color removal rates of 18%, 35%, and 69% for Remazol Brilliant Blue Reactif (λ_{max} = 592 nm; and 25 mg/L), Acid Blue 158 (λ_{max} = 610 nm, and 25 mg/L), and Cibacet Brilliant Blue BG (λ_{max} = 630 nm), respectively, after a 1-h treatment by LiP from *Streptomyces griseosporeus* SN9 were reported by Rekik *et al*. (2015). Shaheen *et al*. (2017) recorded an 80% to 93% decolorization of Sandal reactive dyes by *Ganoderma lucidum* IBL-05 LiP entrapped in Ca-alginate beads. Ollikka *et al*. (1993) investigated the ability of some LiPs from *P. chrysosporium* to decolorize azo, triphenyl methane, heterocyclic, and polymeric dyes. The capability of LiP to decolorize these dyes in the presence of veratryl alcohol as a mediator was confirmed. Cripps *et al*. (1990) did not observe decolorization of Congo red at a pH of 4.5 with crude LiP from a nitrogen-limited culture of *P. chrysosporium*.

Purified LiP obtained from immobilized *P. chrysosporium* completely (100%) decolorized bromo phenyl blue, bromothymol blue and bromocresol green (Table 3) showing increased dye decolorization efficiency. The 96% degradation obtained with

phenol red and 72% with congo red, also reflected a 56% and 44% enhancement respectively, in comparison to the degradation efficiency of the crude enzyme (Table 3), even without the addition of a mediator.

Table 3. Dye Decolorization Efficiency of the Crude and Purified LiP Obtained from the Immobilized *P. chrysosporium*

Compound	λ_{max} (nm)	Degradation (%)		Increase (%)
		Crude LiP	Purified LiP	
Bromophenyl blue	598	53	100	47
Bromothymol blue	616	62	100	38
Bromocresol green	423	60	100	40
Phenol red	443	40	96	56
Congo red	497	28	72	44

Deconstruction of Lignin in the Crop Residues and *in vitro* Incubations

Crop residues are refractory in nature for most solutions and enzymes because of their highly lignified cell walls linked to both hemicellulose and cellulose that form a physical seal around the latter two compounds, which creates an impenetrable barrier. Fungal treatments of fibrous agricultural by-products have a positive effect on the chemical composition. This has proven them to have a high potential to improve the nutritive value of highly lignified ruminant feeds (Tuyen *et al*. 2013). During degradation, this hemicellulose lignin matrix is primarily attacked by white-rot fungi (Martínez *et al*. 2005), which causes a change in the cell wall structure because of delignification and makes the cell contents easily accessible.

However, there is a dearth of studies on the effect of ligninolytic enzyme treatment on straw for ruminants. Rodrigues *et al*. (2008) extracted enzymes from the white-rot fungi *T. versicolor* (TV1 and TV2), *Bjerkandera adusta*, and *Fomes fomentarius* to evaluate their *in vitro* potential and increase the degradation of cell wall components of wheat straw.

Table 4. Changes Obtained in the Fiber Fractions of the Straws upon Treatment with the Purified LiP(T1) Compared with the Untreated Control Straw(C)

Straw	Treatment	% Dry Matter Basis				
		Dry Matter	ADF	NDF	ADL	IVDMD
FM	C	95.85 ± 3.14	39.19 ± 0.36	78.18 ± 0.61	6.98 ± 0.06	40.00 ± 3.65
	T1	93.87 ± 8.76*	34.15 ± 1.62**	63.16 ± 0.223**	5.72 ± 0.07*	58.24 ± 1.06**
LM	C	95.82 ± 2.82	49.19 ± 0.36	79.94 ± 0.07	6.48 ± 0.16	41.83 ± 1.94
	T1	94.03 ± 1.88	31.08 ± 0.12**	74.49 ± 6.20*	5.05 ± 0.21*	54.15 ± 1.78**
BA	C	96.87 ± 3.72	46.13 ± 0.66	80.61 ± 0.08	6.91 ± 0.02	44.92 ± 3.00
	T1	95.02 ± 3.21	45.75 ± 1.60*	77.03 ± 0.20**	4.87 ± 0.04**	64.56 ± 4.22**
BRM	C	94.02 ± 3.03	48.89 ± 0.75	81.58 ± 0.01	6.9 ± 0.09	43.82 ± 1.13
	T1	92.11 ± 1.94*	45.11 ± 0.28*	69.10 ± 1.56**	5.49 ± 0.42*	64.69 ± 2.08**
MS	C	96.43 ± 2.92	49.56 ± 0.90	80.07 ± 0.24	6.23 ± 0.15	42.64 ± 3.22
	T1	94.23 ± 2.28*	40 ± 1.05**	73.65 ± 0.64*	5.71 ± 0.22*	62.57 ± 3.02**
JR	C	96.49 ± 1.92	46.69 ± 0.11	80.66 ± 0.64	7.00 ± 0.44	43.93 ± 1.56
	T1	95.00 ± 3.20	41.57 ± 0.38**	72.02 ± 1.04*	4.13 ± 5.85**	57.20 ± 2.01**
PS	C	95.32 ± 3.10	49.19 ± 1.09	81.58 ± 0.71	8.28 ± 0.21	41.86 ± 3.96
	T1	94.81 ± 2.01	46.84 ± 1.98*	66.05 ± 2.76**	5.62 ± 0.01**	58.50 ± 3.25**
FXM	C	96.31 ± 1.97	48.28 ± 0.46	81.40 ± 0.59	7.80 ± 0.51	43.31 ± 0. 94
	T1	95.11 ± 3.80	45.12 ± 0.63*	69.50 ± 0.71**	6.36 ± 0.03*	63.43 ± 1.16**
PRM	C	96.15 ± 1.86	49.83 ± 0.13	82.38 ± 0.06	6.78 ± 0.12	39.29 ± 0.88
	T1	95.10 ± 3.11	38.44 ± 0.18**	66.69 ± 1.89**	5.68 ± 0.06*	60.56 ± 1.26**

C – untreated control straw; FM- Finger millet; LM- little millet; BA-bajra; BRM barnyard millet; PS- paddy straw; MS -maize stover ; JR - jowar; FXM- foxtail millet and PRM proso millet; the values are the means ± standard deviation of the samples estimated for three replicates; *$P \leq 0.05$; and **$P \leq 0.01$

The results from the cell wall chemical composition analysis showed that the TV2 and BA enzyme extracts decreased the NDF concentration ($P < 0.05$) and that TV1 had a higher activity ($P < 0.05$) towards cellulose. There was an increase in the *in vitro* Neutral Detergent Fiber Digestibility (IVNDFD) caused by treatment of the wheat straw with enzyme extracts from *B. adusta*, TV1, and TV2, and reached a difference of 13% for TV2 ($P < 0.05$) *versus* the untreated control straw. Kumar *et al*. (2015) observed a decrease in the fiber content with an enhancement of the digestibility of straws treated with laccase obtained from *Schizophyllum commune*.

The purified enzyme extract of LiP utilized for treating nine different crop residues by spraying recorded a minimal loss in the dry matter ranging between 1 and 2 units (Table 4). Compared to untreated straws, treatment with LiP resulted in a decrease in NDF content, which ranged from the highest response of 18.02 units being recorded in ragi straw and a minimum decrease of 3.58 units in the BA. With regard to ADF content, little millet recorded the major decrease of 18.11units, while the lowest decrease of 0.38 unit occurred in the case of BA.

Similarly, compared to untreated straws, the highest decrease in ADL contents after treatment with LiP ranged from 2.87 units obtained in JR, followed by 2.66 units in PS, and 2.04 units in BA. The *in vitro* digestibility (IVDMD) of all straws increased after treatment with purified LiP, (Table 4), the response in increase, ranging from a maximum of 21.27 units observed in PRM to a minimum of 12.32 units obtained in LM compared to untreated straws, confirming the potential of LiP in delignification of crop residues.

CONCLUSIONS

1. The results confirmed the secretion of LiP by the wild isolate of *P. chrysosporium*.
2. The LiP obtained from *P. chrysosporium* immobilized on PUF cubes elicited a higher thermostability, pH stability, and dye decolorization efficiency.
3. Treatment of nine crop residues with the purified LiP obtained from *P. chrysosporium* immobilized on PUF cubes had a statistically significant positive effect on their chemical composition and digestibility, which showed that LiP has a high potential to improve the nutritive value of highly lignified ruminant feeds.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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