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Original Article

Purification, kinetic characterization of thermostable multicopper oxidase from the oyster mushroom and its versatility for greener agro-pulp bio bleaching in the paper industry

Manmeet Kaur[1*], Shivani Sharma[1], Inderpal Kaur[2], Harpreet Singh Sodhi[1], Ravish Choudhary[3*], Sezai Ercisli[4], Hafize Fidan[5], Elanur Dasci[6], Riaz Ullah[7], Ahmed Bari[8]

[1] *Department of Microbiology, Punjab Agricultural University, Ludhiana-141004, Punjab, India*
[2] *Department of Biochemistry, Punjab Agricultural University, Ludhiana-141004, Punjab, India*
[3] *Division of Seed Science and Technology, ICAR-Indian Agricultural Research Institute, New Delhi 110012, India*
[4] *Department of Horticulture, Faculty of Agriculture, Ataturk University, 25240 Erzurum, Turkiye*
[5] *Department of Tourism and Culinary Management, Faculty of Economics, University of Food Technologies, 4000 Plovdiv, Bulgaria*
[6] *Department, of Soil Science and Plant Nutrition, Faculty of Agriculture, Atatürk University, 25240 Erzurum, Turkiye*
[7] *Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh, Riyadh Province 11451, Saudi Arabia*
[8] *Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh, Riyadh Province 11451, Saudi Arabia*

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Abstract

Production of a thermostable laccase from *Pleurotus florida* was reported for the first time, both in submerged and solid-state fermentation using agro-industrial residues. This enzyme was purified using ammonium sulphate precipitation (60-90%), Sephadex G-100 and DEAE column ion exchange chromatography, respectively. The laccase was purified to 21.49 fold with an apparent molecular weight of 66 kDa and had an optimal pH of 5 with temperature stability at 60°C. Metal ions such as Cu^{2+} (91.26 µmol/mL/min), Mg^{2+} (68.15 µmol/mL/min), and Fe^{2+} (1.73 µmole/mL/min) enhanced the laccase activity, but Fe^{2+} (1.73 µmol/mL/min) inhibited the enzyme activity. The purified laccase had K_m and V_{max} of 16.68 mM and 26.73 µmole/mL/min for guaiacol as a substrate. The isolated enzyme was characterized by FT-IR which revealed bands at 3655.0/cm, 2894.7/cm, and 1151.7/cm corresponding to primary amines, C-H stretch, and amide –III, respectively. The enzymatic bio bleaching of paddy straw pulp was found to be most effective which resulted in a lowering of kappa number and yellowness by 19.47% and 17.84% whereas an increase in brightness and whiteness by 41.92% and -19.61%. Thus, this might be stated that the crude laccase from *P. florida* can be exploited to reduce the toxic waste load for managing environmental pollution and helps in enhancing the yield and quality of the paper.

Keywords: Biobleaching, Characterization, Enzyme kinetics, Laccase, *Pleurotus florida*

1. Introduction

Earth is a plant-oriented planet and horticulture plants including mushroom has special importance. Horticulture plants are adding value of earth's diversity and fundamental to all life. They include high content of non-nutritive, nutritive, and bioactive compounds. They have been frequently using in biotechnological studies in more recently [1-10].

Laccase (EC 1.10.3.2) is a multicopper oxidase that catalyzes the reduction of molecular oxygen to water by electron oxidation of phenolic and non-phenolic aromatic substrates [11]. It consists of four copper atoms scattered over three redox copper centers: type 1 copper, type 2 copper, and type 3 copper. The type 1 copper center is for the initial oxidation of the substrate, while the type 2 and type 3 copper centers form a trinuclear cluster in which the catalytic process of laccase occurs [12]. This enzyme uses molecular oxygen as an electron acceptor to oxidize its substrates via an electron transfer pathway to unstable free radical intermediates which are then degraded by non-enzymatic mechanisms [13]. *Pleurotus florida*, a white rot fungus, was shown to be the most effective laccase producer with improved redox potential. It has been found that white rot fungi have an enzyme system that breaks down lignin and opens phenyl rings. The broad substrate specificity and catalytic properties of laccase make them useful in a variety of biotechnological processes, including pulp bleaching in the paper industry. Pulp delignification is an

* Corresponding author.
E-mail address: manmeet1-mb@pau.edu (M. Kaur); ravianu1110@gmail.com (R. Choudhary).
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essential step in industrial papermaking.

The residual lignin in the pulp must be removed through a multi-stage bleaching process to brighten the final paper products [14]. An alternative eco-friendly process to bio bleaching with microbial enzymes can be used to reduce pollution and improve paper quality. The enzyme laccase has extraordinary potential to completely transform the pulp and paper industry. The elimination of the lignin-carbohydrate complex, which is formed during the kraft process and serves as a physical barrier in chemical bleaching, is the main goal of the process of pulp delignification with oxidative enzymes [15]. Therefore, the present study was performed to extract, purify and characterize the laccase enzyme from *P. florida* and investigate the potential application of the multicopper oxidase enzyme as a bio bleaching agent in the paper and pulp industry.

2.Material and Methods

2.1. Microorganisms and culture conditions

The *P. florida* was procured from the Dr. HS Garcha Mushroom laboratories, Department of Microbiology, Punjab Agricultural University, Ludhiana, Punjab. The fungus was grown on a potato dextrose agar plate at 25°C for 7 days and kept at 4°C for 30 days before being sub-cultured (periodic transfer).

2.2. Enzyme Production

2.2.1. Laccase production in submerged fermentation

For enzyme synthesis, the fungal spore (*P. florida*) suspension of 1×10^8 spores/ml was inoculated into the Mushroom Minimal Medium (MMM) with the following composition (g/l): L-asparagine (1.60), D-glucose (20.0), KH_2PO_4 (0.46), $MgSO_4.7H_2O$ (0.50), K_2HPO_4 (1.00), thiamine hydrochloride (0.125), agar (20.0) and incubated at 25°C for 15 days [16]. The biomass was filtered and the mycelial mat was crushed in phosphate buffer (pH-7) and centrifuged (10,000 g; 15 min) at 4°C. The supernatant obtained after centrifugation was analyzed for intracellular activities. The broth left after the separation of mycelium was centrifuged, clear supernatant obtained was utilized as the crude extract and the extracellular enzyme activities were determined.

2.2.2. Laccase production in solid state fermentation

The substrate paddy straw was used for the cultivation of *P. florida*. The mature healthy fruit bodies emerged from the spawn-impregnated paddy straw was harvested. Healthy fruiting bodies of mushrooms (200 g) were cut into pieces and then homogenized in 400 ml of ice-cold 50 mM phosphate buffer (pH 7.0). The homogenized suspension was stirred for half an hour at 4ºC and filtered through the doubled layer of clean muslin cloth [17]. The filtrate was centrifuged at 4°C for 15 minutes at 10,000 rpm and the supernatant used as crude enzyme extract for measuring the laccase activity.

2.2.3. Enzymatic assay and protein quantification

The laccase activity was determined spectrophotometrically by evaluating the absorbance change of the reaction mixture using guaiacol as a substrate at 470 nm [18]. The protein content of the samples was determined using BSA as a reference. The absorbance at 280 nm was used to determine the protein content of the column chromatography fractions [19]. The enzyme activity was calculated using the formula as described below:

$$\textbf{Enzyme activity} = \frac{\frac{\Delta A}{\Delta t} \times \frac{Total\ volume\ of\ reaction\ mixture}{Volume\ of\ extract\ taken} \times \frac{1}{\epsilon} \times Volume\ made}{Weight\ of\ sample}$$

Where A= absorbance, t= incubation time, Ɛ= extinction coefficient for guaiacol at 470 (6740/M/cm), total volume of reaction mixture (mL), volume of extract taken (mL), volume made (mL) and weight of sample (g)

2.2.4. Extraction and purification of laccase enzyme from P. florida

The purification procedure was performed with crude intracellular (mycelium) and extracellular (liquid filtrate) extracts obtained from a 14-day-old culture medium showing the highest levels of laccase activity. The crude enzyme was extracted from the fruit bodies of the oyster mushroom, *P. florida*, in 50 mM phosphate buffer (pH 7). It was precipitated with 60-90% ammonium sulfate and kept at 4°C overnight. Centrifuged at (10,000 g, 15 min) and then dissolved in phosphate buffer (pH 7.0) [20]. Gel filtration and ion exchange chromatography were used to further purify the sample. Thereafter, the sample was loaded onto a Sigma-Aldrich Sephadex G-100 column equilibrated with phosphate buffer (pH 7). The protein was eluted at a flow rate of 0.25 mL/min. All active fractions were combined and applied at a flow rate of 0.5 mL/min to a column of diethylaminoethyl cellulose (DEAE cellulose) measuring 25 by 18cm and pre-equilibrated with the same buffer. At this pace, the bound proteins were eluted with various NaCl gradient concentrations (25 mM, 50 mM, 100 mM, 150 mM, 200 mM and 1M).The highly active fractions were combined and stored at 4°C for further characterization studies [21].

2.3. Characterization of partially purified laccase enzyme

2.3.1. Effect of pH and stability

The effect of pH on laccase activity was studied using guaiacol as a substrate using these three buffers: 50 mM glycine-HCl buffer (pH 2), 50 mM sodium tartrate buffer (pH 3-5) and 50 mM potassium phosphate buffer (pH 6.5-7), Tris-HCl buffer (pH 8.2). The enzyme was pre-incubated at 4°C in buffers ranging from pH 3 to 8 to determine pH stability [22]. After the required incubation time, the enzymatic activity was evaluated under optimal assay conditions.

2.3.2. Effect of temperature and thermal stability

Laccase activity was measured at various temperatures (10-100°C) to assess the effect of temperature on enzyme activity. Purified laccase in an optimal buffer was incubated at temperatures ranging from 10-100°C for 150 minutes to evaluate the thermal stability [22]. Under conventional assay conditions, residual activity was determined.

2.3.3. Effect of metal ions on the activity

The influence of many metal ions, including Mn^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+} and Zn^{2+} on laccase activity was investigated. It was assayed by adding divalent metal ions at concentrations ranging from 2 to 10 mM to the reaction mixture and incubating for half an hour. Residual activities

were measured according to the activity assay.

2.3.4. Effect of inhibitors on the activity

Ethylenediaminetetraacetic acid (EDTA), hydrogen peroxide (H_2O_2), sodium azide (NaN_3) and sodium dodecyl sulfate (SDS) as inhibitors were used to study the effect on the enzyme activity. The enzyme was pre-incubated with inhibitors at various concentrations (2mM-10mM) for 15 mins to obtain maximum inhibition in order to study the inhibitory effect on the enzyme [22].

2.3.5. Effect of solvent stability on the activity

Laccase resistance to organic solvents was investigated by incubating the enzyme for 1 hour in various organic solvents such as methanol, dimethyl sulfoxide (DMSO), acetone, chloroform, and ethanol at final concentrations of 2%, 4%, 6%, 8%, and 10% [22]. A standard assay protocol was used to determine the enzyme activity.

2.4. Kinetic properties of the purified laccase

The kinetic properties of laccase towards guaiacol, one of its most common substrates, were determined in the concentration range of 10 mM–100 mM. The isolated enzyme oxidation of the substrate was measured spectrophotometrically at a specific wavelength. K_m and V_{max} of the purified enzyme were calculated using the Lineweaver-Burk transform of the Michaelis-Menten equation [23].

2.5. Molecular characterization of the enzyme through Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS- PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to measure the molecular mass of the predominant laccase. A 12% resolving gel and a 5% stacking gel were used in SDS-PAGE using the standard protocol [24]. Proteins were stained with Coomassie Brilliant Blue R-250 after electrophoresis. The molecular weight markers were used from the pre stained NEXT-GEN Pink ADD protein ladder (10.4-175 kDa).

2.6. Fourier Transfer Infrared Spectroscopy of enzyme

The lyophilized enzyme sample was loaded on the attenuated total reflectance (ATR) crystal in 4000-400cm^{-1} for FTIR measurement of the isolated laccase. For FT-IR investigation, the translucent sample discs, approximately 10µL of the pure enzyme, was encapsulated in 40 mg of KBr pellet [25].

2.7. Effect of purified laccase enzyme on paddy straw pulp

Paddy straw pulp was made by dispersing finely ground oven-dried straw in distilled water. The rice straw pulp samples (oven-dried) at a consistency of 10% (w/v) were inoculated with different enzyme concentrations (2 mL, 4 mL, 6 mL, 8 mL and 10 mL) for a period of 2 h at 60 °C under gentle agitation shaking. The treated pulp was filtered and dried at 50°C for 3 h. Control was maintained under the same conditions without the enzyme addition. The efficiency of the treatment process was measured by the reduction in kappa number [26]. The brightness, whiteness and yellowness of the pulp were determined using a Hunter Scan colorimeter [27]. The absorbance maxima of the filtrate were read at λ_{237} and λ_{465} nm to measure chromophores and hydrophobic compounds [11].

2.8. Statistical analysis

The data for enzymatic bio-bleaching of paddy straw pulp was analyzed statistically using One-way ANOVA IBM SPSS Statistics version 16.0

3. Results

3.1. Partial purification of laccase enzyme from different mycelial growth stages of *P. florida*

3.1.1. Partial purification of intracellular laccase enzyme from oyster mushroom under submerged fermentation

The maximum laccase production from *P.florida* mycelia grown on the minimal mushroom broth was obtained at the end of the 14th day of fermentation with a specific 2.84 U/mg protein activity. Table 1 outlines the laccase enzyme purification processes and other pertinent information. The crude laccase was precipitated with 90% ammonium sulfate saturation resulting in a purification fold (1.61) and recovery of 55.64%. The purification of laccase resulted in a yield of 30.71% of the total units of enzyme activity

Table 1. Summary of purification of laccase enzyme from different stages of *Pleurotus florida.*

Stages	Purification Steps	Volume (mL)	Total protein (mg)	Total activity (U/mL)	Specific activity (U/mg)	Purification fold	Yield (%)
Intracellular	Crude enzyme	500	560	2060	3.67	1	100
	Ammonium sulphate precipitation (90%)	125	182.5	932.5	5.10	1.38	45.26
	Dialysis	52	88.92	688.48	7.74	2.10	33.42
	Sephadex G-100	33	33.99	530.97	15.62	4.25	25.77
	DEAE-Cellulose column	16	9.92	173.92	17.53	4.77	8.44
Extracellular	Crude enzyme	500	895	2825	3.15	1	100
	Ammonium sulphate precipitation (90%)	115	292.1	1580.1	5.40	1.71	55.93
	Dialysis	39	111.54	1601.73	14.36	4.55	56.69
	Sephadex G-100	24	48.48	1185.12	24.44	7.75	41.95
	DEAE-Cellulose column	11	8.58	375.32	43.74	13.88	13.28
Fruiting body	Crude enzyme	500	1275	3970	3.11	1	100
	Ammonium sulphate precipitation (90%)	140	476.8	3936.8	8.25	2.65	99.16
	Dialysis	64	237.44	2611.2	10.99	3.53	65.77
	Sephadex G-100	41	78.72	2102.07	26.7	8.58	52.94
	DEAE-Cellulose column	28	14.84	992.04	66.84	21.49	24.98

initially present in the dialyzed culture filtrate. The dialysate was purified using Sephadex G-100 gel filtration with a purification fold of 16.10%. Column chromatography on DEAE-Cellulose ion exchange resin yielded a 6.68 purification fold, corresponding to a 6.75% final enzyme yield.

3.1.2. Partial purification of extracellular laccase enzyme from oyster mushroom under submerged fermentation

The purification procedure of laccase from the liquid culture filtrate of *P.florida* was summarized in Table 1. The extracellular crude enzyme with an activity of 2176 U/mL was precipitated with 90% ammonium sulphate, and the dialyzed culture filtrate obtained a yield of 43.52%. Sephadex G-100 purified the enzyme with a specific activity of 13.46 U/mg and a purification yield of 24.12%. The last purification step includes the DEAE-cellulose chromatography, which resulted in a purification fold of 15.33 with a specific activity of 42.16 U/mg.

3.1.3. Partial purification of laccase enzyme from the fruiting body of oyster mushroom under solid-state fermentation

The laccase was purified to homogeneity from the *P.florida* fruiting body, and the purification processes are listed in Table 1. The enzyme was purified 25.60 times after the three chromatography processes, yielding a 21.36 per cent total yield. After elution with 25mM-1M NaCl in 0.05 mM sodium acetate buffer (pH 5.5), the elution profile from ion-exchange chromatography on DEAE-Cellulose revealed the laccase activity as a single unique peak (Figure 1). The specific activity of the pooled fractions was 91.93U/mg protein. Furthermore, the eluted fractions' ultraviolet absorbance at 280 nm displayed a single, distinctive peak. As shown in Figure 2, the purified laccase had a single protein band of approximately 66 kDa.

3.2. Biochemical characterization of Laccase

The characteristics parameters were assessed from mushroom fruiting body isolated enzyme, due to the limited protein and enzyme activity at the intracellular and extracellular stages (Table 1).

3.3. Effect of pH on enzyme activity and stability

Laccase pH optimum varies depending on the substrate and its redox potential. The enzyme was active across a wide pH range (3-8), with the highest activity at pH 5 (Figure 3A). Furthermore, it was more active in the acidic zone than in the neutral or alkaline regions. Laccase activity was observed at pH values greater than 5.5, and it gradually declined as the pH increased from 6 to 8. The laccase enzyme was very stable in the assay pH range of 3-8. A modest increase may be seen after 60 minutes of pre-incubation at various pH levels. After 60 minutes of pre-incubation in the various pH conditions, most of the oxidising activity persisted. Furthermore, at optimal pH, the laccase enzyme was more stable, keeping 70.82% of its activity after 150 minutes. In contrast, after 150 minutes of incubation at pH 7 and 8, less than 10% of the laccase activity remained, as shown in Figure 3B. A modest increase may be seen after 60 minutes of pre-incubation at various pH levels. After 60 minutes of pre-incubation in the various pH conditions, a significant proportion of the oxidizing activity was retained.

3.4. Effect of temperature on enzyme activity and stability

The reaction temperature was varied from 10-100°C at pH -5.0 to determine the optimal temperature of purified laccase. At 10°C and 100°C, the least activity was seen. The laccase optimal temperature was 60°C, as indicated in Figure 3C. The isolated enzyme was more stable at 50°C and 60°C, where it retained 67.20 and 64.52 % of its activity after 150 minutes, respectively, and was least stable at 70°C, 90°C, and 100°C, where it determined about 19.20%, 12.34%, and 1.69%, respectively (Figure 3D). At

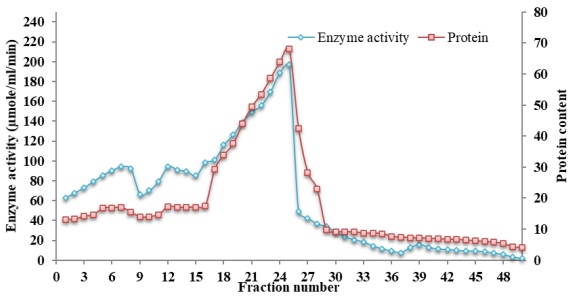

Fig. 1. Elution profile of laccase from *P.florida* fruiting body after ion exchange chromatography using DEAE-Cellulose. The column was equilibrated and eluted with sodium acetate buffer (0.05M, pH-5.5).

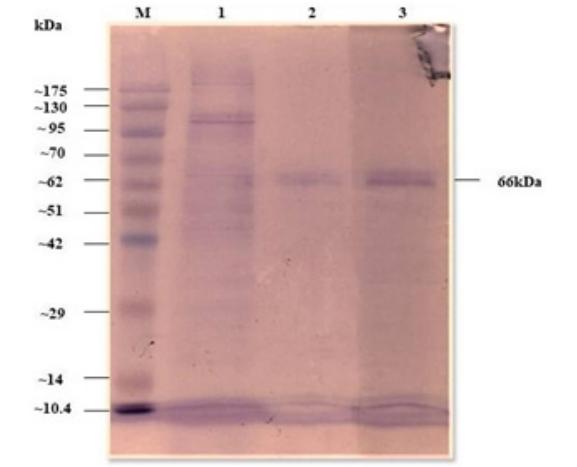

Fig. 2. SDS-PAGE analysis of *Pleurotus florida* laccase. Lanes: (M) standard protein marker ; (1) crude of 90% ammonium precipitated; (2) after Sephadex G-100 gel filtration chromatography; (3) purified laccase after DEAE-cellulose ion exchange column chromatography.

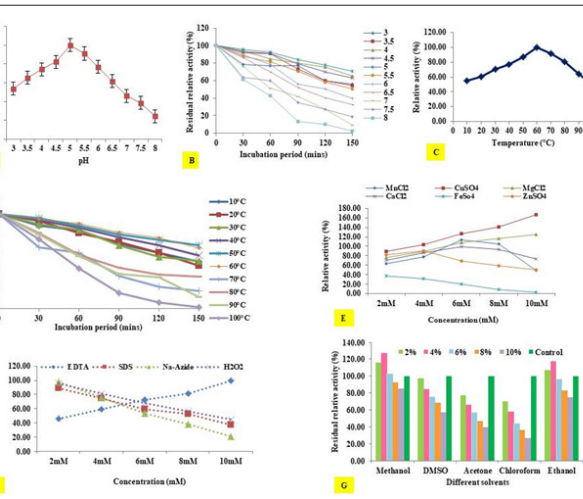

Fig. 3. Effect of various characteristics on the laccase enzyme. (A) pH; (B) pH stability; (C) Temperature; (D) Thermostability; (E) Metal ions; (F) Inhibitors; (G) Organic solvents.

60°C, the oxidising activity toward guaiacol was approximately twice as effective as at 10°C.

3.5. Effect of metal ions on enzyme activity

Several metal ions were assayed at pH 5 and 60°C to determine their effect on *P. florida* laccase activity. The laccase enzyme assay was performed with each metal ion at concentrations ranging from 2mM to 10 mM. The chloride and sulfate salts of these metal ions were used. Figure 3E showed that increasing the concentration of metal ions (2 mM-10 mM) resulted in a steady decrease in enzyme activity, with the exception of copper (Cu^{2+}) and magnesium (Mg^{2+}) which had a somewhat stimulatory effect on enzyme activity. The enzymatic activity was stimulated in the presence of Mn^{2+} (13.79%) at 6 mM, Cu^{2+} (67.11%) and Mg^{2+} (24.79%) at concentrations of 10 mM. Laccase was significantly inhibited by iron (Fe^{3+}) and zinc (Zn^{2+}), which hindered the enzyme activity by 96.83% and 49.75% at 10 mM.

3.6. Effect of inhibitors on enzyme activity

Ethylene diamine tetra acetic acid, sodium dodecyl sulfate, sodium azide, and hydrogen peroxide were used to study the effect of different inhibitors on enzyme activity (Figure 3F). At a concentration of 10mM, the enzyme activity was inhibited by EDTA, which resulted in 99.71% inactivation. However, the majority of the described fungal laccases were inhibited by EDTA. The low accessibility of the structural copper atoms present in the active site, which are necessary for catalytic enzyme function, explained the resistance to this chelator agent. SDS had a considerable inhibitory effect on enzyme activity at 2 mM (89.25%), while at 10 mM, the laccase was inhibited by 62.52%. At a concentration of 10 mM, sodium azide showed a 78.81% inhibitory effect. However, using hydrogen peroxide at a concentration of 2mM resulted in a significant loss of activity (96.08%).

3.7. Effect of solvent stability on enzyme activity

The relative activity of the laccase decreased as the organic solvent concentration was increased from 2% to 10% (v/v), though the enzyme showed the most stability when incubated in 2% and 4% (v/v) organic solvents (Figure 3G). The enzyme retained more than 97.44%, 77.18%, and 70.41% activity in dimethyl sulfoxide, acetone, and chloroform, respectively, at a 2% concentration of the organic solvent. Furthermore, in 4% methanol and ethanol, the laccase retained 127.21% and 117.25% of its activity, respectively. After one hour, the enzyme lost approximately 73.48% and 60.37% activity in chloroform and acetone, respectively, at a concentration of 10% (v/v). Remarkably, the enzyme was more stable in methanol than in other organic solvents, retaining 85% of its residual activity at a concentration of 10% (v/v).

3.8. Kinetic parameters of the purified enzyme

Lineweaver Burk double reciprocal plots of laccase activity at 60°C, using various concentrations of guaiacol as the substrate, were used to determine the kinetic parameters (K_m and V_{max}) of the purified laccase. The isolated laccase K_m and V_{max} values were 16.68mM and 26.73mole/ml/min, respectively, as shown in (Figure 4).

3.9. Fourier Transform Infrared Spectroscopy (FTIR) study of the purified enzyme isolated from fruiting body of *P. florida*

The primary efficient functional groups on purified laccase were observed in this study using FTIR (Figure 5). The primary peaks identified were a broad and strong peak at roughly 3655.0/cm assigned to a non-bonded hydroxyl group (OH stretch). At roughly 3344.9/cm, there were intense peaks assigned to the primary amines related to the N-H bond of amide A of the peptide linkage in the enzyme. At roughly 2894.7/cm, the C-H stretch vibrations for methylene were the most distinctive peak as an organic molecule having at least one aliphatic fragment. The double bond area at 1638.3/cm represented the C=O stretching of the amide I; whereas the peaks at 1540.4/cm, and 1409.6/cm described the C-N stretching of amide II of the peptide linkage. The existence of C-N stretching and N-H bending was indicated by the bands seen in the fingerprint region of 1241.9/cm of the amide III. The band found at 1151.7/cm and 1013.5/cm in the FT-IR spectrum assayed to the C-N stretching. The peroxides were linked to the band at 889.3/cm and 660.3/cm demonstrating out-of-plane N-H bending of the amide V.

3.10. Effect of ligninolytic enzyme isolated from *Pleurotus florida* on paddy straw pulp

Treatment with a concentration of 8 mL resulted in better enzyme efficiency, as summarized in Table 2 and Figure 6.

The paddy straw pulp was pretreated with crude enzyme at variable doses for 2 h at 60°C and pH 7.0. Compared to the untreated sample, kappa number and yellowness decreased with different enzyme doses, while brightness and whiteness improved, indicating the lignin release.

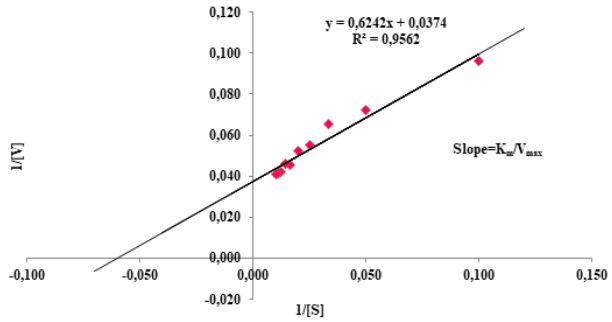

Fig. 4. Lineweaver – Bulk plot of laccase catalyzed oxidation of guaiacol.

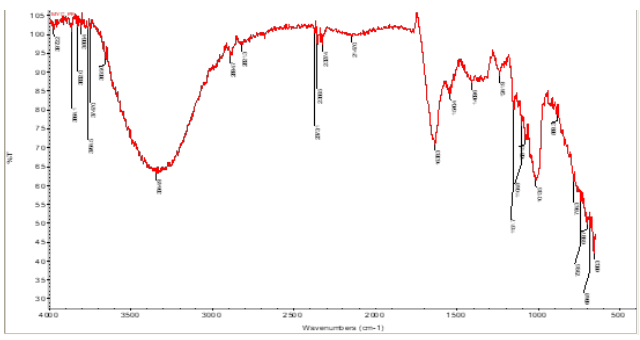

Fig. 5. FT-IR analysis of purified laccase enzyme from the fruiting body of *Pleurotus florida.*

Table 2. Effect of laccase enzyme isolated from *P. florida* in bleaching of paddy straw pulp.

Treatment	Kappa No.	Brightness,% ISO	CIE Whiteness	Yellowness	Chromophoric compounds (λ_{237})	Hydrophobic compounds (λ_{465})
Untreated	24.36±0.46^{a}	38.74±0.29^{b}	-21.74±0.25^{b}	18.67±0.23^{a}	0.084±0.01^{a}	0.182±0.1^{a}
E-1	22.25±0.85^{a,b}	40.76±0.76^{a,b}	-20.20±0.08^{a}	18.21±0.16^{a,b}	0.097±0.04^{a}	0.246±0.21^{a}
E-2	21.54±1.07^{a,b}	41.68±1.12^{a}	-20.13±0.18^{a}	18.09±0.31^{a,b}	0.134±0.09^{a}	0.376±0.06^{a}
E-3	19.84±1.73^{b}	41.15±0.67^{a}	-19.83±0.11^{a}	17.56±0.46^{b}	0.142±0.12^{a}	0.392±0.12^{a}
E-4	19.47±0.78^{b}	41.92±1.05^{a}	-19.61±0.07^{a}	17.84±0.19^{a,b}	0.166±0.08^{a}	0.491±0.15^{a}
E-5	19.49±0.36^{b}	41.83±0.94^{a}	-19.69±0.32^{a}	17.85±0.45^{a,b}	0.169±0.07^{a}	0.497±0.26^{a}

- Treatments; E-1=2mL, E-2=4mL, E-3=6mL, E-4=8mL, E-5= 10mL
- Pulp consistency = 10%; Temperature = 60°C ; pH = 7.0 ; Time= 2 h
- Data represent mean ± SD . The alphabetical letters are significantly different within biobleaching of paddy straw pulp at $P \leq 0.05$ according to Tukey's post hoc test.

Enzyme-treated pulp samples showed the greatest elimination of chromophores and hydrophobic substances.

4. Discussion

This study synthesized, purified, and characterised laccase from the basidiomycetes mushroom *P.florida*. Increased biomass production is critical for mushroom product recovery and biomass generation economics using mycelial and mushroom cultivation-based industrial processes. Although fungal laccases have considerable biotechnological promise, poor enzyme yields and high prices limit commercial usage. Ammonium sulfate precipitation, gel filtration chromatography, and ion-exchange chromatography on DEAE-cellulose were used to purify the laccase produced from *Pleurotus florida*. The brown colour pigment was removed from the enzyme solution using ion-exchange column chromatography (DEAE-Cellulose). *P.florida* intracellular and extracellular enzymes were isolated. The maximal purification folds for intracellular and extracellular purification were 6.68 and 15.33, respectively, after DEAE-cellulose chromatography. However, at a purification fold of 25.60, the enzyme was concentrated in the portion of fruiting body extract absorbed on DEAE-cellulose. This conclusion is consistent with a recent study finding that using DEAE cellulose ion-exchange chromatography, extracellular laccase from *Penicillium chyrsogenum* could be purified to a homogeneity of 29.64-fold [28]. In comparison to several previous reports on laccase-producing fungi, the current study has proven more effective. After DEAE-Cellulose chromatography, laccase isolated from *Hericium coralloides* was purified three times for homogeneity [29]. Meanwhile, Miao et al. [30] used DEAE-Cellulose chromatography to purify laccase from *Tricholoma mongolicum* mycelial extract and obtained a purification fold of 5.2.

SDS-PAGE revealed a single protein band with an estimated molecular mass of 66 kDa, indicating that the isolated laccase from *Pleurotus florida* has a low molecular mass. This finding was comparable to that of *Lentinus squarrosulus*, in which a single protein band with a molecular weight of approximately 66kDa appeared on an SDS-PAGE gel [31]. This fungus has a molecular mass similar to that of *Tricholoma mongolicum* [20]. For illustration, Laccase from *Hericium coralloides* had a molecular weight of 65 kDa [19]. Laccase from *Trametes polyzona* WRF 03 had a molecular weight of 66kDa, equivalent to the observations [32].

In terms of pH and thermostability, the purified laccase from the fruiting body was maximally active at 60°C and

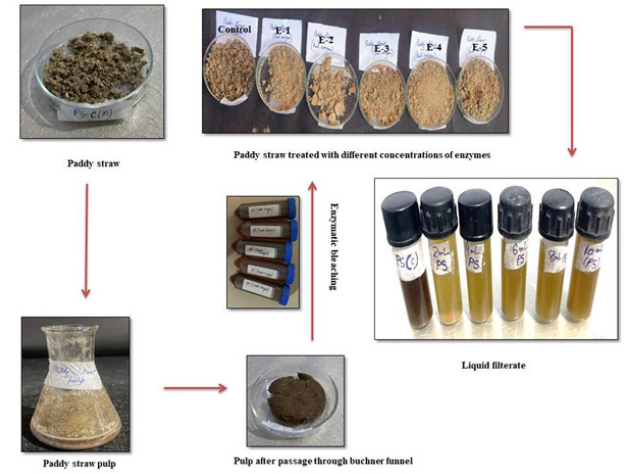

Fig. 6. Schematic representation of enzymatic bio-bleaching of paddy straw.

had its highest activity at pH 5.0. More et al. [33] discovered that the optimum temperature for purified laccase activity from *Pleurotus* spp. was 65°C, and laccase from *Lentinus tigrinus* maintained a similar optimal temperature [34].. Compared to the enzyme from *Marasmius* BBKAV79, which had an optimal temperature of 40°C, the current investigation demonstrated greater optimum temperatures [35]. For example, the laccase activity from *Trichoderma harzianum* utilizing guaiacol as a substrate had a maximum activity at pH 5 [36], while laccase activity from *Cladosporium cladosporioides* had the highest activity at pH 5 [37]. Laccase activity might be reduced at neutral/alkaline pH due to binding a hydroxide anion (laccase inhibitor) to the T2/T3 coppers of laccase, disrupting the internal electron transfer from T1 to T2/T3 centres and therefore inhibiting the enzyme. The impact of pH on laccase stability was investigated in selecting the most appropriate conditions for enzyme purification, application, and storage. After incubation at 4°C for 150 minutes, the isolated laccase from *P. florida* retained 60% of its original activity at pH ranges between 3.0 and 5.0. Moreover, the enzyme maintained 64.52% of its activity at 60°C after incubation for 150 minutes. The activity of the isolated laccase from the ascomycete *Thielavia* sp. was retained over 80% in the pH 5 range after incubation at 4°C for 24 hours [22]. However, they did report that after heating at 60°C for 6 hours, the activity might preserve more than 50% of its initial activity. Ding et al. [38] also discovered that the isolated laccase from *Ganoderma lucidum* was highly stable, retaining 80% of its activity at pH 3.0 and roughly

46% of its activity at 60°C after 80 minutes of incubation. The laccase from *Pleurotus florida* had a high level of stability throughout a wide pH range. It might be a crucial feature for industrial applications. In addition, Laccase from *Marasmius* sp. BBKAV79 was also extremely thermostable, with residual activity of 75.7% stable at pH 5.5 after 60 minutes at 40°C [35]. Laccase activity from *Lentinus squarrosulus* MR13 was steady at pH 5-8.5, while the enzyme activity declined to 34% at 65°C [31]. Chairin et al. [39] found *Trametes polyzona* KURNW027 laccase and *Trametes polyzona* WR710–1 laccase to be stable at 50°C for 1 hour. *Pleurotus florida* laccase enzyme ability to act throughout a wide temperature range of 10–100°C, as well as its stability at 50°C and 60°C, makes it a useful biocatalyst for a variety of industrial applications.

Metal ion's effect on enzyme activity is essential in industrial applications because some metal ions have inhibitory and stimulatory effects on enzyme activity. Metal ions are ubiquitous environmental pollutants that can impact both the production and stability of extracellular enzymes [40]. The findings revealed that all metals investigated lowered laccase activity in different ways, except for Cu^{2+}, Mg^{2+}, and Mn^{2+}, which had a small stimulatory effect on laccase activity. Other fungi have also made similar observations. Castano et al. [41] discovered that copper ions (Cu^{2+}) have a minor stimulatory effect on laccase from *Xylaria* sp. Mukhopadhyay et al. [31] discovered that the activity of isolated laccase from *Lentinus squarrosulus* MR13 increased by 2% in $CuCl_2$ and 5% in the presence of $CuSO_4$ (105.78%) at 1mM concentration. Furthermore, at a concentration of 25 mM, [19] found that Fe^{2+} (70.4%) and Hg^{2+} (5.3%) ions greatly inhibited the laccase, while Mg^{2+} (305.2%) and Al^{3+} (229.5%) ions significantly promoted it.. Laccase has three types of copper sites, as widely known (types I, II, and III). Metal ions bonded near the T1 site of *P. florida* laccase and enhanced competitive inhibition of electron donors by preventing substrate access to the T1 site or inhibiting electron transfer. The enzyme's sensitivity to numerous suspected laccase inhibitors was also investigated, with EDTA, SDS, and H_2O_2 proving to be the most efficient inhibitors of several oxidative enzyme processes at lower concentrations. At greater concentrations (10 mM), sodium azide had a considerably 78.81% inhibitory impact. On the other hand, the extent of inhibition varied substantially depending on the kind and concentration of the examined inhibitors. The findings contradict those of [22], who claimed that utilising the chelating chemical EDTA at concentrations below 10 mM resulted in no substantial loss of laccase activity. [42] studied similar results on partially purified laccase from *Fusarium solani*, indicating that sodium azide completely inhibited the activity, indicating that it functions as an oxidase.

Non-aqueous mediums such as organic solvents are often utilized in biocatalysis [43]. As a result, determining laccase stability in various organic solvents will be valuable in identifying the appropriate reaction media for its implementation in bioprocesses and biotransformation. Since many conversions occur at high concentrations of organic solvents, resistance to water-miscible solvents is pertinent for laccase industrial applicability. In the presence of these chemicals, laccases are extremely sensitive to unfolding. In the presence of 2% dimethyl sulfoxide, acetone, and chloroform, the *P. florida* laccase retained 70-98% of its initial activity. Although the enzyme was stable in many organic solvents, its activity was much lower than in an aqueous solution. Comparable conclusions were found using *F. solani* MAS2 laccase in the presence of 5% acetonitrile and methanol, with 75-80% activity remaining [42].

The purified laccase from *Pleurotus florida* had K_m and V_{max} values of 16.68 mM and 26.73 mol/ml/min, respectively. The estimated K_m and V_{max} showed the laccase efficient oxidation ability toward the substrate. Compared to the value previously reported for *Fomitopsis pinicola* (0.28 mM), the current investigation showed a superior K_m value [40]. More et al. [43] investigated the kinetic characteristics of laccase from *Pleurotus* spp. and found that laccase K_m value for 2,6 DMP was 38.46 mM. Debnath et al. [44] investigated the kinetic characteristics of laccase from *Phoma herbarum* and calculated the K_m value to be 5.217 mM. The low K_m value indicates that the purified laccase has a high affinity for the substrate, which is an essential feature.

Laccase enzyme actions were determined by the functional group present in the active site of the enzyme protein; therefore, it is critical to understand the enzyme's properties. The laccase enzyme was subjected to FT-IR analysis to determine the functional group of the enzyme protein. The amino, carboxylic, and thiol groups, which are present in the active site of the enzyme protein and will react with the substrates, are three major functional groups in the laccase enzyme. Senthivelan et al. [28] observed similar results in the laccase generated by *Penicillium chrysogenum*. Batal et al. [45] found the same characteristics in the isolated laccase from *Pleurotus ostreatus*.

The Kappa number is also one of the most important factors for analysing the effectiveness of enzymes. The kappa number was decreased from 24.36± 0.46 to 19.47±0.78 in the bio-bleached pulp sample (E-4) as compared to control pulp samples, thus showing 20.07% reduction in kappa number after bio-bleaching. The increase in brightness (8.20%) and whiteness (9.79%) and decrease in yellowness (4.44%) were observed during the treatment of paddy straw with enzyme (8 mL). Higher doses of enzymes beyond these values did not further improve the efficiency of bio-bleaching. The pre-bleaching treatment of rice straw pulp using crude laccase enzyme preparation was found to be most effective at enzyme dose of 8 mL. The enzymatic treatment separates lignin-carbohydrate complexes from cellulosic fibers, releasing chromophores, phenolic and hydrophobic chemicals, ultimately resulting in improved optical properties and a significantly reduced kappa number. The optical density of pulp-free filtrate indicated the release of chromophores during enzymatic treatment and the absorbance of filtrate for chromophoric and hydrophobic compounds was observed to be 0.166 and 0.491 in E-4. Hence, due to the hydrolysis of impurities, enzymatic bleaching improved the absorbance of pulp-free filtrates at different wavelengths [36]. The ability of *Trichoderma harzianum* WL1 crude laccase in the treatment of paper industry effluents resulted in the reduction of kappa number (18.4) and brightness (43.9 ISO units) [26]. Similarly, Nagpal et al. [46] observed that enzymatic treatment of rice straw pulp resulted in a 15.29% reduction in pulp kappa number. Moreover, Sridevi et al. [47] observed the release of chromophoric and hydrophobic compounds as 1.842 and 10.34 during the treatment of paper pulp by

xylanase enzyme produced from *Trichoderma asperellum*. Boruah et al. [48] reported that bamboo pulp treated with xylanase produced by *Penicillium meleagrinum* var. *viridifilum* showed that kappa number decreased from 13.50 to 8.50 with the increase in pulp brightness (68-69%).

5. Conclusions

In this study, laccase was purified from the fungus *P. florida* at different stages. The laccase properties showed the maximum enzyme activity at pH 5 and 60°C. In addition, we found that Fe^{2+} was a weak inhibitor, while Cu^{2+} and Mg^{2+} were both strong stimulators. *P. florida* laccase possesses special enzymatic properties that can be beneficial in a variety of industrial processes that require oxidation-reduction reactions. This study concluded that the highest quality pulp is obtained by bleaching rice straw pulp with ligninolytic enzymes. This method of biobleaching has the potential to reduce the consumption of harmful effluents, exposure to bleaching chemicals and eventually chemical pollution.

Author contributions

Manmeet Kaur conducted the experiment, analyzed the data and wrote the manuscript. Inderpal Kaur analyzed the methodology and data. Shivani Sharma and Harpreet Singh Sodhi contributed to the concept and design of the research. Ravish Choudhary, Sezai Ercisli, Hafize Fidan, Elanur Dasci, Riaz Ullah and Ahmed Bari investigated the original draft and checked the last version. The authors confirm the sole responsibility for study conception, design, data collection, analysis of results and manuscript preparation.

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Conflict of interest

All authors declare no conflict of interest.

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