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Purification and Characterization of *Pleurotus florida* Laccase (L1) involved in the Remazol Brilliant Blue R (RBBR) Decoloration

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Abstract

Pleurotus florida produces two extracellular laccase (L1 and L2) isoenzymes and the L1 isoenzyme is dominantly involved in the dye decoloration process. L1 isoenzyme was successfully purified to 6.4 fold with a yield of 36% and had a specific activity of 52.6 U mg^{-1} of protein. The purified laccase was monomeric with an apparent molecular mass of ≈54 kDa. The optimum pH and temperature of the LI isoenzyme was found to be around 5.5 and 50ºC, respectively. L1 isoenzyme showed a half life of 2 h at 60 ºC and at 4 h it retained around 25% residual activity. The kinetic parameters suggest that the order of affinity towards the tested substrates was syringaldazine > ABTS > DMP > guaiacol. Interestingly, L1 isoenzyme was not significantly inhibited by chloroform and benzene, whereas above 50% of laccase activity was inhibited by acetone, dimethyl sulfoxide and methanol.

Keywords: Decoloration, Isoenzyme, Laccase, *Pleurotus florida*, Purification, White-rot fungi.

1 Introduction

Laccases are copper-containing enzymes belonging to the group of blue oxidases. They catalyze the oxidation of various phenolic and inorganic compounds, including diphenols, polyphenols, substituted phenols, diamines, and aromatic amines by a one-electron transfer mechanism using molecular oxygen as the electron acceptor. Laccases are commonly distributed in fungi, higher plants, bacteria, and insects [1]. Their physiological functions differed based on their substrate specificity and isozymes nature in various organisms. Laccases are mainly involved in the detoxification of xenobiotic compounds, lignin biosynthesis, melanin synthesis [2-8]. In structural terms, these enzymes can be either monomeric and multimeric glycoproteins, which may exhibit additional heterogeneity due to variable carbohydrate content or differences in copper content.

Several fungal laccases have been purified [9-11], and some of the laccases encoding genes have been characterized. Several species of genus *Pleurotus* have been described as producers of laccase [12,13]. *Pleurotus florida* produces two extracellular laccase isoenzymes (L1 and L2) and involvement of L2 isoenzyme in the regulation of mycelial growth and yield of the mushroom has been suggested [14], however there was no detailed research on *Pleurotus florida* laccase L1 isoenzyme. In the present study, the dominant extracellular laccase isoenzyme produced (involved in dye decoloration) from *Pleurotus florida* under optimized conditions was purified and enzyme properties were studied.

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2 Materials and methods

2.1 Chemicals and dyes

ABTS, acrylamide, ammonium persulphate, bis-acrylamide, coomassie brilliant blue R-250, Guaiacol, DEAE-Sephacel, 2,6-Dimethoxyphenol, *N*-hydroxybenzotriazole, sephadex G-50, syringaldazine, TEMED and RBBR dye were purchased from Sigma-Aldrich, USA. Protein marker was purchased from Fermentas, Canada and all other chemicals were of analytical grade.

2.2 Microorganism

White-rot fungi *Pleurotus florida* was purchased from National Collection of Industrial Microorganism, National Chemical laboratory (NCL), Pune, India and

was grown on potato dextrose agar (PDA) plates incubated at 28°C for about 7 days. Thereafter, the plates were maintained at 4°C until used. The fungus was sub-cultured every 3 months.

2.3 Laccase production

Laccase production was carried out in the liquid media consisted of 15.21 g glucose, 6.40 g asparagine, 91.78 µM $CuSO_4$ and 100 ml of mineral salt solution (MSS) in 1000 mL of distilled water and pH was adjusted to 5.5. The MSS consisted of 2 g malt extract, 2 g KH_2PO_4, 0.5 g $MgSO_4 \cdot H_2O$, 0.1 g $CaCl_2$ and 0.5 g KCl dissolved in 100 mL of distilled water. 91.78 µM $CuSO_4$ was incorporated into the laccase production media during exponential phase.

2.4 Laccase assay

Laccase activity was measured using ABTS as the substrate at 30°C [15]. The assay mixture contained 100 mM sodium acetate buffer (pH 5.5), 1 mM ABTS, and laccase source. One unit activity was defined as the amount of enzyme that oxidized 1 µmol ABTS per minute. The absorbance increase of assay mixture was monitored at 420 nm (ε_{420} = 36.0 mM^{-1} cm^{-1}) in a UV-visible spectrophotometer (Perkin-Elmer Lambda 25, Germany). The enzyme activities were expressed in U g^{-1} or mL^{-1}.

2.5 Protein estimation

Protein content of the samples was determined according to the method of Lowry et al. [16] using bovine serum albumin (BSA) as a standard.

2.6 Characterization of laccase isoenzymes

In order to identify the number of laccase isoenzymes produced by *Pleurotus florida*, and their decoloration ability, the crude enzyme was subjected to 10% SDS-PAGE. After protein separation on SDS-PAGE, the SDS was removed from the gel by incubating the gel in 0.2% Triton X-100 in 100 mM sodium acetate buffer (pH 5.5) for 10 min. Excess Triton X 100 was removed by washing with 10 mM sodium acetate buffer (pH 5.5). The gel was then incubated in 100 mM sodium acetate buffer (pH 5.5) containing 5 mM guaiacol for the laccase isoenzymes activity detection. For the analysis of dye decoloration by *Pleurotus florida* laccase isoenzymes, the gel was stained with 50 ppm RBBR dye in the presence of 1 mM HBT for 10 min and the dye solution was discarded. After 1 h incubation at room temperature, gel was visualized for decoloration and stained with 5 mM guaiacol to detect the enzyme activity.

2.7 Purification of laccase

The method for the laccase purification was adapted from a protocol described by Das et al. [17] with slight modification. All operations were performed at 4°C unless otherwise mentioned. The culture supernatant 1000 ml was centrifuged at 21,000 × *g* for 30 min and filtered through whatman No. 1 filter paper to remove fine particles. The filtrate was concentrated by Stirred Ultrafiltration Cell (Millipore Corporation, USA) through a membrane filter (membrane molecular weight cut off 10 kDa), until a 20-fold concentration was achieved. The protein was precipitated in two steps. The first step was the addition of ammonium sulfate up to 40% (w/v) saturation at 0 °C and centrifugation at 21,000 x *g* for 30 min. The precipitate was then discarded. As a second step, ammonium sulfate was added to the supernatant to a final concentration of 80% (w/v) saturation at same temperature. After standing in the ammonium sulfate solution for 5 h at 0 °C, the precipitate was collected by centrifugation at 21,000 x *g* for 30 min and resuspended with 100 mM of sodium acetate buffer (pH – 5.5) and dialysed exhaustively against the same buffer for 12 h. The dialysate was applied to anion exchange chromotography DEAE-Sephacel column (30 × 2 cm) pre-equilibrated with 10 mM of sodium acetate buffer (pH – 5.5). After that the enzyme loaded column was washed with 500 ml of the same buffer to remove loosely and unbound sample components. Bound protein was eluted with a linear gradient (0–1 M KCl) in 10 mM of sodium acetate buffer (pH – 5.5) at a flow rate of 1 $mLmin^{-1}$, the eluted fractions were assayed for laccase activity. The active fractions of the laccase peaks were pooled together and dialysed against the same buffer. The dialysate was subjected to size-exclusion chromatography Sephadex G50 (50 x 2 cm) pre-equilibrated with 10 mM of sodium acetate buffer (pH – 5.5). The fractions containing laccase isoenzyme was eluted at a flow rate of 4 mL h^{-1}. Active fractions were pooled together, dialysed against the same buffer. The diaysate was concentrated by lyophilizer (Mini Lyodel Freeze Dryer, India) and stored at -20ºC for further characterization studies.

2.8 Characterization of purified laccase

2.8.1 Influence of pH

The optimum pH of purified laccase activity was determined by incubating the enzyme at different pH varying from 3 to 9 (100 mM citrate buffer pH 3 to 4.5; 100 mM sodium acetate pH 5 to 5.5; 100 mM potassium phosphate pH 6 to 7; 50 mM Tris–HCl pH 7.5 to 9) at 30ºC. For the determination of pH stability, the enzyme solution was incubated at the above pH for different time period. Residual activities of the enzyme were determined spectrophotometrically at 420 nm using ABTS as the substrate.

2.8.2 Influence of temperature

Optimum temperature of purified laccase was investigated by incubating the enzyme from 20 to 80ºC with the increment of 10°C at the optimum pH determined previously. Thermostability of enzyme was determined by incubating the enzyme solution at 40, 60 and 80°C for different time period. Residual activities of enzyme were determined spectrophotometrically at 420 nm using ABTS as the substrate.

2.8.3 Effect of inhibitors

The effects of potential laccase inhibitors and heavy metals were determined by incubating the purified enzyme with various concentrations of inhibitors and measuring the residual activity with ABTS as substrate. Sodium azide, EDTA, L-cysteine, $CaCl_2$, $CuSO_4$, $FeSO_4$,

$HgCl_2$, $MnSO_4$, $NiCl_2$, were incubated with purified laccase at three different concentrations (0.1, 1 and 10 mM) for 60 min at room temperature and the residual enzyme activity was measured spectrophotometrically at 420 nm using ABTS as the substrate.

2.8.4 Effect of solvents

The effects of organic solvents such as acetone, benzene, chloroform, dimethyl sulfoxide and methanol on purified enzyme activity were determined. Enzyme incubated with 10% (v/v) of organic solvents for 60 min at room temperature and residual activity was measured spectrophotometrically at 420 nm using ABTS as the substrate.

2.8.5 Kinetic studies

The Michaelis–Menten coefficient (K_m) values were determined for the substrates syringaldazine (0.005–0.1 mM), ABTS (0.005–0.1 mM), DMP (0.01–1 mM) and guaiacol (0.05–1 mM). The catalytic constant (k_{cat}) for each substrate was determined and the specificity constant (k_{cat}/K_m) was calculated. The wavelengths for laccase activity with the above mentioned substrates were determined spectrophotometrically by allowing the reaction of that substrate with laccase to proceed to completion, performing a spectral scan, and using suitable λ_{max} (Wavelength of maximum absorption).

2.8.6 Molecular weight determination

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed to determine sample purity and the approximate molecular weight of the purified laccase following the method of Laemmli [18] with 4% w/v stacking gel and 10% w/v separating gel. Protein bands were stained with Coomassie brilliant blue R-250, and the approximate molecular weight of the purified laccase was determined along with the standard protein markers (Fermentas, Canada).

2.8.7 Zymogram analysis of laccase

Native PAGE was performed as described by Gabriel [19]. Zymogram analysis for laccase activity was performed after Native-PAGE using 5 mM guaiacol or 5 mM ABTS in 100 mM sodium acetate buffer (pH 5.5) at room temperature.

3. Results and discussion

Many of the white-rot fungi produce more than one laccase isoenzymes [20]. In order to identify the isoenzyme pattern of *Pleurotus florida*, crude laccase was subjected to native and SDS-PAGE followed by zymogram analysis using guaiacol and ABTS oxidation. The native-PAGE (Figure 1a) and SDS-PAGE (Figure 1b (lane 1)) results revealed that two laccase isoenzymes (L1 and L2) were extracellularly produced by *Pleurotus florida*. Similar pattern of laccase isoenzymes was observed by Das *et al*. [21] in a previous study for the same *Pleurotus* sp.. Of these two isoenzymes, L1 was more dominant than L2 isoenzyme. Previously, Sathishkumar et al. [22] reported that the laccase mediated RBBR dye decoloration. Hence, in this study, RBBR was used as a model dye for the decoloration experiment.

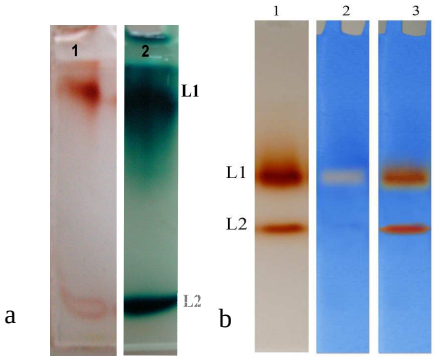

Figure 1. Zymogram analysis of laccase activity and decolorization activity of crude enzyme from *Pleurotus florida*. (a) Zymogram analysis of laccase isoenzymes (L1 and L2) on native-PAGE by guaiacol (Lane 1) and ABTS (Lane 2 oxidation. (b) Zymogram analysis and decolorization activity of laccase isoenzyme on SDS-PAGE. Lane 1. Gel stained with guaiacol; Lane 2. Decolorization of RBBR dye by *Pleurotus florida* laccase isoenzymes; Lane 3. Gel incubated with guaiacol showing the laccase activity at decolorized zone.

For the dye decoloration by *Pleurotus florida* laccase isoenzymes, the gel incubated with RBBR gave a single decolorized band which was corresponding to the L1 isoenzyme after 1 h (Figure 1 (lane 2)). Further, when the decolorized gel was incubated with guaiacol, laccase activity was seen at the decolorized zone (Figure 1 (lane 3)). This result corroborates that the L1 isoenzyme of *Pleurotus florida* laccase was dominantly involved in the dye decoloration process. However there was no visible decolorized band corresponding to the L2 isoenzyme after 1 h. Hence, L1 isoenzyme was selected for further purification and characterization studies.

3.1 Purification of L1 isoenzyme

Extracellular L1 isoenzyme of *Pleurotus florida* laccase was purified to homogeneity by ultrafiltration, ammonium sulphate precipitation, anion exchange and size-exclusion chromatography. In anion exchange chromatography DEAE-Sephacel column, two enzyme peaks were obtained by linear gradient elution. The peaks were around 560 mM (L1 isoenzyme) and 640 mM (L2 isoenzyme) concentrations of KCl (data not shown). Table 1 shows the results of a typical purification of L1 isoenzyme. At the end of the purification process, L1 isoenzyme was purified to 6.4 fold with a yield of 36%. The purified L1 isoenzyme had a specific activity of 52.6 U mg^{-1} of protein using ABTS as substrate under standard assay conditions.

Table 1. Purification profile of *Pleurotus florida* laccase

Purification steps	Volume (ml)	Total laccase activity (U)	Total protein (mg)	Specific activity of laccase (U/mg)	Purification fold	Yield (%)
Crude enzyme (culture filtrate)	1000	5000	610	8.2	1.0	100
Ultrafiltration (10 kDa)	50	4750	335	14.2	1.7	95
Ammonium sulphate precipitation (80%)	20	4010	160	25.1	3.1	80
DEAE-Sephacel						
L1 isoenzyme	10	2370	75	31.6	3.9	47
L2 isoenzyme*	4	973	NE	NE	NE	19
Sephadex G-50	6	1840	35	52.6	6.4	36

* L2 isoenzyme was not part of the present study; NE = Not estimated.

Purified L1 isoenzyme showed a single band in SDS-PAGE after staining with Coomassie brilliant blue R and guaiacol, respectively. The molecular weight of the L1 isoenzyme was calculated to be ≈54 kDa (data not shown). The molecular weight of the laccase (L1 isoenzyme) purified from *Pleurotus florida* falls within the range of 50–80 kDa as evident from the earlier report [23]. Previously, Das et al. [17] reported the molecular weight of purified laccase of *Pleurotus florida* to be 77 KDa. However, our result is in contrast to the report of Das et al. [17]. This variation might be due to the differences between strains, different ecological origin and culture conditions.

3.2 Influence of pH and temperature on enzyme stability

Enzyme stability is most important for biotechnological applications. The stability of laccase could be obviously having a substantial effect on the oxidation rate, hence it should be considered.

3.2.1 pH

The influence of pH on enzyme activity was determined at different pH ranging from 3 to 9 (Figure 2a). The optimum pH for the LI isoenzyme of *Pleurotus florida* laccase was found to be pH 5.5, which was quite similar to the of *Pleurotus florida* laccase studied by Das et al. [21] and *Pleurotus pulmonarius* [24] using ABTS as the substrate. However most of the laccase required an acidic pH for its optimal activity, these include laccases from *Agaricus blazei* (pH 2) [25], *Agaricus dispansus* (pH 4) [26], *Ganoderma lucidum* (pH 4) [27], *Panus tigrinus* (2.7–3.2) [28], *Pleurotus eryngii* (pH 3–5) [26], *Clitocybe maxima* (pH 3) [23]. Maximum activity of enzyme was found to be between pH 4.5 and 7. In the case of enzyme stability, L1 isoenzyme was almost stable at low acidic to neutral pH (5 to 6) incubated at room temperature (Figure 2b). However, the enzyme was completely inactivated at pH 3 and 9 within 90 min. ***3.2.2***

3.2.2 Temperature

The temperature dependence of the L1 isoenzyme of *Pleurotus florida* laccase activity is depicted in Figure 3a. The optimum temperature of enzyme was found to be pH 50ºC using ABTS as substrate, which was quite similar to other laccases from *Pleurotus ostreatus* [29], *Cantharellu cibarius* [26] and *Coriolus hirsutus* which manifests a temperature optimum at 45°C [30].

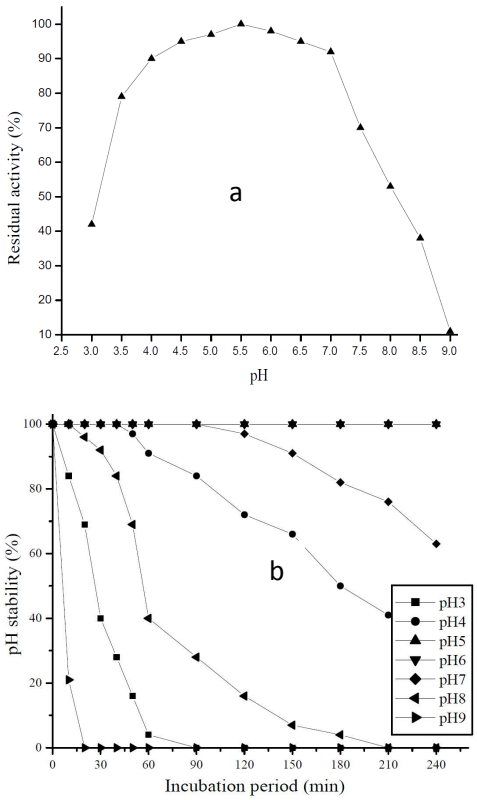

Figure 2. Influence of pH on *Pleurotus florida* laccase (L1 isoenzyme). (a) Optimum pH; (b) pH stability.

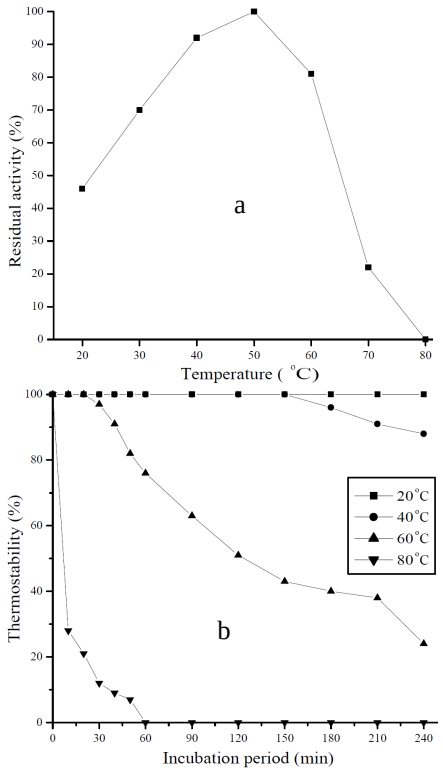

Figure 3. Influence of temperature on *Pleurotus florida* laccase (L1 isoenzyme). (a) Optimum temperature; (b) Thermostability stability

Youn et al. [31] reported the optimum temperature for laccase of *Pleurotus ostreatus* was 30–35ºC. On the other hand, laccases from *Ganoderma lucidum*, *Pleurotus eryngii* and *Trametes giganteum* manifest a temperature optimum at 70°C [27].

Some of the white-rot fungal laccases have been described as thermostable, even though most laccases are not active at higher than 50ºC [24]. For example *Pleurotus ostreatus* showed a half life of 30 min at 60 ºC [29] and *Pleurotus eryngii* retained 10% residual activity at 60ºC after 30 min incubation [32]. However *Pleurotus florida* laccase (L1) showed a half life of 2 h at 60ºC and at 4 h it retained around 25% residual activity (Figure 3b) which indicates its better thermal stability than other *Pleurotus* species laccases. The laccase activity decreased rapidly at 80ºC and the complete inactivation occurred within 1 h incubation.

3.3 Kinetic studies

As mentioned in the methods, the purified laccase was characterized in terms of its Michaelis constant (K_m), catalytic constants (k_{cat}) and specificity constant (k_{cat}/K_m) with four different substrates, namely syringaldazine, ABTS, guaiacol and DMP (Table 2). The enzyme showed the lowest K_m value of 21 µM toward syringaldazine. The catalytic efficiency analysed as k_{cat}/K_m showed that ABTS was more efficiently oxidized, followed by syringaldazine, DMP and guaiacol. The kinetic parameters suggest that the order of affinity toward the tested substrates was syringaldazine > ABTS > DMP > guaiacol. The k_{cat} value for ABTS (1,121 S^{-1}) was higher than those found for several other white-rot fungal laccases, such as those of *Trametes multicolor* (510 S^{-1}) [33], *Coriolus hirsutus* (260 S^{-1}) [30], *Picnoporus cinnabarinus* (920 S^{-1}) [34] and *Phellinus ribis* (8.0 × 104 min^{-1}) [35].

Table 2. Kinetic parameters for *Pleurotus florida* laccase (L1)

Substrate	Wavelength λ_{max}	ε_{max} M^{-1} cm^{-1}	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m
Syringaldazine	525	65,000	21	364	17.3
ABTS	420	36,000	38	1,121	29.5
DMP	470	35,640	210	1466	6.98
Guaiacol	435	6,400	550	3310	6.02

The enzyme activity assay was performed at 30 °C. The molecular weight of ≈54 kDa was used to calculate the k_{cat} values. All values were calculated by the linear regression (correlation coefficient ≥ 0.98) of double reciprocal plots, 1/v *versus* 1/[S], from every set of triplicate measurements.

Pleurotus florida laccase desired ABTS as the perfect substrate similar to other fungal laccases [36]. Purified laccase was also found to oxidize typical laccase substrates. For the phenolic compounds, the oxidizing activity was observed in the above said order. This suggests that the substituted methoxy groups play an important role [37]. Comparison of the K_m values for ABTS (38 µM) with those *Pleurotus ostreatus* (90 µM), *Pleurotus sajor-caju* (56 µM), *Rigidoporus lignosus* (80 µM), *Panaeolus papilionaceus* (50 µM), *Coprinus friesii* (41 µM), *Myceliophthora thermophila* (96 µM), *Scytalidium thermophilium* (89 µM) and *Agaricus blazei* (62 µM) [25] shows that *Pleurotus florida* laccase exhibits a higher affinity for ABTS but apparently lower for syringaldazine.

3.4 Effect of inhibitors and organic solvents on laccase

Table 3 shows the effects of several chemical compounds on L1 isoenzyme of *Pleurotus florida* laccase activity. The effects of several laccase inhibitors were determined with ABTS as a substrate in 50 mM sodium acetate buffer (pH 5.5). Potential laccase inhibitor sodium azide completely inhibited purified L1 isonzyme even at 0.1 mM concentration, whereas other compounds required at higher concentration than that of sodium azide for complete inhibition. Sugumaran [38](1995) reported sodium azide a common inhibitor of metalloenzymes strongly inhibit the laccase activity.

The binding of sodium azide to the type 2 and 3 copper sites affects internal electron transfer, thus inhibiting the activity of the laccase. These findings are in keeping with the general properties of laccase from a diverse range of fungal sources [39]. The laccase activity was slightly inhibited by chelating agent EDTA at high concentration (10 mM). However, the enzyme was stable

up to 1 mM concentration of EDTA, which is similar to previous report of *Pleurotus florida* [17].

Table 3. Effect of inhibitors on *Pleurotus florida* laccase (L1)

Chemical compounds	Concentration (mM)	Inhibition[a] (%)	Activation[a] (%)
Sodium azide	0.1	100	-
	1.0	100	-
	10	100	-
EDTA	0.1	-	-
	1.0	-	-
	10	11	--
L-cysteine	0.1	-	-
	1.0	40	-
	10	100	-
$CaCl_2$	0.1	-	-
	1.0	-	-
	10	-	-
$CuSO_4$	0.1	-	3
	1.0	-	16
	10	-	21
$FeSO_4$	0.1	-	-
	1.0	-	-
	10	-	-
$HgCl_2$	0.1	-	-
	1.0	-	-
	10	16	-
$MnSO_4$	0.1	-	-
	1.0	-	-
	10	23	-
$NiCl_2$	0.1	-	-
	1.0	-	-
	10	-	-

[a]Values reported are the means of values from three independent experiments, with a maximal sample mean deviation of ± 5%.

Some of the laccases purified from other white-rot fungi are inhibited by 1 mM EDTA [39]. However, some exception have been previously described, such as the laccases of *Pleurotus ostreatus* [40], *Marasmius quercophilus* [41], *Pleurotus florida* [17] and *Phellinus ribis* [35], where inhibited only at high concentrations of EDTA. L-cysteine effectively inhibited 40% of enzyme at 1 mM concentration. This result was similar to the other white-rot fungal laccases [42].

Table 4. Solvent stability of *Pleurotus florida* laccase (L1)

Solvent	Concentration (%)	Stability (%)
Acetone	10	41
Benzene	10%	95
Chloroform	10%	98
Dimethyl sulfoxide	10%	46
Methanol	10%	37

Reactions were incubated at room temperature for 60 min and residual activity measured with ABTS as substrate.

Purified laccase (L1 isoenzyme) activity was significantly stimulated by Cu^+ by 3, 16 and 21% at 0.1, 1.0 and 10 mM concentrations, respectively (Table 3). This may be due to the filling of type-2 copper binding sites with copper ions [43]. Enzyme activity was slightly inhibited by Hg^+ and Mn^+ at 10 mM concentration (Table 3). In the case of Fe^+, Ca^+ and Ni^+ there was no inhibition observed on enzyme activity up to 10 mM concentration. Table 4 shows the results of organic solvent stability of purified laccase. Interestingly, *Pleurotus florida* laccase was not significantly inhibited by chloroform and benzene, whereas above 50% of laccase activity was inhibited by acetone, dimethyl sulfoxide and methanol.

4 Conclusions

In conclusion, *Pleurotus florida* produced two (L1 and L2) extracellular isoenzymes and the L1 isoenzyme is dominantly involved in the dye decoloration process. Because of the trouble free purification procedure, high yield, thermostability and organic solvent stability; the *Pleurotus florida* laccase can be used for industrial and bioremediation processes.

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