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PURIFICATION AND CHARACTERIZATION OF LACCASE SECRETED BY *Phellinus linteus* MTCC-1175 AND ITS ROLE IN THE SELECTIVE OXIDATION OF AROMATIC METHYL GROUP

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A laccase from the culture filtrate of *Phellinus linteus* MTCC-1175 has been purified to homogeneity. The method involved concentration of the culture filtrate by ammonium sulphate precipitation and an anion exchange chromatography on DEAE-cellulose. The SDS-PAGE and native-PAGE gave single protein band indicating that the enzyme preparation was pure. The molecular mass of the enzyme determined from SDS-PAGE analysis was 70 kDa. Using 2,6-dimethoxyphenol, 2,2'[azino-bis-(3-ethylbonzthiazoline-6-sulphonic acid) diammonium salt] (ABTS) and 4-hydroxy-3,5-dimethoxybenzaldehyde azine as the substrates, the K_m, k_{cat} and k_{cat}/K_m values of the laccase were found to be 160 μM, 6.85 s^{-1}, 4.28 × 10^4 M^{-1} s^{-1}, 42 μM, 6.85 s^{-1}, 16.3 × 10^4 M^{-1} s^{-1} and 92 μM, 6.85 s^{-1}, 7.44 × 10^4 M^{-1} s^{-1}, respectively. The pH and the temperature optima of the *P. linteus* MTCC-1175 laccase were 5.0 and 45°C, respectively. The activation energy for thermal denaturation of the enzyme was 38.20 kJ/mole/K. The enzyme was the most stable at pH 5.0 after 1 h reaction. In the presence of ABTS as the mediator, the enzyme transformed toluene, 3-nitrotoluene and 4-chlorotoluene to benzaldehyde, 3-nitrobenzaldehyde and 4-chlorobenzaldehyde, respectively.

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Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) is a polyphenol oxidase, which belongs to the superfamily of multicopper oxidases [1, 2] and catalyzes [3–5] the four-electron reduction of molecular oxygen to water. Laccases are dimeric or tetrameric glycoproteins. To perform their catalytic functions, laccases use Cu atoms that are distributed at the 3 different copper centres. They are called blue (type-1), normal (type-2) and coupled binuclear (type-3) copper centres and differ in their EPR characteristics [6, 7]. The organic substrate is oxidized by one electron at the active site of the laccase generating a reaction radical which further reacts non-enzymatically. The electron is received at type-1 Cu centre and shuttled to the trinuclear cluster where oxygen is reduced to water.

Ortho- and para-diphenols, aminophenols, polyphenols, polyamines, lignins, and arylamines and some of the inorganic ions are the substrates for laccases. The ability of laccases to catalyze the oxidation of various phenolic compounds, coupled to the reduction of molecular oxygen to water makes them valuable from the point of view of commercial applications [4, 8–10]. The biotechnological importance of laccases has increased after the discovery that substrate range for oxidizable reaction could be further extended in the presence of small readily oxidizable molecules called mediators [11, 12]. During the last two decades, laccases have turned out to be the most promising enzymes for industrial uses [9, 10] having applications in food, pulps, paper, textile, and cosmetics industries and in synthetic organic chemistry [13–16].

Laccases purified from different sources exhibit different properties and are suitable for many applications [3–6]. Thus, there is a scientific need to study laccases from new sources so that laccases with novel properties could be found.

Moreover, there are reports in the literature that the fungal strains which secrete blue laccases in liquid growth media, secrete yellow laccases when grow in the media containing solid lignocellulosic substrates like wheat straw [17, 18]. The yellow laccases lack the absorbance band around 610 nm found in case of blue laccases. Moreover, yellow laccases do not require the presence of mediator molecules for the oxidation of non-phenolic compounds [18].

Phellinus linteus MTCC-1175 is a white rot fungus growing on logs and its laccase has not been purified so far. It is not known which type of laccase (blue or yellow) is secreted when fungus grows in medium containing solid lignin-containing substrates. The aim of the research was to purify laccase from *P. linteus* MTCC-1175, to determine its type and to study physicochemical properties. Results reported have shown that *P. linteus* MTCC-1175 secretes blue laccase when grows in the presence of solid lignin-containing natural substrate, wheat straw, and this enzyme is suitable

for the conversion of aromatic methyl group to aldehyde group in the presence of mediator ABTS.

MATERIALS AND METHODS

Materials. 4-Hydroxy-3,5-dimethoxybenzaldehyde azine (syringaldazine), 4-chlorotoluene and DEAE-cellulose were obtained from Sigma (USA). 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) and 2,6-dimethoxy phenol (DMP) were purchased in Fluka Chemi (Switzerland). All other chemicals used in these investigations were obtained either from Himedia laboratory Ltd. (India) or from Merck (Germany) and used without further purifications. The chemicals used in the gel electrophoresis of the protein samples and the protein molecular weight markers were purchased from Bangalore Geni Pvt. Ltd., (India).

The fungal strain and its growth. *Phellinus linteus* MTCC-1175 was procured from the Microbial Type Culture Collection Center and Gene Bank, Institute of Microbial Technology, Chandigarh, (India) and maintained on agar slants as reported in MTCC Catalogue of strains-2000 [19]. The growth medium for the fungus contained (g/L): malt extract – 20.0 and agar – 20.0; the pH was adjusted to 6.5.

In order to detect the extracellular secretion of the laccase by *P. linteus* MTCC-1175, the liquid growth medium reported by Coll et al. [20] was used. This medium consisted of (g/L): glucose – 10.0, asparagine – 1.0, yeast extract – 0.5, $MgSO_4 \cdot 7H_2O$ – 0.01 and $FeSO_4 \cdot 7H_2O$ – 0.01. The liquid growth medium was supplied with natural lignin substrates like coir dust, corn cob, wheat straw, saw dust or bagasse particles. Each of these substrates was prepared by adding 0.5 g to 25 mL of growth medium in 100 mL culture flasks which were sterilized. The sterilized growth media were inoculated with small pieces of the *P. linteus* MTCC-1175 mycelia (0.5 × 0.5 cm) under aseptic conditions and the fungal cultures were grown under stationary culture conditions at 25°C in a biological oxygen demand (BOD) incubator. In order to monitor the production of the laccase in the liquid culture medium, 0.5 mL aliquots of the growth medium were withdrawn at the regular intervals of 24 h and filtered through sterilized Millipore filter 0.22 μm (USA). The filtered extract was analyzed for the activity of the laccase using DMP as the substrate by the method [21] given below in assay section. The optimal time for extracellular secretion of the *P. linteus* MTCC-1175 laccase in the liquid medium was determined by plotting the enzyme activity (unit/mL) in the growth medium against the number of days after inoculation of the fungal mycelia. Each point on the curve was an average of 3 measurements. The growth medium for the control experiment did not contain natural lignolytic substrate. In order to optimize the conditions for maximum production of the laccase by *P. linteus* MTCC-1175, the amount of the best enzyme inducer, wheat-straw particles, was varied from 100 to 1000 mg in 25 mL of the growth medium. The amount of the inducer in the growth medium which gave the maximum of the enzyme activity was taken as the optimal.

Enzyme assay. The assay solution using DMP as the substrate [20] contained 1.0 mM DMP in 50 mM sodium malonate buffer (pH 4.5) at 37°C, using ABTS as the substrate [22] contained 0.5 mM ABTS in 0.1 M sodium acetate buffer (pH 5.0) at 25°C and using syringaldazine as the substrate [23] contained 0.1 mM syringaldazine in 50 mM sodium phosphate buffer (pH 6.0) at 50°C. In case of DMP, the reaction was monitored by measuring OD_{468} and using the molar extinction coefficient [20] value of 49.6 mM^{-1} cm^{-1}. In case of ABTS, the reaction was assayed by measuring OD_{420} and using the molar extinction coefficient [22] value of 36.0 mM^{-1} cm^{-1}. In case of syringaldazine, the reaction was performed by measuring OD_{530} and using molar extinction coefficient [23] value 64.0 mM^{-1} cm^{-1}. The UV/Vis spectrophotometer Hitachi (Japan) model U-2900 fitted with electronic temperature control unit was used for absorbance measurement. One enzyme unit produced 1 μM of the product per min under the specified assay conditions.

Purification of laccase. For the purification of the laccase, *P. linteus* MTCC-1175 was grown in ten 100 mL culture flasks each containing 25 mL of growth medium with 400 mg of the inducer, wheat-straw particles, under stationary culture conditions in a BOD incubator at 30°C. The maximum activity of the laccase was revealed on 9 day after the inoculation of the fungal mycelia. On the 9 day, mycelia from all flasks were removed by filtration through 4 layers of cheese cloth. The culture filtrate was saturated up to 30% with ammonium sulphate and centrifuged at 5480 × *g* and 4°C for 20 min. The precipitate was discarded and the supernatant was saturated up to 85% by further addition of ammonium sulphate. The resulting suspension was centrifuged at the same conditions and the supernatant was discarded. The precipitate was dissolved in 100 mM Na-acetate buffer (pH 4.5) and dialyzed against 10 mM Na-acetate buffer (pH 4.5) with 3 changes of buffer during 8 h. Thirty mL of dialyzate containing 2.7 mg/mL protein was concentrated using Amicon concentrator cell model 8200 (USA) and loaded on the DEAE-cellulose column (size 0.75 × 18.0 cm) which was pre-equilibrated with 10 mM Na-acetate/acetic acid buffer (pH 4.0) with flow rate 15 mL/h. The column was washed with 100 mL of the same buffer. The enzyme was eluted by applying linear gradient of 1 M NaCl in the same buffer (50 mL buffer + 50 mL buffer with 1.0 M NaCl). The fractions of 5.0 mL were collected and analyzed for the laccase activity [20]. The protein was measured by the Lowry method. All fractions containing laccase activity were combined and concentrated by Amicon concentrator cell model 8200 (USA) to 8 mL. The enzyme was stored in 10 mM Na-ace-

tate/acetic acid buffer (pH 4.0) at 4°C. The enzyme did not loose any activity for one month under these conditions.

SDS-PAGE analysis. The purity of the enzyme preparation was checked by using SDS-PAGE [24]. The molecular weight markers were phosphorylase (97.4 kDa), bovine serum albumin (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soyabean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa). Gel was run at a constant current 20 mA. The molecular weight was determined by the Weber and Osborn method [25].

Native-PAGE and zymogram analysis. The native PAGE of the purified enzyme was done using the Schagger and von Jajow method [26]. The composition of resolving and stacking gels was similar to those used in SDS-PAGE except that SDS was absent. The reference protein was bovine serum albumin (66.0 kDa). Two sets of native gels were done. One set was strained with Coomassie brilliant blue R-250 and the other set was used for zymogram [27] preparation. For the preparation of zymogram 100 mM DMP solution was made in 10 mM Na-acetate buffer (pH 4.0). The native gel was dipped in zymogram solution for 5 min and a brown band appeared. The zymogram was removed from the DMP solution and washed thrice with the same buffer at the intervals of 5 min.

Steady state enzyme kinetics. The steady state kinetics of the purified *P. linteus* MTCC-1175 laccase was studied using DMP, ABTS, and syringaldazine as the substrates following the methods as mentioned in the assay section. K_m and k_{cat} values for the enzyme were determined from the linear regression of double reciprocal plots. The pH and temperature optima of the enzyme were determined by measuring the steady state velocities of the enzyme catalyzed reaction in the solutions of varying pH/temperature keeping the other parameters fixed and drawing graphs of steady state velocities versus the variable parameter.

Temperature optimum and thermal stability. Temperature optimum of the *P. linteus* MTCC-1175 laccase was detected in 1 mL of reaction solution containing 1.0 mM DMP in 100 mM sodium malonate buffer (pH 4.5) with varying temperature from 30 to 70°C. Thermal stability of the *P. linteus* MTCC-1175 laccase was tested by incubating an enzyme aliquot in the same reaction solution at a particular temperature for 60 min, assaying its residual activity and plotting the residual activity against temperature. The rate constants for the denaturation of the enzyme at different temperatures were determined by keeping the laccase aliquots at fixed temperatures and assaying the enzyme activity at regular intervals of time and plotting residual activity against time. The rate constants were calculated from $t_{1/2}$ values using the equation $k = 0.693/t_{1/2}$. Energy of activation for thermal denaturation of laccase was calculated from Arrhenius plot.

pH optimum and pH stability. pH optimum of the enzyme was determined in 1 mL of reaction solution containing 1.0 mM DMP in 100 mM sodium malonate buffer with varying pH (from 3.0 to 6.0). pH stability of the *P. linteus* MTCC-1175 laccase was tested by incubating enzyme aliquot at a particular pH for 60 min, assaying its residual activity and plotting the residual activity against pH. In this case 1 mL of the same reaction solution with varying pH from 4.0 to 6.0 was used.

Biotransformations in the presence of ABTS as mediator. The biotransformation of toluene to benzaldehyde [28, 29] was done in 15 mL of 100 mM Na-acetate buffer (pH 4.5) containing 0.1 mM ABTS as mediator and 40 μg of purified laccase mixed with 20 mM toluene in 20 mL of dioxane. The mixture was stirred vigorously for 30 min at room temperature in a 100 mL conical flask. The reaction solution was extracted thrice with 40 mL of ethylacetate and 20 μl of the ethyl acetate extract was injected in Waters HPLC Model 600E system (Waters, USA) using Spherisorb C18 column (5 UV, 4.5 × 250 mm). Methanol was used for elution with the flow rate of 0.5 mL/min. The detection of sample was made using Waters UV detector model 2487 (USA) at 254 nm.

The biotransformations of 3-nitrotoluene to 3-nitrobenzaldehyde and 4-chlorotoluene to 4-chlorobenzaldehyde were also studied using the method described above except that the stirring was done for 60 and 90 min, respectively.

RESULTS AND DISCUSSION

Figure 1 shows the secretion of the laccase of *P. linteus* MTCC-1175 in the liquid growth medium supplied with various lignin-containing natural substrates like corn cob, coir dust, saw dust, wheat straw or bagasse particles. The control experiment had the same medium composition but without the supplement. It is obvious that the addition of lignin-containing natural substrates in the medium enhanced the secretion of the laccase. The maximum of extracellular secretion of the *P. linteus* MTCC-1175 laccase was observed the case of using wheat-straw particles. In order to optimize the process, secretion of the enzyme in presence of different amounts of wheat-straw particles was studied. The results are shown in Fig. 2. The maximum level of the laccase was revealed in the medium containing 400 mg of the wheat-straw particles per 25 mL of the culture medium.

The purification procedure of the laccase of *P. linteus* MTCC-1175 from the liquid culture filtrate is summarized in Table, and the elution profile of the laccase activity from DEAE cellulose column is shown in Fig. 3. The enzyme was bound to DEAE-cellulose in 10 mM Na-acetate/acetic acid buffer (pH 4.0) and eluted by the linear gradient of NaCl in the range 0.32 to 0.73 M. The pooled and concentrated fractions containing laccase gave 16.5-fold purification with

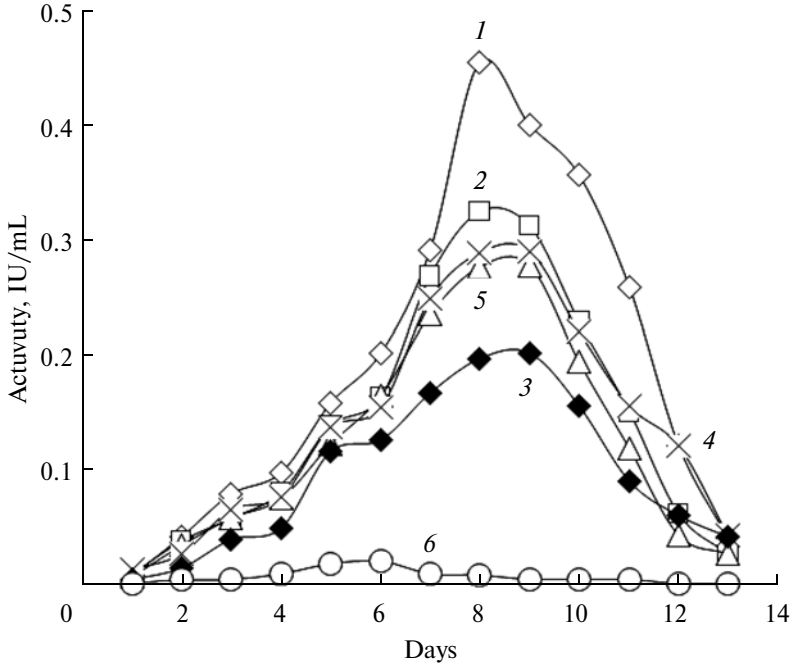

Fig. 1. Secretion of the *P. linteus* MTCC-1175 laccase in the liquid growth medium supplemented with different natural lignin-containing substrates: *1* – wheat straw; *2* – corn cob; *3* – bagasse; *4* – saw dust; *5* – coir dust and *6* – control.

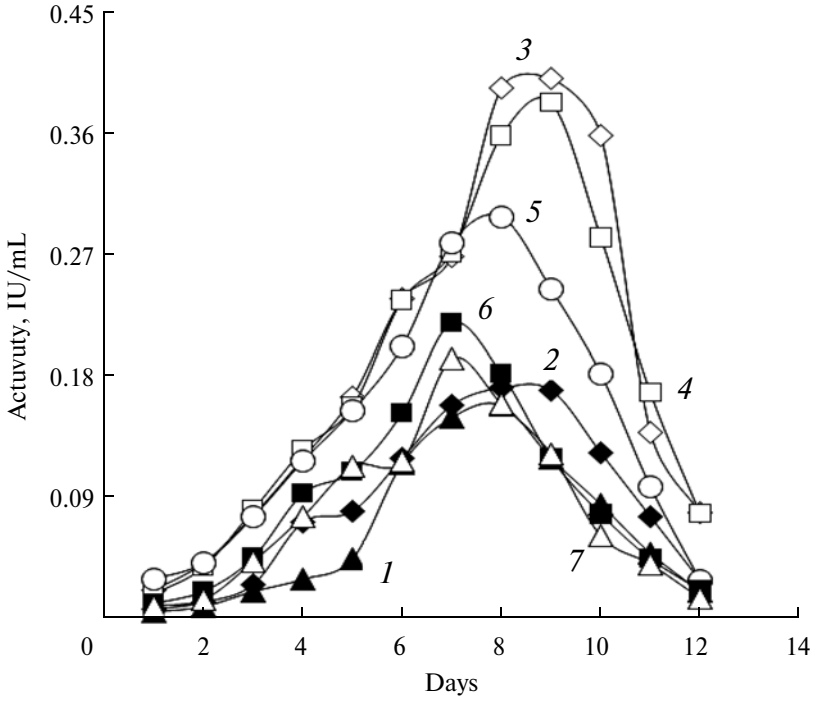

Fig. 2. Optimization of the *P. linteus* MTCC-1175 laccase secretion in liquid growth medium supplemented with different amounts of wheat-straw particles (mg/25 mL): *1* – 100; *2* – 200; *3* – 400; *4* – 500; *5* – 600; *6* – 800 and *7* – 1000.

32.1% recovery of the enzyme activity. The concentrated enzyme sample was analyzed by SDS-PAGE, native PAGE and zymogram preparation (Fig. 4). The appearance of single protein band in SDS-PAGE indicated that the enzyme sample was pure (Fig. 4a). Native PAGE confirmed the purity of the enzyme (Fig. 4b). The

Purification of the *P. linteus* MTCC-1175 laccase

Step	Volume, mL	Activity, IU/mL	Protein, mg/mL	Specific activity, IU/mg	Total protein, mg	Total activity, IU	Purification, -fold	Yield, %
Culture filtrate	150	0.39	1.1	0.36	165.0	58.65	1	100.0
Ammonium sulphate precipitation	20	2.32	3.0	0.77	60.0	46.30	2.2	79.0
Dialysis	30	1.50	1.0	1.50	30.0	45.00	4.3	77.0
DEAE-cellulose	8	2.35	0.4	5.87	3.2	18.80	16.5	32.1

position of the purified laccase in the zymogram (Fig. 4c) of native PAGE coincides with the position of the single protein band of purified laccase in the native PAGE (Fig. 4b). The molecular mass of the purified laccase of *P. linteus* MTCC-1175 determined from the analysis of SDS-PAGE [25] was 70 kDa. It is compared to the molecular masses of laccases reported in the literature [4] which are in the range 43–383 kDa.

The purified *P. linteus* MTCC-1175 laccase had the characteristic colour of the blue laccases. Its UV/Vis spectrum is shown in Fig. 5. In the insert one can see the absorbance around 600 nm found in the cases of blue laccases. There are reports in the literature [18], that usually fungal strains which secrete blue laccases in the liquid growth media, secrete yellow laccase when grown in the medium containing solid natural lignin substrates like wheat straw, coir dust, bagasse etc. The results obtained with the *P. linteus* MTCC-1175 laccase indicated that blue laccases could be secreted even in the growth medium containing solid natural lignin substrates.

The Michaelis-Menten and double reciprocal plots for the purified laccase of *P. linteus* MTCC-1175 were drawn using DMP, ABTS and syringaldazine as the variable substrates. The K_m, k_{cat} and k_{cat}/K_m values determined from the double reciprocal plots using DMP, ABTS and syringaldazine as the substrates were 160 μM, 6.85 s^{-1} , 4.28×10^4 M^{-1} s^{-1}, 42 μM, 6.85 s^{-1}, 6.3×10^4 M^{-1} s^{-1} and 92 μM, 6.85 s^{-1}, 7.44×10^4 M^{-1} s^{-1}, respectively. The range of K_m values reported [4] for fungal laccases using DMP, ABTS, and syringaldazine as the substrates are 8–14720 μM, 4–770 μM and 3–3400 μM, respectively. However, the K_m value of the *P. linteus* MTCC-1175 laccase for DMP compares well with the K_m of enzyme

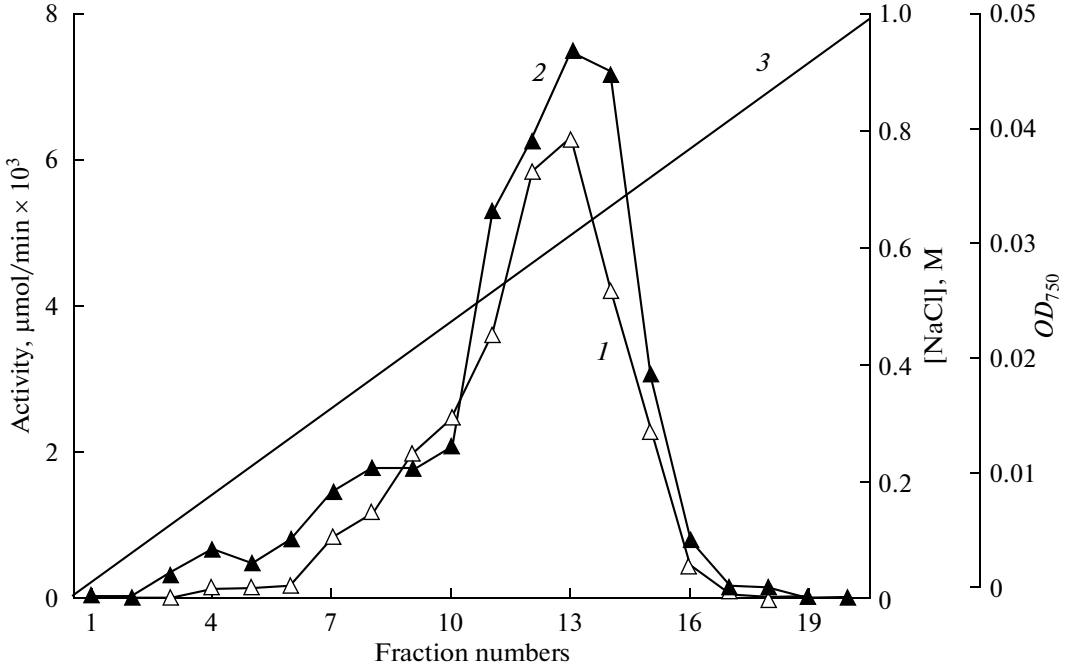

Fig. 3. Elution profile of the *P. linteus* MTCC-1175 laccase from the DEAE-cellulose column: *1* – activity; *2* – protein and 3 – NaCl.

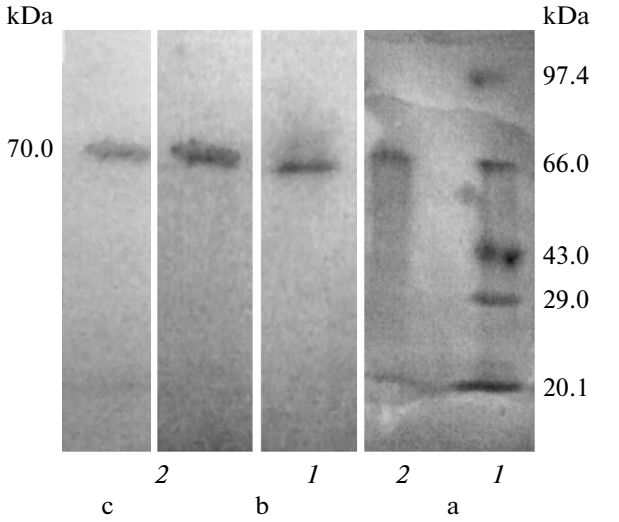

Fig. 4. SDS-PAGE (a), native-PAGE (b) and zymogram (c) of the *P. linteus* MTCC-1175 purified laccase. Lane *1* – molecular weight markers; lane *2* – purified laccase.

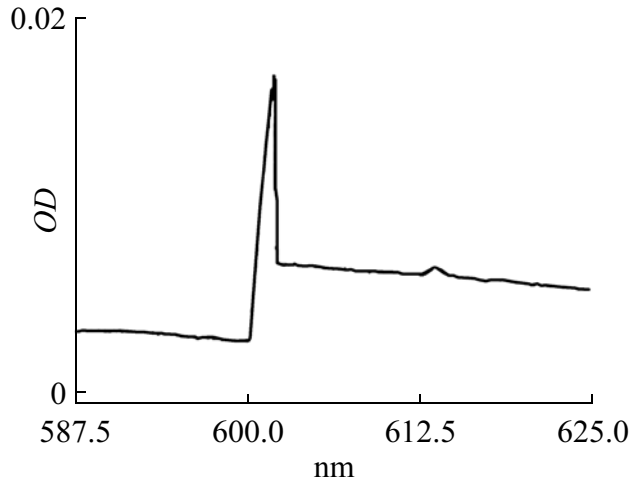

Fig. 5. UV-Vis spectrum of the *P. linteus* MTCC-1175 purified laccase (4.5 μM laccase in 10 mM Na-acetate/acetic acid buffer, pH 4.5).

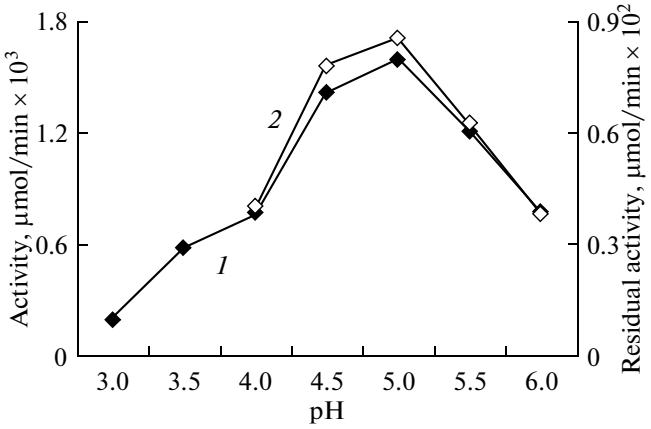

Fig. 6. Determination of pH optimum (*1*) and pH stability (*2*) of the *P. linteus* MTCC-1175 purified laccase.

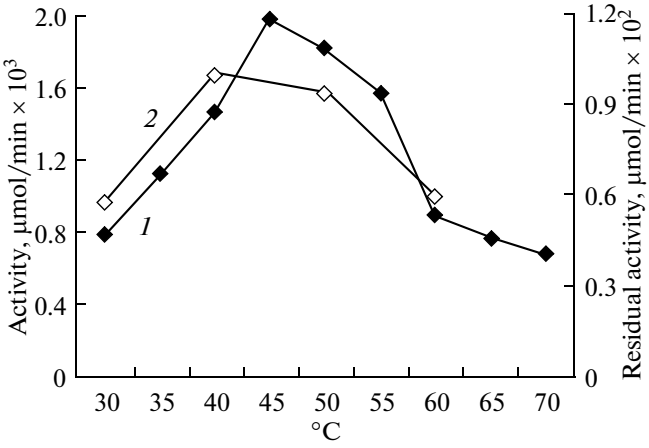

Fig. 7. Determination of temperature optimum (1) and thermal stability (*2*) of the *P. linteus* MTCC-1175 purified laccase.

purified from *Armillaria mellea* Lac I [4]. The K_m value of this enzyme using ABTS as substrate is similar to the K_m of laccases purified from *Coprinus friesll, Daedalea quercina* and *Trametes versicolor* [4]. The K_m value of this laccase for syringaldazine is comparable with the K_m of enzyme purified from *Pleurotus ostreatus* POXA 3b [4]. The value of k_{cat} determined for the purified *P. linteus* MTCC-1175 laccase has been found to be low in comparison to the values of k_{cat} reported for other fungal laccases [4]. It is worth mentioning over here that the catalytic performances of laccases span several orders of magnitude for different substrates and are characteristic for specific proteins [4].

The changes of the activity of the purified *P. linteus* MTCC-1175 laccase with the variation of pH of the reaction solution are shown in Fig. 6. The determined pH optimum of the enzyme was 5.0. The pH optima reported in the literature [4] for laccases using DMP as the substrate are in the range 3.0–8.0 pH units. The pH optimum of the purified *P. linteus* MTCC-1175 laccase compares well with pH optima of laccases *of C. friesll*, *Hericium echinareum*, *Phellinus ribis*, *P. ostreatus* POXA 1W, *Ophiostoma novo-ulmi* and *Marasmius quercophilus* [4]. The results of the pH stability of the laccase studied are shown in Fig. 6 where the residual activities of the enzyme have been plotted against the different pHs for which the enzyme has been exposed for 1 h. It was revealed that the enzyme had maximum stability at pH 5.0.

The dependence of the activity of the purified *P. linteus* MTCC-1175 laccase on variation of the temperature of the reaction solution is shown in Fig. 7. The cal-

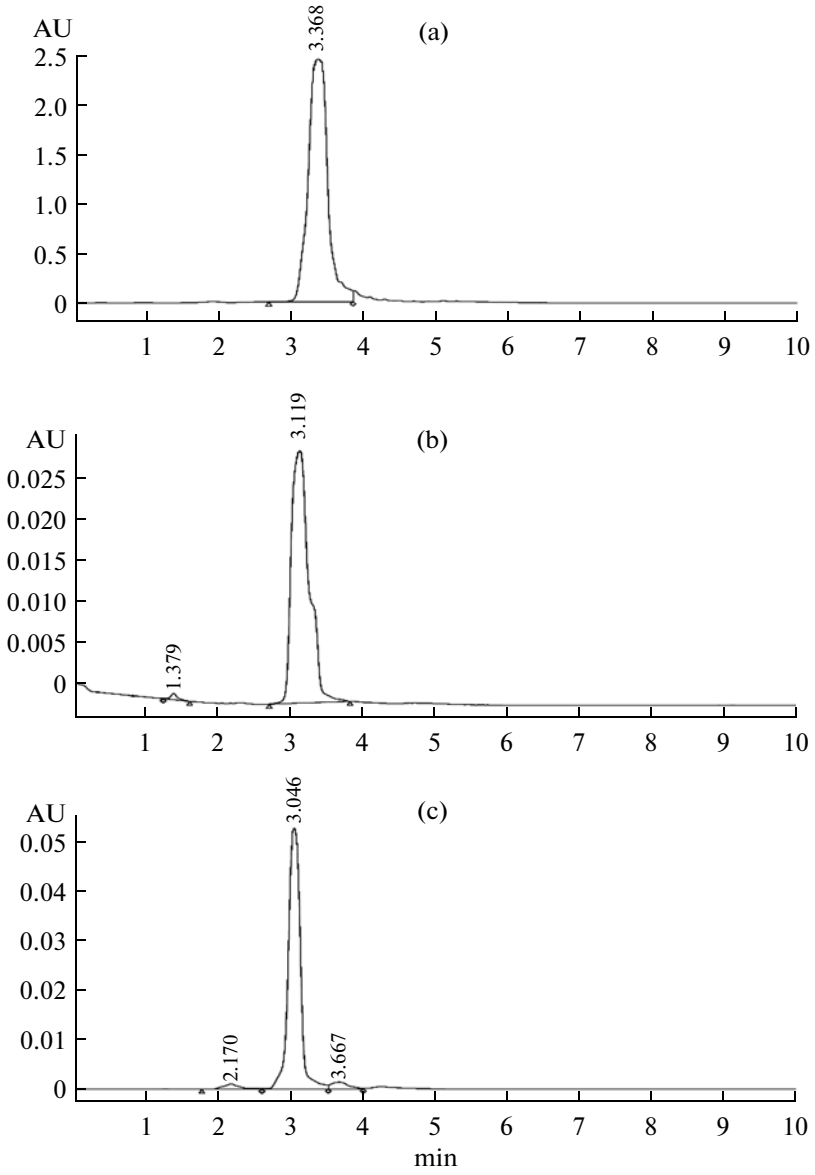

Fig. 8. Transformation of toluene to benzaldehyde by the laccase of *P. linteus* MTCC-1175 in presence of mediator. (a) and (b) the HPLC of the standard samples of toluene and bensaldehyde respectively, (c) the chromatogram of the ethyl acetate extract of the product formed by the enzyme with toluene in presence of mediator molecule.

culated temperature optimum was 45°C. The range of temperature optima reported for other laccases are 25–80°C [4]. The purified laccases of *Coriolus hirsutus, Thelephora terrestris* and *Volvariella volvacea* also have temperature optima at 45°C [4]. The results of the thermal stability of the purified laccase studied are shown in Fig. 7 where the residual activities of enzyme have been plotted against the temperatures for which the enzyme has been exposed for 1 h. The activation energy of the thermal denaturation of the purified *P. linteus* MTCC-1175 laccase has been found to be 38.2 kJ/mol/K.

One of the applications of the laccases in organic synthesis is the selective oxidation of aromatic methyl group to the aldehyde without further oxidation to carboxylic group. Blue laccases catalyze this reaction in presence of ABTS or 1-hydroxybenzotriazole as mediators [28, 29]. The applications of the purified *P. linteus* MTCC-1175 laccase for the above conversion were tested in presence of ABTS diammonium salts [28] using toluene, 3-nitrotoluene and 4-chlorotoluene as the substrates. The results of the bioconversion of toluene to benzaldehyde are shown in Fig. 8. Figure 8a is the HPLC chromatogram of the ethyl acetate extract of the product formed by the reaction of the enzyme with toluene in the presence of mediator molecule. Figure 8b and c are the chromatograms of the standard samples of benzaldehyde and toluene, respectively. The retention time of the standard samples of toluene and benzaldehyde were 3.4 and 3.1 min, respectively. Thus, the retention time of the product of the enzyme-catalyzed

reaction (3.1) coincided with that of benzaldehyde showing that the product was benzaldehyde. Yield of the extracted benzaldehyde was found to be 94%.

The similar type of reactions were done for 3-nitrotoluene bioconversion to 3-nitrobenzaldehyde and 4-chlorotoluene bioconversion to 4-chlorobenzaldehyde in the presence of ABTS as the mediator molecule. The retention time of the standard samples of 3-nitrotoluene, 4-chlorotoluene, 3-nitrobenzaldehyde and 4-chlorobenzaldehyde were 6.6, 7.3, 5.9 and 6.2 min, respectively. Thus, the retention time of the products of the enzyme catalyzed reaction (5.9 and 6.2 min) revealed that the products of enzyme catalyzed reaction were 3-nitrobenzaldehyde and 4-chlorobenzaldehyde. In these cases, yield of the extracted 3-nitrobenzaldehyde and 4-chlorobenzaldehyde was 98% and 80%, respectively.

This communication reports the purification of a laccase from a new fungal source, *P. linteus* MTCC-1175, which can be used for the selective transformation of aromatic methyl group to aldehyde group in presence of diammonium salt of ABTS as the mediator. The reaction is eco-friendly, requires less than 2 h and the yield of the product is more than 80%.

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