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Purification and Characterization of Laccase Secreted by *L. lividus*

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Abstract The culture conditions for maximum secretion of laccase by *Loweporus lividus* MTCC-1178 have been optimized. The laccase from the culture filtrate of *L. lividus* MTCC-1178 has been purified to homogeneity. The molecular weight of the purified laccase is 64.8 kDa. The enzymatic characteristics like K_m, pH, and temperature optimum using 2,6-dimethoxyphenol have been determined and found to be 480 μM, 5.0, and 60 °C, respectively. The K_m values for other substrates like catechol, m-cresol, pyrogallol, and syringaldazine have also been determined and found to be 230, 210, 320, and 350 μM, respectively.

Keywords Laccases · *L. lividus* · Lignolytic enzymes · Metalloenzymes · Cu containing enzymes · Lignolytic fungi

Laccases [E.C.1.10.3.2] are multicopper enzymes belonging to the group of blue oxidases [1–3]. They catalyze the oxidation of a variety of phenolic compounds as well as diamines and aromatic amines with concomitant reduction of molecular oxygen to water [4]. A general reaction scheme has been proposed as [1–5]:

$$4\,RH + O_2 \rightarrow 4\,R + 2H_2O$$

Laccases are widely distributed in higher plants [6, 7] and fungi [6–19], and laccase-like activity has been observed in some bacteria [19–21], insects [22], and wasp venom [23]. Though the role of laccase in living systems is not fully understood, it is an enzyme of varied biotechnological applications [24–27].

In view of the broad biotechnological applications of laccases [24–27], there is a scientific need to identify different sources of laccases having diverse properties so that suitable laccases for various applications [24–27] could be identified. Keeping these points in view, the authors have initiated studies on the characteristics of laccase produced by indigenous lignolytic fungal strains. In this communication, the secretion of laccase by a

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new fungal strain *Loweporus lividus* MTCC-1178 has been reported. The culture conditions for maximum production of laccase in the liquid culture medium of *L. lividus* MTCC-1178 have been optimized. A simple procedure for the purification of laccase from the culture filtrate of *L. lividus* MTCC-1178 has been developed, and the characteristics of laccase like K_m, pH, temperature optima, and thermal stability have been determined.

Materials and Methods

Syringaldazine (2-hydroxy-3, 5-dimethoxybenaldehyde azine) and DEAE cellulose were from Sigma Chemical Company, St. Louis, USA. 2,6-Dimethoxyphenol (DMP) was from Fluka, Chemi new Ulm, Switzerland. All other chemicals used in these investigations were either from Himedia Laboratory Ltd, Mumbai or from E. Merck (India) Ltd. Werli Road Mumbai and were used without further purifications. The chemicals used in the gel electrophoresis of the protein samples were from Geni Pvt. Ltd. Bangalore.

The fungal strain was procured from the Microbial Type Culture Collection Center and Gene Bank, Institute of Microbial Technology, Chandigarh, India and was maintained on agar slant as reported in the MTCC Catalogue of Strains—2000 [28]. The growth medium for the fungal strain *L. lividus* MTCC-1178 consisted of malt extract 20.0 g and agar 20.0 g in 1.0 L double distilled water, and the pH of the medium was 6.5.

The liquid culture medium GAE reported by Coll et al. [29] was used for screening the fungal strain for the production of extracellular laccase in the culture medium. This medium consisted of glucose 10.0 g, asparagine 1.0 g, yeast extract 0.5 g, and $MgSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot 7H_2O$, 0.01 g in 1.0 L of double distilled water. The above liquid culture medium containing natural lignin substrates like coir dust, corn cob, wheat straw, saw dust, and bagasse particles was separately prepared by adding 0.5 g of one of the natural lignin substrates to 25 mL of growth medium in 100 mL culture flasks which were sterilized. The sterilized growth medium was inoculated with a small piece of mycelium (0.5 cm×0.5 cm) under aseptic condition, and the fungal culture was grown under stationary culture condition at 30 °C in a BOD incubator. In order to monitor the production of laccase in the liquid culture medium, 0.5 mL aliquots of the growth medium were withdrawn at regular intervals of 24 h and filtered through a sterilized millipore filter of 0.22 μm. The filtered extract was analyzed for the activity of laccase using DMP and syringaldazine as the substrates. The assay solution of 1.0 mL consisted of the substrates DMP containing 1 mM 2,6-dimethoxyphenol in 50 mM sodium malonate buffer, pH 4.5 at 37 °C and syringaldazine containing 0.1 mM syringaldazine in 50 mM sodium phosphate buffer, pH 6.0 at 50 °C. In the case of DMP, the reaction was monitored by measuring the absorbance change at λ=468 nm and using the molar extinction coefficient value of 49.6 mM^{-1} cm^{-1}. In the case of syringaldazine, the reaction was monitored by measuring the absorbance change at λ=530 nm and using the molar extinction coefficient [30] value of 64.0 mM^{-1} cm^{-1}. The UV/Vis spectrophotometer Hitachi (Japan) model U-2000 fitted with an electronic temperature control unit was used for absorbance measurement. The least count of absorbance measurement was 0.001 absorbance unit. One enzyme unit produced 1 μmol of the product per minute under the specified assay conditions.

Extracellular secretion of the laccase in the liquid culture medium by *L. lividus* MTCC-1178 was determined by plotting the enzyme unit per milliliter of the growth medium against the number of days after inoculation of the fungal mycelia. Each point on the curve is an average of three measurements. The growth medium for the control experiment has the same composition except that no natural lignolytic substrate has been added. The best

inducer of laccase was identified with the help of drawing secretion curves using different inducers. In order to optimize the conditions for maximum production of laccase by *L. lividus* MTCC-1178 in the liquid culture medium, the amount of the best inducer (bagasse in this case) was varied from 100 to 1,000 mg in 25 mL of the growth medium. In this case also the enzyme unit per milliliter of the growth medium was plotted against the number of days after the inoculation of the fungal strain. The amount of the inducer in the growth medium which gave the maximum height of the enzyme activity peak was taken as the optimal amount of the inducer.

For the purification of laccase, *L. lividus* MTCC-1178 was grown in 10×25 mL sterilized growth medium in ten 100 mL culture flasks containing an optimal amount of the inducer (bagasse in this case) under stationary culture condition in a BOD incubator at 30 °C. The maximum activity of the laccase appeared on the ninth day of inoculation of the fungal mycelia. On the ninth day, all the cultures in the ten flasks were pooled, and mycelia were removed by filtration through four layers of cheese cloth. The culture filtrate was saturated up to 30% with ammonium sulfate and centrifuged using refrigerated centrifuge Sigma (Germany) model 3K-30 at 12,500 rpm (10,354×*g*) for 20 min at 4 °C. The precipitate was discarded and the supernatant was saturated up to 80% by further addition of ammonium sulfate. The resulting suspension was centrifuged using the same process of centrifugation and the supernatant was discarded. The precipitate was dissolved in 10 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 6.5) and dialyzed against 10 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 6.5) in a volume ratio of 1:1,000, with three changes at intervals of 8 h. A 10-mL enzyme sample containing 1.0 mg/mL protein was loaded on to a DEAE column (size 1.0 cm×37 cm) which was pre-equilibrated with 10 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 6.5), and the flow rate was 18 mL/h. The column was washed with 60 mL of the same buffer. The enzyme was eluted by applying linear gradient of 1.0 M NaCl in the same buffer (70 mL buffer+70 mL buffer with 1.0 M NaCl). The fractions of 9.0 mL size were collected and analyzed for laccase activity and protein concentration. The active laccase fractions were pooled and concentrated by solid sucrose to 5.0 mL. The protein estimation was done by the Lowery method [31].

The K_m values were determined by measuring steady state velocities of the laccase-catalyzed reaction at different substrate concentrations and drawing double reciprocal plots [32, 33]. The pH optimum was determined by measuring the steady state velocities of laccase-catalyzed reaction in the solutions having different pH values and plotting steady state velocities against the pH values of the reaction solutions. Similarly, the temperature optimum was determined by measuring the steady state velocities in reaction solutions at different temperatures and plotting the steady state velocities against temperatures of the reaction solutions. The thermal stability was determined at different temperatures by allowing the enzyme to stay at that temperature and monitoring the activity at intervals of 1 h and plotting a graph of activity against the time duration for which the enzyme has been exposed to that temperature. The substrate specificity of the purified laccase was determined by measuring steady state velocities of the laccase-catalyzed reaction using 2,6-dimethoxyphenol, catechol, m-cresol, pyrogallol, and syringaldazine at various concentrations and drawing double reciprocal plots and calculating K_m values for all the above substrates.

Results and Discussion

L. lividus MTCC-1178, a white rot causing fungi, has been isolated from the logs of *Shorea robusta* [28] (family name Dipterocarpaceae). Since it causes white rot, secretion of

lignolytic enzymes like ligninperoxidase, Mn peroxidase, and laccase is expected in its liquid culture growth medium. The screening of the liquid culture growth medium containing natural lignolytic substrates like coir dust, corn cob, wheat straw, and saw dust and bagasse for extracellular secretion of ligninperoxidase, Mn peroxidase, and laccase has given positive results (unpublished results). It is worth mentioning that Saparrat et al. [34] have also screened the liquid culture growth medium of an Argentinean lignolytic fungal strain *L. lividus* CLPS 289 isolated from rotten wood of a subtropical rainforest for the extracellular secretion of lignolytic enzymes, but they have not been able to detect these enzymes under their liquid culture conditions though extracellular ABTS oxidizing activity in the solid medium of the same fungal strain has been reported [35]. The above differences may be due to different culture conditions used by the two different research groups of workers or may be due to different strains.

Figure 1 shows the secretion of laccase in the liquid culture growth medium amended with various lignin containing natural substrates like corn cob, coir dust, saw dust, wheat straw, and bagasse particles and inoculated with *L. lividus* MTCC-1178. The control experiment has similar medium composition as the experimental one except the absence of the natural lignolytic substrate. It is obvious from the figure that lignin containing natural substrates when present in the liquid culture growth medium enhanced the secretion of laccase. This enhancement of extracellular secretion of laccase is maximum in the case of the growth medium containing bagasse particles. The results of optimization of culture conditions for maximum secretion of laccase by *L. lividus* MTCC-1178 have been shown in Fig. 2. It is obvious from Fig. 2 that the maximum laccase secretion occurs in the liquid culture growth medium containing 800 mg of bagasse particles in a culture volume of 25 mL at 30 °C under stationary culture condition. The maximum level of the laccase secreted in the culture medium was 0.26 IU/mL.

The purification chart for the purification of the laccase from the liquid culture filtrate of *L. lividus* MTCC-1178 is shown in Table 1. The method gives 67.6-fold purification with

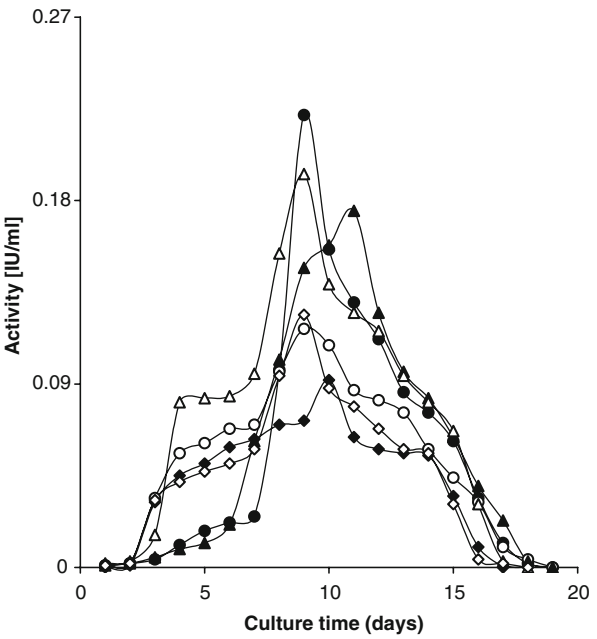

Fig. 1 Secretion of laccase by *Loweporus lividus* MTCC-1178 in the liquid culture medium supplemented with different lignin containing natural substrates: bagasse (*filled circle*), coir dust (*open circle*), corn cob (*filled triangle*), wheat straw (*open triangle*), saw dust (*open square*), control (*filled square*)

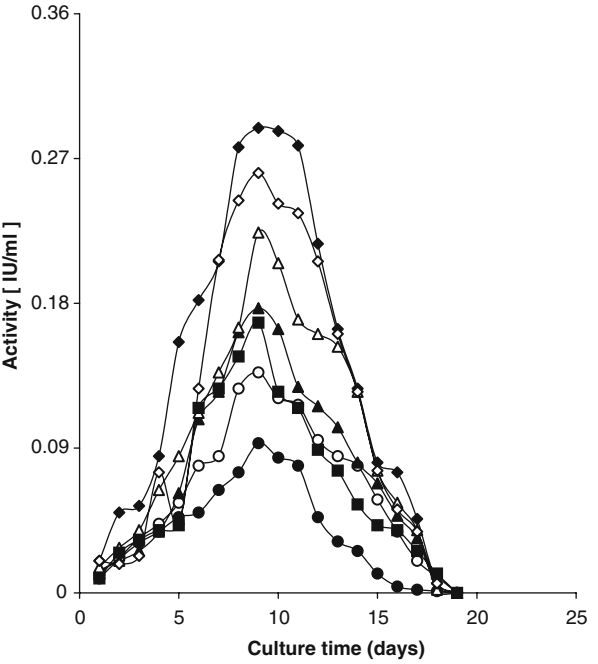

Fig. 2 Optimization of laccase by *Loweporus lividus* MTCC-1178 in the liquid culture medium supplemented with different concentrations of bagasse particles: 100 mg (*filled circle*), 200 mg (*open circle*), 400 mg (*filled triangle*), 500 mg (*open triangle*), 600 mg (*filled square*), 800 mg (*open square*), 1,000 mg (*filled diamond*)

8.7% recovery of the enzyme activity. The elution profile of the laccase activity from the DEAE cellulose column is shown in Fig. 3. A single activity peak on anion exchange chromatography indicates that there are no multiple isozyme forms of laccase in the culture filtrate. Moreover, the enzyme activity peak coincides with one of the protein peak indicating that the enzyme might be pure. The results of SDS-PAGE are shown in Fig. 4. The presence of single bands in lanes 2 and 3 clearly shows that the enzyme preparation is pure and the calculated relative molecular mass is 64.8 kDa. The relative molecular masses of laccases purified from the culture filtrates of *Ganoderma lucidium*, *Chaetomium thermophilium*, *Neurospora crassa*, and *Gaeumannomyces graminis* var. *tritici* are 68, 77, 64.8, and 60 kDa, respectively.

The double reciprocal plots using 2,6-dimethoxyphenol as variable substrate are shown in Fig. 5. Similar plots using catechol, m-cresol, pyrogallol, and syringaldazine as the variable substrates were also made but are not given here. The calculated K_m values for the above substrate were 230, 210, 320, and 350 μM, respectively. The K_m values for the laccase purified from *G. graminis* var. *tritici* using 2,6-dimethoxyphenol, catechol, and

Table 1 Purification chart.

Step no.	Steps	Volume (mL)	Activity (IU/mL)	Protein (mg/mL)	Total activity	Total protein	Specific activity (IU/mg)	Purification fold	Percent recovery
1	Crude enzyme	250	0.055	2.975	13.75	743.7	0.018	1	100
2	Ammonium sulfate	10	0.456	1.0	4.52	10.0	0.456	25.33	32.72
3	DEAE	2	0.504	0.85	1.2	1.70	1.41	67.6	8.7

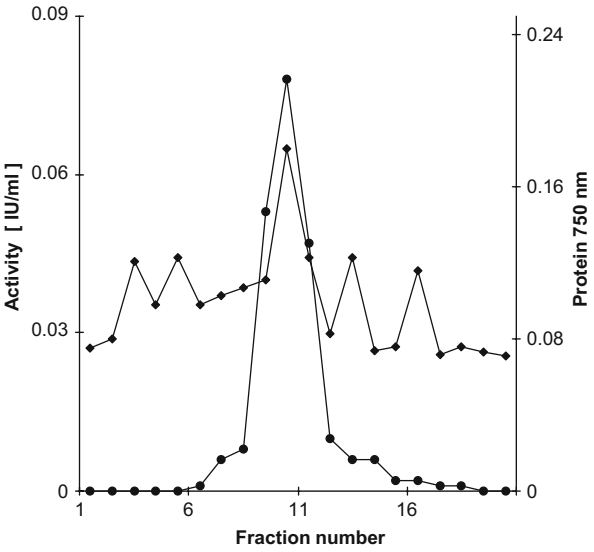

Fig. 3 Elution profile from the DEAE cellulose column, activity (*filled circle*) protein (*filled square*)

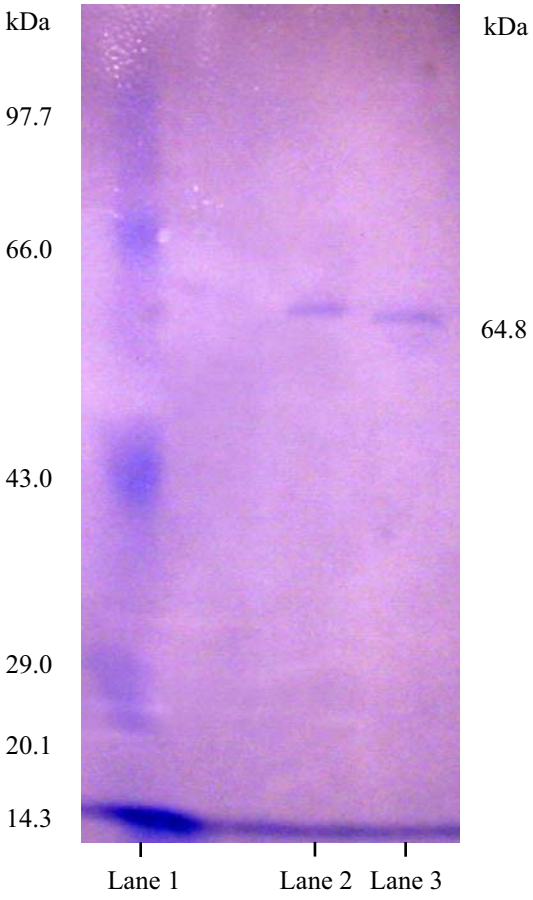

Fig. 4 SDS-polyacrylamide gel electrophoresis. Molecular wt. markers (lane 1) purified laccase (lane 2, lane 3)

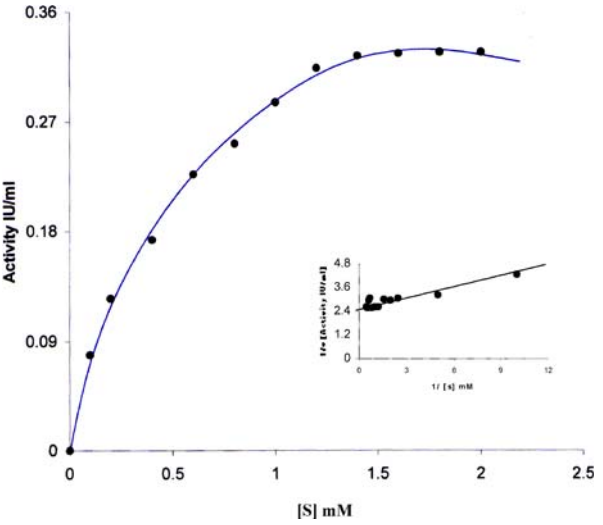

Fig. 5 The double reciprocal plot 2,6-dimethoxyphenol as substrate. The calculated K_m value for DMP is 480 μM. Similar results are obtained for the other substrates catechol, m-cresol, pyrogallol, and syringaldazine, so the double reciprocal plots for the other substrates are not given here. The calculated K_m values for substrate catechol, m-cresol, pyrogallol, and syringaldazine were 230, 210, 320, and 250 μM, respectively

pyrogallol are 26, 250, and 310 μM, respectively. Thus, the purified laccase from *L. lividus* MTCC-1178 has very low affinity for 2,6-dimethoxyphenol as compared to the laccase purified from *G. graminis* var. *tritici* whereas the affinities of the both laccases for catechol and pyrogallol are of the same order of magnitude.

The variation of the activity of the laccase with the variation of pH of the reaction solution is shown in Fig. 6 using 2,6-dimethoxyphenol as the substrate. It is obvious from the figure that the pH optimum of the purified laccase is 5.0, but the enzyme retains more than 50% of its maximum activity in the pH range of 3.5–5.5. The pH optima of the laccase purified from *G. lucidium*, *C. thermophilium*, and *G. graminis* var. *tritici* reported in the literature [35–38] are 3.5, 6.0, and 4.5, respectively. Thus, the pH optimum of the laccase purified from the culture filtrate of *L. lividus* MTCC-1178 lies in the region reported for the pH optima of laccases purified from the culture filtrates of other fungal strains. The variation of the activity of the purified laccase with temperature is shown in Fig. 7. The activity is maximum at 60 °C and the enzyme has more than 50% of its maximum

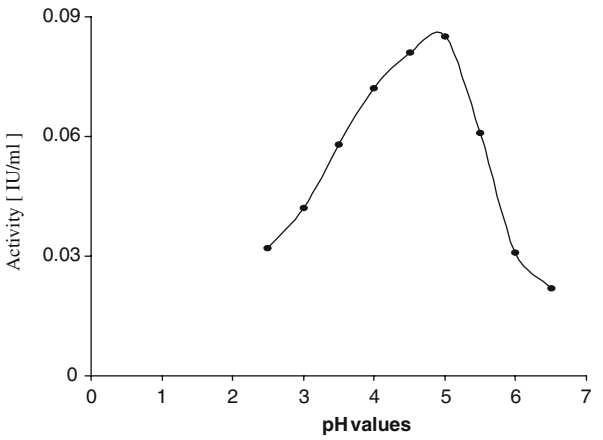

Fig. 6 Variation of activity of laccase of *Loweporus lividus* MTCC-1178 with variation of reaction pH

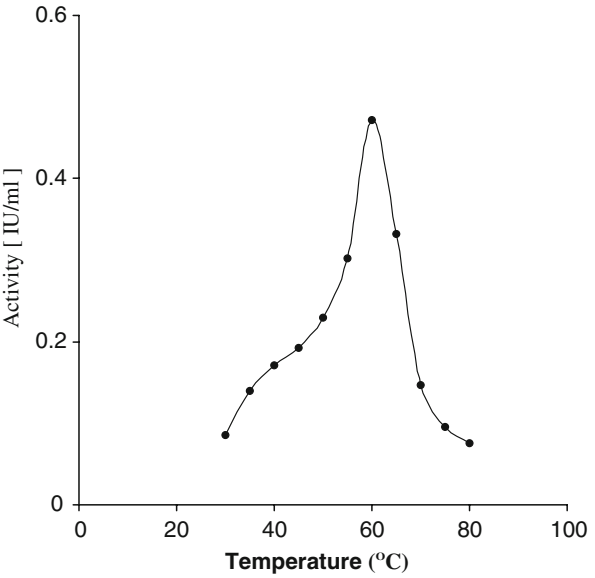

Fig. 7 Variation of activity of laccase of *Loweporus lividus* MTCC-1178 with variation of reaction temperature

activity at temperature above 40 °C. The temperature optima of the laccases purified from the culture filtrates of *G. lucidium*, *Pleurotus eryngii*, *Daedalea quercina*, and *Streptomyces cyaneus* CECT-3335 are 40, 65, 60, and 70 °C, respectively. Thus, the temperature optimum of the purified laccase is also in the range of the temperature optima reported for the other fungal laccases. The results of the studies on the thermal stability of laccase are shown in Fig. 8 where the activities of the enzyme have been plotted against time intervals for which the enzyme has been exposed to different temperatures. As shown in Fig. 8, it follows that the enzyme is stable at 60 °C for 2 h.

In conclusion, this communication reports a laccase which is easy to purify using only one ion exchange chromatography step, but the characteristics of this laccase are similar to other reported fungal laccases.

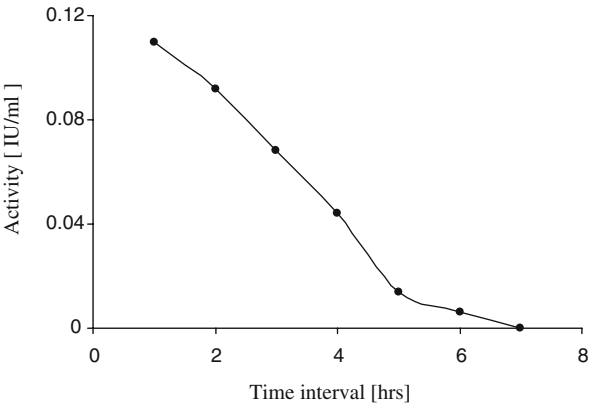

Fig. 8 Thermal stability of the purified laccase

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