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PURIFICATION AND CHARACTERISATION OF LIGNIN PEROXIDASE FROM *Pycnoporus sanguineus* MTCC-137

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Extracellular secretion of lignin peroxidase from *Pycnoporus sanguineus* MTCC-137 in the liquid culture growth medium amended with lignin containing natural sources has been shown. The maximum secretion of lignin peroxidase has been found in the presence of saw dust. The enzyme has been purified to homogeneity from the culture filtrate of the fungus using ultrafiltration and anion exchange chromatography on DEAE-cellulose. The purified lignin peroxidase gave a single protein band in sodium dodecylsulphate polyacrylamide gel electrophoresis corresponding to the molecular mass 40 kDa. The K_m, k_{cat} and k_{cat}/K_m values of the enzyme using veratryl alcohol and H_2O_2 as the substrate were 61 μM, 2.13 s^{-1}, 3.5×10^4 M^{-1} s^{-1} and 71 μM, 2.13 s^{-1}, 3.0×10^4 M^{-1} s^{-1} respectively at the optimum pH of 2.5. The temperature optimum of the enzyme was 25°C.

The lignin decomposing basidiomycete white rot fungi secrete a hemeprotein, lignin peroxidases (**LiP**, EC 1.11.1.7) which in presence of H_2O_2 degrades lignin and lignin model compounds [1]. The sequence of catalysis is given below:

$$\text{LiP } (Fe^{3+})\ P + H_2O_2 \rightarrow \text{LiP-I}(Fe^{4+} - O)P^{+\cdot} + H_2O, \quad (1)$$

$$\text{LiP-I } (Fe^{4+}\text{-O})\ P^{+\cdot} + R \rightarrow \text{LiP-II } (Fe^{4+}\text{-O})P + R^{\cdot}, \quad (2)$$

$$\text{LiP-II } (Fe^{4+}\text{-O})\ P + R + 2H^+ \rightarrow \text{LiP } (Fe^{3+})P + R^{\cdot} + H_2O, \quad (3)$$

where R is the organic substrate and P is porphyrin. LiP compound I (LiP-I) carries both oxidizing equivalents of H_2O_2, one as an oxyferryl (Fe^{4+}-O) center and one as a porphyrin π cation radical ($P^{+\cdot}$), whereas LiP compound II (LiP-II) carries only one oxidizing equivalent. The substrate R is oxidized by compound I to an aryl cation radical which with subsequent nonenzymatic reactions yield the final products. LiP is a biotechnologicaly important enzyme having wide potential applications (I) in delignification of lignocellulosic materials [2] which are seen as an alternative to the depleting oil reserves, (II) in the conversion of coal to low molecular mass fractions [3] which could be used as a feed stock for the production of commodity chemicals, (III) in biopulping and biobleaching [4] in paper industries, (IV) in removal of recalcitrant organic pollutants [5–9] and (V) in the enzymatic polymerization [10] in polymer industries. Keeping in view the biotechnological potential of LiP, the authors have initiated enzymatic studies on the LiP from indigenous fungal strains. LiP of *Phanerochaete chyrosoporium* has been extensively studied [11–13] and LiP of *Trametes versicolor* [12], *Pleurotus ostreatus* [13], *Polyporus ostroiformis* [14], *Ganoderma lucidum* [15], *Aspergillus terreus* [16], *Fusarium oxysporum* [16], *Pencillium citrinum* [16], *Rizopus nigricans* [17], *Pleurotus sajor-caju* [18], *Abortiporus biennis* [19], *Pestalotia bicolor* [19], *Heterobasidium annosum* [19], *Gloephyllum striatum* [19], *Loweporus lividus* [19] have been reported. The fungal strain *Pycnoporus sanguineus* MTCC-137 is a white rot fungus isolated from *Shorea robusta* and is reported to secrete laccase but its lignin peroxidase has not been reported so far. Our research interest in lignin peroxidases has prompted us to screen this fungal strain for the secretion of lignin peroxidase. The culture condition for the maximum secretion of the enzyme has been optimized. The enzyme has been purified and enzymatic characteristics like K_m, pH and temperature optima have been determined.

MATERIALS AND METHODS

Veratryl alcohol, which is 3,4-dimethoxy benzyl alcohol was from Aldrich (USA). Nitriloacetate was from Sigma Chemical Co. (USA). The chemicals used in gel electrophoresis including protein molecular weight markers were procured from Bangalore Genei Pvt. Ltd. (India). All other chemicals were either from CDH (Delhi) or Loba Chemie (Mumbai) or SD Fine Chem Limited (Worli, Mumbai) and were used without further purification.

Fungal strain. The fungal strain was procured from Microbial Type Culture Collection Center and Gene Bank, Institute of Microbial Technology, Chandigarh and was maintained on agar slants [20]. The medium used for agar slants for the fungal strain *Pycnoporus*

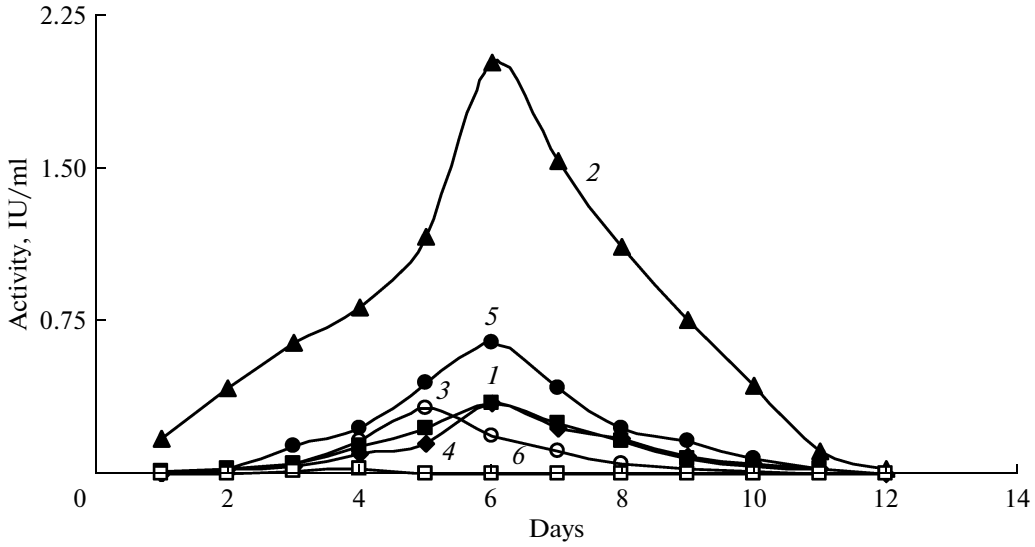

Fig. 1. Secretion of ligninperoxidase by *P. sanguineus* MTCC-37 in the liquid culture medium supplemented with different natural lignin containing substrates: coirdust (*1*), sawdust (*2*), bagasse (*3*), corn-cob (*4*), wheat straw (*5*), and without substrate.

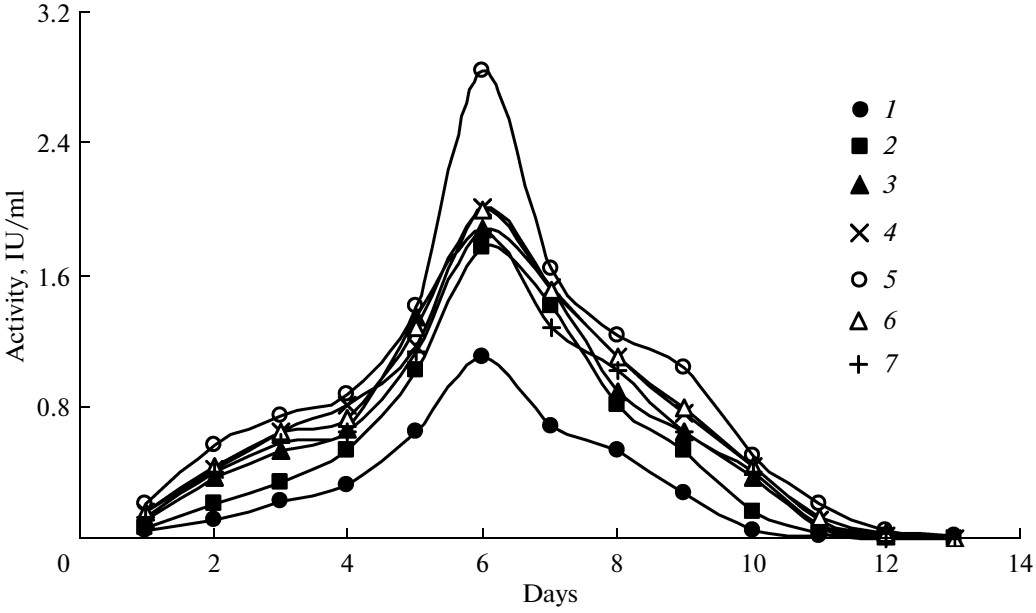

Fig. 2. Optimisation of secretion of ligninperoxidase in the liquid culture medium amended with sawdust (*1–7*) by the fungal strain *P. sanguineus* MTCC-37.
1 – 100, *2* – 200, *3* – 400, *4* – 500, *5* – 600, *6* – 800, *7* – 1000 mg.

sanguineus MTCC-137 consisted of (g/l): glucose – 10, yeast extract – 5.0 and agar – 15.0, dissolved in double distilled water.

Enzyme assay. The lignin peroxidase activity has been assayed [11] by monitoring the formation of veratraldehyde spectrophotometrically at $\lambda = 310$ nm using veratryl alcohol as a substrate with UV/VIS spectrophotometer Hitachi (Japan) model U-2000, which was fitted with electronic temperature control unit. Molar extinction coefficient value 9300 $M^{-1}\,cm^{-1}$ for veratraldehyde was used to calculate the enzyme unit. One unit (IU) of lignin peroxidase was defined as the amount of enzyme, which converts one μM of veratryl alcohol to veratraldehyde under the standard assay condition. The least count of absorbance measurement was 0.001 absorbance unit.

Secretion and optimization. For the secretion of lignin peroxidase, the fungal strain was grown in a medium

Table 1. Purification steps

Steps	Total volume	Protein, mg/ml	Activity, U/ml	Specific activity, U/mg	Total protein, mg	Total activity, U	Purification fold	Recovery, %
1. Crude enzyme	720	0.20	0.28	1.41	144	203.04	1.00	100
2. Concentrated enzyme	10	1.43	3.12	2.17	14.30	31.16	1.53	15.30
3. Dialysed enzyme	18	0.51	1.35	2.64	9.18	24.30	1.87	11.96
4. DEAE cellulose	30	0.23	0.74	3.19	6.90	22.0	2.26	10.85

containing (g/l): glucose – 10.0, ammonium tartrate – 1.32, KH_2PO_4 – 0.2, $MgSO_4 \cdot 7H_2O$ – 50 mg, $CaCl_2$ – 10.0 mg, thiamine – 10.0 μg/l and 1.0 ml of a solution containing (g/l): $MgSO_4 \cdot 7H_2O$ – 3.0, $MnSO_4 \cdot H_2O$ – 0.5, NaCl – 1.0, and (mg/l): $FeSO_4 \cdot 7H_2O$ – 100, $CoCl_2 \cdot 6H_2O$ – 185, $CaCl_2$ – 80, $ZnSO_4 \cdot 7H_2O$ – 180, $CuSO_4 \cdot 5H_2O$ – 10.0, $AlK(SO_4)_2$ – 10.0, H_3BO_3 – 10.0, $Na_2MoO_4 \cdot 2H_2O$ – 12.0 and nitrilotriacetate – 1.5 g. The pH of basal medium was adjusted to 4.5 with 20 mM dimethyl succinate.

Extracellular secretion of lignin peroxidase in the liquid culture medium by *P. sanguineus* MTCC-137, was determined by inoculating a small piece of mycelia of size 5 mm diameter aseptically in 100 ml sterilized culture flask containing 25 ml of the growth medium amended with 500 mg of lignin containing natural substrates like coir-dust, sawdust, bagasse particles, corncob, wheat-straw and one control, the growth medium of which was not amended with natural lignin containing substrate. The culture was grown under stationary condition in a BioChemical Oxygen Demand Incubator (B.O.D., India) at 30°C. 0.5 ml of the growth medium was withdrawn at the regular interval of 24 h, was filtered through Millipore (France) Millex-GS filter (0.22 μm) unit and was assayed using the method described above [11]. Enzyme unit/ml was plotted against the number of days of growth and the best inducer was decided on the basis of the enzyme activity peak height.

In order to optimise the culture conditions for maximum production of lignin peroxidase by the above mentioned fungal strain, the amount of the best inducer sawdust was varied from 100 to 1000 mg in 25 ml of the growth medium as mentioned above. In this case also the enzyme units/ml 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.

Purification. The enzyme was purified by growing the fungal strain in thirty sterilized 100 ml culture flasks each containing 25 ml of the liquid culture growth medium containing optimal amount of sawdust 600 mg. The maximum activity appeared in the growth medium on the 5th day after inoculation of the fungal mycelia. When the maximum activity appeared in the culture flasks, the cultures of all flasks were

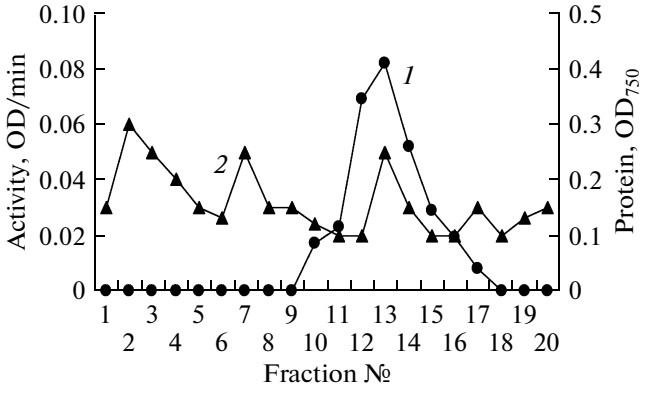

Fig. 3. Elution profile of the lignin peroxidase from DEAE cellulose column.
1 – the activity, D_{310}; *2* – protein, D_{750}.

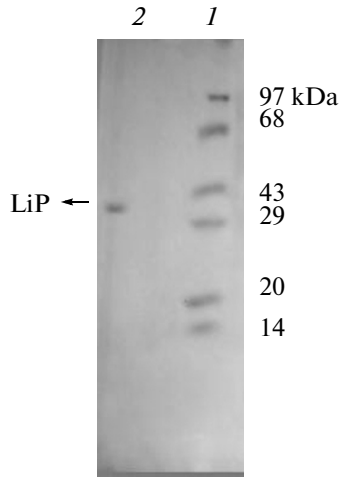

Fig. 4. SDS–PAGE analysis results of the lignin peroxidase. Lane *1* – the molecular weight markers (from top): phosphorylase (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soya bean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa); Lane *2* – the purified ligninperoxidase (20 μg).

Table 2. The enzymatic characteristics for lignin peroxidases from different sources using veratryl alcohol and H_2O_2 as the substrates

Fungal strains	K_m for veratryl alcohol, μM	K_m for H_2O_2, μM	Temperature, °C	pH
Phanerochaete chrysosporium	60	80	26	3.0
Penicillium citrinum	69	64	30	4.0
Fusarium oxysporium	64	72	25	2.3
Aspergillus terreus	60	80	22	2.0
Loweporus lividus	58	83	24	2.6
Gloeophyllum sepiarium	55	75	25	2.5
*Pycnoporus sanguineus**	61	71	25	2.5

* Present study.

pooled, mycelia was removed by filtration through 4 layers of cheese cloth. The culture filtrate 720 ml with 0.282 IU/ml activity was concentrated with Amicon Concentration Cell Model 8200 (USA) using PM-10 ultrafitration membrane with molecular weight cut-off value 10 kDa to 10 ml. The concentrated enzyme was dialysed against 1000 times excess of 5 mM sodium succinate buffer pH 5.5 overnight at 20°C. The dialysed enzyme was loaded on a DEAE column of 1 cm diameter and 16 cm height which was preequilibrated with the same buffer. The adsorbed enzyme was washed with 50 ml of the same buffer and was eluted by applying NaCl gradient (0–200 mM; 50 ml/50 ml). Fractions (5 ml) were collected and analysed for lignin peroxidase activity [11] and protein concentration [21]. The active fractions were combined and concentrated with the Amicon Concentration Cell Model 8200, and there after with Model-3 using ultrafiltration membrane PM10. The concentrated enzyme was stored at 4°C and was used for further studies. The enzyme did not loose activity for two months under these conditions.

SDS-Polyacramide gel electrophoresis. The homogeneity of the enzyme preparation was checked by SDS–PAGE analysis using the method of Weber and Osborn [22]. The separating gel was 12% acrylamide in 0.375 M Tris-HCl buffer pH 8.8 and stacking gel was 5% acrylamide in 0.063 M Tris-HCl buffer 6.8. Proteins were visualized by staining with Coomassie Brilliant Blue R – 250.The molecular weight markers were phosphorylase (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soya bean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa). Gel was run at a constant current of 20 mA using Technosource (Mumbai, India) mini vertical gel electrophoresis equipment model Monokin.

Steady state enzyme kinetics. The steady state enzyme kinetics of the purified lignin peroxidase was studied by measuring steady state rate of formation of veratraldehyde spectrophotometrically at 310 nm and using the molar extinction coefficient value of 9300 M^{-1} cm^{-1} using veratryl alcohol as the substrate. The K_m and V_{max} values for the substrate veratryl alcohol were determined by measuring the steady state velocity of the enzyme catalysed reaction at different concentrations of veratryl alcohol (range 0.1–2.5 mM) at the fixed enzyme saturating H_2O_2 concentration 0.4 mM in

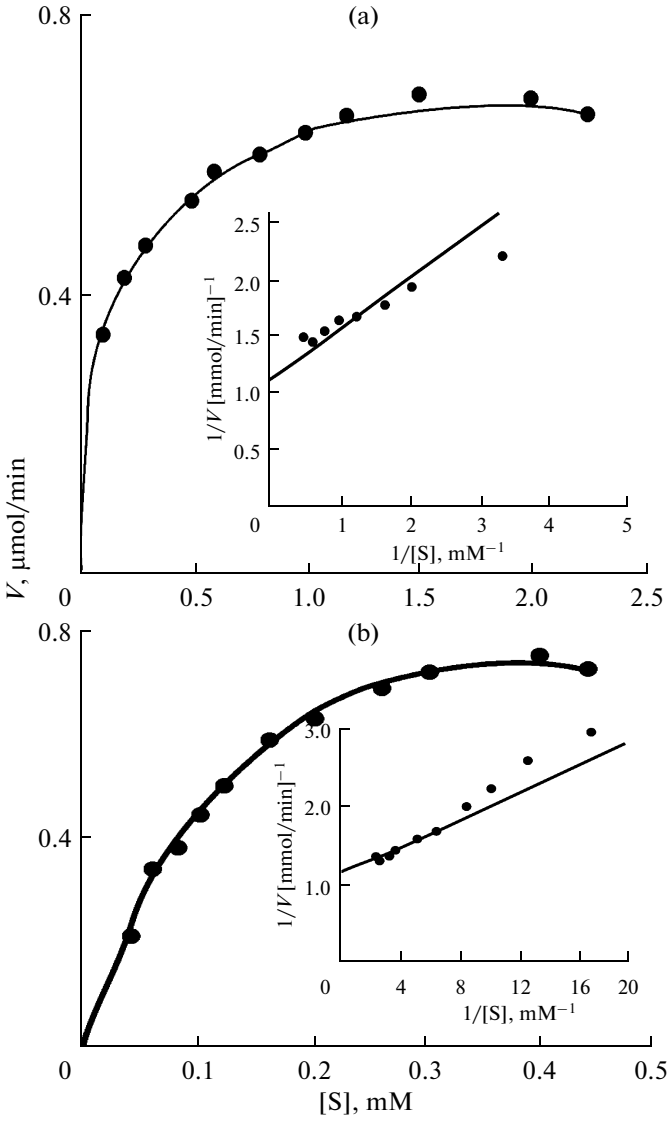

Fig. 5. Michaelis–Menten and double reciprocal plots for veratryl alcohol (a) and for H_2O_2 (b) as the substrate.

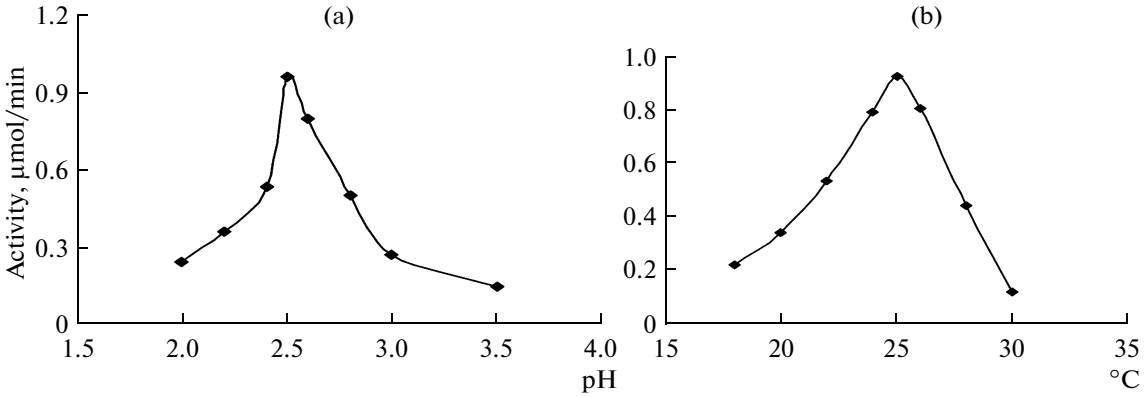

Fig. 6. The pH (a) and temperature (b) optima of the purified lignin peroxidase.

50 mM sodium tartrate buffer pH 2.5 at 25°C. The K_m and V_{max} were calculated by the linear regression analysis of the double reciprocal plot.The same procedure was adopted for the determination of K_m and V_{max} for H_2O_2 except that H_2O_2 concentration was varied in the range 0.05–0.5 mM at the fixed enzyme saturating concentration 2 mM of veratryl alcohol.

The pH optimum of the purified lignin peoxidase was determined by measuring the steady state velocity of the enzyme catalysed reaction in reaction solutions of varying pH values (range 2.0–3.5 pH) at the fixed temperature of 25°C. 1 ml reaction mixture consists of 0.4 mM H_2O_2, 2 mM veratryl alcohol, in 50 mM sodium tartrate buffer at variable pH. The pH optimum was calculated by plotting steady state velocity at different pH versus pH of the reaction solution. A similar procedure was adopted for the determination of the temperature optimum except that the steady state velocity was determined at the solutions of varying temperature (18°C to 30°C) at a fixed pH of 2.5. 1 ml reaction mixture consists of 0.4 mM H_2O_2, 2 mM veratryl alcohol in 50 mM sodium tartrate buffer pH 2.5 at variable temperature.

RESULTS AND DISCUSSION

The fungal strain was screened for the secretion of lignin peroxidase in its liquid culture growth medium containing separately sawdust, coirdust, wheatstraw, corncob, bagasse particles and a control experiment in which non of the above mentioned substrates were added. It is obvious from the Fig. 1 that lignin containing natural substrates induce the secretion of lignin peroxidase and sawdust is the best inducer. The culture conditions for the maximum secretion of lignin peroxidase in its liquid culture growth medium were optimized by varying the amount of the best inducer sawdust. The results are shown in Fig. 2. Results show that 600 mg of sawdust contained in 25 ml culture medium in 100 ml culture flask gave the maximum secretion.

The procedure for the purification of lignin peroxidase from *P. sanguineus* cuture filtrate has already been described in material and method section. The results of purification procedure are summarized in Table 1. It involved concentration by ultrafiltration and an ion exchange chromatography on anion exchanger DEAE-cellulose. The elution profile of the lignin peroxidase from DEAE cellulose is shown in the Fig. 3. The active fractions were combined, concentrated and analysed by SDS-PAGE for the purity of the enzyme. The results of SDS-PAGE analysis are shown in Fig. 4 in which lane *1* contains protein molecular weight markers and lane *2* contains the purified lignin peroxidase. The presence of single protein band in lane 2 clearly showed that the enzyme was pure. The molecular mass calculated from the SDS-PAGE analysis was 40 kDa. The value of molecular mass of the purified lignin peroxidase lies in the range (38–46 kDa) of molecular masses reported [11] for the different isoenzymes secreted by *Phanerochaete chrysosporium*. In presence of saw-dust in the liquid culture growth medium, *P. sanguineus* secretes only one form of lignin peroxidase where as in case of *P. chrysosporium* 10 isoenzymes of varying molecular masses and specific activities have been reported [11].

The Michaelis Menten and double reciprocal plots using veratryl alcohol and H_2O_2 as variable substrates are shown in Fig. 5. 1 ml reaction mixture consists of 0.1–2.5 mM veratryl alcohol as the variable substrate, 0.4 mM H_2O_2 in 50 mM sodium tartrate buffer pH 2.5 at 25°C or 1ml reaction mixture consists of 0.05–0.5 mM H_2O_2 as the variable substrate, 2 mM veratryl alcohol, in 50 mM sodium tartrate buffer pH 2.5 at 25°C.

The K_m, k_{cat} and k_{cat}/K_m values for veratryl alcohol and H_2O_2 are 61 μM, 2.13 s^{-1}, 3.5×10^4 M^{-1} s^{-1} and 71 μM, 2.13, 3.0×10^4 $M^{-1}s^{-1}$ respectively. The K_m values for the lignin peroxidase of *P. chrysosporium* using veratryl alcohol and H_2O_2 as the variable substrates have been reported [11] to be 60 and 80 μM respectively. The k_{cat}

and k_{cat}/K_m values calculated from the available data in the literature for different isozymes of lignin peroxidase of *P. chrysosporium* have also been found in the same range (3.5×10^4 to 17.5×10^4 $M^{-1} s^{-1}$) assuming that all isozymes have the same K_m value for veratryl alcohol and H_2O_2 of 60 and 80 μM as reported in the literature [11]. The pH and temperature optima (Fig. 6) of the purified lignin peroxidase were also in the same range as the values reported for the lignin peroxidases from other sources given in Table 2.

In conclusion, this communication reports a lignin peroxidase of *P. sanguineus* MTCC-137 secreted in culture medium containing sawdust. Only one form of the enzyme is secreted in the culture medium and the enzymatic properties of the enzyme are similar to the properties reported for lignin peroxidases of *P. chrysosporium* ATCC-24725.

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REFERENCES

1. Edwards, S.L., Raag, R., Wariishi, H., and Michael, H.G., *Proc. Natl. Acad. Sci. USA,* 1993, vol. 90, pp. 750–754.
2. Harley, B.S., Brodo, P.M.A., and Senior P.J., Proceeding of Royal Society Discussion Meeting on Utilisation of Lignocellulosic Wastes, Cambridge: Cambridge Univ. Press, 1988.
3. Catcheside, D.E.A. and Ralph, J.P., *Appl. Microbiol. Biotechnol.,* 1999, vol. 53, pp. 16–24.
4. Eriksson, K.E. and Kirk, T.K., *FEMS Microbiol. Rev.*, 1994, vol. 13, pp. 351–364.
5. Bumpus, J.A., Tien, M., Wright, D., and Aust, S.D., *Science*, 1985, vol. 228, pp. 1434–1436.
6. Kwant, S.S. and Chang, J.K., *Biotechnol. Letters,* 1998, vol. 20, pp. 569–572.
7. Satwinder, S.M., Rajesh, G., Chand, P., and John, F.K., *J. Chem. Technol. Biotechnol.*, 1998, vol. 73, pp. 292–296.
8. Levin, L., Papinutti, L., and Forchiassin, F., *Biores. Technol.*, 2004, vol. 94, pp. 169–176.
9. Cenek, N., Katerina, S., Pavla, E., Tomas, C., Aparna, K., Elke, L., and Vaclav, S., *Soil Biol. Biochem.*, 2004, vol. 36, pp. 1545–1551.
10. Hiroshi, U. and Shiro, K., *CHEMTECH*, 1999, vol. 29, p. 22.
11. Tien, M. and Kirk, T.K., *Methods in Enzymology*, 1988, vol. 161, pp. 238–249.
12. Vyas, B.R.M., Vole, J., and Saaek, V., *Folia Microbiol.*, 1994, vol. 39, pp. 235.
13. Kang, S., Shin, K.S., Han, Y.H., Youn, H.D. and Hah, Y.C., *Biochem. Biophys. Acta*, 1993, vol. 1163, p. 158.
14. Satyahari, D., Tapas K. Maity, and Bhattacharyya, B.C., *Appl. Environ. Microbiol.*, 1994, vol. 60, pp. 4216–4218.
15. *Perumal, K.* and Kalaichelvan, P.T., *Indian. J. Expt. Biol.*, 1996, vol. 34, pp. 1121–1125.
16. Meera, K., Yadav, R.S.S., and Yadav, K.D.S., *Indian. J. Expt. Biol.* 2002, vol. 40, pp. 802–806.
17. Shanmugan, V. and Yadav, K.D.S., *Indian. J. Microbiol.*, 1997, vol. 37, pp. 105–106.
18. Shanmugan, V. and Yadav, K.D.S., *Indian. J. Expt. Biol.*, 1996, vol. 34, pp. 1164–1165.
19. Patel, V.K., Yadav, R.S.S., and Yadav, K.D.S., *Indian J. Biotechnol.*, 2007, vol. 6, pp. 553–556.
20. Microbial Type Culture Collection, Catalogue of Strains-2000, 5-th Ed., Chandigarh, India., Institute of Microbial Technology, 2000.
21. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., *J. Biol. Chem.*, 1951, vol. 193, pp. 265–275.
22. Werber, K. and Osborn, M., *J. Biol. Chem*, 1969, vol. 244, pp. 4406–4412.