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PURIFICATION, CHARACTERISATION AND COAL DEPOLYMERISATION ACTIVITY OF LIGNIN PEROXIDASE FROM *Lenzitus betulina* MTCC-1183

© 2012 M. Yadav, S. K. Singh, and S. Yadava

D.D.U. Gorakhpur University, Gorakhpur, 273 009, India

e-mail: drmeerayadav@rediffmail.com

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Lignin peroxidase from the culture filtrate of *Lenzitus betulina* MTCC-1183 has been purified to homogeneity using concentration by ultrafiltration and anion exchange chromatography on DEAE cellulose. The molecular weight of the purified lignin peroxidase using SDS-PAGE analysis was 43 kDa. Specific activity of the enzyme was 29.58 IU/mg. The K_m values for veratryl alcohol and H_2O_2 for the purified enzyme were 54 μM and 81 μM, respectively. The k_{cat} value of the purified enzyme was 2.3 s^{-1} using 3,4-dimethoxybenzyl alcohol as the substrate. The optimal conditions for the lignin peroxidase assay were detected at pH 2.4 and 22°C. Thermal stability of the purified enzyme has also been studied and its activation energy for deactivation was 287 kJ/mol. The purified lignin peroxidase depolymerised humic acid in presence of H_2O_2. Depolymerisation of coal by the *L. betulina* MTCC-1183 has been demonstrated using humic acid as a model of coal.

The lignin decomposing white-rot basidiomycetes secrete a hemeprotein, lignin peroxidases (LiP, E.C. 1.11.1.14) which in presence of H_2O_2 degrade lignin and lignin model compounds [1]. Different steps involved in the catalysis by LiP are

$$\mathrm{LiP[Fe(III)P] + H_2O_2 \longrightarrow LiP\text{-}I[Fe(IV)P^{+\bullet}] + H_2O} \quad (1)$$

$$\mathrm{LiP\text{-}I[Fe(IV)P^{+\bullet}] + RH \longrightarrow LiP\text{-}II[Fe(IV)P] + R^{+\bullet}} \quad (2)$$

$$\mathrm{LiP\text{-}II[Fe(IV)P] + RH_+2H^+ \longrightarrow LiP[Fe(III)P] + R^{+\bullet} + H_2O,} \quad (3)$$

where R is the organic substrate and P is the porphyrin. The 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 ($P^{+\bullet}$), whereas LiP compound II (LiP-II) carries only one oxidizing equivalent.

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 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 [11–13]. In a previous communication, secretion of LiP from 6 indigenous fungi, namely *Abortiporus biennis, Pestalotia bicolor, Heterobasidium annosum, Gloephyllum striatum, Loweporus lividus*, *Lenzitus betulina,* in their liquid culture growth media amended with lignin-containing natural substances has been reported [14]. However, LiP from these fungal strains have not been purified and characterized. The aim of the study was to purify and characterize LiP from the liquid growth medium of *Lenzitus betulina* MTCC-1183. Its enzymatic characteristics like K_m, pH and temperature optima and thermal stability using veratryl alcohol and H_2O_2 as the substrate have been determined. Depolymerisation of coal by the purified enzyme of *L. betulina* MTCC-1183 has been demonstrated using humic acid as a model of coal.

MATERIALS AND METHODS

Chemicals. Veratryl alcohol, which is 3,4-dimethoxybenzyl alcohol was purchased from Aldrich (USA). Nitriloacetate was from Sigma Chemical Co. (USA). All the chemicals including molecular weight markers 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) and were procured from Bangalore Genei Pvt. Ltd. (India). All other chemicals were obtained either from CDH (India) or Loba Chemie (India) or s.d. Fine-Chem. Ltd. (India) and were used without further purification.

Fungal strain. The fungal strain *Lenzitus betulina* MTCC-1183 was procured from Microbial Type Culture Collection Center and Gene Bank, Institute of microbial technology, Chandigarh (India) and was

maintained on agar slants using growth medium consisted (g/l): malt extract – 20.0 and agar – 20.0 dissolved in double distilled water.

For production of LiP, the fungus was grown in a medium containing (g/l): glucose – 10, ammonium tartrate – 1.32, KH_2PO_4 – 0.2, (mg/l): $MgSO_4 \cdot 7H_2O$ – 0.05, $CaCl_2$ – 0.01, (μg/l): thiamine – 10 and 1 ml of a solution containing (g/l): $MgSO_4 \cdot 7H_2O$ – 3.0, $MnSO_4 \cdot H_2O$ – 0.5, NaCl – 1.0, $FeSO_4 \cdot 7H_2O$ – 0.1, $CoCl_2 \cdot 6H_2O$ – 0.185, $CaCl_2$ – 0.08, $ZnSO_4 \cdot 7H_2O$ – 0.18, $CuSO_4 \cdot 5H_2O$ – 0.01, $AlK(SO_4)_2$ – 0.01, H_3BO_3 – 0.01, $Na_2MoO_4 \cdot 2H_2O$ – 0.012 and nitrilotriacetate – 1.5. The pH of basal medium was adjusted to 4.5 with 20 mM dimethyl succinate.

Enzyme was prepared by growing the fungi in 25 ml of sterilized culture media in 100 ml conical flasks containing natural lignin substrate. Flasks were inoculated with mycelia of 1cm diameter under aseptic conditions and the fungal culture was grown under stationary culture at 30°C. Attempts to scale up production via a proportional increase in both culture volume and flask size or with the use of shallow pans resulted in lower activity [15].

Enzyme assay. The LiP activity [15] has been assayed by monitoring the formation of veratraldehyde using veratryl alcohol as a substrate at $\lambda = 310$ nm with UV/VIS spectrophotometer Hitachi (Japan) model U-2000, which was fitted with electronic temperature control unit. Molar extinction coefficient 9300 $M^{-1} cm^{-1}$ was used for veratraldehyde to calculate the enzyme unit. The activity of Mn-peroxidase was determined by monitoring the absorbance change at $\lambda = 240$ nm due to formation of Mn (II) lactate and using $MnSO_4$ as the substrate [16]. The activity of laccase [17] was analysed using 2,6-dimethoxyphenol (DMP) as the substrate. The assay solution of 1 ml consisted of 1 mM DMP in 50 mM sodium malonate buffer, pH 4.5 at 37°C. The reaction was monitored by measuring the absorbance change at $\lambda = 468$ nm and using the molar extinction coefficient of 49.6 $mM^{-1} cm^{-1}$. One unit of the enzyme was defined as the amount of enzyme, which converts one μmole of substrate to product under the standard assay conditions. The least count of absorbance measurement was 0.001 absorbance unit.

Purification of the enzyme. The secretion of LiP in the liquid culture medium by *L. betulina* MTCC-1183 has been reported and the culture conditions for maximum secretion have already been optimized [14]. The fungus was grown under the reported optimal conditions in 100 ml culture flasks containing 25 ml of the liquid culture growth medium under stationary conditions in a biological oxygen demand incubator at 30°C.

The maximum LiP activity appeared on 5th day after inoculation of mycelia. Culture medium of all flasks were pooled, filtered through 4 layers of cheese cloth and 1200 ml of culture filtrate with 0.50 IU/ml activity was concentrated to 10 ml with Amicon Concentration Cell Model 8200 (USA) using PM10 ultrafitration membrane (India). The concentrated enzyme was dialyzed against 1000-fold excess of 5.0 mM sodium succinate buffer (pH 5.5) at 20°C overnight. The dialyzed enzyme was loaded on a DEAE column (1 × 16 cm) which was preequilibrated with the same buffer. After washing with 50 ml of the same buffer the adsorbed enzyme was eluted by applying a linear gradient of 0–200 mM NaCl (50 ml of buffer +50 ml of buffer containing 200 mM NaCl). 5 ml fractions were collected and analyzed for the LiP activity [15] and protein concentration was determined by the method Lowry et al. using bovine serum albumin as a standard. The active fractions were combined and concentrated with the Amicon Concentration Cell Model 8200, and then with Model-3 using ultrafiltration membrane PM10 to 1 ml. The concentrated enzyme was stored at 4°C. The enzyme did not loose activity for 2 months under these conditions.

SDS-PAGE. The homogeneity of the enzyme preparation was checked by SDS-PAGE using the method of Weber and Osborn [18]. The separating gel with 12% acrylamide in 0.375 M Tris-HCl buffer (pH 8.8) and stacking gel with 5% acrylamide in 0.063 M Tris-HCl buffer (pH 6.8) were used. Gel was run at a constant current of 20 mA [20]. Proteins were visualized by silver staining [19].

Enzymatic characteristics. The K_m values of the purified LiP for veratryl alcohol was determined by measuring steady state velocities of the enzyme catalyzed reactions at different substrate concentrations (0.02–2.5 mM) at a fixed H_2O_2 concentration (0.4 mM). The values of K_m and V_{max} were calculated by the linear regression of the double reciprocal plots [20]. The K_m value for H_2O_2 was detected following the same procedure by varying H_2O_2 concentration (0.006–0.6 mM) at a fixed enzyme saturating concentration of veratryl alcohol (2.0 mM). The pH optimum was determined by measuring the steady state velocities of the LiP catalyzed reaction at different pH values (1.8–3.5) in 50 mM disodium tartrate/tartaric acid buffer. The optimum value of pH was detected by plotting steady state velocity against the pH of the reaction solution. The temperature optimum of the enzyme was determined by measuring the steady state velocities of reaction in the range from 15 to 35°C and plotting the steady state velocities against temperatures.

Thermal stability of the enzyme has been assessed by measuring steady state velocities of enzyme catalyzed reaction at different time intervals at fixed temperatures. The values of deactivation rate constants were detected by the $t_{1/2}$ of the deactivation curves. The energy of activation of the deactivation process was determined by Arrhenius plot of log k vs 1/T.

Coal depolymerisation activity. The coal depolymerizing activity of the purified LiP was assessed by measuring the decrease of OD_{450} and the increase of

Table 1. Purification of LiP from *L. betulina* MTCC-1183.

Steps	Total volume	Protein, mg/ml	Activity, U/ml	Specific activity, U/mg	Total protein, mg	Total activity, U	Purification, fold	Recovery, %
Crude enzyme	1200	0.10	0.50	5.00	120	600	1	100
Concent-rated enzyme	10	0.90	9.56	10.62	9.0	95.6	2.12	15.9
Dialysed enzyme	16	0.65	7.55	11.62	10.4	120.8	2.32	20.13
DEAE-cellulose chromatography	15	0.12	3.55	29.58	1.80 (6.60)*	53.25 (160.50)*	5.92	8.87 (26.6)*

* The values given in brackets are calculated on the basis of 16 ml of dialyzed sample but only 5 ml of 16 ml dialyzed sample has been loaded on DEAE cellulose column.

OD_{360} [21] when the enzyme and H_2O_2 was added to the humic acid solution. The reaction solution consisted of 200 μl of humic acid (1 mg/ml), 100 μl of freshly prepared 0.4 mM H_2O_2, 200 μl of 50 mM of sodium tartrate buffer (pH 2.5) and 450 μl of double distilled water and maintained at 25°C. The reaction was started by the addition of 50 μl of the enzyme solution. Absorbance was observed at the intervals of 20 s. A plot of absorbance vs time was made.

Screening of the fungi for coal depolymerisation was performed in Petri dishes containing Tein and Kirk medium [11] or modified Kirk and Czapek-Dox agar medium [21]. Tein and Kirk medium contained (g/l): humic acid – 1.0, glucose – 10.0, malt extract – 10, peptone – 2.0, yeast extract – 2.0, L-Asp – 1.0, KH_2PO_4 – 2.0, $MgSO_4 \cdot 7H_2O$ – 1.0, agar – 20.0, (mg/l): thiamine-HCl – 1.0. Modified Kirk medium contained (g/l): humic acid – 1.0, glucose – 0.2, yeast extract – 0.05, 2,2–dimethyl succinate – 2.2, ammonium tartrate – 0.5, KH_2PO_4 – 2.0, $MgSO_4 \cdot 7H_2O$ – 0.5 g, $CaCl_2$ – 0.1, agar – 28. Czapek-Dox agar medium consisted (g/l): humic acid – 1.0, glucose – 0.2, yeast extract – 0.05, $NaNO_3$ – 0.5, K_2HPO_4 – 1.0, $MgSO_4 \cdot 7H_2O$ – 0.5, KCl – 0.5, $FeSO_4 \cdot 7H_2O$ – 0.5, agar – 18.0. The plates were inoculated with the *Lenzitus* mycelia and incubated at 25°C in the dark for 2 weeks. Decolorisation of the dark brown agar around the fungal growth area was observed periodically. A piece of mycelium of diameter 1 cm taken from the decolorized zone was suspended in 1 ml of double distilled water, filtered through Millex-GS 0.22 μm Millipore filter unit and the filtrate was used for the assay of LiP, Mn-peroxidase and laccase.

RESULTS AND DISCUSSION

The purification procedure of LiP from the culture filtrate of the fungus *L. betulina* MTCC-1183 is summarized in Table 1 and the elution profile of the enzyme activity from the DEAE-cellulose column is given in Fig. 1. All the LiP fractions were combined and concentrated and the enzyme sample obtained was analyzed by SDS-PAGE analysis (Fig. 2). The presence of single protein band in lane 2 of Fig. 2 indicates that the purified enzyme is homogeneous. The molecular weight of the purified enzyme calculated from SDS-PAGE analysis was 43.0 kDa. The molecular weights of different isozymes of LiP purified from the culture filtrate of the fungus *Phanerochaete chryososporium* ATCC-24725 [15] have been reported in the range 38.0 to 46.0 kDa where as for 2 isozymes of *P. chryososporium* ATCC-20696 [22] and *Pleurotus sajor caju* MTCC-141 [24] they were 38.0 kDa and 40.0 kDa, respectively. Thus, the molecular weight of

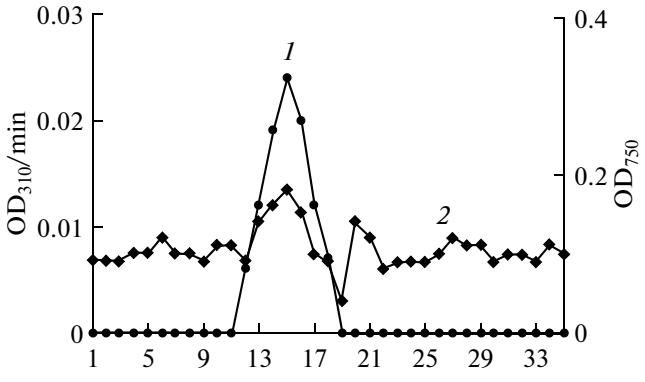

Fig. 1. Typical elution profile of LiP from *L. betulina* MTCC-1183 after DEAE column. *1* – the enzyme activity at 310 nm; *2* – protein at 750 nm.

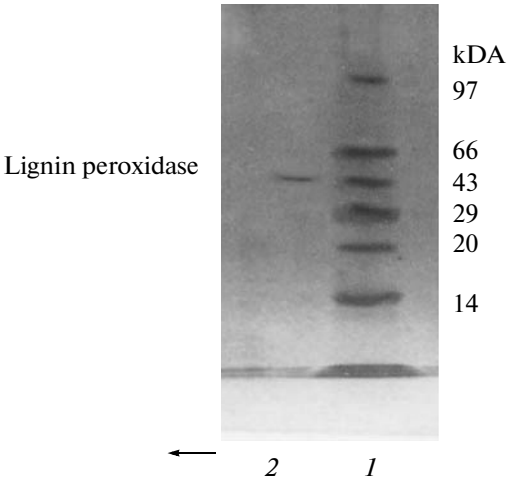

Fig. 2. SDS – PAGE of LiP from *L. betulina* MTCC-1183. *1* – the molecular weight markers; *2* – the purified LiP (50 µl, 1.55 U/ml).

the LiP purified from the culture filtrate of *L. betulina* MTCC-1183 has been found to be in the same range as for other fungal strains [15, 22]. It is important that the purification procedure described here for the LiP is simpler than the procedures reported for the enzyme from other fungi [15, 22]. Moreover, we have only one activity peak after DEAE cellulose indicating that under the conditions used for the growth of the fungus only one form of the enzyme is produced where as, in cases of purification of LiP from other sources multiple forms of isozymes have been detected [15, 22]. The specific activity of purified LiP of *L. betulina* MTCC-1183 has been found to be 29.58 IU/mg.

Enzymatic characteristics. The Michaelis-Menten and double reciprocals plots using veratryl alcohol and H_2O_2 as the variable substrates at the fixed enzyme saturating concentration are shown in Figs. 3a and 3b, respectively. The calculated K_m values for veratryl alcohol and H_2O_2 were 54 µM and 81 µM, respectively. For comparison, the K_m values for veratryl alcohol and H_2O_2 for the LiP purified from the culture filtrates of other fungi are given in Table 2. They were found to be in the same range. The calculated k_{cat} value for the purified enzyme was 2.3 s^{-1} for veratryl alcohol as the substrate. In order to check that the purified enzyme is a LiP and not a versatile peroxidase, it was analysed for Mn-peroxidase activity [16] and this activity was not revealed.

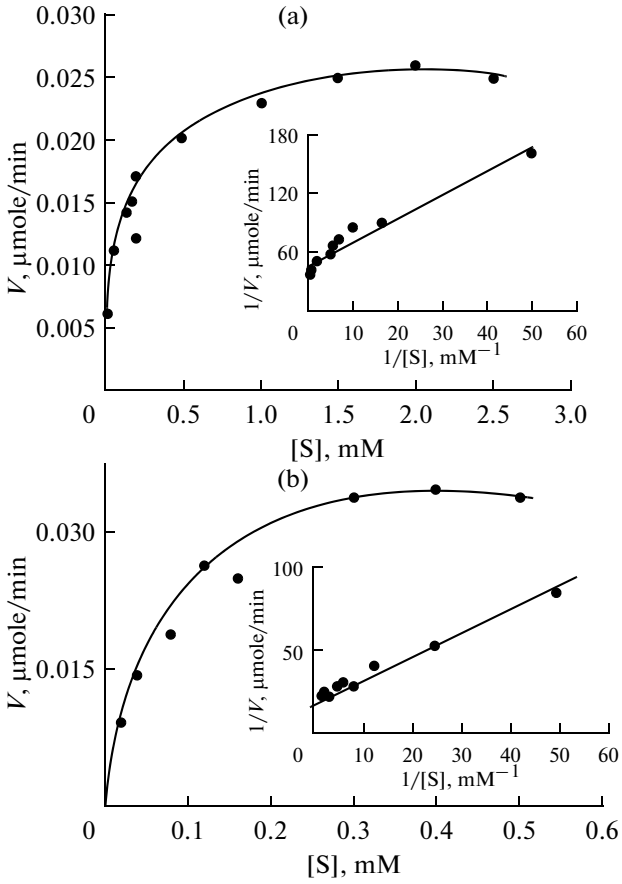

Fig. 3. Michelis-Menten and double reciprocal plots for LiP from *L. betulina* MTCC-1183. 1 ml reaction mixture consisted of (0.02–2.5) mM veratryl alcohol (a) or (0.006–0.6) mM H_2O_2 (b) as the variable substrate, 0.4 mM H_2O_2 (a) or 2.0 mM veratryl alcohol (b) in 50 mM sodium tartrate buffer pH 2.5 at 25°C.

The variation of the steady state velocity of the purified LiP with pH is shown in Fig. 4a. 1 ml of reaction mixture consisted of 2.0 mM veratryl alcohol, 0.4 mM

Table 2. K_m values for fungal LiP calculated using veratryl alcohol or H_2O_2 as the substrate

Fungal strains	K_m for veratryl alcohol, µM	K_m for H_2O_2, µM	Temp., °C	pH
Phanerochaete chrysosporium [15]	60	80	26	3.0
Penicillium citrinum [11]	69	64	30	4.0
Fusarium oxysporum [11]	64	72	25	2.3
Aspergillus terreus [11]	60	80	22	2.0
Pleurotus sajor caju [24]	57	80	30	3.0
Loweporus lividus [26]	55	75	25	2.5
L. betulina MTCC-1183	54	81	22	2.4

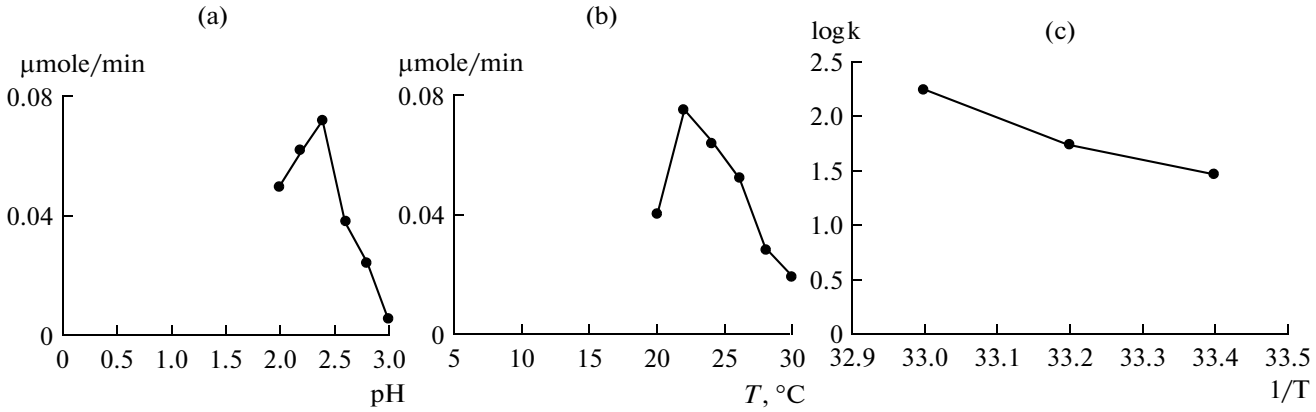

Fig. 4. Dependence of the LiP from *L. betulina* MTCC-1183 on pH (a) and temperature (b). Thermal stability of the LiP from *L. betulina* MTCC-1183 at different time at fixed temperature of reaction (c).

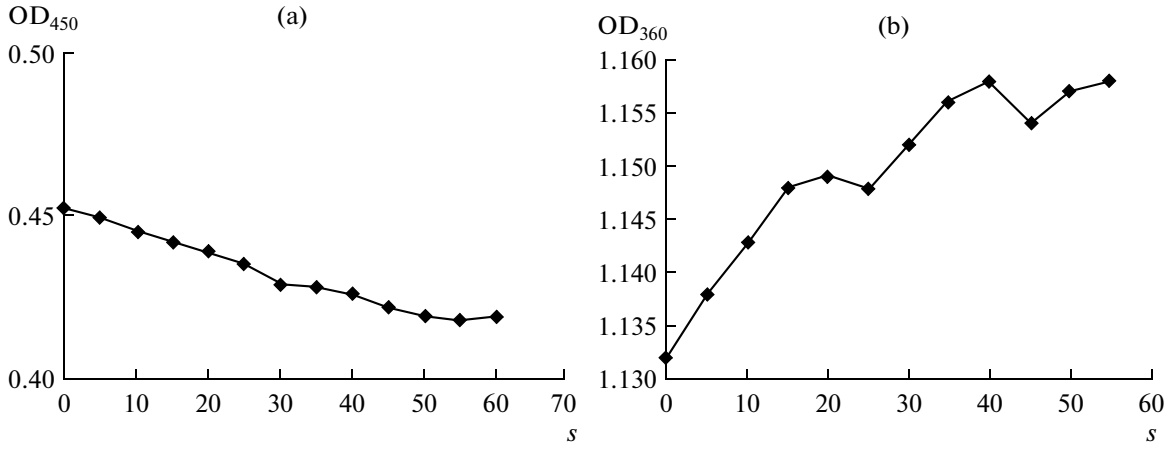

Fig. 5. Coal depolymerising activity of the LiP from *L. betulina* MTCC-1183 assayed by measuring decrease of OD_{450} (a) and increase of OD_{360} (b).

H_2O_2 in 50 mM sodium tartrate buffer at 25°C and pH changed from 1.8 to 4.5. The calculated pH optimum was 2.4. The pH optima of LiP purified from other fungal sources are given in Table 2 and the most of them are located in the range from 2.0 to 4.0 though a few enzymes have pH optima in the alkaline range [13, 25]. Effect of temperature on the steady state velocity of the purified LiP is shown in Fig. 4b. 1 ml of reaction mixture consisted of 2.0 mM veratryl alcohol, 0.4 mM H_2O_2 in 50 mM sodium tartrate buffer at pH 2.5 and temperature changed from 15° to 35°C. Temperature optima of LiP purified from other fungi are also given in Table 2 and they are in the range from 22 to 30°C. Thus, the pH and temperature optima of the purified enzyme are located in the same range as reported for the other fungal strains.

The Arrhenius plot for the determination of energy of activation for the thermal deactivation process of the enzyme is shown in Fig. 4c. 1 ml of reaction mixture consisted of 2.0 mM veratryl alcohol and 0.4 mM H_2O_2 in 50 mM sodium tartrate buffer (pH 2.5). The calculated energy of activation was 287 kJ/mol. For LiP of other fungi this parameter is not available in the literature for comparison.

Humic acid degradation. The results of depolymerisation of humic acid by the purified LiP of *L. betulina* MTCC-1183 are shown in Fig. 5. Fig. 5a shows the depolymerization of the dark brown humic acid fraction (decrease of OD_{450}) and Fig. 5b shows the formation of yellowish colored fulvic-acid-like compounds (increase of OD_{360}) by purified LiP of *L. betulina* MTCC-1183. Thus, it has been shown that LiP caused a disappearence of high molecular mass coal fractions and the formation of smaller ones. These investigations have clarified that the LiP from *L. betulina* MTCC-1183 is involved in coal depolymerisation. In 1989 Wondrack et

Fig. 6. Decolourisation of humic acid by *L. betulina* MTCC-1183 in modified Kirk medium containing humic acid (1g/l).

al. [23] first demonstrated the oxidizing effect of lignin peroxidase from *P. chrysosporium* on coal polymers in aqueous solution. Mechanism of breakdown is still to be investigated. Due to the large size of lignin as well as coal humic acids, they cannot be taken up into the hyphae, thus an extracellular enzymatic attack has to be assumed. These enzymes are able to form reactive radicals (aryl cation radicals) within the lignin polymer, which can lead to a complete breakdown of the high molecular mass structure.

In order to see if the fungus it self depolymerises humic acid when it was grown on the medium containing humic acid, experiments were performed. The results are shown in Fig. 6. It was found that *L. betulina* MTCC-1183 decolorized the humic acid in modified Kirk medium to maximum extent as compared to the Czapek-Dox agar medium and Tien and Kirk medium. The colour of humic acid containing agar was dark brown which changed to yellow around the fungal growth area. The bleaching effect was stable for 3 weeks at 25°C. It has already been demonstrated [21] that the decolorization of agar medium containing coal humic acids is a suitable and easy method for the rapid detection of coal depolymerizing fungi. The bleaching effect around the growth area of fungi appears due to a breakdown of high molecular mass coal fractions to low molecular mass ones. It was assumed that fulvic-acids-like compounds were formed during depolymerization of coal.

The analysis of a small portion of the decolourized zone of the fungal growth region revealed the presence of the LiP and laccase activity (0.25 U/ml) where as no activity of Mn-peroxidase was found. Thus, LiP is involved at least partially in the depolymerisation of humic acid by *L. butilina* MTCC-1183.

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