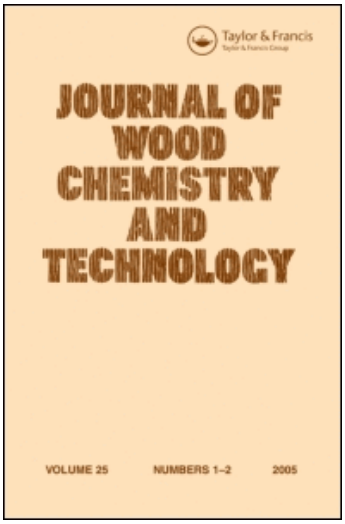

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Purification and Characterization of Lignin Peroxidase from *Pleurotus sajor caju* MTCC–141

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Abstract: The culture conditions for extracellular secretion of lignin peroxidase by *Pleurotus sajor caju* MTCC–141 in the liquid culture growth medium amended with lignin containing natural substrates have been studied. Secretion of lignin peroxidase has been found to be maximum in the presence of bagasse. Lignin peroxidase from the liquid culture filtrate has been purified to homogeneity. Two isozymes having relative molecular masses 38 and 40 kDa have been isolated. The enzymatic characteristics like Km, pH, and temperature optima of the major isozyme (40 KDa) has been determined using veratryl alcohol, n-propanol, and H_2O_2 as the substrate. The Km values for veratryl alcohol, n-propanol, and H_2O_2 have been found to be 57 μM, 500 μM, and 80 μM, respectively. The pH and temperature optima of lignin peroxidase have been found to be 3 and 30°C, respectively. The inhibition of the enzyme activity by sodium azide has been studied and it has been found to be uncompetitive, with KI value of 4 mM.

Keywords: Heme enzyme, lignin peroxidase, lignolytic enzyme, peroxidase, *Pleurotus sajor caju*

INTRODUCTION

Lignin peroxidase [LiP; E.C.1.11.1.14] oxidizes high redox-potential compounds such as veratryl alcohol, methoxy benzene, and nonphenolic lignin model dimmers[1] in the presence of H_2O_2. The catalytic cycle consists of three steps, as follows:

$$\text{LiP}\,(\text{Fe}^{3+})\ \text{P} + \text{H}_2\text{O}_2 \rightarrow \text{LiP-I}\,(\text{Fe}^{4+}\text{-O})\ \text{P}^{\cdot} + \text{H}_2\text{O} \qquad (1)$$

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

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

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 radical $R^{\cdot}$, which further reacts non-enzymatically to yield the final products. The structural and functional aspects of lignin peroxidase have been studied.[2] Lignin peroxidases have potential applications in (i) delignification of lignocellulosic materials,[3] which are seen as an alternative to the depleting oil reserves, (ii) in the conversion of coal to low molecular mass fractions,[4] which could be used as feed stock for the production of commodity chemicals, (iii) in biopulping and biobleaching[5] in paper industries, (iv) in removal of recalcitrant organic pollutants,[6–8] and (v) in the enzymatic polymerization[9] in polymer industries. Although a large number of lignolytic microorganisms have been reported,[10–13] the lignolytic enzymes of only a few micro-organisms have been purified and characterized.[1] The fungal strain *P. sajor caju* has been reported[14] to be lignolytic. However, the secretion of LiP by this fungal strain has not been reported. In this communication we have reported the secretion of lignin peroxidase by *P. sajor caju* MTCC–141 in the liquid culture media containing natural lignin substrates like coirdust, sawdust, corncob, bagasse particles, and wheat straw.

The culture conditions for maximum secretion of lignin peroxidase in the liquid medium containing bagasse by *P. sajor caju* have been optimized. A simple procedure for purification of lignin peroxidase from the culture filtrate has been developed. The enzymatic characteristics like Km, pH, and temperature optima of the enzyme have been determined. Inhibition of the enzyme by sodium azide has also been studied.

MATERIALS AND METHODS

Chemicals

Veratryl alcohol (3,4-dimethoxy benzyl alcohol) was from Aldrich (Wisconsin, USA). Dimethyl succinate and nitrilotriacetate were from Sigma Chemical Co. (St. Louis, USA). All other chemicals were either obtained from CDH (Delhi) or from Loba Chemie (Mumbai) and were used without further purifications.

Fungal Strains and Preparation

Pleurotus sajor caju MTCC–141 was procured from MTCC Centre and Gene Bank, Institute of Microbial Technology, Chandigarh. The fungal strain was

maintained on Tien and Kirk medium,[15] which consisted of glucose (10 g), malt extract (10 g), peptone (2 g), yeast extract (2 g), L-asparagine (1 g), KH_2PO_4 (2 g), $MgSO_4 \cdot 7H_2O$ (1 g), thiamine-HCl (1 mg), and agar (20 g) dissolved in double distilled water (1 L).

For the production of lignin peroxidase,[16] the fungal strain was grown in a medium containing 10 g glucose, 1.32 g ammonium tartrate 0.2 g KH_2PO_4, 50 mg $MgSO_4 \cdot 7H_2O$, 10 mg $CaCl_2$, 10 μg thiamine per liter, and 1 ml of a solution containing per liter 3 g $MgSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 1 g NaCl, 100 mg $FeSO_4 \cdot 7H_2O$, 185 mg $CoCl_2 \cdot 6H_2O$, 80 mg $CaCl_2$, 180 mg $ZnSO_4 \cdot 7H_2O$, 10 mg $CuSO_4 \cdot 5H_2O$, 10 mg $AlK(SO_4)_2$, 10 mg H_3BO_3, 12 mg $Na_2MoO_4 \cdot 2H_2O$, and 1.5 g nitrilotriacetate. The pH of basal medium was adjusted to 4.5 with 20 m*M* dimethyl succinate. Growth media containing natural lignin sources like coirdust, sawdust, corncob, bagasse particles, and wheat straw were separately prepared by adding 0.5 g of one of the natural lignin sources to 20 ml of growth medium in 100-ml culture flasks, which were sterilized. The sterilized growth medium was inoculated with mycelia of 1 cm diameter under aseptic condition and the fungal culture was grown under stationary culture condition at 25°C in an incubator.

The liquid culture medium containing bagasse particles gave the best production of lignin peroxidase. The culture conditions were further optimized for maximum production of lignin peroxidase by varying the amount of bagasse particles in the culture medium.

The enzyme was purified by growing the fungal strain in culture media 30 × 25 ml in 100-ml culture flasks under the optimal culture conditions for maximum production of the lignin peroxidase.

On the 5th day of inoculation, when lignin peroxidase activity reached maximum value, the cultures were pooled, filtered through four layers of cheese cloth and culture filtrate volume 800 ml with 0.192 IU/ml activity was concentrated with Amicon Concentration Cell Model 8200 using PM10 ultrafitration membrane with molecular wt. cut off value 10 kD to 10 ml. The concentrated enzyme was dialyzed against 1000 times excess of 5 mM sodium succinate buffer pH 5.5 over night at 20°C. The dialyzed enzyme was loaded on a DEAE column,[17] which was pre-equilibrated 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). The active fractions were combined, concentrated with Amicon Concentration Cell Model 8200, and there after with Amicon concentration Cell Model-3 using ultrafiltration membrane PM10. The concentrated enzyme was stored in a fridge at 4°C and was used for further studies. The enzyme retains full activity for two months under these conditions.

SDS-Polyacramide Gel Electrophoresis

The homogeneity of the enzyme preparation was checked by SDS–PAGE using the method of Weber and Osborn.[18] 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 silver staining. The molecular weight markers were Phosphorylase (97.4 kDa), Bovine serum albumin (68 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29 kDa), and Lysozyme (14.3 kDa) and were procured from Bangalore Genei Pvt. Ltd. (Bangalore, India). Gel was run at a constant current of 20 mA.[19]

Enzyme Activity Assay and Characterization

The activity of lignin peroxidase was assayed by the method reported by Tien and Kirk[15] using veratryl alcohol as the substrate and monitoring the formation of veratraldehyde spectrophotometrically at $\lambda = 310$ nm using molar extinction coefficient value of 9300 $M^{-1}cm^{-1}$. The reaction solution 1 ml consisted of veratryl alcohol 2 mM, H_2O_2(freshly prepared) 0.4 mM in 50 mM of sodium tartrate buffer pH 2.5 at 25°C. The reaction was started by adding 50 μl of the enzyme solution. The spectrophotometer used was Hitachi (Japan) Model U–2000 and was fitted with an electronic temperature control unit. The least count of absorbance measurement was 0.001 absorbance unit.

The enzymatic characteristics of the enzyme like K_m, pH, and temperature optima were determined using veratryl alcohol and *n*-propanol[20,21] as the substrates and monitoring the formation of veratraldehyde and propionaldehyde spectrophotometrically at wavelengths 310 and 300 nm, respectively. The values of molar extinction coefficients of veratraldehyde and propionaldehyde used for the calculation of the enzyme units were 9300 $M^{-1}cm^{-1}$ and 20 $M^{-1}cm^{-1}$, respectively. For determination of K_m, steady state velocities of the enzyme-catalyzed reaction were determined at different concentrations of the substrate and K_m was calculated from the double reciprocal plot of 1/[V] versus 1/[S], where [V] is the steady state velocity of the enzyme-catalyzed reaction and [S] is the concentration of the substrate taken. For determination of pH optimum, the steady state velocity of the enzyme-catalyzed reaction was determined at different pH values and a plot of V versus pH was drawn. The pH range from 1.5 to 4.5 was maintained using tartaric acid/disodium tartrate buffer (50 m*M*). For determination of temperature optimum, steady state velocity of the enzyme-catalyzed reaction was determined at different temperatures and a plot of V versus temperature was drawn.

Inhibition Studies by Sodium Azide

Two sets of experiments have been performed for inhibition of lignin peroxidase by sodium azide. In one set of experiments, the steady state velocity of lignin peroxidase–catalyzed reaction has been measured at different concentrations of sodium azide and a graph has been plotted in velocity versus azide

concentration. In another set of experiments, double reciprocal plots at different concentrations of sodium azide have been plotted to decide the nature of inhibition.

RESULTS AND DISCUSSION

The extracellular production of lignin peroxidase was observed in the liquid culture growth media amended with lignin containing natural substrates, like corncob, coirdust, sawdust, wheat straw, and bagasse inoculated with mycelia of *P. sajor caju* MTCC–141.The control experiment was run in parallel, which has similar medium composition except the natural lignolytic substrate. The results indicate that the presence of lignin containing natural substrates in liquid media enhanced the extracellular secretion of lignin peroxidase as shown in Figure 1 and has been summarized in Table 1.

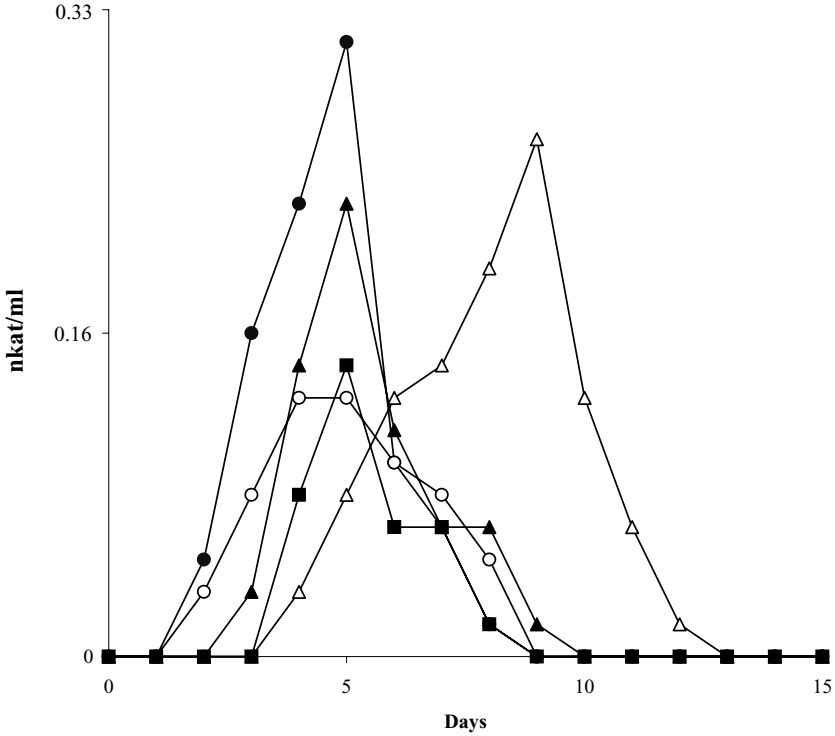

Figure 1. Secretion of lignin peroxidase by *P. sajor caju* in the liquid culture medium supplemented with different natural lignin containing substrates. [(a) Coirdust (▲); (b) Sawdust (Δ); (c) Bagasse (●); (d) Corn-cob (○); (e) Wheat straw (■); and (f) without substrate (□)].

Table 1. Culture conditions for maximum secretion of ligninperoxidase

S. no. Fungal strain	Best inducer per 25 mL liquid culture	Maximum production	Maximum level of activity (μm ole/min)
1. *Pleurotus sajor caju* MTCC-141	Saw dust	9th day	0.016
	Wheat straw	5th day	0.009
	Corn cob	5th day	0.008
	Coir dust	5th day	0.014
	Bagasse	5th day	0.019
	None	not found	0.000

The order of induction of lignin peroxidase production in the liquid culture medium by *P. sajor caju* was bagasse > sawdust > coirdust > wheat straw > corncob > control. The results of optimization of culture conditions for maximum production of lignin peroxidase by *P. sajor caju* is shown in Figure 2 in which maximum level of enzyme units secreted in the liquid culture medium containing different amounts of bagasse particles have been plotted against the amount of bagasse particles.

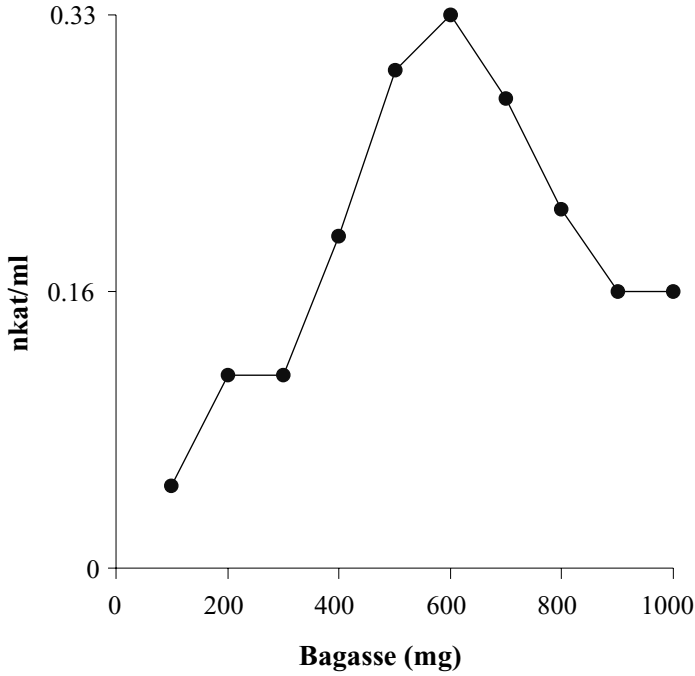

Figure 2. Optimization of secretion of lignin peroxidase with respect to the amount of bagasse particles present in the liquid culture medium (25 ml).

Table 2. Purification chart

No.	Steps	Total vol.	Protein (mg/ml)	Activity (U/ml)	Specific (U/mg)	Total protein	Total activity	Purification fold	% recovery
1	Crude enzyme	800	0.09	0.192	2.13	72	153.6	1	100
2	Concentrated enzyme	10	0.6	10.0	16.6	6	100.0	7.79	65.10
3	Dialyzed enzyme	18	0.33	5.54	16.8	5.94	99.72	7.88	64.91
4	DEAE (only 1 ml of the dialyzed sample was loaded)	15	0.01	0.120	12	0.15	1.8	5.63	21.06
		15	0.012	0.182	15.1	0.18	2.73	7.08	31.86

It is obvious that the maximum production of lignin peroxidase by *P. sajor caju* occurs at 600 mg bagasse particle in a culture volume of 25 ml at 25°C under stationary culture conditions.

The enzyme was purified by the procedure given in the Materials and Methods section. The purification chart is given in Table 2.

The elution profile from DEAE column is shown in Figure 3, which shows that the activity is eluted in two major protein peaks. This is not an unusual result because in case of purification of lignin peroxidase from *Phanerochaete chrysosporium*, ten isozymes have been reported.[15] Figure 4 shows the results of the SDS–PAGE experiment, which was done to check the purities of the two isoenzymes. The appearance of single protein bands under silver staining in case of both the isoenzymes clearly indicates that both the isozymes are pure and have molecular weight nearly 38 kDa and 40 kDa.

Michaelis–Menten and double reciprocal plots for the major isozyme of ligninperoxidase of *P. sajor caju* using veratryl alcohol, n-propanol, and hydrogen peroxide as the variable substrates are shown in Figures 5, 6, and 7, respectively. Thus lignin peroxidase from *P. sajor caju* followed Michaelis–Menten kinetics and calculated Km values of the enzyme for veratryl alcohol, *n*-propanol, and H_2O_2 were 57 μM, 500 μM, and 76 μM, which are of the same order of magnitude as Km values reported[15] for the lignin peroxidase of *Phanerochaete chrysosporium* as shown in a comparative chart (Table 3).

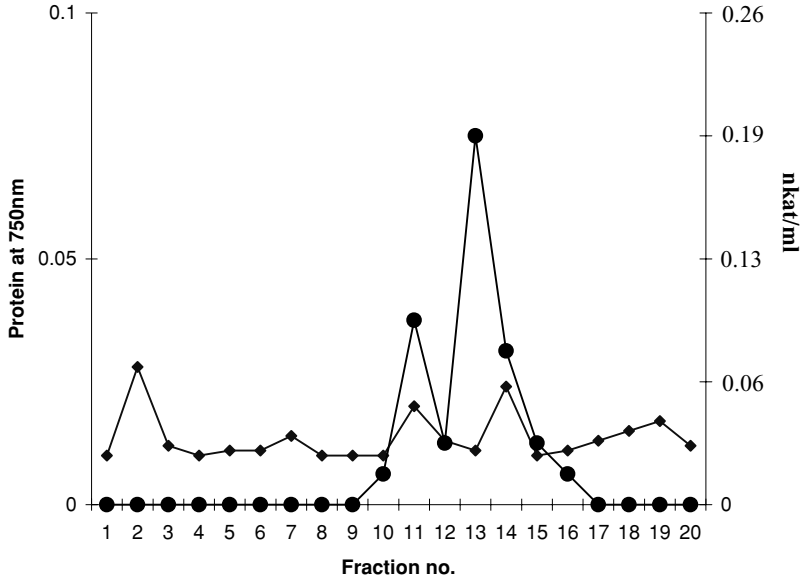

Figure 3. Typical elution profile of lignin peroxidase from DEAE column. (●) activity–profile and (♦) protein–profile.

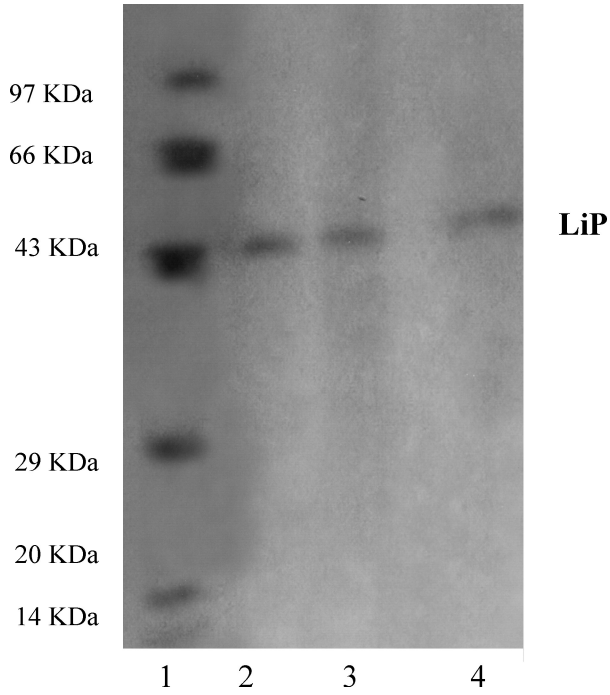

Figure 4. SDS–PAGE Lane 1 contains the molecular weight markers (from top): Phosphorylase (97.4 kD), Bovine serum albumin (68 kD), Ovalbumin (43 kD), Carbonic anhydrase (29 kD) and Lysozyme (14.3 kD). Lane 2 and 3 contains the purified lignin peroxidase (Isoenzyme 38 kDa). Lane 4 contains the purified lignin peroxidase (Isoenzyme 40 kDa).

Figure 8 shows the variation of activities of lignin peroxidase of *P. sajor caju* with variation of pH of the reaction medium. The pH optima is found to be 3, which is also the same as reported[15] for the lignin peroxidase of *Phanerochaete chrysosporium*. Variation of activities of lignin peroxidase of *P. sajor caju* with temperature are shown in Figure 9. The temperature optima of this enzyme is 20°C, which is lower than that reported[15] for the lignin peroxidase of *Phanerochaete chrysosporium*.

Inhibition of Lignin Peroxidase by Sodium Azide

Two sets of experiments have been done for studying the inhibition of lignin peroxidase by sodium azide. In one set of experiments steady state velocity of lignin peroxidase–catalyzed reaction has been measured in presence of different concentrations of sodium azide. It is shown in Figure 10. It

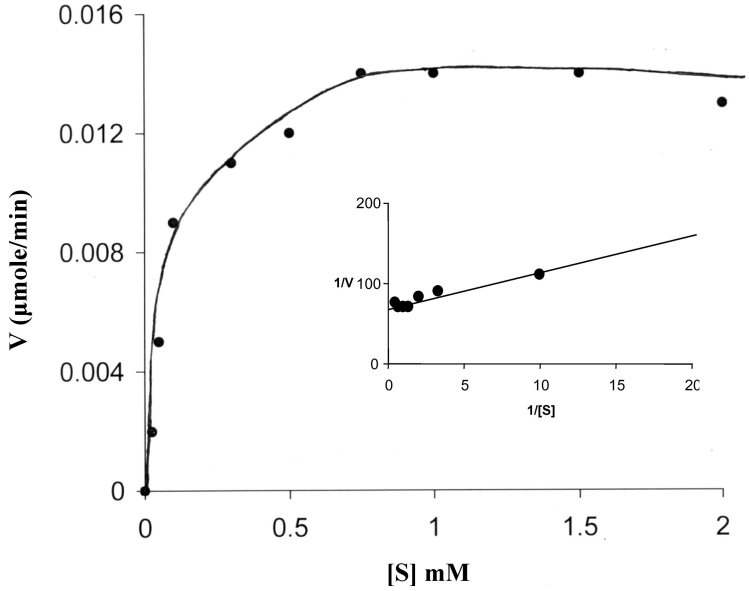

Figure 5. Michaelis–Menten and double reciprocal plots for lignin peroxidase of *P. sajor caju* using veratryl alcohol as the variable substrate.

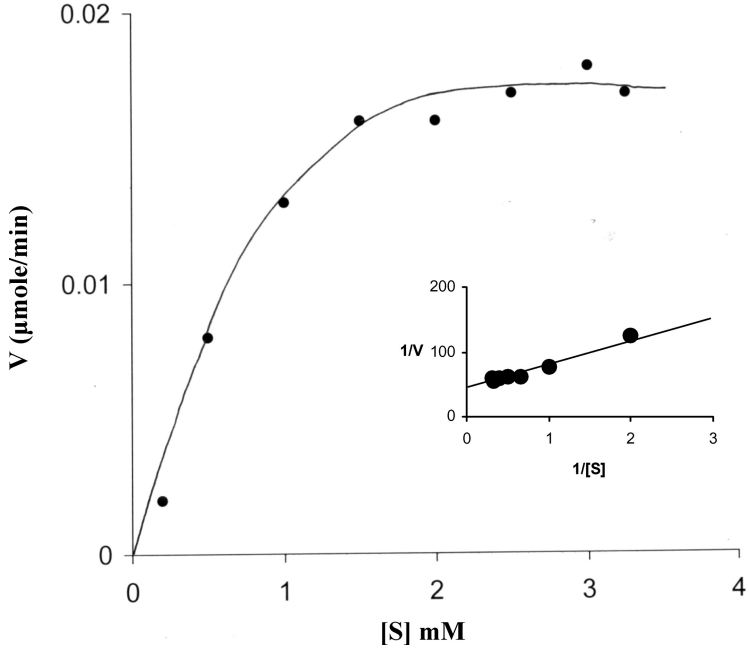

Figure 6. Michaelis–Menten and double reciprocal plots for lignin peroxidase of *P. sajor caju* using n-propanol as the variable substrate.

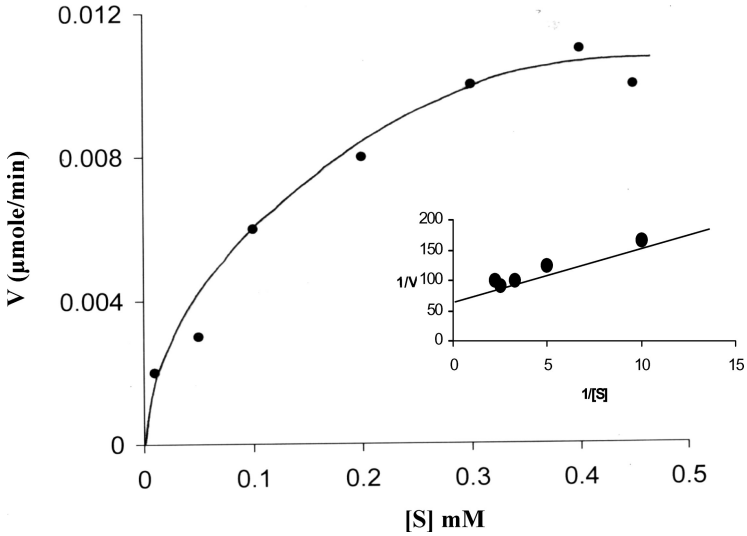

Figure 7. Michaelis–Menten and double reciprocal plots for lignin peroxidase of *P. sajor caju* using H_2O_2 as the variable substrate.

is obvious from the figure that sodium azide inhibits the activity of lignin peroxidase.

In the other set of experiments, the studies have been made to decide the nature of inhibition. In these experiments, double reciprocal plots have been made in the presence of different fixed concentrations of sodium azide (as shown in Figure 11). These plots are nearly parallel straight lines showing that the inhibition is uncompetitive with inhibition constant K_I value of 4 mM.

Thus this communication reports a simple method for purification of lignin peroxidase, a biotechnologically important enzyme from the liquid culture medium of *P. sajor caju* not yet reported in the literature.

Table 3. Comparative chart

Species	Km for Veratryl alcohol	Km for n-propanol	Km for H_2O_2	pH	Temperature
Phanerochaete chrysosporium	60 μM	500 μM	80 μM	3	26°C
Pleurotus sajor-caju	57 μM	500 μM	76 μM	3	20°C

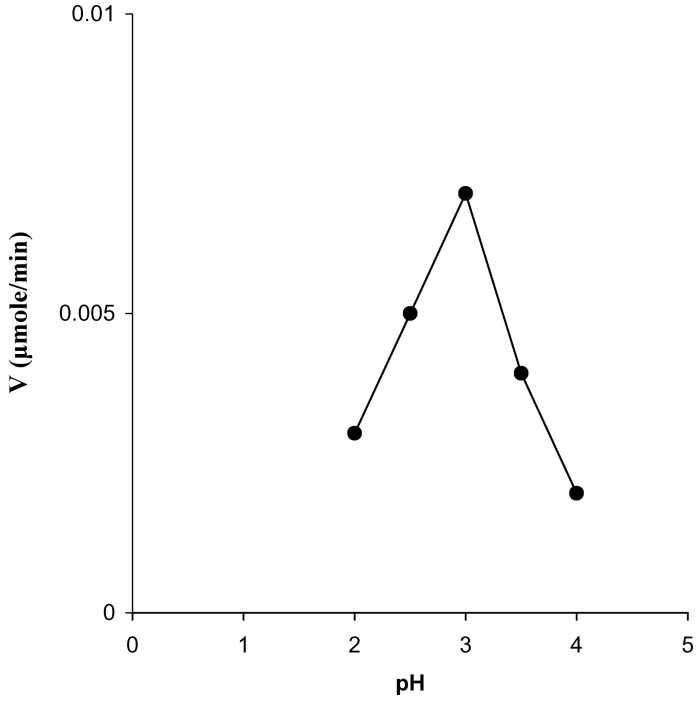

Figure 8. Activity–pH profile of the purified lignin peroxidase.

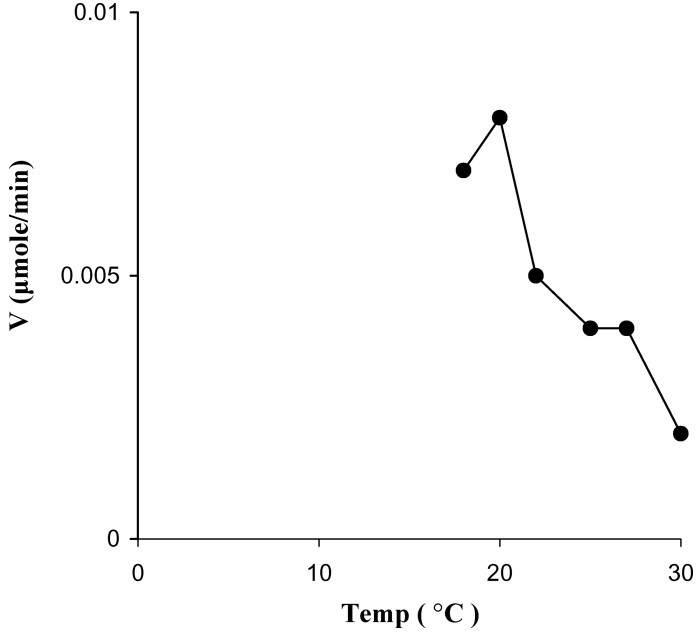

Figure 9. Activity–temperature profile of the purified lignin peroxidase.

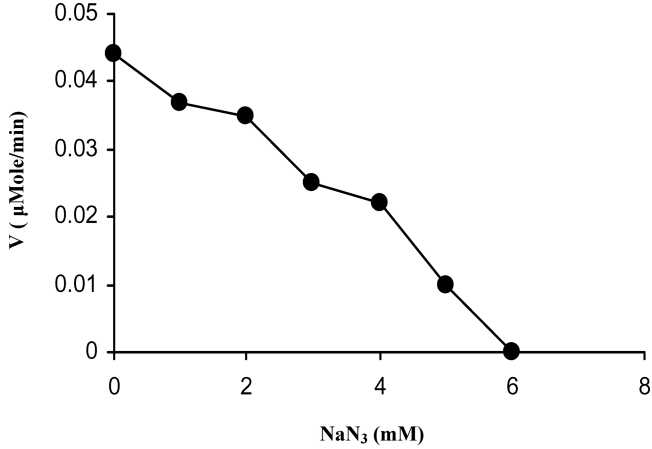

Figure 10. Inhibition of lignin peroxidase by sodium azide.

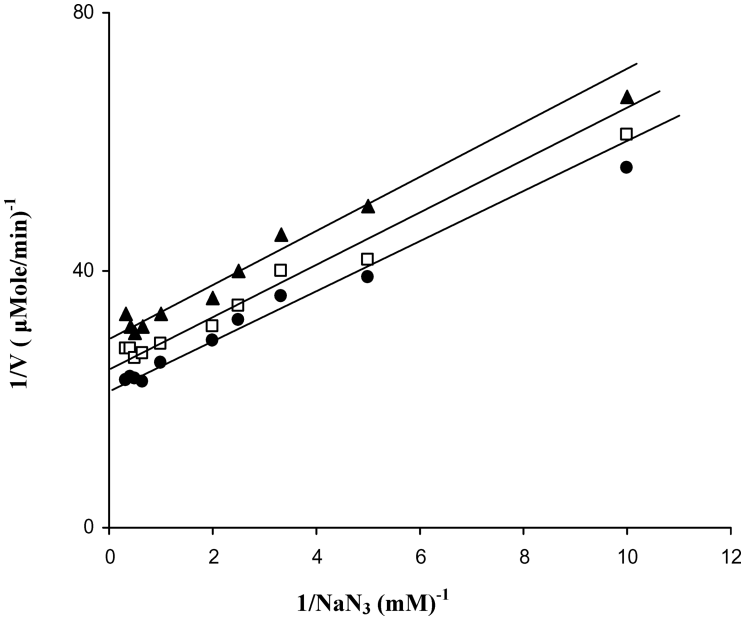

Figure 11. Double reciprocal plots for lignin peroxidase in presence of different fixed concentration of sodium azide.

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