Purification of lignin peroxidase from *Hexagona tenuis* MTCC-1119 and its kinetic properties in aqueous medium containing miscible organic solvents

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Lignin peroxidase from culture filtrate of a new lignolytic fungal strain *Hexagona tenuis* MTCC-1119 has been purified to homogeneity using a simple procedure. The molecular wt. of the enzyme has been found to be 48 kDa using SDS-PAGE analysis. The enzymatic characteristics like $K_m$, $pH$ and temperature optima of the enzyme using veratryl alcohol, $n$-propanol and $H_2O_2$ as substrate has been determined. The $K_m$ values have been found to be 70 μM, 80 μM and 530 μM for veratryl alcohol, $H_2O_2$ and $n$-propanol respectively. The $pH$ and temperature optima were 3 and 30°C respectively. The enzyme retains 50% of its activity in 10% of the water miscible organic solvents acetone, dioxane, diethyl ether, acetonitrile and dimethyl formamide in aqueous medium. The inhibition of the enzyme by acetone has been found to be reversible and uncompetitive with inhibition constant $K_I$ values 2.72 mM, 2.96 mM and 0.34 mM using veratryl alcohol, hydrogen peroxide and $n$-propanol as the substrates respectively.

**Keywords:** Lignin peroxidase, veratryl alcohol, *Hexagona tenuis, n*-propanol

The immense potential of enzymes as catalysts in organic synthesis is well documented. This potential has been enhanced by the use of enzymes as catalysts in non-aqueous media. This potential is further enhanced due to the enzymes exhibiting new properties like the ability to catalyse reactions which are not catalysed in aqueous media, enhance thermostability, molecular memory and radically altered selectivity in non-aqueous media. One of the obvious advantages of using enzymes in organic solvents is the solubilities of the substrates in such media which are not soluble in pure aqueous medium. In spite of tremendous potential of enzyme catalysis in non-aqueous or mixed aqueous solvents, enzyme kinetics in such media has rarely been studied. In the present communication, purification of lignin peroxidase from the culture filtrate of a new lignolytic fungal strain *Hexagona tenuis* MTCC-1119 has been achieved.

**Materials and Methods**

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

**Fungal strain and enzyme preparation**

The indigenous lignolytic fungal strain *Hexagona tenuis* MTCC-1119 was procured from MTCC Centre and Gene Bank, Institute of Microbial Technology, Chandigarh. The fungal strain was maintained on Tien and Kirk medium which consisted of glucose (10 g), malt extract (10 g), peptone (2 g), yeast extract (2 g), L-asparagine (1 g), KH$_2$PO$_4$ (2 g), MgSO$_4$.7H$_2$O (1 g), thiamine-HCl (1 mg) and agar (20 g) dissolved in double distilled water (1 L). For the production of lignin peroxidase, the fungal strain was grown in a medium containing 10 g glucose, 1.32 g ammonium tartrate, 0.2 g KH$_2$PO$_4$, 50 mg MgSO$_4$.7H$_2$O, 10 mg CaCl$_2$, 10 μg thiamine per litre and 1 mL of a solution containing per liter 3 g MgSO$_4$.7H$_2$O, 0.5 g MnSO$_4$.H$_2$O, 1 g NaCl, 100 mg FeSO$_4$.7H$_2$O, 185 mg CoCl$_2$.6H$_2$O, 80 mg CaCl$_2$, 180 mg ZnSO$_4$.7H$_2$O, 10 mg CuSO$_4$.5H$_2$O, 10 mg AlK(SO$_4$)$_2$, 10 mg H$_3$BO$_3$, 12 mg Na$_2$MoO$_4$.2H$_2$O and 1.5 g nitrilotriacetate. The $pH$ of basal medium was adjusted to 4.5 with 20 mM dimethyl succinate. The culture conditions for maximum production of lignin peroxidase *Hexagona tenuis* MTCC-1119 have been optimized by Yadav et al. The same culture conditions were used for growing the fungal strain for the purification of lignin peroxidase. The sterilized culture media 20 mL in 100 mL flask × 60 were inoculated with 1.0 mL of spores suspension (spore
density $5 \times 10^6$ spores/mL) under aseptic condition and the fungal cultures were grown under stationary culture condition in a BOD maintained at 30°C.

On fifth day of inoculation when lignin peroxidase activity reached maximum value, the cultures were pooled, mycelia were removed by filtration through four layers of cheese cloth and culture filtrate $\approx 1.2$ L with 0.086 IU/mL activity was concentrated with Amicon Concentration Cell Model 8200 using PM10 ultrafiltration membrane with molecular wt. cut-off value 10 kDa to 10 mL. The concentrated enzyme was dialysed against 1000 times excess of 5 mM sodium succinate buffer $p_H 5.5$ overnight at 20ºC. The dialysed enzyme was loaded on a DEAE column size 1 x 16 cm 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; 50 mL + 50 mL=100 mL). 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 using the method of Weber and Osborn. The separating gel was 12% acrylamide in 0.375 M Tris-HCl buffer $p_H 8.8$ and stacking gel was 5% acrylamide in 0.063 M Tris-HCl buffer 6.8. Proteins were visualized by staining with Coomassie Blue R-250. The molecular weight markers were Phosphorylase (97.4 kDa), Bovine serum albumin (66 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29 kDa), Soyabean trypsin inhibitor (20.1 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 (Ref 15).

**Enzyme activity assay**

The activity of lignin peroxidase was assayed by the method reported by Tien and Kirk 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$_2$O$_2$ (freshly prepared) 0.4 mM in 50 mM of sodium tartrate buffer $p_H 2.5$ at 25°C. The reaction was started by adding 50 μL of the enzyme solution of 0.203 IU/mL. The spectrophotometer used was Hitachi (Japan) Model U-2000 and was fitted with electronic temperature control unit. The least count of absorbance measurement was 0.001 absorbance unit. One enzyme unit is the amount of the enzyme which produces 1μmole of the product per minute under the assay condition specified above.

**Effect of organic solvents on catalytic activity of lignin peroxidase**

The effects of organic solvents acetone, dioxane, acetonitrile, dimethyl formamide and diethyl ether were studied by measuring the activities of the enzyme containing 0, 5, 10, 15, 20 percent of the organic solvents in the aqueous reaction solutions mentioned above for activity assay for lignin peroxidase. A graph was plotted showing relative activity versus percentage of organic solvent in the aqueous medium. The inhibition of the enzyme activity by acetone was studied by drawing reciprocal plots in presence of 0, 5, 10 percent of acetone in the aqueous reaction solutions. The effect of acetone on pH and temperature optima were studied by plotting activity versus pH and activity versus temperature curves in presence of 0, 5 and 10 percent acetone.

**Results and Discussion**

Yadav et al. have reported the secretion of lignin peroxidase by indigenous lignolytic fungal strain |Hexagona tenuis| MTCC-1119 in the liquid culture medium containing wheat straw as natural lignin substrate. The culture conditions have already been optimized for maximum secretion of lignin peroxidase by |Hexagona tenuis| MTCC-1119. The maximum activity appeared on the 5th day of inoculation of the fungal spores. The enzyme was purified by the procedure described in the material and method section. The purification chart is given in the Table I.

The typical elution profile from DEAE column is shown in Figure 1 which shows that the activity is eluted in a single peak. The result of SDS-PAGE shown in Figure 2 shows only one major enzyme band indicating that the enzyme is pure. The molecular weight of the enzyme has been found to be 48 kDa.

The results of steady state kinetic studies on the purified enzyme using veratryl alcohol, H$_2$O$_2$ and
n-propanol as variable substrates are shown in Figures 3 (a), (b) and (c) respectively. The calculated K_m values are 70 μM, 80 μM and 530 μM respectively for veratryl alcohol, H_2O_2 and n-propanol. The enzymatic characteristics of lignin peroxidase of *Phanerochaete chrysosporium* ATCC-24725 have been widely studied\textsuperscript{12}. The K_m values using the above substrate for the lignin peroxidase of *Phanerochaete chrysosporium* have been reported to be 60 μM (Ref 12), 80 μM (Ref 12) and 500 μM (Ref 18) respectively indicating that enzymatic characteristics of lignin peroxidase of *Hexagona tenuis* MTCC-1119 are very similar to lignin peroxidase of *Phanerochaete chrysosporium*. The activity-pH and activity-temperature profiles of lignin peroxidase of *Hexagona tenuis* are shown in Figures 4 and 5 respectively. The pH and temperature optima of this enzyme are 3 and 30°C respectively. These values of pH and temperature optima are greater than those reported\textsuperscript{12} for lignin peroxidase of *Phanerochaete chrysosporium*, the values being 2.5 and 26°C respectively.

The effects of water miscible organic solvents acetone, dioxane, dimethylformamide, acetonitrile and diethyl ether on the activity of the lignin peroxidase of *Hexagona tenuis* are shown in Figure 6. The activity of the enzyme is completely inhibited by nearly 20% of these solvents in aqueous medium but it reverted back if the concentrations of these solvents were decreased in the reaction medium showing that the inhibition is reversible. In order to determine the nature of inhibition, double reciprocal plots were drawn in presence of 0%, 5%, 10% of acetone in aqueous medium using veratryl alcohol, n-propanol and H_2O_2 as substrates as shown in Figure 7. The double reciprocal plots shown in Figure 7 (a), (b) and (c) are parallel lines showing that the inhibition by acetone is uncompetitive. The calculated inhibition constants K_I are 2.72 mM, 2.96 mM and 0.34 mM using veratryl alcohol,

### Table I — Purification Chart

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<tr>
<th>S. No</th>
<th>Steps</th>
<th>Total Vol (mL)</th>
<th>Protein (mg/mL)</th>
<th>Activity (U/mL)</th>
<th>Specific Activity (U/mg)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (U)</th>
<th>Purification Fold</th>
<th>% Recovery</th>
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<tr>
<td>1</td>
<td>Crude enzyme</td>
<td>0.03</td>
<td>0.08</td>
<td>2.68</td>
<td>38.40</td>
<td>103.20</td>
<td>1</td>
<td>100</td>
<td></td>
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<tr>
<td>2</td>
<td>Concentrated enzyme</td>
<td>0.25</td>
<td>4.14</td>
<td>16.56</td>
<td>2.50</td>
<td>41.40</td>
<td>6.17</td>
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<tr>
<td>3</td>
<td>Dialysed enzyme</td>
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<td>1.80</td>
<td>18.00</td>
<td>1.72</td>
<td>30.60</td>
<td>7.46</td>
<td>29.60</td>
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<tr>
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<td>DEAE</td>
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<td>0.87</td>
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<td>0.08</td>
<td>4.14</td>
<td>16.56</td>
<td>16.56</td>
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</tbody>
</table>

Figure 1 — A typical elution profile of the enzyme from DEAE column. (■) activity profile at 310 nm and (●) protein profile at 750 nm

Figure 2 — SDS – PAGE analysis of the purified enzyme. Lane 1 contains the molecular weight markers (from top): Phosphorylase (97.4 kDa), Bovine serum albumin (66 kDa), Ovalbumin (43 kDa), Soyabean trypsin inhibitor (20.1 kDa), Carbonic anhydrase (29 kDa) and Lysozyme (14.3 kDa). Lane 2 contains the purified lignin peroxidase.
Figure 3 — Michaelis-Menten plots and double reciprocal plots for the purified lignin peroxidase (a) using veratryl alcohol as the substrate, (b) using n-propanol as the substrate and (c) using H2O2 as the substrate.

Figure 4 — Activity – pH profile of the purified lignin peroxidase.

Figure 5 — Activity – temperature profile of the purified lignin peroxidase.
hydrogen peroxide and \( n \)-propanol as the substrates respectively. In case of \( n \)-propanol as the substrate, the inhibition constant of acetone is lower than the \( K_m \) value of the enzyme showing that the enzyme has greater affinity for acetone (the inhibitor) than \( n \)-propanol (the substrate). In case of the other two substrates, the enzyme has greater affinities for the substrates than acetone, the inhibitor. It is worth mentioning that the enzyme has lower affinity for propanol (\( K_m \) value 530 µM) than veratryl alcohol (\( K_m \) value 70 µM) and hydrogen peroxide (\( K_m \) value 80 µM).

The activity-pH profile and activity-temperature profile in presence of 0%, 5% and 10% acetone were drawn using veratryl alcohol as substrate (figures not shown here). Though the activity is decreased in presence of acetone, pH and temperature optima were unaffected by the presence of acetone.

Thus in this communication, purification of lignin peroxidase from the culture filtrate of a new indigenous lignolytic fungal strain *Hexagona tenuis* MTCC-1119 to homogeneity has been achieved using a simple procedure. The enzymatic characteristics of

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**Figure 6** — The effect of water miscible organic solvents on the activity of the purified lignin peroxidase acetone (■), dioxane (□), dimethylformamide (▲), acetonitrile (⊙) and diethyl ether (●) on the activity of lignin peroxidase of *Hexagona tenuis.*

**Figure 7** — The effect of acetone on the double reciprocal plots using (a) veratryl alcohol (■) \( n \)-propanol and (c) \( H_2O_2 \) as the substrates. 0% acetone (⊙), 5% acetone (■), 10% acetone (●).
the enzyme like Km, pH and temperature optima have been determined indicating that enzymatic characteristics of lignin peroxidase of *Hexagona tenuis* MTCC-1119 are very similar to lignin peroxidase of *Phanerochaete chrysosporium*. Effects of water miscible organic solvents on the enzymatic characteristics have been studied. In conclusion, the enzyme could retain up to nearly 50% activity in 10% of these organic solvents in aqueous media. Thus these reaction media could be used for biotransformations involving lignin peroxidases.

**Acknowledgements**

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