Lignin peroxidases of some indigenous ligninolytic fungi: secretion and enzymatic characteristics

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Abstract Secretion and enzymatic characteristics of lignin peroxidases from *Gloeophyllum sepiarium* MTCC 1170, *Cladosporium herbarum* MTCC 346, *Lenzites betulina* MTCC 1183, *Daedalea flavidula* MTCC 145, *Hexagonia teruis* MTCC 1119 and *Coirolopsis floccosa* MTCC 1177 ligninolytic fungal strains have been reported. Secretion of lignin peroxidase by these ligninolytic fungal strains have been found to be in the range of 0.86 to 3.0 enzyme unit per ml of the culture medium. The enzymatic characteristics like K_m , pH and temperature optima of all the lignin peroxidases of the above fungal strains have been determined using veratryl alcohol and H_2O_2 as the variable substrates. The K_m values using veratryl alcohol as the substrate were found to be 65.0 μ M, 58.5 μ M, 63.0 μ M, 54.5 μ M, 54.6 μ M and 61.0 μ M respectively. The K_m values using H_2O_2 as the substrate were found to be 88.0 μ M, 86.0 μ M, 71.0 μ M, 67.0 μ M, 80.0 μ M and 78.0 μ M respectively. The pH optima values for lignin peroxidases of the above ligninolytic fungal strains were found to be 2.5, 2.4, 2.4, 2.25, 2.5 and 2.8 respectively, where as the temperature optima values were 25°C, 24°C, 25°C, 23°C, 24°C and 25°C respectively.

Keywords Lignin peroxidases · Heme peroxidases · Ligninolytic enzymes · Ligninolytic fungi

Introduction

Lignin Peroxidase [E.C.1.11.1.14] is a biotechnologically important enzyme which has application potential in delignification of lignocellulosic materials [1, 2], in the conversion of high molecular mass fraction coal to low molecular mass fraction coal [3] so that it could be used as feed stock for the production of commodity chemicals, in biopulping [4] and biobleaching [5] in paper industries, in removal of recalcitrant organic pollutants [6–10], and in the enzymatic polymerisation [11] in polymer industries. Considering the application potentials of lignin peroxidases, we have initiated studies on identification of indigenous sources of lignin peroxidases and the enzymatic characteristics of the enzymes present in these sources so that the enzymes suitable for various applications could be identified. The secretion of lignin peroxidase by these ligninolytic fungal strains have been demonstrated, the culture conditions for maximum production of lignin peroxidases in cases of these fungal strains have been optimized and the enzymatic characteristics like K_m , pH and temperature optima of lignin peroxidases produced by these ligninolytic fungal strains have been determined.

Veratryl alcohol (3,4-dimethoxy benzyl alcohol) was from Aldrich (Wisconsin, USA). Nitroacetate was from Sigma Chemical Company (St. Louis USA). All other chemicals were either from CDH (Delhi) or from Loba Chemie (Mumbai) or from s.d. fine-chem Limited (Worli road, Mumbai) and were used without further purification.

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The fungal strains were procured from Microbial Type Culture Collection Center and Gene Bank, Institute of Microbial Technology, Chandigarh and were maintained on agar slants. The growth medium [12] for agar slants for the fungal strains *Gloeophyllum sepiarium* MTCC 1170, *Lenzites betulina* MTCC 1183, *Coirolopsis floccosa* MTCC 1177 consisted of growth medium [12] no. 65 which contained malt extract 20.0 g and agar 20.0 g in 1.0 L double distilled water and pH of medium was adjusted 7.5. The growth medium [10] for fungal strain *Daedalea flavida* MTCC 145 consisted of growth medium [12] no. 8 which contained yeast extract 5.0 g, glucose 10.0 g and agar 15.0 g in 1.0 L double distilled water, pH was 5.8. Similarly the growth medium [12] for agar slants *Hexagonia teruis* MTCC 1119 and *Cladosporium herbarum* MTCC 346 consisted of growth medium [12] no. 97 and 7 respectively. Growth medium [12] no. 97 contained malt extract 20.0 g, peptone 5.0 g and agar 15.0 g in 1.0 L double distilled water and growth medium [12] no. 7 contained Potato (scrubbed and diced) 200.0 g, Dextrose 20.0 g and agar 15.0 g in 1.0 L double distilled water. [Boiled diced potatoes in 500 mL of double distilled water until thoroughly cooked. Filtered through cheese cloth and added double distilled water to filtrate upto 1.0 L. Added agar to the filtrate and dissolved by boiling. Removed from heat and added glucose (dextrose) pH of the medium was 5.6].

These microorganisms were tested for extracellular secretion of lignin peroxidase in liquid culture growth medium. The growth medium consisted of 10g of glucose, 1.32 g of ammonium tartrate, 0.2 g of KH_2PO_4 , 50 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg of CaCl_2 , 10g of thiamine-HCl per liter and 1 ml of a solution containing per litre 3 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1 g of NaCl, 100 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 185 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 80 mg of CaCl_2 , 180 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 10 mg of $\text{AlK}(\text{SO}_4)_2$, 10 mg of H_3BO_3 , 12 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ and 1.5 g of nitrilotriacetate. Growth medium [13] containing natural lignin substrates like corn-cob, coir-dust, saw-dust, wheat-straw and bagasse particles were separately prepared by adding 0.5 g of one of the natural lignin substrate to 25 mL of growth medium in 100 mL culture flasks which were sterilized. The sterilized growth medium was inoculated with small piece of mycelium (0.5×0.5 cm) under aseptic condition and the fungal culture was grown under stationary culture condition at 30°C in a BOD. The aliquots (0.5 mL) of the growth medium were withdraw at the regular intervals of 1 day and were filtered through Millipore Millex-GS 0.22 μM filter unit. The lignin peroxidase activity [13, 14] was assayed using veratryl alcohol as the substrate at $\lambda = 310$ nm with UV/VIS spectrophotometer Hitachi (Japan) model U-2000, which was fitted with electronic

temperature control unit. The enzyme unit was calculated using molar extinction coefficient value of $9300 \text{ M}^{-1}\text{cm}^{-1}$ for veratraldehyde. The least count of absorbance measurement was 0.001 absorbance unit.

The secretion curve for lignin peroxidase was obtained by plotting enzyme unit/mL of the growth medium against the number of days for which the fungal strain has been allowed to grow. Each point on the curve is an average of triplicate measurements. The percentage standard deviation was less than 5%. The growth media of control experiments have same compositions as others except that no natural ligninolytic substrates have been added.

Optimization of lignin peroxidase in liquid culture growth medium was done as a function of concentration of the lignin containing natural substrates, the same growth medium used as in case of secretion. Culture flasks containing growth medium with different concentrations of lignin containing natural substrates were set up. All culture flasks were sterilized and cooled. A small piece of mycelium (0.5×0.5 cm) of a particular fungal strain was added in each culture flasks having different concentrations of lignin containing natural substrates under aseptic conditions. The cultures were allowed to grow under stationary condition at 30°C in a BOD. 0.5 mL aliquots of the growth medium at 1 day intervals were withdrawn and filtered through Millipore Millex-GS 0.22 μM filter unit. The filtered growth medium was analysed for the presence of lignin peroxidase per mL of growth medium using veratryl alcohol as the substrate. The enzyme unit per mL of the growth medium was plotted against the number of days for culture flasks containing different concentrations of the lignin containing natural substrates.

The enzymatic characteristics like K_m , pH and temperature optima were determined using veratryl alcohol and H_2O_2 as the substrates.

The K_m values for veratryl alcohol and H_2O_2 were determined from the double reciprocal plots of $1/[V]$ Vs $1/[S]$, where $[V]$ is the steady state velocity of the enzyme catalysed reaction and $[S]$ is the concentration of the substrate. The pH optimum value was determined by plotting the steady state velocity of the enzyme catalysed reaction against different pH value. The pH range from 1.8 to 3.5 was maintained using tartaric acid/ disodium tartrate buffer (50 mM). The temperature optimum value was determined by plotting the steady state velocity of the enzyme catalysed reaction against different temperatures. In all cases, triplicate measurements were made for a single condition and the average value of the measurements was calculated.

Fig. 1 (A), (B), (C), (D), (E), and (F) in which enzyme units/mL of the growth media have been plotted against the time of growth showed the extracellular production

Fig. 1 Secretion of lignin peroxidase by (A) *Gloeophyllum sepiarium* MTCC 1170 (B) *Cladosporium herbarum* MTCC 346 (C) *Lenzites betulina* MTCC 1183 (D) *Daedalea flavida* MTCC 145 (E) *Hexagonia teruis* MTCC 1119 (F) *Coirolopsis floccosa* MTCC 1177 (a) Corn-cob (♦) (b) Coir-dust (■) (c) Saw-dust (▲) (d) Wheat straw (●) (e) Bagasse (○) (f) Control (◇).

of lignin peroxidases in the liquid culture growth media amended with various lignin containing natural substrates like corn-cob, coir-dust, saw-dust, wheat-straw and bagasse inoculated with *Gloeophyllum sepiarium* MTCC 1170, *Cladosporium herbarum* MTCC 346, *Lenzites betulina* MTCC 1183, *Daedalea flavida* MTCC 145, *Hexagonia teruis* MTCC 1119 and *Coirolopsis floccosa* MTCC 1177 respectively. In each case, control experiments having simi-

lar medium composition as the uncontrolled one except the absence of natural ligninolytic substrate were set. It is obvious from the figures that lignin containing natural substrates when present in the liquid media enhanced the extracellular production of lignin peroxidases. This enhancement of extracellular lignin peroxidase is maximum in case of the growth medium containing coir-dust, saw-dust, coir-dust, wheat-straw and coir-dust in cases of *Gloeoph-*

yllum sepiarium MTCC 1170, *Cladosporium herbarum* MTCC 346, *Lenzites betulina* MTCC 1183, *Daedalea flavida* MTCC 145, *Hexagonia teruis* MTCC 1119 and *Coirolopsis floccosa* MTCC 1177 respectively.

Fig. 2 (A), (B), (C), (D), (E), and (F) showed the optimisations of the culture conditions for the maximum secretion

of lignin peroxidases in *Gloeophyllum sepiarium* MTCC 1170, *Cladosporium herbarum* MTCC 346, *Lenzites betulina* MTCC 1183, *Daedalea flavida* MTCC 145, *Hexagonia teruis* MTCC 1119 and *Coirolopsis floccosa* MTCC 1177 respectively. The results of the optimisations of the culture conditions for the maximum production of lignin peroxi-

Fig. 2 Optimisation of lignin peroxidase by (A) *Gloeophyllum sepiarium* MTCC 1170 (B) *Cladosporium herbarum* MTCC 346 (C) *Lenzites betulina* MTCC 1183 (D) *Daedalea flavida* MTCC 145 (E) *Hexagonia teruis* MTCC 1119 (F) *Coirolopsis floccosa* MTCC 1177 in the liquid culture medium amended with different amounts of coir dust, saw dust, saw dust, coir dust, wheat straw and coir dust. (a) 100mg (●) (b) 200mg (■) (c) 400mg (▲) (d) 500mg (×) (e) 600mg (○) (f) 800mg (Δ) (g) 1000mg (+).

dase in all the six fungal strains are summarised in Table 1. The table indicates the best inducer of the lignin peroxidase in per 25 mL of the liquid culture medium to give maximum enzyme units and also the maximum level of enzyme units produced in case of each of the fungal strains. It is obvious from the Table 1 that *Hexagonia teruis* MTCC 1119 and *Coirolopsis floccosa* MTCC 1177 are the best secretors of lignin peroxidases under the studied conditions and the maximum enzyme units/mL of liquid culture medium are achieved with wheat straw and coir dust as inducers at 0.8g/25 mL and 0.8g/25 mL of liquid cultures respectively. Under these conditions, the maximum levels of lignin peroxidases achieved in case of *Hexagonia teruis* MTCC 1119 and *Coirolopsis floccosa* MTCC 1177 are 2.9 IU/mL and 3.0 IU/mL respectively. *Phanerochaete Chrysosporium* ATCC-24725 has been widely used for the purification of lignin peroxidase [14]. The reported [14] enzyme unit without adding natural ligninolytic substrates in liquid culture medium is 0.075 IU/mL. The significance of our result is that *Hexagonia teruis* MTCC 1119 and *Coirolopsis floccosa* MTCC 1177 are relatively better secretors of lignin

peroxidases and can be used for commercial production of lignin peroxidases. By applying specific biotechnological strategies secretion of lignin peroxidase by *Hexagonia teruis* MTCC 1119 and *Coirolopsis floccosa* MTCC 1177 can be further increased as has been done [15–18] in case of *Phanerochaete Chrysosporium* ATCC-24725.

Figures 3 and 4 show the Michaelis-Menten and double reciprocal plots for lignin peroxidase of *Coirolopsis floccosa* MTCC 1177 using veratryl alcohol and H_2O_2 as the variable substrates. These plots for lignin peroxidases of other five fungal strains namely *Gloeophyllum sepium* MTCC 1170, *Cladosporium herbarum* MTCC 346, *Lenzites betulina* MTCC 1183, *Daedalea flavigra* MTCC 145 and *Hexagonia teruis* MTCC 1119 have also been drawn but not shown here because of similarity with Figs. 3 and 4. The K_m values for lignin peroxidases of all the six fungal strains have been determined using veratryl alcohol and H_2O_2 as the variable substrates and results obtained are summarized in Table 2. It is obvious from the Table 2 that the K_m values of the lignin peroxidases produced by six indigenous ligninolytic fungal strains are 65.0 μ M, 58.5 μ M, 63.0 μ M, 54.5 μ M, 54.6 μ M

Table 1 Optimal culture conditions for maximum secretion of lignin peroxidases

S. No.	Fungal Strain	Best inducer with amount per 25 mL liquid culture	Days of Maximum production	Maximum level of activity per mL in enzyme unit
1.	<i>Lenzites sepiaria</i> MTCC 1170	Coir dust (0.6 g)	4 th	1.69
2.	<i>Cladosporium herbarum</i> MTCC 346	Saw dust (0.5g)	7 th	0.86
3.	<i>Lenzites betulina</i> MTCC 1183	Saw dust (0.8g)	7 th	1.72
4.	<i>Daedalea flavigra</i> MTCC 145	Coir dust (0.4g)	5 th	2.30
5.	<i>Hexagonia teruis</i> MTCC 1119	Wheat straw (0.8g)	9 th	2.90
6.	<i>Coirolopsis floccosa</i> MTCC 1177	Coir dust (0.8g)	5 th	3.00

Fig. 3 Michaelis–Menten and double reciprocal plot for the lignin peroxidase of *Coirolopsis floccosa* MTCC 1177 using veratryl alcohol, as the variable substrate.

Fig. 4 Michaelis–Menten and double reciprocal plot for the lignin peroxidase of *Coirolopsis floccosa* MTCC 1177 using H_2O_2 , as the variable substrate.

and 61.0 μ M for veratryl alcohol and 88.0 μ M, 86.0 μ M, 71.0 μ M, 67.0 μ M, 80.0 μ M and 78.0 μ M for H_2O_2 , which are in the same range of the reported K_m values 60.0 μ M for

Fig. 5 Variation of the activities of the lignin peroxidases of different fungal strains with variation of reaction pH (a) *Lenzites sepiaria* MTCC 1170 (●) (b) *Cladosporium herbarum* MTCC 346 (■) (c) *Lenzites betulina* MTCC 1183 (▲) (d) *Daedalea flavida* MTCC 145 (×) (e) *Hexagonia teruis* MTCC 1119 (○) (f) *Coirolopsis floccosa* MTCC 1177 (□).

Fig. 6 Variation of the activities of the lignin peroxidases of different fungal strains with variation of reaction temperature (a) *Lenzites sepiaria* MTCC 1170 (●) (b) *Cladosporium herbarum* MTCC 346 (■) (c) *Lenzites betulina* MTCC 1183 (▲) (d) *Daedalea flavida* MTCC 145 (×) (e) *Hexagonia teruis* MTCC 1119 (○) (f) *Coirolopsis floccosa* MTCC 1177 (□).

Table 2 Enzymatic characteristics like K_m , pH and temperature optima of lignin peroxidases

S. No.	Fungal strain	K_m for veratryl alcohol (μ M)	K_m for H_2O_2 (μ M)	pH optimum	Temperature optimum ($^{\circ}$ C)
1.	<i>Lenzites sepiaria</i> MTCC 1170	65.0	88.0	2.5	25
2.	<i>Cladosporium herbarum</i> MTCC 346	58.5	86.0	2.4	24
3.	<i>Lenzites betulina</i> MTCC 1183	63.0	71.0	2.4	25
4.	<i>Daedalea flavida</i> MTCC 145	54.5	67.0	2.25	23
5.	<i>Hexagonia teruis</i> MTCC 1119	54.6	80.0	2.5	24
6.	<i>Coirolopsis floccosa</i> MTCC 1177	61.0	78.0	2.8	25

veratryl alcohol and 80.0 μ M for H_2O_2 of *Phanerochaete Chrysosporium* [14] ATCC-24725.

Figures 5 and 6 show the variation of activities of lignin peroxidases of *Gloeophyllum sepiarium* MTCC 1170, *Cladosporium herbarum* MTCC 346, *Lenzites betulina* MTCC 1183, *Daedalea flavida* MTCC 145, *Hexagonia teruis* MTCC 1119 and *Coirolopsis floccosa* MTCC 1177 with variation of pH and temperature of the reaction medium respectively. The results of pH and temperature optima of all the above mentioned six fungal strains have been determined and achieved results are also summarized in Table 2. It is clearly seen from the Table 2 that results obtained for pH optima values are 2.5, 2.4, 2.4, 2.25, 2.5 and 2.8 and temperature optima values are 25 $^{\circ}$ C, 24 $^{\circ}$ C, 25 $^{\circ}$ C, 23 $^{\circ}$ C, 24 $^{\circ}$ C and 25 $^{\circ}$ C for the six indigenous ligninolytic fungal strains, which are also in the same range as reported pH optimum 2.8 and temperature optimum 24 $^{\circ}$ C of *Phanerochaete Chrysosporium* [14] ATCC-24725.

Thus the indigenous ligninolytic fungal strain *Hexagonia teruis* MTCC 1119 and *Coirolopsis floccosa* MTCC

1177 have been found better secretors of lignin peroxidases, which can be used in place of *Phanerochaete Chrysosporium* ATCC-24725 and the enzymatic characteristics of the lignin peroxidase so obtained is nearly similar to *Phanerochaete Chrysosporium* ATCC-24725.

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