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Secretion and characterisation of ligninperoxidases by some new indigenous lignolytic fungi

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ABSTRACT

Secretion and enzymatic characteristics of ligninperoxidases from *Coriolus versicolor* MTCC 138, *Trametes hirsuta* MTCC 136, *Phellinus linteus* MTCC 1175, *Volvariella volvacea* MTCC 957, *Phenerochaete chrysosporium* MTCC 787, *Pleurotus ostreatus* MTCC 1803, *Polyporous velutinus* MTCC 1813, *Daedalea elegans* MTCC 1812, *Pleurotus sajor caju* MTCC 141, *Pleurotus sapidus* MTCC 1807 and *Agaricus campestris* MTCC 972 lignolytic fungi have been reported. Secretion of ligninperoxidase by these lignolytic fungi have been found to be in the range of 1.07 to 4.42 enzyme unit per ml of the culture medium. The enzymatic characteristics like Km, pH and temperature optima of ligninperoxidases from *Coriolus versicolor* MTCC 138, *Trametes hirsuta* MTCC 136, *Phellinus linteus* MTCC 1175, *Volvariella volvacea* MTCC 957, *Phenerochaete chrysosporium* MTCC 787, *Pleurotus ostreatus* MTCC 1803, *Polyporous velutinus* MTCC 1813, *Daedalea elegans* MTCC 1812, *Pleurotus sajor caju* MTCC 141, *Pleurotus sapidus* MTCC 1807 and *Agaricus campestris* MTCC 972 fungi have been determined using veratryl alcohol and H_2O_2 as the substrates. The values of K_m using veratryl alcohol as the substrate have been found to be 61.0 µM, 65.0 µM, 59.0 M, 66.0 µM, 63.0 µM, 57.0 mM, 60.0 µM, 61.0 µM, 74.0 µM, 62.0 µM and 71.0 µM respectively. The values of K_m using H_2O_2 as the substrate have been found to be 75.0 µM, 60.0 M, 65.0 µM, 70.0 µM, 90.0 µM, 75.0 µM, 80.0 µM, 80.0 µM, 75.0 µM, 60.0 µM, and 90.0 µM respectively. The *pH* optima values for ligninperoxidases of the above fungi have been found to be 2.4, 2.4, 2.5, 2.6, 2.6, 2.5, 2.5, 2.6, 2.5, 2.6 and 2.8 respectively, where as the temperature optima values were 25 °C, 25 °C, 24 °C , 25 °C, 25 °C, 25 °C, 26 °C, 25 °C, 25 °C, 25 °C and 25 °C respectively.

Key words: Lignin peroxidase, heme peroxidase, lignolytic enzyme.

INTRODUCTION

Ligninperoxidases[1] [E.C.1.11.1.7] are biotechnologically important enzymes having applications in delignification of lignocellulosic materials[2], in the conversion of high molecular mass fraction to low molecular mass fraction[3] that could be used as feed-stock for the production of commodity chemicals, in biopulping and biobleaching in paper industries[4], in bioremediation[5-9] and in enzymatic polymerization[10]. Keeping in view the biotechnological potential of ligninperoxidases, the authors intiated enzymatic studies on the ligninperoxidases of indigenous lignolytic fungi[11] which have been isolated, purified, identified and deposited at MTCC Center and Gene Bank, Institute of Microbial Technology, Chandigarh. In this communication the authors report the secretion of ligninperoxidase in the liquid culture media of the lignolytic fungi *Coriolus versicolor* MTCC 138, *Trametes hirsuta* MTCC 136, *Phellinus linteus* MTCC 1175, *Volvariella volvacea* MTCC 957, *Phenerochaete chrysosporium* MTCC 787, *Pleurotus ostreatus* MTCC 1803, *Polyporous velutinus* MTCC 1813, *Daedalea elegans* MTCC 1812, *Pleurotus sajor caju* MTCC 141, *Pleurotus sapidus* MTCC 1807 and *Agaricus campestris* MTCC 972 .

The culture conditions for maximum production of ligninperoxidase in the liquid cuture media of the above fungi have been optimized and the enzymatic characteristics like K_m for veratryl alcohol & H_2O_2 and *p*H & temperature optima of the ligninperoxidases produced by the above fungi have been determined.

MATERIAL AND METHODS

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

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 medium[12] used for agar slants for the fungi *Phenerochaete chrysosporium* MTCC 787 consisted of growth medium no. 43 which contained malt extract 20.0g, glucose 20.0g, peptone 1.0g and agar 20.0g in 1.0 L double distilled water. The medium[12] used for agar slants for the fungi *Pleurotus sajor caju* MTCC 141, *Pleurotus sapidus* MTCC 1807, *Agaricus campestris* MTCC 972, *Pleurotus ostreatus* MTCC 1803 and *Volvariella volvacea* MTCC 957 consisted of growth medium no. 7 which contained Potato (scrubbed and diced) 200.0g, Dextrose 20.0g and agar 15.0g in 1.0L double distilled water. Boiled diced potatoes in 500mL of double distilled water until throughly cooked. Filter through cheese cloth and added double distilled water to filterate upto 1.0 L . Added agar to the filtrate and dissolved by boiling. Remove from heat and added glucose (dextrose) *p*H of the medium was 5.6 and for the fungal strains *Trametes hirsuta* MTCC 136 and *Coriolus versicolor* MTCC 138 consisted of growth medium no.8 which contained yeast extract 5.0g, glucose 10.0g and agar 15.0g in 1.0 L double distilled water, *p*H was 5.8 while the fungal strains *Phellinus linteus* MTCC 1175, *Daedalea elegans* MTCC 1812 and *Polyporous velutinus* MTCC 1813 consisted of growth medium no. 65 which contained malt extract 20.0g and agar 20.0g in 1.0 L double distilled water and *p*H of medium was adjusted 7.5.

These microorganisms were tested for secretion of ligninperoxidase in liquid culture medium. The growth medium consisted of 10g of glucose, 1.32g of ammonium tartrate, 0.2 g of KH_2PO_4, 50 mg of $MgSO_4.7H_2O$, 10 mg of $CaCl_2$,10g of thiamine-HCl per liter and 1 ml of a solution containing per litre 3 g of $MgSO_4.7H_2O$, 0.5 g of $MnSO_4.H_2O$, 1 g of NaCl, 100 mg of $FeSO_4.7H_2O$, 185 mg of $CoCl_2$. $6H_2O$, 80 mg of $CaCl_2$, 180 mg of $ZnSO_4$. $7H_2O$, 10 mg of $CuSO_4$. $5H_2O$, 10 mg of $AlK(SO_4)_2$, 10 mg of H_3BO_3, 12 mg of Na_2MoO_4. $2H_2O$ 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 and aliquots of 0.5 mL of the growth medium were withdraw at the regular intervals of 24 hrs and were filtered through Millipore Millex-GS 0.22 µM filter unit. The ligninperoxidase activity[11,13] has been assayed by monitoring the formation of veratraldehyde spectrophotometrically at µ=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 of ligninperoxidase was defined as the amount of enzyme, which converts one m mole of veratryl alcohol to veratraldehyde under the standard assay condition. The least count of absorbance measurement was 0.001 absorbance unit.

Extracellular secretion of ligninperoxidase in the liquid culture medium by *Coriolus versicolor* MTCC 138, *Trametes hirsuta* MTCC 136, *Phellinus linteus* MTCC 1175, *Volvariella volvacea* MTCC 957, *Phenerochaete chrysosporium* MTCC 787, *Pleurotus ostreatus* MTCC 1803, *Polyporous velutinus* MTCC 1813, *Daedalea elegans* MTCC 1812, *Pleurotus sajor caju* MTCC 141, *Pleurotus sapidus* MTCC 1807 and *Agaricus campestris* MTCC 972 were determined plotting the enzyme unit/mL of the growth medium against the number of days after inocubation of the fungal mycelia. Each point on the curve is an average of the three measurements. The growth medium control experiment has the same composition as others expect that no natural lignolytic substrates have been added. The best inducers of ligninperoxidase secretion were identified with the help of secretion curve.

In order to optimise the culture conditions for maximum production of ligninperoxidase by the above mentioned fungal strains. The amount of the best inducers were varied from 100 mg to 1000 mg in 25 ml of the growth medium. In this case also the enzyme units per 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.

Ligninperoxidases for further research work were prepared by growing the fungal cultures (5×25 ml) in five culture flasks (100ml) containing optimal amount of the inducers under stationary culture condition in a BOD at 30° C.The maximum activity appeared from 4 to 6 days after inoculation of fungal mycelia. On maximum day of activity, cultures in all the five flasks were pooled, filtered through four layers of cheese cloth and stored at 4^0 C. The enzyme did not loose appreciable activities for one month under these condition.

Table 1 : Optimal culture conditions for maximum secretion of ligninperoxidases

S. No.	Fungal Strain	Best inducer with amount per 25 mL liquid culture	Maximum production	Maximum activity (IU/ml)
1.	*Coriolus versicolor* MTCC 138	Saw dust (0.5g)	5th	2.13
2.	*Trametes hirsuta* MTCC 136	Saw dust (0.5g)	4th	3.98
3.	*Phellinus linteus* MTCC 1175	Saw dust (0.5g)	5th	1.94
4.	*Volvariella volvacea* MTCC 957	Wheat straw (0.8g)	5th	4.42
5.	*Phenerochaete chrysosporium* MTCC 787	Saw dust (0.5g	6th	1.07
6.	*Pleurotus ostreatus* MTCC 1803	Coir dust (0.8g	5th	1.84
7.	*Polyporous velutinus* MTCC 1813	Saw dust (0.5g	4th	1.30
8.	*Daedalea elegans* MTCC 1812	Saw dust (0.5g	4th	1.94
9.	*Pleurotus sajor caju* MTCC 141	Wheat straw (0.8g)	5th	2.98
10.	*Pleurotus sapidus* MTCC 1807	Coir dust (0.8g	6th	3.94
11.	*Agaricus campestris* MTCC 972	Wheat straw (0.8g)	6th	4.20

Table 2: Enzymatic characteristics like K_m, *p*H and temperature optima of ligninperoxidases

S. No.	Fungal strain	K_m for veratryl alcohol (µM)	K_m for H_2O_2 (µm)	*p*H Optimum	Temperature optimum (°C)
1.	*Coriolus versicolor* MTCC 138	61	75	2.4	25
2.	*Trametes hirsuta* MTCC 136	65	60	2.34	25
3.	*Phellinus linteus* MTCC 1175	59	65	2.5	24
4.	*Volvariella volvacea* MTCC 957	66	70	2.6	25
5.	*Phenerochaete chrysosporium* MTCC 787	63	90	2.6	25
6.	*Pleurotus ostreatus* MTCC 1803	57	75	2.5	25
7.	*Polyporous velutinus* MTCC 1813	60	80	2.5	26
8.	*Daedalea elegans* MTCC 1812	61	80	2.6	25
9.	*Pleurotus sajor caju* MTCC 141	72	75	2.5	25
10.	*Pleurotus sapidus* MTCC 1807	62	60	2.6	25
11.	*Agaricus campestris* MTCC 972	71	90	2.8	25

The enzymatic characteristics of ligninperoxidases like $K_{m,}$ *p*H and temperature optima were determined using veratryl alcohol and H_2O_2 as the substrates.

The K_m values were determined by measuring steady state velocities of ligninperoxidases catalysed reactions at different substrate concentrations and drawing double reciprocal plots[14], The *p*H optimum was determined by measuring the steady state velocities of ligninperoxidase catalysed reaction in the reaction solutions having different *p*H values and plotting steady state velosity against the *p*H values. Similarly, the temperature optimum were determined by measuring the steady state velocities in reaction solutions at different temperatures and plotting the steady state velocities against temperatures of the reaction solutions.

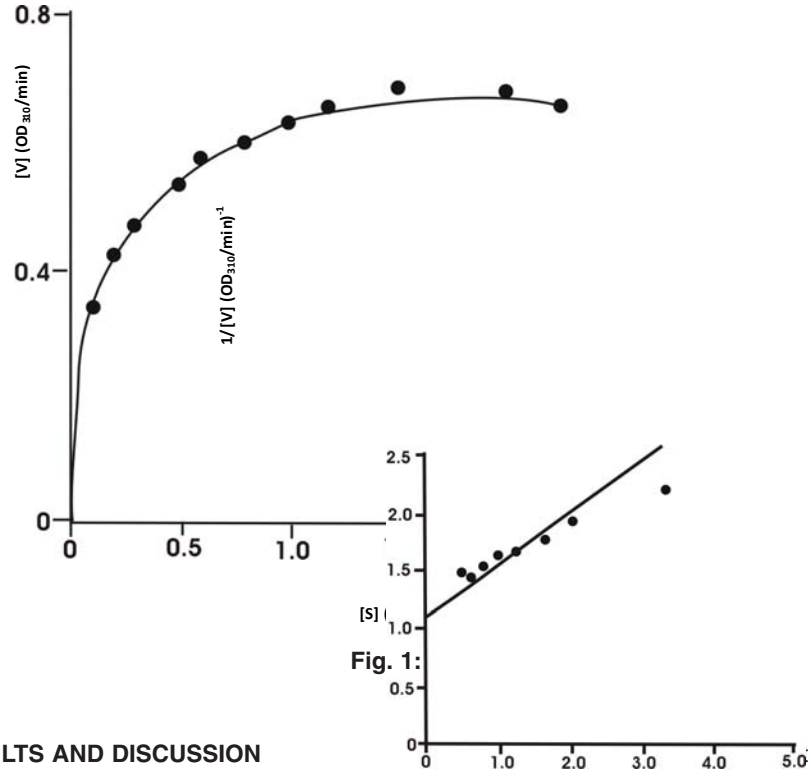

Fig. 1:

RESULTS AND DISCUSSION

The extracellular production of ligninperoxidases were observed 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 individualy with *Coriolus versicolor* MTCC 138, *Trametes hirsuta* MTCC 136, *Phellinus linteus* MTCC 1175, *Volvariella volvacea* MTCC 957, *Phenerochaete chrysosporium* MTCC 787, *Pleurotus ostreatus* MTCC 1803, *Polyporous velutinus* MTCC 1813, *Daedalea elegans* MTCC 1812, *Pleurotus sajor caju* MTCC 141, *Pleurotus sapidus* MTCC 1807 and *Agaricus campestris* MTCC 972. In each case, control experiment had the natural lignolytic substrate. The results show that the presence of lignin containing natural substrates in liquid media enhanced the extracellular secretion of lignin peroxidases as shown in Fig 1.0 (in case of MTCC 957). The enhancement of extracellular ligninperoxidase was the maximum in the presence of saw dust for *Coriolus versicolor* MTCC 138, *Trametes hirsuta* MTCC 136, *Phellinus linteus* MTCC 1175, *Phenerochaete chrysosporium* MTCC 787, *Polyporous velutinus* MTCC 1813 and *Daedalea elegans* MTCC 1812, in the presence of wheat straw for *Volvariella volvacea* MTCC 957, *Pleurotus sajor caju* MTCC 141 and *Agaricus campestris* MTCC 972, coir-dust for *Pleurotus ostreatus* MTCC 1803 and *Pleurotus sapidus* MTCC

1807. The figures for the extracellular production of ligninperoxidases by other species were similar, therefore not given here.

The results of optimisation of the culture conditions for the maximum secretion of ligninperoxidase in cases of all the eleven fungal strains are summarized in Table 1.

It is clear that *Volvariella volvacea* MTCC 957 was found to be the best secretor of lignin peroxidase (4.42 IU/mL) in the presence of saw dust in the liquid culture media. Reported enzyme unit in liquid culture medium without adding natural lignolutic substrates was 0.075 IU/mL[13] in case of *Phanerochacte Chrysosporium* ATCC-24725, which has been widely used in case of purification of ligninperoxidase. The present results can be compared with the indigenous strain of *P. ostreotus* MTCC 142, in which the maximum level of ligninperoxidase[15] secretion was reported as 3.50 IU/mL. This clearly shows that *Volvariella volvacea* MTCC 957 is relatively better secretor of ligninperoxidase and can be used for commertial production of ligninperoxidase.

The enzymatic characteristic, like K_m, *pH* and temperature optima, determined for fungal strains used are summerised in Table 2.

Thus it is clear that the enzymatic characteristics of the ligninperoxidases produced by indigenous fungal strains are in the same range as the enzymatics characteristics reported by *Phanerochaete Chrysosporium* ATCC-24725[13].

Therefore, it can be concluded that the indigenous Lignolytic fungal strain *Volvariella volvacea* MTCC 957, which has been found better secretor of ligninperoxidase and can be used in place of *Phanerochaete Chrysosporium* ATCC-24725 and *P. ostreotus* MTCC 142[11]. Further, the enzymatic characteristics of the ligninperoxidases obtained by indigenous Lignolytic fungal strains will not be much different from those of *Phanerochaete Chrysosporium* ATCC-24725 and *P. ostreotus* MTCC 142.

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REFERENCES

1. Steven L Edwards , Reeta Raag , Hiroyuki Wariishi & Michael H Gold , Crystal structure of lignin peroxidase, *Proc Natl Acad Sci USA,* **90**: 750-754 (1993).
2. Harley B S, Brodo P M A & Senior P J, Proceeding of Royal society discussion meeting on utilisation of lignocellulosic wastes, *Cambridge University Press*, (1988).
3. Catcheside D E A & Ralph J P, Biological processing of coal, *Appl. Microbiol Biotechnol,* **52:**16-24 (1999).
4. Eriksson K E & Kirk T K, Biopulping: an overview of developments in an environmentally safe paper making technology, *FEMS Microbiol Rev*, **13**: 351-364 (1994).
5. Bumpus J A, Tien M, Wright D & Aust S D, Oxidation of persistant environmental pollutants by a white – rot fungus, *Science*, **228**: 1434-1436 (1985)
6. Kwant Soo Sin & Chang- Jin Kim, Decolorisation of artificial dyes by peroxidase from the white-rot fungus *Pleurotus ostreatus, Biotechnology letters,* **20:** 569-572 (1998).
7. Satwinder S Marwaha, Rajesh Grover, Chand Prakash & John F Kennedy, Continuous biobleaching of black liquor from the pulp and paper industry using an immobilised cell system, *J* Chem *Technol Biotechnol*, **73**: 292-296 (1998).
8. Levin L, Papinutti L and Forchiassin F, Evalution of Argentinean white-rot fungi for their ability to produce ligninmodifying

enzymes and decolorize industrial dues, *Bioresource Technology*, **94:**169-176 (2004)

9. N Cenek, S Katerina, E Pavla, C Tomas, K Aparna, L Elke and S Vaclav, Lignolytic fungi in bioremediation: extracellular enzyme production and degradation rate, *Soil Biology* and *Biochemistry*, **36**:1545-1551(2004)
10. Hiroshi Uyama & Shiro Kobayashi, Enzymatic polymerization yield useful polyphenols, *CHEMTECH*, October, 22 (1999)
11. Meera Kumari, RSSYadav & KDSYadav Secretion of ligninperoxidase by *Penicillium citrinum, Fusarium oxysporum and Aspergillus terreus, Ind.J.Expt.Biol.* **40**: 802-806 (2002)
12. Microbial Type Culture Collection, Catalogue of Strains-2000, Fifth Edition, Institute of Microbial Technology Sector 39-A, Chandigarh.
13. M Tien and T K Kirk, Ligninperoxidase of *Phanerochaete Chrysosporium, Methods in Enzymology*, **161**: 238-249 (1988)
14. Alan Fersht, Structure and mechanism in protein science, W.H. Freeman and Company, New York, (1998)
15. VKPatel, RSS Yadav & KDS Yadav, Enzymatic characteristics of ligninperoxidases of indigenous lignolytic fungal strains Part-I, *Indian Journal of Biotechnology,* **6**: 553-556 *(* 2007).