

Biotransformation of Aromatic Methyl Groups to Aldehyde Groups Using Laccase of *Gloephyllum Stratum* MTCC-1117

RAM SAHAY (✉ rs_chemistry@rediffmail.com)

Research

Keywords: Lignolytic fungi, *Gloephyllum stratum*, 2,6-dimethoxyphenol, Cu-enzyme, Multicopper oxidases

Posted Date: June 17th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-34178/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Laccases has been produced by white rot fungi are involved in lignin containing natural substrates wheat-straw, bagasse, saw-dust, corn cob and coir dust particle on the production of laccase enzyme in the aqueous cultivation medium of *Gloephyllum stratum* MTCC1117. The approach involved concentration of aqueous filtrate by ultrafiltration and anion exchange chromatography on DEAE (diethyl aminoethyl cellulose). From SDS-PAGE analysis the molecular mass of the purified enzyme is 57 kDa. The K_m and k_{cat} values of the laccase are found to be 18 μM and 0.34 s^{-1} using 2,6-dimethoxyphenol as the substrate, giving a k_{cat}/K_m value is $1.70 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. The pH and temperature optimum were 4.5 and 40 °C respectively. The purified enzyme has yellow colour and does not show absorption band around 610 nm found in blue laccases. Moreover the conversion of methylbenzene to benzaldehyde in the lack of mediator molecules, property exhibited by yellow laccases.

Introduction

Laccase (benzenediol: oxygen oxidoreductase, E.C. 1.10.3.2) a family of blue multicopper oxidases are capable of oxidizing a wide range of aromatic compound with concomitant reduction of molecular oxygen to water [1–3]. Laccases are widely distributed in higher plants and fungi (basidiomycetes) while laccase like activities have also been found in some insects [4] and bacteria [5]. However, most laccases are of fungal origin, especially from the class of white rot fungi.

Correspondence to: R. Sahay

E-mail: rs_chemistry@rediffmail.com

Laccases are involved in several physiological functions, such as lignin biosynthesis, plant pathogenesis, insect sclerotisation and degradation of lignocellulosic materials. It is well recognized that laccases are involved in both polymerization and depolymerisation processes of lignin [1]. Laccase are seen as industrially interesting enzymes because they have as biological catalysts, laccases have been demonstrated to possess potential in several industrial applications including bleaching of paper pulp, bioremediation, enzymatic removal of phenolic compounds in beverages, enzymatic modification of fiber dye bleaching [2, 6], biosensors [7].

It is a dimeric or tetrameric glycoprotein which contains four copper atoms per monomer distributed in three redox sites [8] termed as type-1 (T1) or blue copper, type-2 (T2) or normal copper and type-3 (T3) or coupled binuclear copper. Type-2 and type-3 copper form a trinuclear cluster. The organic substrate is oxidized by one electron at the active site of the laccase, generating a reaction radical which further reacts non-enzymatically. The electron is received at type-1 copper and it is shuttled to the trinuclear cluster where as oxygen is reduced to water. A general reaction scheme has been proposed as:



Ortho and para-diphenols, aminophenol, polyphenols, polyamines, lignin and arylamines and some of the inorganic ions are the substrates for laccases. It is the most promising enzyme of oxidoreductases group for industrial applications [8–9]. The biotechnological importance of laccases has increased after the discovery that oxidizable reaction substrate range could be further extended in the presence of small readily oxidizable molecules called mediators [10–11]. During the last two decades, laccases have turned out to be the most promising enzymes for industrial uses [8–9] having applications in food, pulp and paper, textile, cosmetics industries and in synthetic organic chemistry [12–15]. Laccases purified from different sources exhibit different properties and are suitable for different applications. Keeping these

points in view, the authors have initiated studies on purification, characterization and biotechnological applications of laccases from different sources [16–18]. *G. stratum* MTCC-1117 is a white rot fungus isolated from the twigs of *Ficus bengalensis* [19]. Being a white rot fungus, it is expected to secrete lignolytic enzymes. The special status of the laccase among lignolytic enzymes has prompted the authors to screen this fungus for the secretion of the laccase. In addition, there are reports [20–21] that blue laccase-secreting fungal strains secrete yellow laccases in the presence of culture media containing natural lignin-containing substrates but studies on yellow laccases are rare [22–25]. Moreover, blue laccases transform methylbenzene to benzaldehyde in the presence of mediator molecules [26–27]. Conversion of methylbenzene to benzaldehyde in the absence of mediator molecules has not been reported so far. The objective of this communication is to purify and characterize a laccase from the liquid culture growth medium of *Gloephyllum stratum* MTCC-1117 containing natural lignin-containing substrate wheat straw particles and to demonstrate the conversion of methylbenzene to benzaldehyde by the laccase in the absence of mediator molecules.

Material And Methods

Syringaldazine (3,5-Dimethoxy-4-hydroxybenzaldehyde azine) and DEAE (diethyl aminoethyl) Cellulose were from Sigma Chemical Company, St. Louis USA. DMP (2,6-dimethoxy phenol), and ABTS 2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) was from Fluka, Chemi new Ulm, Switzerland. The chemicals used in the gel electrophoresis of protein samples were from Geni Pvt. Ltd. Bangalore. All other chemicals used in these investigations were either from Himedia laboratory Ltd, Mumbai or from E. Merck (India) Ltd. Werli Road Mumbai and were used without further purifications.

The fungal strain was procured from the Microbial Type Culture Collection Center and Gene Bank, Institute of Microbial Technology, Chandigarh and was maintained on agar slant as reported in MTCC Catalogue of strains-2000 [19]. The growth medium for the fungal strain *G. stratum* MTCC-1117 consisted of malt extract 20.0 g and agar 20.0 g in 1.0 L Milli-Q water and pH of the medium was 6.5.

The liquid culture medium reported by Coll et al [28] was used for screening the fungal strain for the production of extracellular laccase in the liquid culture medium. This medium consisted of yeast extract 0.5 g, asparagine 1.0 g, glucose 10.0 g, $MgSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot 7H_2O$ 0.01 g in 1.0 L of Milli-Q water. The liquid culture medium containing natural lignin substrate like wheat straw, saw dust, coir dust, corn cob, and bagasse particle were separately prepared by adding 0.5 g of one of the natural lignin substrates to 25 ml of the above mentioned growth medium in 100 ml culture flasks which were sterilized. The sterilized growth medium was inoculated with small piece of mycelium (0.5 cm × 0.5 cm) under aseptic condition and the fungal culture was grown under stationary culture condition at 30 °C in a BOD incubator. In order to monitor the production of laccase in the liquid culture medium 0.5 mL aliquots of the growth medium were withdrawn at a regular intervals of 24 hrs and filtered through sterilized Millipore filter 0.22 mm. The filtered extract was analyzed for the activity of the laccase using DMP as the substrate by the method [28] given below in the assay section. Secretion of the laccase in the liquid culture medium by *G. stratum* MTCC-1117 was determined by plotting the enzyme unit per milliliter of the growth medium against the number of days after inoculation of the fungal mycelia. Each point on the curve is an average of the three measurements. The growth medium for the control experiment has the same composition except that no natural lignolytic substrate has been added. In order to optimize the condition for maximum production of the laccase by *G. stratum* MTCC-1117 in the liquid culture medium, the amount of best inducer wheat straw particle was varied from 100-1000 mg in 25 mL of the growth medium. The amount of the inducer in the growth medium which gives the maximum height of the enzyme activity peak was taken as the optimal amount of the inducer.

Enzyme assay

The assay solution 1.0 mL for ABTS as the substrate [28] contained 0.5 mM ABTS in 0.1 M sodium acetate buffer pH 5.0 at 25 °C for DMP as the substrate [29] 1.0 mL solution contained 1.0 mM DMP in 50 mM sodium malonate buffer pH 4.5

at 37 °C and for Syringaldazine (3,5-dimethoxy-4-hydroxybenzaldehyde azine) as the substrate [30] contained 0.1 mM syringaldazine in 50 mM sodium phosphate buffer pH 6.0 at 50 °C. In case of ABTS, the reaction was monitored by measuring the absorbance change at $\lambda=420$ nm and using the molar extinction coefficient [28] value of $36.0 \text{ mM}^{-1} \text{ cm}^{-1}$. In case of DMP, the reaction was monitored by measuring the absorbance change at $\lambda=468$ nm and using the molar extinction coefficient [29] value of $49.6 \text{ mM}^{-1} \text{ cm}^{-1}$. In case of syringaldazine, the reaction was monitored by measuring the absorbance change at $\lambda=530$ nm and using molar extinction coefficient [30] value $64.0 \text{ mM}^{-1} \text{ cm}^{-1}$. The UV/Vis spectrophotometer Hitachi (Japan) model U-2000 fitted with electronic temperature control unit was used for absorbance measurement. The least count of absorbance measurement was 0.001 absorbance unit. One enzyme unit produced 1 μmol of the product per min under the specified assay conditions.

Purification of laccase enzyme

For the purification of the laccase, *G. stratum* MTCC-1117 was grown in ten 100 mL culture flasks each containing 25 mL sterilized growth medium containing optimal amount 800 mg of the inducer wheat straw particles under stationary culture condition in a BOD incubator at 30 °C. The maximum activity of the laccase appeared on seventh day of the inoculation of the fungal mycelia. On the seventh day, all the cultures in the ten flasks were pooled, mycelia were removed by filtration through four layers of cheese cloth. The culture filtrate was concentrated by using Amicon concentration cell model-8200 with PM-10 ultrafiltration membrane, molecular weight cut-off value 10 kDa. The concentrated enzyme was dialysed against 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer (pH 6.5) in a volume ratio 1:1000 with three changes at the intervals of 8 h. 10-millilitre enzyme sample containing 1.87 mg/mL protein was loaded on to the DEAE column (size 1.0 \times 38.0 cm) which was pre-equilibrated with 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer (pH 6.5), and the flow rate was 18 mL/h. The column was washed with 60 mL of the same buffer. The enzyme was eluted by applying linear gradient of 0.0 to 1.0 M NaCl in the same buffer (50 mL buffer + 50 mL buffer with 1.0 M NaCl). The fractions of 5.0 mL size were collected and analyzed for the laccase activity [28]. The protein estimation was done by the lowery method [31]. All laccase-active fractions were combined and concentrated by putting it in a dialysis bag and covering the dialysis bag with powdered sucrose at 4 °C.

SDS-PAGE Analysis

The purity of the enzyme preparation was checked by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [32]. The molecular weight marker were 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. (Bangalore, India). Gel was run at a constant current 20 mA [33]. The molecular weight was determined by the Weber and Osborn method [34].

UV/Vis Spectrum of the Purified Enzyme

The UV/Vis spectrum of the purified laccase 0.56 mg/mL was recorded in 50 mM sodium phosphate buffer pH 6.5 at 30 °C, using UV/Vis spectrophotometer Hitachi (Japan) model U-2000 as mentioned in the assay section.

Steady-State Enzyme Kinetics

The steady-state enzyme kinetics of the purified laccase was studied using DMP as the substrate following the method as mentioned in the assay section [29]. K_m and k_{cat} values for the enzyme were determined from the linear regression of double reciprocal plot. The pH and temperature optima of the enzyme were determined by measuring the steady-state velocities of the enzyme catalyzed reaction in the solutions of varying pH/temperature keeping the other parameters fixed and drawing graphs of steady-state velocities versus the variable parameter.

Native-PAGE and Zymogram Analysis

The native polyacrylamide gel electrophoresis of purified enzyme was done using the reported method [35]. The composition of resolving and stacking gel was similar to that used in SDS-PAGE, except SDS was absent. The molecular weight marker was the bovine serum albumin 66 kDa. Two sets of native gel were done. One set was stained with Coomassie Brilliant Blue R-250 and the other set was used for zymogram[36] preparation. For the preparation of zymogram, 100-mM DMP solution was made in 10 mM sodium tartrate buffer pH 5.0. The native gel was dipped in zymogram solution for 5 min, and a brown band appeared. The zymogram was removed from the DMP solution and was washed thrice with 10 mM sodium tartrate buffer pH 5.0 at the interval of 5 min.

Oxidation of Veratryl Alcohol to Veratraldehyde

This study was made by recording the UV/Vis spectra at zero time and after 30 min of 1.0 mL of the solution containing 0.10 mM veratryl alcohol, and 10.6 μg of the laccase in 100 mM sodium malonate buffer pH 4.5 at 30 °C. The time course of the conversion of veratryl alcohol to veratraldehyde was monitored by observing absorbance increase at 310 nm due to veratraldehyde [36] formation in the solution of the above composition.

Selective oxidation of the aromatic methyl group to aldehyde in the absence of mediators

The bioconversion of methylbenzene to benzaldehyde [26-27] was done in 2 mL of 10 mM sodium phosphate buffer pH 6.5 containing 100 μL methylbenzene and 11.6 μg of purified laccase in the absence of any mediator kept in a 25-mL conical flask which was stirred vigorously for 3 min. The reaction solution was extracted thrice with 2 mL of n-hexane 20-microlitres of the n-hexane extracted was injected in Waters HPLC Model 600E using spherisorb C_{18} 5 UV, 4.5 \times 250 mm column. The eluant phase was methanol/ water in the ratio 1:1 (v/v) at the flow rate of 1.0 mL/min. The detection was made using Waters UV detector model 2487 at $\lambda=254$ nm.

Results And Discussion

The results of the secretion of laccase by *G. stratum* MTCC-1117 in the liquid culture growth medium natural lignin containing substrate wheat straw, bagasse, corn cob, saw dust and coir dust and a control in which no natural lignin containing substrate has been added are shown in Fig. 1. Though the laccase was secreted in the liquid culture medium in the absence of natural lignin-containing substrates, the level of enzyme units secreted was low in comparison to the levels of enzyme units secreted in the presence of the natural lignin-containing substrates. The maximum level of enzyme units secreted in the liquid culture medium was in the presence of wheat straw particles. In order to optimize the secretion of the laccase in the presence of wheat straw particles, secretion of the laccase in the presence of different amounts of wheat straw particles were studied. The results are shown in Fig. 2. The maximum level of the laccase was secreted in the liquid culture medium containing 800 mg of the wheat straw particles per 25 mL of the culture medium.

The results of the purification of the laccase from the culture filtrate of the fungal strain are summarized in Table 1. It involved concentration by ultrafiltration and an ion exchange chromatography on DEAE cellulose. The elution profile of the enzyme from DEAE cellulose column is shown in Fig. 3. The enzyme bound to DEAE cellulose in 10 mM sodium phosphate buffer pH 6.0 and was eluted by the linear gradient of NaCl in the range 300 to 500 mM. The fraction numbers 6 to 10 were combined and concentrated to 1 mL by keeping the combined portion in a dialysis bag and covering the bag with powdered sucrose at 4 °C. The concentrated enzyme sample was analysed by SDS-PAGE, zymogram and native PAGE preparation. The results of SDS-PAGE, zymogram and native PAGE preparation are shown in Fig. 4A-C, respectively. In Fig. 4A, lane 1 contains molecular weight markers and lane 2 contains the purified enzyme. The appearance of single protein band in SDS-PAGE indicates that the enzyme sample is pure. In Fig. 4B lane 1 a single protein band in the purified enzyme in the zymogram analysis. Figure 3C of native PAGE coincides with the position of the purified laccase in the

native PAGE. The relative molecular mass of the purified laccase determined from the analysis of SDS-PAGE was 57 kDa. The relative molecular masses of laccases purified from the culture filtrate of *Ganoderma lucidum*, *Chaetomium thermophilum*, *Neurospora crassa*, and *Gaeumannomyces graminis var. tritici* are 68, 77, 64.8 and 60 kDa [28, 38–40] respectively. Thus the relative molecular mass of *G. stratum* MTCC-1117 laccase is in the same range as reported for other fungal laccases [41].

The Michaelis-Menten and double reciprocal plots for the purified enzyme using DMP as the variable substrate [28] are shown in Fig. 5A, B respectively. The K_m and k_{cat} values determined from the double reciprocal plot using DMP as the substrate of the enzyme were 18 μM and 0.34 s^{-1} , respectively, giving k_{cat}/K_m $1.70 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. The range of K_m values reported [41] for fungal laccases using DMP as the substrate are 8–14,720 μM . However the K_m value for this laccase compares well with the K_m of laccase purified from *Armillaria mellea* Lac-1 [42]. The value of k_{cat} determined for the purified laccase has been found to be low in comparison to the values of k_{cat} reported for other fungal laccases [42]. It is worth mentioning over here that the catalytic performances of laccases span several orders of magnitude for different substrates and are characteristic for specific proteins [42]. The results of the variation of the activity of the purified laccase with the variation of pH of the reaction solution are shown in Fig. 6. The determined pH optimum of the enzyme was 4.0. The pH optima reported in the literature [42] for laccases using DMP as the substrate are in the range 3.0–8.0 pH units. The pH optimum of the purified laccase compares well with pH optima of laccases of *Ceriporiopsis subvermispora* L1, *C. subvermispora* L2, *Daedalea quercina* and *Lentinula edodes* LCC 1 [42].

The results of variation of the activity of the purified laccase as a result of variation of the temperature of the reaction solution of enzyme catalysed reaction are shown in Fig. 7. The calculated temperature optimum was 35 °C. The range of temperature optima reported for other laccases are 25–80 °C. The laccases of *Pleurotus ostreatus* POXA 3b and *P. ostreatus* POXC also have temperature optima at 35 °C [42]. The purified laccase does not possess a blue colour as visually observed in cases of blue laccases. Instead, it has a yellow colour. It has already been reported in the literature that some fungal strains, which secrete blue laccase in submerged liquid cultures, secrete blue laccases when grown in solid-state fermentation containing natural lignin substrate, wheat straw [20]. The yellow laccases lack absorption band around 610 nm observed in cases of blue laccases. The UV/Vis spectrum of the purified laccase was recorded and is shown in Fig. 8. The authors could not detect the blue absorption band around 610 nm in the purified laccase spectrum, indicating that it is a yellow laccase. It has also been reported that yellow laccases which oxidize non-phenolic substrates without the presence of electron transfer mediators [20–21] which are essential for the oxidation of non-phenolic substrates by blue laccases. The purified laccase was tested for its property of the oxidation of veratryl alcohol, a non-phenolic substrate which is not oxidized by blue laccases in the absence of the mediator molecules. The results are shown in Fig. 8. Figure 8 (a) is the spectrum of a solution containing veratryl alcohol and the purified enzyme just after addition of the enzyme. Figure 8 (b) is the spectrum of the same solution after 30 min of the addition of the purified enzyme. Veratryl alcohol has very low absorption at $\lambda = 310 \text{ nm}$ whereas the oxidation product of veratryl alcohol, veratraldehyde, has absorption maximum at $\lambda = 310 \text{ nm}$. The comparison of the spectra **a** and **b** given in Fig. 8 clearly shows that the enzyme has oxidized veratryl alcohol to veratraldehyde in the absence of any mediator molecule. The time course of the oxidation of veratryl alcohol to veratraldehyde monitored by increase in absorbance at $\lambda = 310 \text{ nm}$ due to the formation of veratraldehyde is shown in Fig. 8 (c) as the insert. Thus the purified laccase exhibits the property exhibited by yellow laccases and can be termed as a yellow laccase.

One of the applications of the laccases in organic synthesis is in the selective oxidation of the aromatic methyl group to the corresponding aldehyde. The chemical routes of this conversion are inconvenient because the reaction has to be stopped at the aldehyde stage so that carboxylic acid could not be formed. Moreover, they require drastic reaction conditions which pollute the environment. The conversion done with laccase occurs under milder conditions, yield is > 90% and the process is ecofriendly. Laccases are the preferred enzymes because they cycle on oxygen and generate no

side products except water. The use of blue laccases for this purpose has been studied [26–27] but they require mediator molecules like ABTS [27] and 1-hydroxy-1Hbenzotriazole (HOBT) [28]. As has been discussed above, the purified laccase oxidizes veratryl alcohol, a non-phenolic substrate to veratraldehyde without the presence of the mediator molecules. The potential of the purified laccase as a biocatalyst for the conversion of aromatic methyl group to the corresponding aldehyde in the absence of the mediator molecule was tested using methylbenzene as the substrate. The results are shown in Fig. 8. Figure 8a is the HPLC chromatogram of the n-hexane extract of the product formed by the reaction of the enzyme with methylbenzene in the absence of any mediator molecule. Figure 8b, c is the chromatograms of the standard samples of benzaldehyde and methylbenzene, respectively. The retention time of the standard sample of methylbenzene was 4.05 min, and the retention time of the standard sample of benzaldehyde was 2.96 min. Thus the retention time of the product of the enzyme-catalysed reaction 3.09 coincides with the retention time of benzaldehyde 2.96 min, showing that the product of enzyme-catalysed reaction is benzaldehyde. Figure 8 clearly demonstrates that the purified enzyme catalyses the oxidation of methylbenzene to benzaldehyde in the absence of mediator molecules like ABTS and HOBT as reported in cases of blue laccases [27–28] for the same conversion. The purified enzyme is a better biocatalyst than blue laccases for the above conversion because it does not require the mediator molecules for the above conversion.

Conclusions

Secretion of laccase was studied in presence of different lignin containing substrate (wheat straw, saw dust, coir dust, corn cob, and bagasse) in aqueous culture medium. The maximum level of laccases secreted in the liquid culture medium (25 mL) was in the presence of 800 mg wheat straw particles. Thus in this communication a yellow laccase from a new fungal strain *G. stratum* MTCC-1117 has been purified and characterized. Purified yellow laccases catalyse the oxidation of methylbenzene to benzaldehyde in the absence of mediator molecules like ABTS and HOBT [27–28]. Thus purified yellow laccase is a better biocatalyst than blue laccases.

Declarations

Availability of data and materials

Author RS have procured and interpreted the data himself from experiment performed in the Chemistry lab Maharana Pratap P.G. College, Jungle Dhusan Gorakhpur. The materials required for the experiment were available in the lab.

Ethics approval and consent to participate:

Not applicable

Consent for publication

Author is agree to publish the manuscript in the “Journal of Biological Research-Thessaloniki”.

Competing interests

The authors declare that they have no competing interests.

Author's Contribution's

The author RS himself performs all the experiments and the result reported in the manuscript.

Acknowledgement

The authors Dr. R. Sahay thankfully acknowledges the Department of Chemistry Maharana Pratap P. G. College J. D. Gorakhpur-273014, India.

References

1. Thruston C, The structure and function of fungal laccases. 1994; 140:19-26.
2. Gianfreda L, Xu F, Bollag JM, Laccases: A Useful Group of Oxidoreductive. Enzymes 1999;J3:1-2.
3. Ullah MA, Bedford CT, Evans CS, Reactions of pentachlorophenol with laccase from *Coriolus versicolor*. Applied Microbiology biotechnology. 2000;53:230-234.
4. Thomas BR, Yonekura M, Morgan TD, Czapla TH, Hopkins TL, Kramer KL, A trypsin solubilized laccase from pharate pupal integument of the tobacco hornworm, *Manduca sexta*. *Manduca sexta*. Insect Biochem. 1989;19:611-622.
5. Alexandre G, Zhulin IB, Laccases Are Widespread in Bacteria. Trends Biotechnol. 2000;18:41-42.
6. Abadulla E, Tzanov T, Costa S, Robra KH, Cavaco-Paulo A, Gubitz GM, Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsute*. Applied Environmental Microbiology. 2000;66:3357-3362.
7. Kulys J, Viziunaite R, Amperometric biosensors based on recombinant laccases for phenols determination. Biosens Bioelectron. 2003;18:319-325.
8. Wandrey C, Liese A, & Kihumbu D, Industrial biocatalysis: past, present and future. Organic Process Research and Development. 2000;4(4):285-
9. Couto SR, & Herrera JLT, Industrial and biotechnological applications of laccases: A Review. Biotechnological Advances. 2006;24:500-
10. Acunzo DF, & Galli C, First evidence of catalytic mediation by phenolic compounds in the laccase induced oxidation of lignin models. Journal of European Biochemistry. 2003;27:3634-
11. Morozova OV, Shumakovich GP, Shleev SV, & Yaropolov YAI, Laccase mediator systems and their applications: A review. Applied Biochemistry and Microbiology. 2007;43:523-
12. Coniglio A, Galli C, & Gentili P, Oxidation of amides by laccase generated aminoxyl radicals. Journal of Molecular Catalysis B: 2008;50(1):40-49.
13. Mikolasch A, Niedermeyer THJ, Lalk M, Witt S, Sefeld S, Hammer E, Novel penicillins synthesized by biotransformation using laccase from *Trametes spec*. *Chemical and Pharmaceutical Bulletin*. 2006;54(5):632-
14. Mikolasch A, Niedermeyer THJ, Lalk M, Witt S, Seefeldt S. Hammer E, Novel cephalosporins synthesized by amination of 2,5-Dihydroxybenzoic acid derivatives using fungal laccases II. *Chemical and Pharmaceutical Bulletin*. 2007; 55(3):412-
15. Mikolasch A, Hammer E, Jonas U, Popowski K, Stielow A, & Schaner F, Synthesis of 3-(3,4-dihydroxyphenyl)-propionic acid derivatives by N-coupling of amines using laccase. 2002;58:7589-7593.
16. Sahay R, Yadav RSS, & Yadav KDS, Purification and Characterization of laccase Secreted by *lividus*. Applied Biochemistry and Biotechnology. 2009;157:311-320.
17. Sahay R, Yadav RSS, & Yadav KDS, Purification and Characterization of extracellular laccase secreted by *Pleurotus sajor-caju* MTCC 141. Chinese Journal of Biotechnology. 2008;24(12):2068-
18. Sahay R, Yadav RSS, Yadav S, & Yadav KDS, A Laccase of *fomes durissimus* MTCC-1173 and its role in the conversion of methylbenzene to benzaldehyde. Applied Biochemistry and Biotechnology. 2012;166:563-575.
19. Catalogue of strains (5th edition). Microbial Type Culture Collection and Gene Bank Institute of Microbial Technology. Chandigarh p.60. 2000.

20. Leontievsky AA, Vares T, Lankinen P, Shergill JK, Pozdnyakova N, Myasoedova NM, Blue and yellow laccases of ligninolytic fungi. *FEMS Microbiology Letters*. 1997;156:9-
21. Leontievsky A, Myasoedova N, Pozdyakova N, & Goloveva L, Yellow laccase of *Panus tigrinus* oxidizes nonphenolic substrates without electron transfer mediators. *FEBS Letters*. 1997;413:446-
22. Couto SR, & Herrera JLT, Industrial and biotechnological applications of laccases: A Review. *Biotechnology Advances*. 2006;2:500-
23. Osma J, Toca-Herrera JL, Rodriguez-Couto S, Uses of laccases in the food industry:A Review. *Enzyme Research*. 2010;ID918761,doi: 4061/2010/918761.
24. Brizwani K, Rigdon A, Vadlani PV, Fungal laccases: production, function and applications in food processing. *Enzyme Researc* 2010;ID 149748, doi:10.4061/2010/149748.
25. Desai SS, & Nityanand C, Microbial Laccases and their Applications: A Review. *Asian Journal of Biotechnology*. 2011;13(2):98-
26. Potthest A, Rosenau T, Chen CL, & Gratz J, Selective enzymic oxidation of aromatic methyl groups to aldehydes. *Jouranal of Organic Chemistry*. 1995;60: 4320-
27. Fritz L, & Kunath B, Synthesis of aromatic aldehydes by laccase mediator assisted oxidation. *Tetrahedron Letters*. 1998;39:5955-
28. Coll MP, Fernandez-Abalos JM, Villomueva JR, Somtamaria R., & Perez P, Purification and characterization of a phenoloxidase (laccase) from the lignin-degrading basidiomycete PM1 (CECT-2971). *Applied and Environmental Microbiology*. 1993;59:2607-
29. Bourbonnais R, & Paice MG, Oxidation of non-phenolic substrates An expanded role for laccase in lignin *Applied Microbial Biotechnology*. 1993;36:823-827.
30. Chefetz B, Chen Y, & Hador Y, Purification and characterization of laccase from *chaetomium thermophilum* and its role in humification. *Applied and Environmental Microbiology*. 1998;64:3175-
31. Lowry OH, Rosebrough NJ, Farrand AL, & Randall RJ, Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*. 1951;193(1):265-
32. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. 1970;227:680-685.
33. Anonymous, Polyacrylamide gel electrophoresis: laboratory techniques, Pharmacia, Laboratory Separation Division: Uppsala. 1984.
34. Weber K, & Osborn M, The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. *Journal of Biological Chemistry*. 1969;244:4406-
35. Schagger, & Jajow, H, Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Analytical Biochemistry*. 1991; 199:223-
36. Zouri-Mechichi H, Mechichi T, Dhouib SS, Martinez AT, & Martinez MJ, Laccase purification and characterization from *Trametes troglia*. Isolated in Tunisia: decolorization of textile dyes by the purified enzyme. *Enzyme and Microbial Technology*. 2006;39(1):141-
37. Tein M, & Kirk TK, Lignin peroxidase of *Phanerochaete chrysosporium*. *Methods in Enzymology*. (1988;161:238-
38. Solomon EI, Machonkin TE, Sundaram UM, MultiCopper Oxidases Messerschmidt A. ed., World Scientific Singapore. 1997; pp:103-
39. Mario SCN, Margarita AM, Tournier BHA, Extracellular ABTS oxidizing activity of autochthonous fungal strains from argentina in solid medium. *Revista Iberoamericana de Micologia*. 2000;17(2):64-
40. Mario SCN, Martinez AM, Cabello MN, Screening for ligninolytic enzymes in autochthonous fungal strains from Argentina isolated from different substrate. *Revista Iberoamericana de Micologia*. 2002;19:181-

41. Edens WA, Goins TQ, Dooley D, Purification and characterization of a secreted laccase of *gaeumannomyces graminis*. Applied and Environmental Microbiology. 1999;65:3071-3074.
42. Baldrian P, Fungal laccases occurrence and properties. FEMS Microbiology Reviews. 2006;30:215-242.

Tables

Table-1: Purification chart

S. No.	Steps	Volume (mL)	Activity (IU/mL)	Protein (mg/mL)	Total Activity (IU)	Total Protein (mg)	Specific activity (IU/mg)	Purification fold	% Recovery
1.	Crude Enzyme	500	0.053	2.15	26.5	1075	0.024	1	100
2.	Amicon Concentration	20	0.127	1.97	2.54	39.4	0.064	2.66	9.58
3.	DEAE	2	0.987	3.85	1.97	7.70	0.255	10.66	7.43

Figures

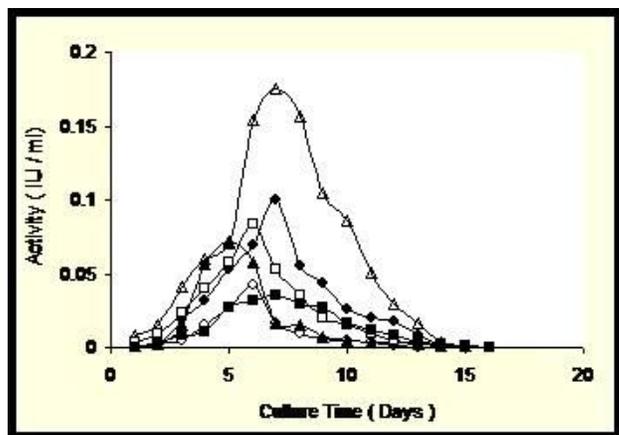


Figure 1

Secretion of laccase by *Gloeophyllum stratum* MTCC-1117 in the liquid culture medium supplemented with different natural lignin containing substrate, Wheat straw (Δ), Corn cob (◻), Baggasse (○) Coir dust (◊) Saw dust (▽) Control (○)

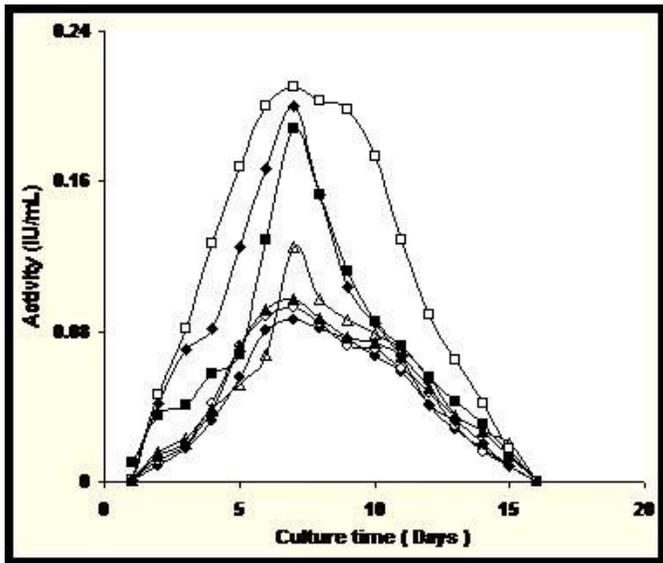


Figure 2

Optimization of laccase by *Gloephyllum stratum* MTCC-1117 in the liquid culture medium supplemented with different concentration of wheat straw particles, 100 mg (), 200 mg (), 400 mg (), 500 mg (), 600 mg (), 800 mg (), 1000 mg ()

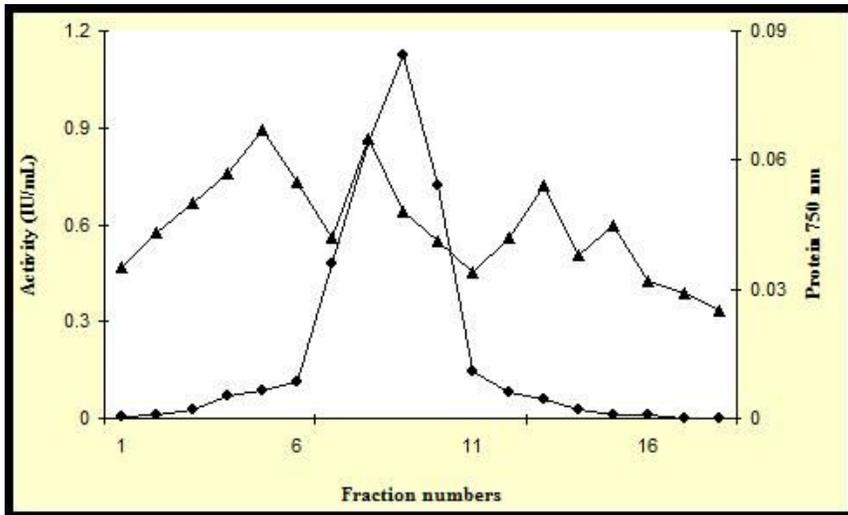


Figure 3

Elution profile of the enzyme from DEAE cellulose column: activity () and protein ()

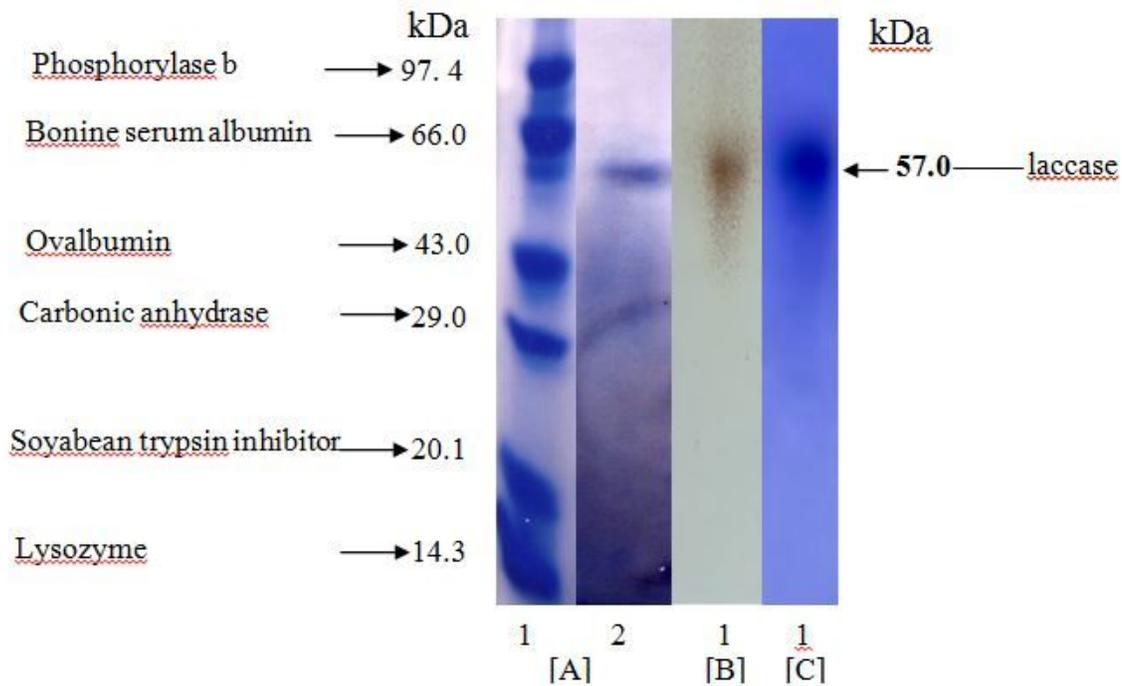


Figure 4

SDS-PAGE [A], Zymogram [B] and NATIVE-PAGE [C] of the purified laccase. [A] Molecular weight markers (lane-1), Purified enzyme 25 μ g (lane-2) [B] Zymogram 25 μ g of pure enzyme stained with 2,6-Dimethoxyphenol. [C] Native gel purified enzyme 25 μ g.

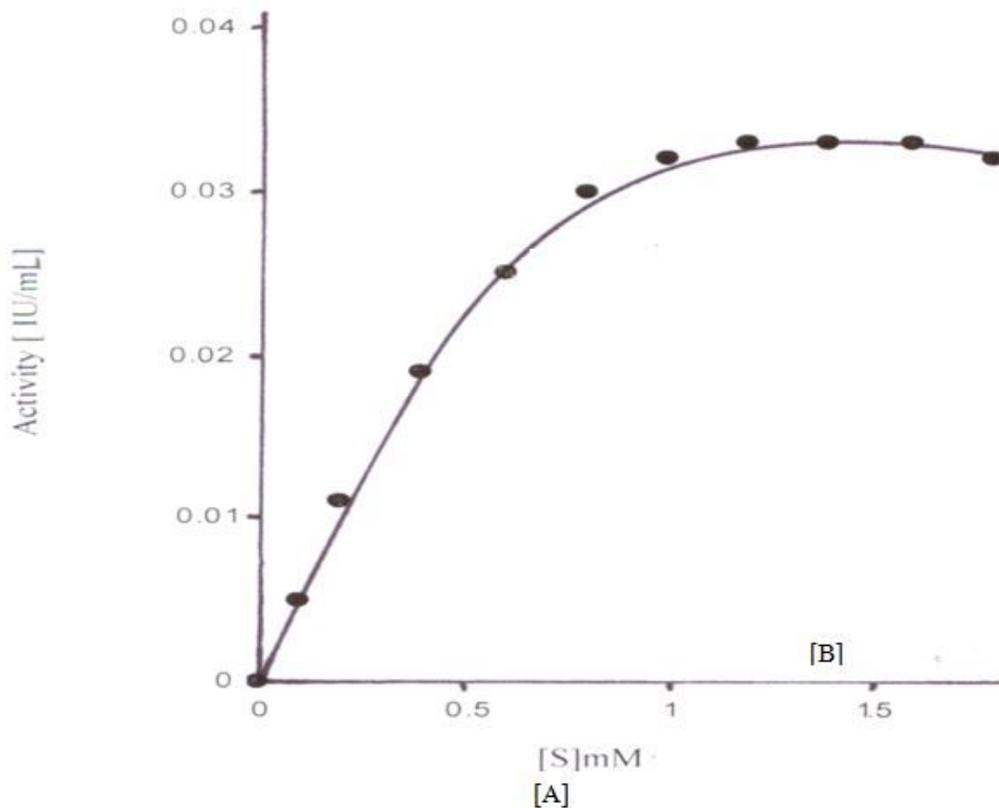


Figure 5

Michaelis-Menten curve [A] and double reciprocal pots [B] for laccase enzyme of *G. stratum* MTCC-1117 using 2,6-dimethoxyphenol as the variable substrate. In A, B 1.0 mL reaction solution contained 1.0 mM DMP 4.3 μ g of the enzyme in 50 mM sodium malonate buffer pH 4.5 at 30 $^{\circ}$ C.

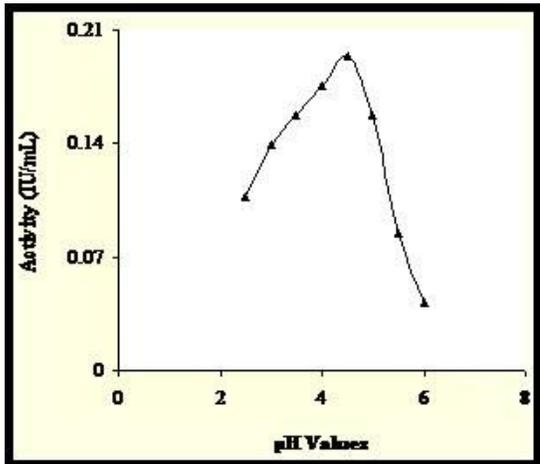


Figure 6

Determination of pH optima, 1.0 mL reaction solution contained 1.0 mM DMP in varying pH from 2.5 to 6.0 in 50 mM sodium malonate buffer at 30 $^{\circ}$ C added 4.3 μ g of the laccase enzyme.

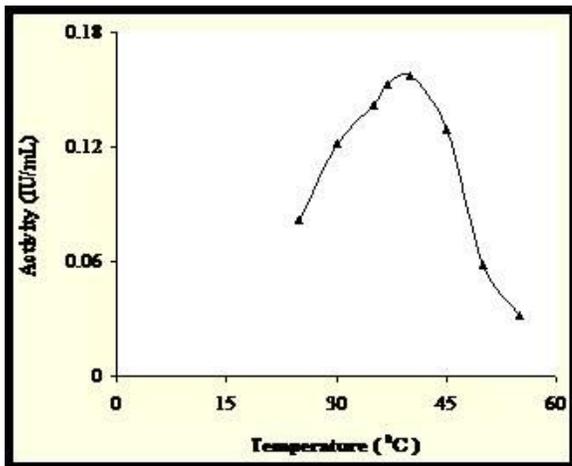


Figure 7

Determination of temperature optima, 1.0 mL reaction solution contained 1.0 mM DMP in 50 mM sodium malonate buffer at pH 4.5 with varying temperature from 25 to 50 $^{\circ}$ C and added 4.3 μ g of the laccase enzyme.

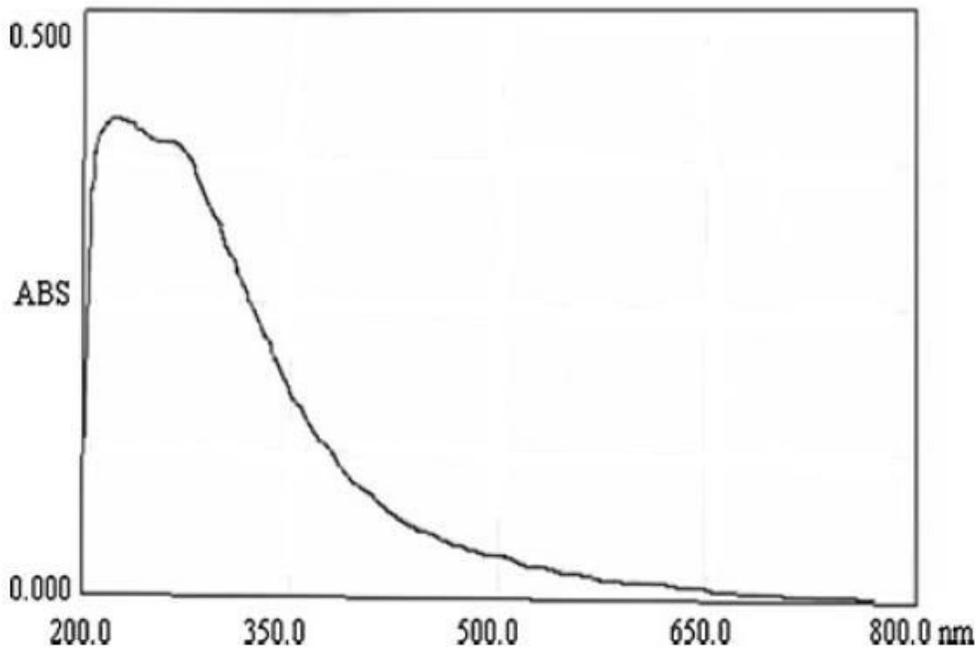


Figure 8

Spectrum of the purified purified laccase enzyme 0.55 mg in 1.0 mL 50 mM sodium phosphate buffer pH 4.5 at 30 °C.

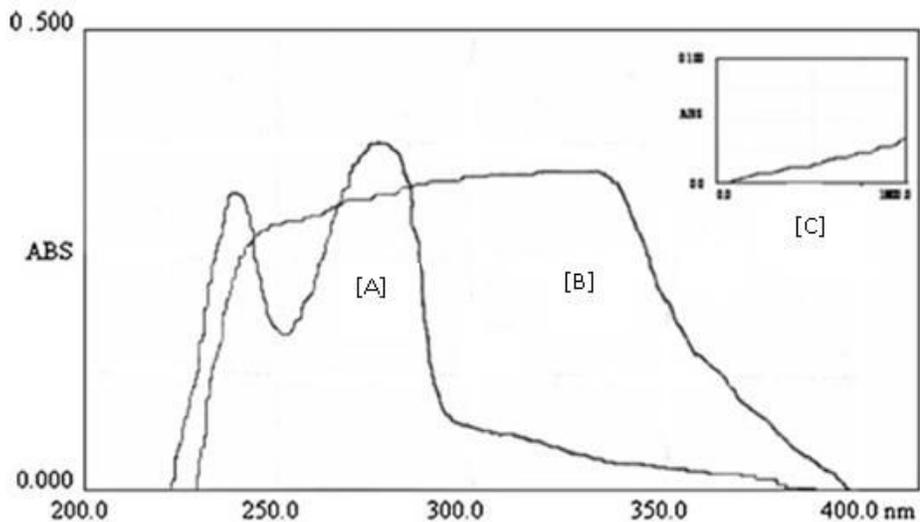


Figure 9

[A] The conversion of veratryl alcohol to veratraldehyde by the purified laccase. Spectrum of 1.0 mL solution containing 0.10 mM veratryl alcohol and 10.6 µg of the enzyme in 100 mM sodium malonate buffer pH 4.5, temperature 30 °C at zero time. [B] Spectrum of 1.0 mL solution containing 0.10 mM veratryl alcohol and 10.6 µg of the enzyme in 100 mM sodium malonate buffer pH 4.5, temperature 30 °C after 30 min. [C] Increase of absorbance at 310 nm due to the conversion of veratryl alcohol to veratraldehyde with time (c).

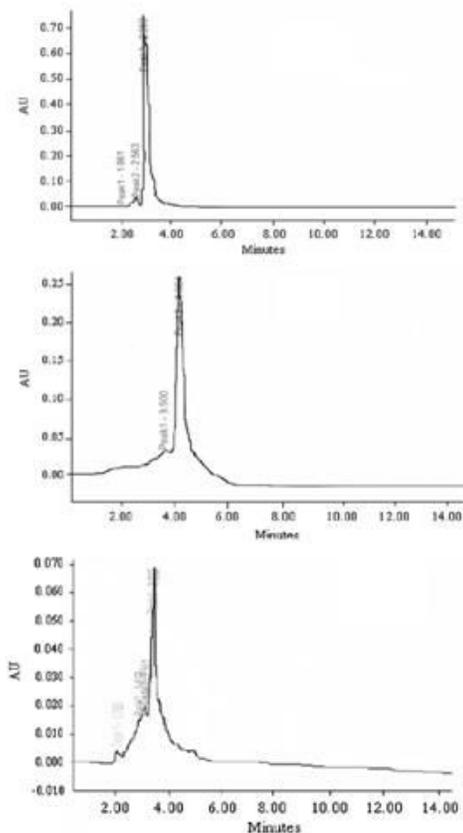


Figure 10

Biotransformation of methylbenzene to benzaldehyde by the enzyme of *G. stratum* MTCC-1117. [A] The chromatogram of the pure benzaldehyde. [B] The chromatogram of the pure methylbenzene [C] The chromatogram of the product of enzymatic reaction with methylbenzene. The reaction solution contained 100 μ L of methylbenzene and 4.3 μ g of the enzyme in 2 mL 10 mM sodium phosphate buffer pH 6.5 at 30 $^{\circ}$ C was stirred for 3 min and extracted thrice with 2 mL of n-hexane and 20 μ L was injected in HPLC.