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Application of crude laccase of *Xylaria polymorpha* MTCC-1100 in selective oxidation of aromatic methyl group to aldehyde group

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ABSTRACT

The chemical routes of oxidation of methyl group to its aldehyde is inconvenient because once a methyl group is attacked, it is likely to be oxidized to the carboxylic acid and it is very difficult to stop the reaction at the aldehyde stage. Fungal laccases can be used for such oxidation reaction and the reaction can be completed sharply within 1-2 hrs. We have used the crude laccase obtained from the fungal strain *Xylaria polymorpha* MTCC-1100 for the selective biotransformations of toluene, 3-nitrotoluene and 4-chlorotoluene to benzaldehyde, 3-nitrobenzaldehyde and 4-chlorobenzaldehyde, respectively. In all cases ABTS is used as mediator molecule. Main purpose to do such transformations from crude laccase is to save the time consumed in purification process and avoid the drastic conditions. Such transformations are also environmentally safe. HPLC technique is used for comparative studies of these transformed products with their standards.

KEYWORDS

Laccase;
Xylaria polymorpha;
Lignolytic enzymes;
Metalloenzymes.

INTODUCTION

Laccase [benzenediol: oxygen oxidoreductase; E.C. 1.10.3.2] is a polyphenol oxidase, which belongs to the superfamily of multicopper oxidases[1,2] and catalyzes[3-5] the four electron reduction of molecular oxygen to water. Laccases are dimeric or tetrameric glycoproteins. To perform their catalytic functions, laccases depend on Cu atoms that are distributed at the three different copper centres (Figure 1) viz. Type-1 or blue copper centre, Type-2 or normal copper centre and Type-3 or coupled binuclear copper centres, differing in their characteristics electronic paramagnetic resonance (EPR) signals[6,7]. 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 type1 Cu and is shuttled to the trinuclear cluster where oxygen is reduced to water.

Ortho and para diphenols, aminophenols, polyphenols, polyamines, lignins, and arylamines and some of the inorganic ions are the substrates for laccases. The ability of laccases to catalyze the oxidation of various phenolic as well as non-phenolic compounds, coupled to the reduction of molecular oxygen to water makes it valuable from the point of view of their commercial applications[4,8-10]. The biotechnological importance of laccases have increased after the discovery that oxidiz-

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able reaction substrate range could be further extended in the presence of small readily oxidizable molecules called mediators[11,12]. During the last two decades, laccases have turned out to be the most promising enzymes for industrial uses[9,10] having applications in food, pulps, paper, textile, and cosmetics industries and in synthetic organic chemistry[13-16].

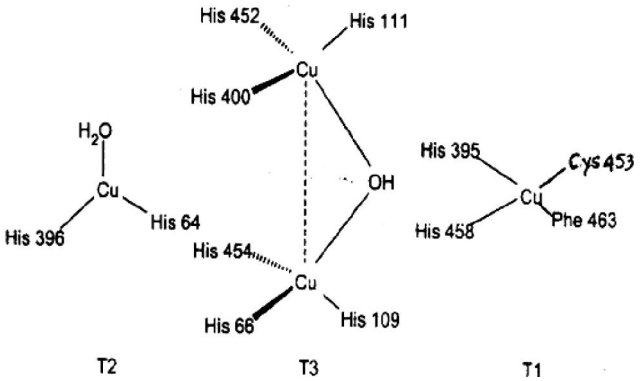

Figure 1 : Three different types of copper centre in laccase to perform their catalytic functions

The objective of this communication is to obtain the crude laccase from the liquid culture growth medium containing natural lignin substrate saw dust of *Xylaria polymorpha* MTCC-1100 and to demonstrate the selective biotransformations of toluene, 3-nitrotoluene and 4-chlorotoluene to corresponding benzalaldehyde, 3-nitrobenzalaldehyde and 4-chlorobenzalaldehyde in presence of ABTS as mediator molecule. The main advantage of this work is that there is no need of drastic conditions. Such biotransformation is environmentally safe and time saving due to use of crude laccase which is also very effective for such transformations.

EXPERIMENTAL

Materials

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

The fungal strain and its growth

The fungal strain was procured from the Microbial Type Culture Collection Center and Gene Bank, Institute of Microbial Technology, Chandigarh, (India) and was maintained on agar slant as reported in MTCC Catalogue of strains-2000[17]. The growth medium for the fungal strain *Xylaria polymorpha* MTCC-1100 consisted of malt extract 20.0 g, peptone 5.0 g, and agar 15.0 g in 1.0 L Milli-Q water.

In order to detect the extracellular secretion of the laccase by *Xylaria polymorpha* MTCC-1100, the liquid culture growth medium reported by Coll et al.[18] was used. This medium consisted of glucose 10.0 g, asparagine 1.0 g, yeast extract 0.5 g, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$, 0.01 g in 1.0 L of Milli-Q water. The above liquid culture growth medium containing natural lignin substrates like coir dust, corn cob, wheat straw, saw dust and bagasse particles were separately prepared by adding 0.5 g of one of the natural lignin substrates to 25 mL of growth medium in 100 mL culture flasks which were sterilized. The sterilized growth media were inoculated with small pieces of mycelia (0.5 cm × 0.5 cm) under aseptic condition and the fungal cultures were grown under stationary culture conditions at 25 °C in a biological oxygen demand (BOD) incubator. In order to monitor the production of the laccase in the liquid culture medium, 0.5 mL aliquots of the growth medium were withdrawn at the regular intervals of 24 hrs and filtered through sterilized Millipore filter 0.22 μm. The filtered extract was analyzed for the activity of the laccase using DMP as the substrate by the method[19] given below in assay section. Extracellular secretion of the laccase in the liquid culture medium by *Xylaria polymorpha* MTCC-1100 was determined by plotting the enzyme unit/mL of the growth medium against the number of days after inoculation of the fungal mycelia. In order to optimize the conditions for maximum production of the laccase by *Xylaria polymorpha* MTCC-1100 in the liquid culture medium, the amount of the best inducer saw dust were varied from 100 mg to 1000 mg in 25 mL of the growth medium. 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.

Enzyme assay

The assay solution 1.0 mL for DMP as the substrate[18] contained 1.0 mM DMP in 50 mM sodium malonate buffer pH 4.0 at 37 °C. The reaction was monitored by measuring the absorbance change at λ=468 nm and using the molar extinction coefficient[18] value of 49.6 mM^{-1} cm^{-1}. The UV/Vis spectrophotometer Hitachi (Japan) model U-2900 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 μMole of the product per minute under the specified assay conditions.

Biotransformations in the presence of ABTS

The biotransformation of toluene to benzaldehyde[20,21] was done in 7 mL of 100 mM sodium acetate buffer pH 4.0 containing 20 mM toluene in 15 mL of dioxane, 0.1 mM ABTS and 350 μL of crude laccase kept in a 100 mL conical flask which was stirred vigorously for 45 minutes (completion of the reaction is confirmed by the UV/Vis spectrophotometer Hitachi (Japan) model U-2900). The reaction solution was extracted thrice with 20 mL of ethyl acetate. 20 μL of the ethyl acetate extract was injected in Waters HPLC Model 600E using spherisorb C_{18} 5 UV, 4.5×250 mm column. The eluant phase was methanol at the flow rate of 0.5 mL/min. The detection was made using Waters UV detector model 2487 at λ=254 nm.

The oxidation of 3-nitrotoluene to 3-nitrobenzaldehyde and 4-chlorotoluene to 4-chlorobenzaldehyde were also studied using the same method described above except time of stirring the reaction solutions are 60 and 90 minutes respectively in these cases.

RESULTS AND DISCUSSION

The experiment, to know the maximum secretion of the laccase in the liquid culture growth medium amended with various lignin containing natural substrates like corn cob, coir dust, saw dust, wheat straw and bagasse particles, was done and inoculated with *Xylaria polymorpha* MTCC-1100. The control experiment has similar medium composition except that the natural lignin containing substrate was absent. The extracellular secretion of the laccase is maximum in the case of the growth medium containing saw dust. In order to optimize the secretion of the laccase in the presence of saw dust, secretion of the laccase in presence of different amounts of saw dust were studied. The maximum level of the laccase was secreted in the liquid culture medium containing 500 mg of the saw dust per 25 mL of the culture medium. The crude laccase obtained from the liquid culture growth medium with natural lignin substrate, saw dust, as described above is used for the selective transformations of aromatic methyl group to the corresponding aldehyde group.

One of the best application 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 methyl groups are preferably converted into carboxylic acids and it becomes very difficult to stop the reaction at aldehyde stage. Moreover, they require drastic reaction conditions which pollute the environment. The conversion done with crude laccase occurs under milder conditions, yield is > 80% and the process is ecofriendly. The use of crude laccases for this purpose has been studied[22,23] in the presence of mediator molecules like 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS)[22]. The potential of the crude laccase as a biocatalyst for the conversion of aromatic methyl group to the corresponding aldehyde group in the presence of the mediator molecule was tested using toluene, 3-nitrotoluene and 4-chlorotoluene as the substrates. The results are shown in Figures 2-5.

Figures 2(a),(b) and (c) are the UV-visible spectra for showing the conversions of toluene, 3-nitrotoluene and 4- chlorotoluene to benzaldehyde, 3- nitrobenzaldehyde and 4- chlorobenzaldehyde, respectively in the presence of mediator molecule, ABTS[20,21]. In Figures 2(a), (b) and (c), spectrum (i) shows the spectrum of the reaction solution with ABTS as mediator molecule before the addition of enzyme and spectrum (ii) shows the spectrum of reaction solution with ABTS after the addition of crude laccase

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representing the completion of the reactions at t= 45, 60 and 90 minutes for Figures 2(a), (b) and (c), respectively.

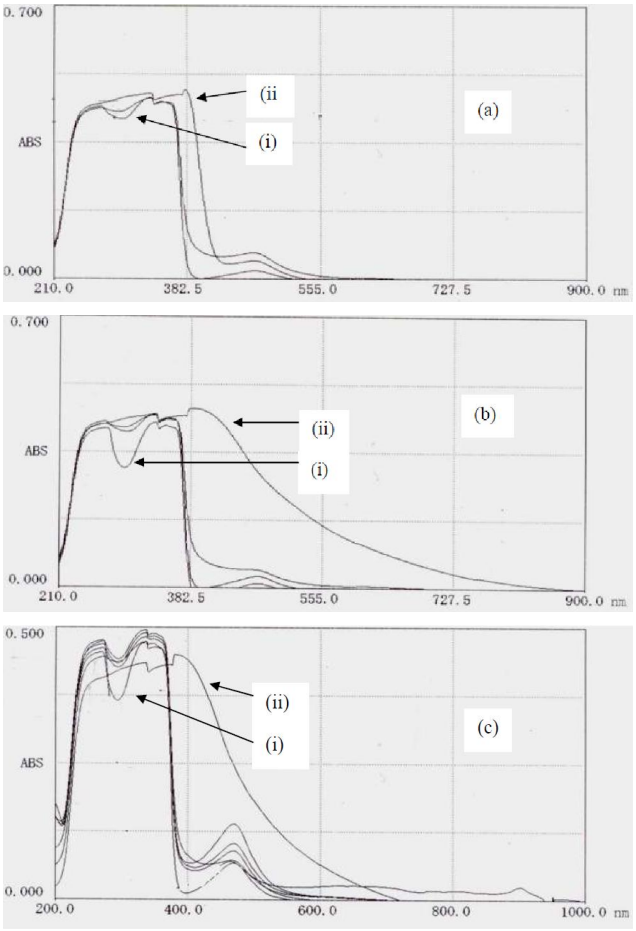

Figure 2 : UV-Visible spectra of the different biotransformation reactions; (a) Spectra of the biotransformation of toluene to benzaldehyde; (b) Spectra of the biotransformation of 3-nitrotoluene to 3-nitrobenzaldehyde; (c) Spectra of the biotransformation of 4-chlorotoluene to 4-chlorobenzaldehyde, (i) Spectrum of reaction solutions with ABTS before the addition of crude laccase in each case, (ii) Spectrum of reaction solutions with ABTS at 45, 60, and 90 minutes after the addition of crude laccase

Figure 3(a) is the HPLC chromatogram of the ethylacetate extract of the product formed by the reaction of the enzyme with toluene in the presence of ABTS as mediator molecule. Figures 3(b), (c) are the chromatograms of the standard samples of benzaldehyde and toluene, respectively. The retention time of the standard sample of toluene was 3.37 min. and the retention time of the standard sample of benzaldehyde was 3.12 min. Thus the retention time of the product of the enzyme-catalysed reaction 3.10 coincides with the retention time of benzaldehyde 3.12 min., confirming that the product of enzyme-catalysed reaction is benzaldehyde.

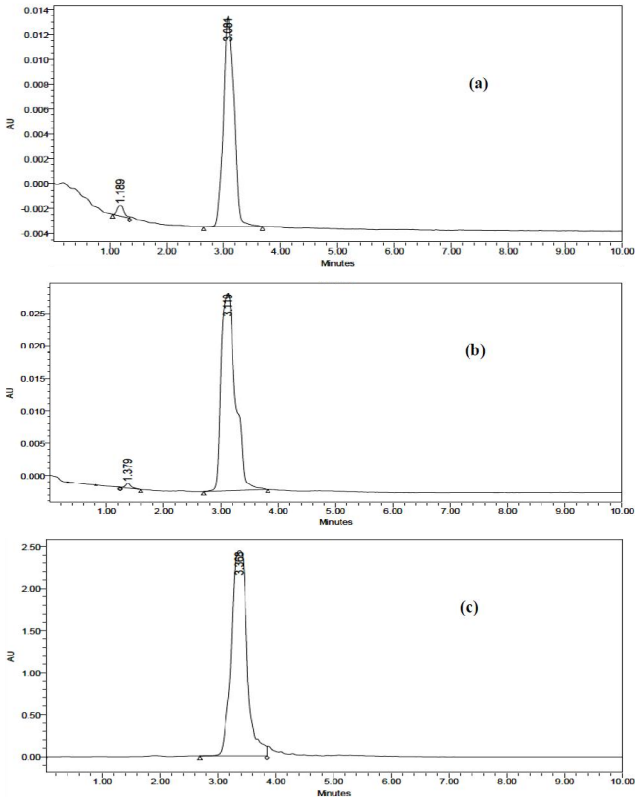

Figure 3 : Transformation of toluene to benzaldehyde by the laccase of *Xylaria polymorpha* MTCC-1100 in presence of mediator; (a) The chromatogram of the product of enzymatic reaction with toluene. The reaction solution contained 20 mmol of methylbenzene, 15 ml dioxane, and 7 mL sodium acetate buffer (pH 4.0) at room temperature was stirred for 45 minutes and extracted thrice with ethyl acetate and 20 µL was injected in HPLC; (b) The chromatogram of the pure benzaldehyde; (c) The chromatogram of the pure toluene

Figure 4(a) is the HPLC chromatogram of the ethylacetate extract of the product formed by the reaction of the enzyme with 3-nitrotoluene in the presence of ABTS as mediator molecule. Figures 4(b), (c) are the chromatograms of the standard samples of 3-nitrobenzaldehyde and 3-nitrotoluene, respectively. The retention time of the standard sample of 3-nitrotoluene was 6.58 min. and the retention time of the standard sample of 3-nitrobenzaldehyde was 5.91 min. Thus the retention time of the product of the enzyme-catalyzed reaction 5.86 coincides with the retention time of 3-nitrobenzaldehyde 5.91 min., confirming that the product of enzyme-catalysed reaction is 3-nitrobenzaldehyde.

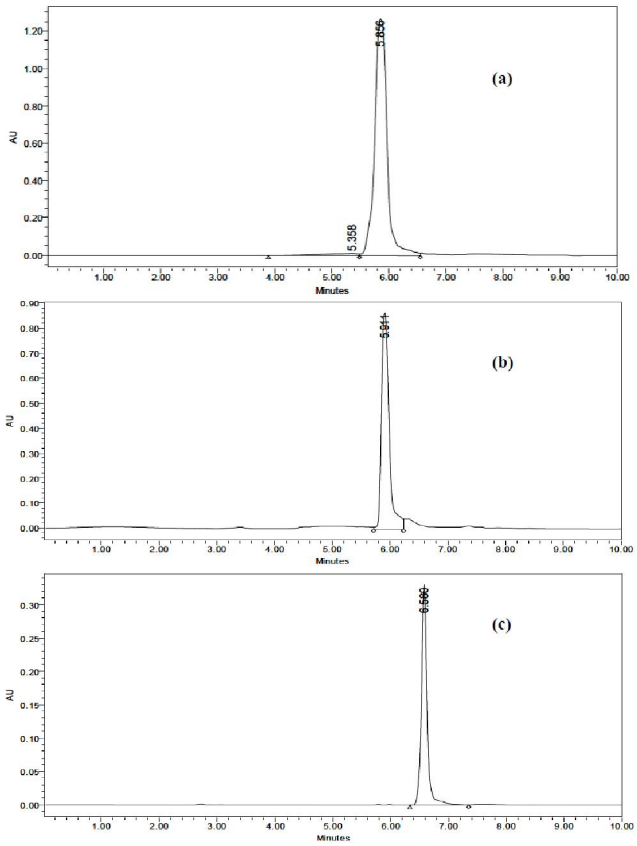

Figure 4 : Transformation of 3-nitrotoluene to 3-nitrobenzaldehyde by the laccase of *Xylaria polymorpha* MTCC-1100 in presence of mediator; (a) The chromatogram of the product of enzymatic reaction with 3- nitrotoluene. The reaction solution contained 20 mmol of 3-nitrotoluene, 15 ml dioxane, and 7 mL sodium acetate buffer (pH 4.0) at room temperature was stirred for 45 minutes and extracted thrice with ethyl acetate and 20 µL was injected in HPLC; (b) The chromatogram of the pure 3-nitrobenzaldehyde; (c) The chromatogram of the pure 3-nitrotoluene

Figure 5(a) is the HPLC chromatogram of the ethyl acetate extract of the product formed by the reaction of the enzyme with 4-chlorotoluene in the presence of ABTS as mediator molecule. Figures 5(b), (c) are the chromatograms of the standard samples of 4-chlorobenzaldehyde and 4-chlorotoluene, respectively. The retention time of the standard sample of 4-chlorotoluene was 7.33 min. and the retention time of the standard sample of 4-chlorobenzaldehyde was 6.25 min. Thus the retention time of the product of the enzyme-catalysed reaction 6.20 coincides with the retention time of 4-chlorobenzaldehyde 6.25 min., confirming that the product of enzyme-catalyzed reaction is 4-chlorobenzaldehyde. Figures 3-5 clearly demonstrates that the crude enzyme catalyses the conversions of toluene, 3- nitrotoluene and 4- chlorotoluene to benzaldehyde, 3- nitrobenzaldehyde and 4- chlorobenzaldehyde, respectively in the presence of mediator molecule ABTS[20,21].

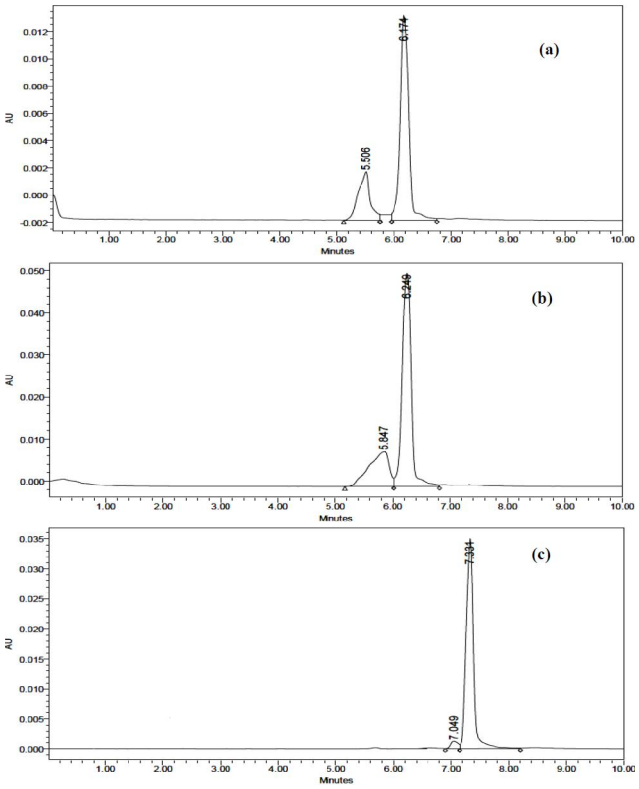

Figure 5 : Transformation of 4-chlorotoluene to 4-chlorobenzaldehyde by the laccase of *Xylaria polymorpha* MTCC-1100 in presence of mediator; (a) The chromatogram of the product of enzymatic reaction with 4- chlorotoluene. The reaction solution contained 20 mmol of 4-chlorotoluene, 15 ml dioxane, and 7 mL sodium acetate buffer (pH 4.0) at room temperature was stirred for 90 minutes and extracted thrice with ethyl acetate and 20 µL was injected in HPLC; (b) The chromatogram of the pure 4-chlorobenzaldehyde; (c) The chromatogram of the pure 4-chlorotoluene

CONCLUSIONS

Thus, this communication reports the selective oxidation of aromatic methyl group to aldehyde group in the presence of ABTS as mediator molecule with crude laccase obtained from the fungal strain *Xylaria polymorpha* MTCC-1100 and clearly demonstrates that there is no need of purification, which is a tedious and very time consuming process, for such transformation. The comparative studies of HPLC chromatograms have been done here for the transformed products with their standards.

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