ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2014, Vol. 40, No. 3, pp. 288–292. © Pleiades Publishing, Ltd., 2014.

Role of Laccase from *Coriolus versicolor* MTCC-138 in Selective Oxidation of Aromatic Methyl Group[1]

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Received August 30, 2013; in final form, September 23, 2013

Abstract—Now a day, laccases are the most promising enzymes in the area of biotechnology and synthesis. One of the best applications of laccases is the selective oxidation of aromatic methyl group to aldehyde group. Such transformations are valuable because it is difficult to stop the reaction at aldehyde stage. Chemical methods used for such biotransformations are expensive and give poor yields. But, the laccase-catalyzed biotransformations of such type are non-expensive and yield is excellent. Authors have used crude laccase obtained from the liquid culture growth medium of fungal strain *Coriolus versicolor* MTCC-138 for the biotransformations of toluene, 3-nitrotoluene, and 4-chlorotoluene to benzaldehyde, 3-nitrobenzaldehyde, and 4-chlorobenzaldehyde, respectively, instead of purified laccase because purification process requires much time and cost. This communication reports that crude laccase can also be used in the place of purified laccase as effective biocatalyst.

Keywords: laccase, Coriolus versicolor, mediator, HPLC, biotransformation

DOI: 10.1134/S1068162014020034

INTRODUCTION

Laccase [EC 1.10.3.2] belongs to a group of polyphenol oxidases containing copper atoms in the catalytic center, which catalyze the reduction of molecular oxygen to water [1–3]. It was first reported in Japanese lacquer tree *Rhus vernicifera* [4]. Little is known about higher plant laccases, probably due to their presence in cell walls. Laccases are the lygnolytic enzymes and abundantly occur in the fungal systems [5], mainly in ascomycetes, deuteromycetes, and basidiomycetes; its production in lower fungi has never been demonstrated. They occur in fungal causative agents of the soft rot, in most, white rot causing fungi, soil saprophytes, and edible fungi. These laccase producing fungi are generally called wood degrading fungi. White rot fungi are the highest producers of the laccases, but litter decomposing and ectomicorrhizal fungi also secrete laccases. Almost all white rot fungi are laccase producers [5–7], except for *Phanerochaete chrysosporium*. Some examples of the fungi which produce laccases are *Pleurotus ostreatus, Coriolus sanguineus, Trametes hirsuta, T. vercicolor, T. villosa, Coriolopsis polyzona, Phlebia radiate, Podospora anserine, Lentinus tigrinus, Pleurotus eryngii, Fomes durrisimus* MTCC-1173, *Pleurotus sajor caju* MTCC-141, and *Trametes trogii*. Laccase is also reported in bacteria, e.g. *Azospirillum lipoferum* [8], as well as in wasp venom [9].

However, it should not be generalized that only the fungal system has ability to produce the laccases. Several reports can be referred to in the literature on production of laccase in ascomycetes, such as *Gaeumannomyces graminis* [10], *Magnaporthe grisea* [11], *Ophiostoma novo-ulmi* [12], *Mauginella* [13], *Melanocarpus albomyces* [14], *Monocillium indicum* [15], *Neurospora crassa* [16], and *Podospora anserine* [17]. In addition to plant pathogenic species, laccase production was also reported for some soil ascomycete species from the genera *Aspergillus, Curvularia*, and *Penicillium* [18–20] and in some freshwater ascomycetes [21].

The ability of laccases to catalyze 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 [2, 22–24]. The importance of laccases has increased after the discovery of mediators [25, 26]. During the last two decades, laccases have turned out to be the most promising enzymes for industrial uses [23, 24] having applications in food, pulps, paper, textile, cosmetics industries, and in synthetic organic chemistry [27–30].

Selective oxidation of aromatic methyl group to its aldehyde group has been done previously [31–33] using purified laccase. Purification of enzyme is a long process that needs high-cost chemical materials and takes much time. In the present communication, crude laccase obtained from liquid culture growth medium containing natural lignin substrate, coir dust,

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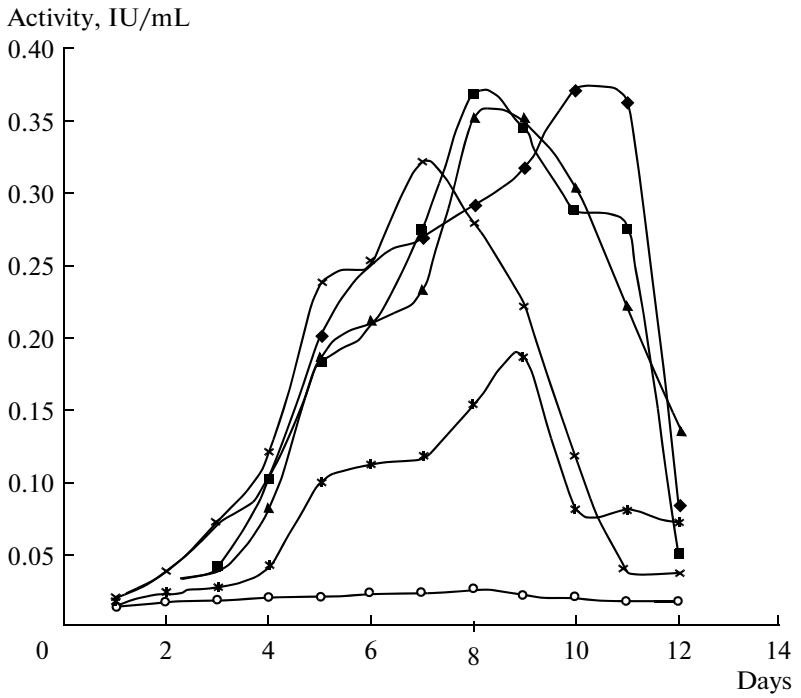

Fig. 1. Secretion of laccase by *Coriolus versicolor* MTCC-138 in the liquid culture medium supplemented with different natural lignin containing substrates saw dust (×), corn cob (■), wheat straw (▲), coir dust (◆), bagasse (✳), and control (○).

of *C. versicolor* MTCC-138 was used to demonstrate selective oxidation of aromatic methyl group in the presence of ABTS as mediator molecule. The purpose of using crude laccase for such biotransformations was to prove that the crude laccase is also an effective biocatalyst like purified laccase and there is no need of high-cost materials in order to obtain the crude laccase. Thus, the novelty of this communication is in the use of crude laccase obtained from *C. versicolor* MTCC-138, which was not reported so far, for such selective oxidation reactions.

RESULTS AND DISCUSSION

The experiment to define 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 performed with *Coriolus versicolor* MTCC-138. The control experiment had similar medium composition except for the natural lignin containing substrate being absent. The extracellular secretion of laccase was maximum in the case of the growth medium containing coir dust (Fig. 1). In order to optimize secretion of laccase, it was studied in the presence of different amounts of coir dust. The maximum level of laccase was secreted in the liquid culture medium containing 500 mg of the coir dust per 25 mL of the culture medium (Fig. 2). The crude laccase obtained from liquid culture growth medium with a natural lignin substrate, coir dust, as described above, was used for selective biotransformation of aromatic methyl group to the corresponding aldehyde group.

Selective transformations of aromatic methyl group to its aldehyde group are, generally, a difficult task in organic synthesis. The chemical routes of this transformation are 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. Transformation of aromatic methyl group to aldehyde group is one of the best applications for laccases, who do it sharply within 1–3 h. The conversion done with crude laccase occurs under milder conditions, the yield is >85%, and the process is ecofriendly. The use of laccases for this purpose has been studied in the presence of a mediator molecule 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) [31]. Toluene, 3-nitrotoluene, and 4-chlorotoluene were successfully converted to benzaldehyde, 3-nitrobenzaldehyde, and 4-chlorobenzaldehyde, respectively, in the presence of ABTS as a mediator molecule.

High performance liquid chromatography (HPLC). In above enzyme catalyzed biotransformations, all products formed are easily available and simple. So, authors have used HPLC technique to confirm the actual product formation by comparing the HPLC chromatograms of standard aldehyde compounds with the enzymatically transformed compounds. The retention time of the standard sample of toluene was 4.21 min and the retention time of the standard sample

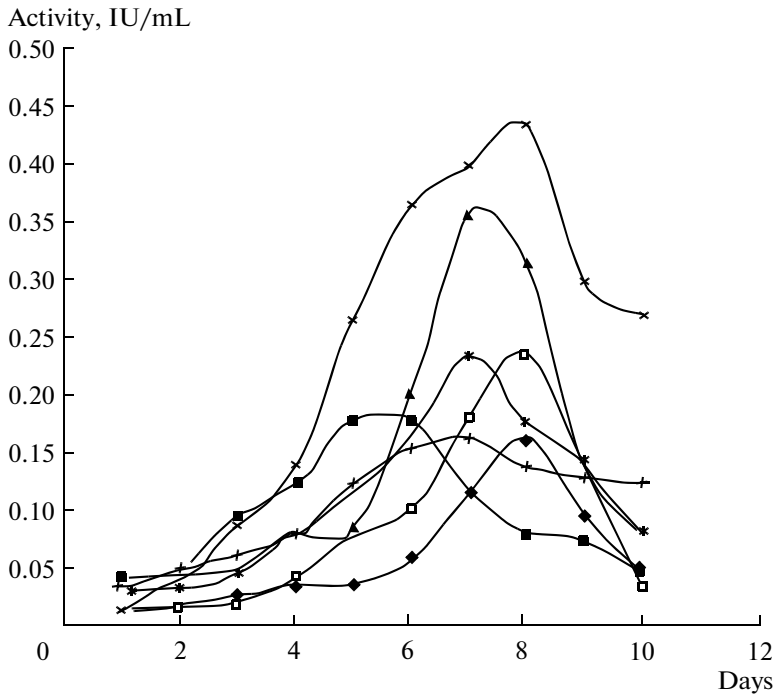

Fig. 2. Optimization of laccase secretion by *Coriolus versicolor* MTCC-138 in liquid culture medium supplemented with different amounts of coir dust: 100 mg (◆), 200 mg (□), 400 mg (▲), 500 mg (×), 600 mg (✳), 800 mg (■), and 1000 mg (+).

of benzaldehyde was 3.52 min. The retention time of the product of the enzyme-catalyzed reaction (3.50 min) coincided with the retention time of benzaldehyde (3.52 min), confirming that the product of enzyme catalyzed reaction was benzaldehyde. The yield of the product was found to be 100%.

The similar type of reactions were done for 3-nitrotoluene bioconversion to 3-nitrobenzaldehyde and 4-chlorotoluene bioconversion to 4-chlorobenzaldehyde in the presence of ABTS as a mediator molecule. The retention time of the standard samples of 3-nitrotoluene, 4-chlorotoluene, 3-nitrobenzaldehyde and 4-chlorobenzaldehyde were 6.58, 7.33, 5.91, and 6.25 min, respectively. Thus, the retention time of the products of the enzyme catalyzed reaction (5.96 and 6.23 min) revealed that the products of enzyme catalyzed reaction were 3-nitrobenzaldehyde and 4-chlorobenzaldehyde. In these cases, yield of the extracted 3-nitrobenzaldehyde and 4-chlorobenzaldehyde were 100 and 89%, respectively.

Infrared (IR) spectroscopy. Identification and characterization of products obtained during enzymatic reaction were analyzed on the basis of IR-results. IR results obtained for the expected benzaldehyde are the following: band at νC–H = 3008 cm^{-1} was due to the aromatic C–H stretching, aldehydic C–H stretching band came at νC–H = 2760 cm^{-1}, while conjugated aldehydic C=O stretching band came at νC=O = 1708 cm^{-1}. A peak at 1345 cm^{-1} was due to the aldehydic C–H bending. The results demonstrate that the product was benzaldehyde.

For expected product 3-nitrobenzaldehyde, following spectral data were obtained. Band at νC–H = 3010 cm^{-1} was due to the aromatic C–H stretching while band at νC–H = 2900 cm^{-1} was due to the aldehydic C–H stretching. Conjugated aldehydic C=O band appeared at νC=O = 1740 cm^{-1}. Two bands obtained at νN=O = 1500 cm^{-1}, 1305 cm^{-1} were due to the asymmetric N=O stretching and symmetric N=O stretching, respectively. A peak at 1390 cm^{-1} was due to the aldehydic C–H bending.

For expected product 4-chlorobenzaldehyde, IR-results obtained are illustrated below. Appearance of a band at νC–H = 3025 cm^{-1} was due to the C–H stretching while appearance of a band at νC–H = 2830 cm^{-1} was due to the aldehydic C–H stretching. Conjugated aldehydic C=O band appeared at νC=O = 1730 cm^{-1}. A peak at 1385 cm^{-1} was due to the aldehydic C–H bending.

^{1}H NMR ($CDCl_3$, 300 MHz, ppm). ^{1}H NMR spectral data obtained for the expected product, i.e. benzaldehyde, shows a singlet δ = 9.10 ppm (1H) due to the aldehydic proton, a doublet δ = 7.83 ppm (2H) due to ortho proton, a triplet δ = 7.36 ppm (2H) due to meta proton and a triplet δ = 7.52 ppm (1H) due to para proton.

^{1}H NMR spectral data obtained for the expected product, i.e. 3-nitrobenzaldehyde, shows a singlet

$\delta = 9.56$ ppm (1H) due to the aldehydic proton, a singlet $\delta = 8.38$ (1H) due to the proton located in-between CHO and NO_2 groups, a doublet at 8.12 (1H) due to the proton para to the CHO group, a triplet $\delta = 7.95$ (1H) due to proton meta to the CHO group, and a doublet 8.10 (1H) due to the proton ortho to the CHO group.

^{1}H NMR spectral data obtained for the expected product, i.e. 4-chlorobenzaldehyde, shows a singlet $\delta = 9.13$ ppm (1H) for aldehydic proton, a doublet 7.87 (2H) ppm for protons ortho to the CHO group, and a doublet 7.35 (2H) for the protons meta to the CHO group.

In this way, all the above described results of HPLC, IR and 1H NMR, collectively and clearly demonstrate that the enzymatically transformed products were benzaldehyde, 3-nitrobenzaldehyde and 4-chlorobenzaldehyde.

Thus, this communication reports the selective oxidation of aromatic methyl group to aldehyde group in the presence of ABTS with crude laccase obtained from the fungal strain *C. versicolor* MTCC-138 and demonstrates that crude laccase is also an effective biocatalyst, which can be used in the place of purified laccase, successfully, for such oxidation reactions.

EXPERIMENTAL

Materials. 4-Chlorotoluene was from Sigma Chemical Company, St. Louis (USA). 2,2'-Azinobis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) and 2,6-dimethoxyphenol (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.

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 [34]. The growth medium for the fungal strain *Coriolus versicolor* MTCC-138 consisted of yeast extract, 5.0 g, glucose, 10.0 g, and agar, 15.0 g, in 1.0 L Milli-Q water.

In order to detect the extracellular secretion of laccase by *Coriolus versicolor* MTCC-138, liquid culture growth medium reported by Coll et al. [35] was used. This medium consisted of glucose, 10.0 g, asparagines, 1.0 g, yeast extract, 0.5 g, $MgSO_4 \cdot 7H_2O$, and $FeSO_4 \cdot 7H_2O$, 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 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 laccase in the liquid culture medium, 0.5 mL aliquots of the growth medium were withdrawn at regular intervals of 24 hrs and filtered through sterilized Millipore filter 0.22 μm. The filtered extract was analyzed for the activity of laccase using DMP as the substrate by the method [35] given in assay section. Extracellular secretion of laccase in the liquid culture medium by *C. versicolor* MTCC-138 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 laccase by *C. versicolor* MTCC-138 in the liquid culture medium, the amount of the best inducer coir dust was varied from 100 mg to 1000 mg in 25 mL of the growth medium. The amount of the inducer in the growth medium that 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 [35] contained 1.0 mM DMP in 50 mM sodium malonate buffer, pH 4.5, at 37°C. The reaction was monitored by measuring the absorbance change at $\lambda = 468$ nm and using the molar extinction coefficient [35] 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 μmol of the product per minute under the specified assay conditions.

Biotransformations in the presence of ABTS as mediator. The biotransformation of toluene to benzaldehyde [31–33] was performed in 14 mL of 100 mM sodium acetate buffer, pH 4.5, containing 20 mM toluene in 30 mL of dioxane, 0.1 mM ABTS and 500 μL of crude laccase kept in a 250 mL conical flask which was stirred vigorously for 30 minutes (completion of the reaction was confirmed by spectrophotometry measurement on the UV/Vis spectrophotometer Hitachi (Japan) model U-2900). The reaction solution was extracted twice with 40 mL of ethyl acetate. Aliquot of 20 μL of the ethyl acetate extract was injected in Waters HPLC Model 600E using spherisorb C18 column (5 UV, 4.5 × 250 mm). The mobile phase was consisted methanol, flow rate was 0.5 mL/min. The detection was performed using a Waters UV detector model 2487 at $\lambda = 254$ nm.

Biotransformations of 3-nitrotoluene to 3-nitrobenzaldehyde and 4-chlorotoluene to 4-chlorobenzaldehyde were also studied using the same method as described above, except for the fact that times of stirring of the reaction solutions were one and two hours, respectively, in these cases.

All the synthesized products were extracted from their reaction solutions with ethyl acetate and these

extracted products were identified and characterized by HPLC, IR, and ^{1}H NMR techniques. Since yields of the products were 100% except 4-chlorobenzaldehyde, where yield was 89%, we did not purify the products any further.

ACKNOWLEDGMENTS

The authors acknowledge the financial support of CSIR-HRDG, New Delhi for the award of JRF and SRF (NET), award no. 09/057(0201)/2010-EMR-I to Mr. Pankaj Kumar Chaurasia. Dr. S.K Singh is also thankful to CSIR-HRDG, New Delhi, for the award of RA, award no. 09/057(0206)/2012-EMR-I. The authors are thankful to Prof. K.D.S. Yadav, Emeritus Scientist, CSIR, New Delhi, for his helpful discussions.

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