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Syntheses of aromatic aldehydes by laccase without the help of mediators

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This communication reports the selective bioconversions of substituted toluenes to substituted benzaldehydes without the help of any mediators by purified laccase of indigenous fungal strain *Fomes durissimus* microbial type culture collection (MTCC)-1173. Molecular mass of laccase purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was found to be 74.86 kDa (~75 kDa). By using this purified laccase, selective bioconversions of 3-nitrotoluene to 3-nitrobenzaldehyde, 2-fluorotoluene to 2-fluorobenzaldehyde, 4-fluorotoluene to 4-fluorobenzaldehyde, 2-chlorotoluene to 2-chlorobenzaldehyde and 4-chlorotoluene to 4-chlorobenzaldehyde have been done without the help of mediator molecules within 1–2 hrs at room temperature and pressure with high yields (>90%). All the above bioconversions are good examples of green chemistry.

Keywords: laccase; *Fomes durissimus*; selective bioconversions; green chemistry

1. Introduction

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. To perform their catalytic functions, laccases depend on Cu atoms that are distributed at the three different copper centers, namely, type-1 or blue copper center, type-2 or normal copper center, and type-3 or coupled binuclear copper centers, differing in their characteristics electronic paramagnetic resonance 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 nonenzymatically. The electron is received at type1 Cu and is shuttled to the trinuclear cluster where oxygen is reduced to water.

The ability of laccases to catalyze the oxidation of various phenolic as well as nonphenolic compounds, coupled with 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 has increased after the discovery of small readily oxidizable molecules called mediators (*11*, *12*) that extend the substrate range for laccases. 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 (*13–19*).

It was previously reported that blue laccases transform aromatic methyl group to aromatic aldehyde group in the presence of mediator molecules (*17*, *18*), but yellow laccases can do it without the help of any mediator molecules (*19*). Only conversion of toluene to benzaldehyde was done previously by using yellow laccase without the help of any mediator (*19*). Thus, the main objective of this communication was to demonstrate the conversions of different substituted toluenes to corresponding benzaldehydes by considering 3-nitrotoluene, 2-fluorotoluene, 4-fluorotoluene, 2-chlorotoluene, and 4-chlorotoluene as reacting substrates in the absence of mediator molecules using pure laccase of *Fomes durissimus* Microbial type culture collection-1173. Authors have purified the laccase from *F. durissimus* Microbial type culture collection (MTCC)-1173 in order to demonstrate the above conversions. Novelty of this manuscript was in the syntheses of substituted benzaldehydes without the help of any mediators at room temperature and pressure.

2. Results and discussion

Selective bioconversion of aromatic methyl group to aldehyde group is one of the best reactions of laccases in organic syntheses. The chemical routes of these conversions are inconvenient because methyl groups are preferably converted into carboxylic acids, and it is very difficult to stop the reaction at aldehyde stage. Moreover, they require drastic reaction conditions which pollute the environment. The conversion done

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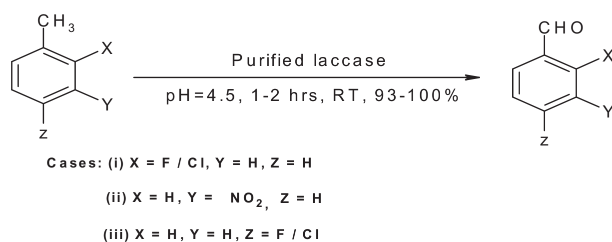
with laccase occurs under milder conditions, yield is >90%, and the process is ecofriendly. The use of pure laccase for this purpose has been studied (*17*, *18*) in the presence of mediator molecules like 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (*17*), but the role of laccase for such conversions in the absence of mediator molecule was done only for conversion of toluene to benzaldehyde (*19*), and its role was not reported for other substituted toluenes.

The role of purified laccase obtained from *F. durissimus* MTCC-1173 as an effective biocatalyst for the conversion of aromatic methyl group to the corresponding aldehyde group in absence of mediator molecules was tested using substituted toluenes as 3-nitrotoluene, 2-fluorotoluene, 4-fluorotoluene, 2-chlorotoluene, and 4-chlorotoluene, as substrates given in Scheme 1.

The purity of the laccase sample from *F. durissimus* MTCC-1173 was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (*20*, *21*). Since, laccase from this source has already been purified and characterized by Sahay et al. (*19*), authors have checked the purity of laccase by using SDS-PAGE analysis for the purpose of their synthetic work. The results of SDS-PAGE analysis are shown in Figure 1. In Figure 1, lane a contains purified laccase, while lane b contains the different molecular weight markers. Appearance of single protein band in SDS-PAGE (lane a) indicated that the enzyme sample was pure. The molecular mass of the purified laccase determined from the analysis (*24*) of SDS-PAGE was 74.86 (~75) kDa.

This enzyme has been purified for the purpose of syntheses of different substituted benzaldehydes from their respective substituted toluenes. These laccase-catalyzed syntheses of 3-nitrobenzaldehyde, 2-fluorobenzaldehyde, 4-fluorobenzaldehyde, 2-chlorobenzaldehyde, and 4-chlorobenzaldehyde have been done in absence of mediator molecules from 3-nitrotoluene, 2-fluorotoluene, 4-fluorotoluene, 2-chlorotoluene, and 4-chlorotoluene, respectively. Yield of



Scheme 1. Reaction of different substituted toluenes with purified laccase of *F. durissimus* MTCC-1173 in absence of mediators at room temperature.

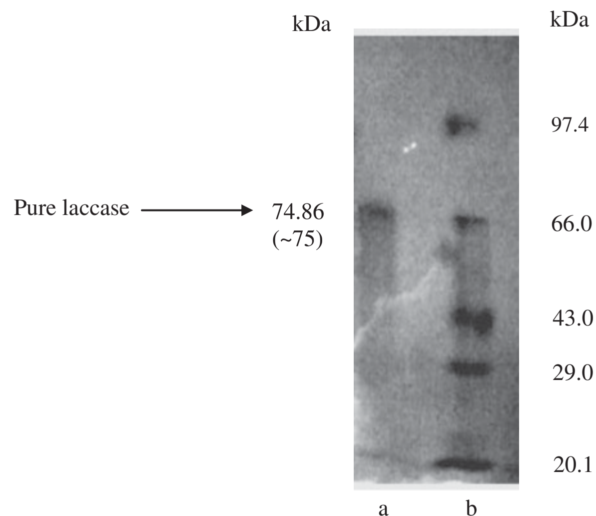


Figure 1. SDS-PAGE. (a) Pure laccase, (b) standard molecular weight markers.

all the synthesized products was good and given in Table 1 with reactants and products. Tests of this laccase for the selective oxidation of aromatic methyl group of xylenes, methyl-substituted naphthalene, ethyl benzene, etc., have also been done, but these reactions were either not successful or gives very low products yield (<15%) in the absence of mediator molecule due to which these reactions were not describes in the manuscript.

2.1. *High-performance liquid chromatography (HPLC)*

In the above selective enzyme-catalyzed bioconversions, 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 samples of 3-nitrotoluene, 2-fluorotoluene, 4-fluorotoluene, 2-chlorotoluene, 4-chlorotoluene, 3-nitrobenzaldehyde, 2-fluorobenzaldehyde, 4-fluorobenzaldehyde, 2-chlorobenzaldehyde, and 4-chlorobenzaldehyde were 6.58, 6.89, 7.23, 6.93, 7.33, 5.91, 5.98, 6.05, 6.21, and

Table 1. Enzymatically synthesized different benzaldehydes from their respective toluenes in the absence of mediators with yields.

Reactants	Products	Yield (%)
3-Nitrotoluene	3-Nitrobenzaldehyde	98
2-Fluorotoluene	2-Fluorobenzaldehyde	93
4-Fluorotoluene	4-Fluorobenzaldehyde	95
2-Chlorotoluene	2-Chlorobenzaldehyde	95
4-Chlorotoluene	4-Chlorobenzaldehyde	96

6.25 min, respectively. Thus, the retention time of the products of the enzyme-catalyzed reactions (5.95, 5.99, 6.09, 6.20, and 6.31 min) revealed that the products of the enzyme-catalyzed reactions were 3-nitrobenzaldehyde, 2-fluorobenzaldehyde, 4-fluorobenzaldehyde, 2-chlorobenzaldehyde, and 4-chlorobenzaldehyde.

2.2. Infrared (IR) spectroscopy (KBr, ν, cm^{-1})

All the enzyme-catalyzed products were identified by IR results. For expected product 3-nitrobenzaldehyde, IR results were 3067, 2841, 1700, 1515, 1325, and 1371 cm^{-1}, while for expected product 2-fluorobenzaldehyde, 4-fluorobenzaldehyde, 2-chlorobenzaldehyde, and 4-chlorobenzaldehyde, IR results obtained were approximately same as given here: ~3057, ~2836, ~1705, and ~1363 cm^{-1}.

In the above IR results, band near 2836–2841 cm^{-1}, due to the aldehydic C–H stretching, and ~1700–1705, due to conjugated aldehydic C=O stretching, confirm the products formation.

2.3. Proton nuclear magnetic resonance spectroscopy ($CDCl_3$, 300 MHz, ppm)

^{1}H NMR spectral data obtained for the all the expected products are given below:

For 3-nitrobenzaldehyde: 9.49 (s, 1H), 8.35 (s, 1H), 8.29 (db, 1H), 7.96 (t, 1H), and 8.18 (db, 1H)

For 2-fluorobenzaldehyde and 2-chlorobenzaldehyde: ~9.52 (s, 1H), ~7.85 (db, 1H), ~7.71 (db, 1H), ~7.18 (t, 1H) and ~ 6.89 (t, 1H).

For 4-fluorobenzaldehyde and 4-chlorobenzaldehyde: ~9.43 (s, 1H), ~7.78 (db, 2H) and ~7.41 (db, 2H).

In all the expected products, a singlet near $\delta \sim$ 9.49 ppm (1H) was due to the aldehydic proton which confirms the conversion of aromatic methyl group to aromatic aldehyde group in the absence of mediator molecules.

In this way, all the above described results of HPLC, IR, and ^{1}H NMR (figures of chromatograms and spectra has been omitted because all the products were simple and well known), clearly demonstrate that the enzymatically transformed products were 3-nitrobenzaldehyde, 2-fluorobenzaldehyde, 4-fluorobenzaldehyde, 2-chlorobenzaldehyde, and 4-chlorobenzaldehyde.

2.4. Conclusions

This communication shows the successful laccase-catalyzed bioconversions of substituted toluenes to corresponding benzaldehydes in the absence of mediator molecules by purified laccase from *F. durissimus* MTCC-1173 which were not reported so far without mediator molecules. Yields were excellent, and all the syntheses were done at room temperature without using any drastic reaction conditions.

3. Experimental

3.1. Materials

4-Chlorotoluene, 4-fluorotoluene, 3-nitrotoluene, and diethyl amino ethyl (DEAE) cellulose were from Sigma Chemical Company, St. Louis (USA). 2-Fluorotoluene, 2-chlorotoluene, and 2,6-dimethoxy phenol (DMP) were from Fluka, Chemi new Ulm (Switzerland). The chemicals used in the gel electrophoresis of the protein samples were from Bangalore Geni Pvt. Ltd., Bangalore (India).

3.2. The fungal strain, its growth, and purification of laccase

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 (22).

For the purification of the laccase, *F. durissimus* MTCC-1173 (19) was grown in ten 100 mL culture flasks each containing 25 mL sterilized growth medium reported by Coll et al. (23) (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) with optimal amount of 800 mg of the best inducer bagasse particles (19), under stationary culture condition in a biological oxygen demand incubator at 30°C. The maximum activity of the laccase appeared on ninth day (19) of the inoculation of the fungal mycelia. On the ninth day, all the cultures in the 10 flasks were pooled, and mycelia were removed by filtration through four layers of cheese cloth. The culture filtrates were concentrated using Amicon concentration cell model 8200 with PM-10 ultrafiltration membrane, and molecular weight cut-off value was 10 kDa. The concentrated enzymes were dialyzed against 10 mM Na-acetate buffer (pH 4.5) in a volume ratio of 1:1000 with three changes at intervals of 8 hrs. The dialyzed enzyme sample was loaded on to the DEAE column (size 0.75 cm × 18.0 cm) which was pre-equilibrated with 10 mM sodium acetate/acetic acid buffer (pH 4.5) and the flow rate was 15 mL/hr. The column was washed with 100 mL of the same buffer. The enzyme was eluted by applying linear gradient of M NaCl in the same buffer (75 mL buffer + 75 mL buffer with 1.0 M NaCl). The fractions of 5.0 mL size were collected and analyzed for the laccase activity (23).

All laccase active fractions were combined and concentrated by Amicon concentrator cell model 8200 and then by model-3 to 3 mL. The enzyme was stored in a refrigerator in 10 mM sodium acetate/acetic acid buffer pH 4.0. The enzyme does not lose any activity for one month under these conditions.

3.3. SDS-PAGE analysis

The purity of the enzyme preparation was checked by using SDS-PAGE (*20*). The resolving gel was 12% acrylamide in tris/HCl buffer pH 8.8, and the staking gel was 5% acrylamide in tris/HCl buffer pH 6.8. The molecular weight markers 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). Gel was run at a constant current 20 mA (Polyacrylamide Gel Electrophoresis, 1984) (*21*). The molecular weight was determined by Weber and Osborn method (*24*).

3.4. Enzyme assay

The assay solution 1.0 mL for DMP as the substrate (*23*) 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 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 μmol of the product per minute under the specified assay conditions.

3.5. Bioconversions in the absence of mediators

The bioconversion of 2-chlorotoluene to 2-chlorobenzaldehyde (*17–19*) was done in 15 mL of 100 mM sodium acetate buffer pH 4.5 containing 20 mM toluene, 25 mL dioxane, and 500 µL of purified laccase (the activity of concentrated laccase was 0.553 IU/mL) kept in a 100-mL conical flask which was stirred vigorously for 90 minutes (the completion of the reaction is confirmed by the UV/Vis spectrophotometer Hitachi (Japan) model U-2900). The reaction solution was extracted thrice with n-hexane. Twentymicroliter of the ethyl acetate extract was injected in Waters HPLC Model 600E using spherisorb C_{18} 5 UV, 4.5 × 250 mm column. The eluent 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 bioconversions of 3-nitrotoluene to 3-nitrobenzaldehyde, 4-chlorotoluene to 4-chlorobenzaldehyde, 2-fluorotoluene to 2-fluorobenzaldehyde, 4-fluorotoluene to 4-fluorobenzaldehyde were also studied using the same method described above, except that the times of stirring the reaction solutions were 75, 90, 120, and 120 minutes, respectively.

Since only small amounts of chemical auxiliaries are applied which remain in the aqueous phase after extraction of the aldehydes with an organic solvent (ethyl acetate), very pure compounds are obtained requiring no further purification. During these oxidations, no side reactions occur because of the high specificity of laccase as biocatalyst. Thus, authors have used ethyl acetate as an organic solvent for the extraction of products and found the almost pure benzaldehydes and substituted benzaldehydes in high yields (average yield was 93%).

All the synthesized products were identified and characterized by HPLC, IR, and ^{1}H NMR techniques and given in the results and discussion section.

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References

(1) Hoegger, P.J.; Kilaru, S.; Jomes, T.Y.; Thacker, J.R.; Kuees U. *FEBS J.* **2006**, *273*, 2308–2326.
(2) Messerschmidt, A. *Multi-Copper Oxidases*; World Scientific: Singapore, **1997**.
(3) Riva, S. *Trends in Biotechnol.* **2006**, *24* (5), 219–226.
(4) Baldrian, P. *FEMS Microbiol. Rev.* **2006**, *30*, 215–242.
(5) Dwivedi, U.N.; Singh, P.; Pandey, V.P.; Kumar, A.J. *Mol. Cat. B: Enzymatic.* **2011**, *68* (2), 117–128.
(6) Solomon, E.I.; Baldwin, M.J.; Lowery, M.D. *Chem. Rev.* **1992**, *92*, 521–542.
(7) Bento, I.; Armenia, M.; Corrondo, P.F.; Lindloy. J. *Biol. Inorg. Chem.* **2006**, *11*, 919–927.
(8) Wandrey, C.; Liese, A.; Kihumbu, D. *Org. Proc. Res. Develop.* **2000**, *4* (4), 285–290.
(9) Couto, S.R.; Harrera, J.L.T. *Biotechnol. Advan.* **2006**, *24*, 500–513.

(10) Xu, F. *Ind. Biotechnol*. **2005**, *1*, 38–50.
(11) Acunzo, D.F.; Galli, C. J. *Europ. Biochem*. **2003**, *270*, 3634–3640.
(12) Morozova, O.V.; Shumakovich, G.P.; Shleev, S.V.; Yaropolov, Y.I. *Biochem. Microbiol*. **2003**, *43*, 523–535.
(13) Coniglio, A.; Galli, C.; Gentili, P.J. Mol. *Cat. B: Enzymatic*. **2008**, *50* (1), 40–49.
(14) Mikolasch, A.; Niedermeyer, T.H.J.; Lalk, M.; Witt, S.; Seefeld, S.; Hammer, E.; Schauer, F.; Gesell, M.; Hessel, S.; Julich, W.D.; Lindoquist, U. *Chem. Pharmaceut. Bullet*. **2006**, *54* (5), 632–638.
(15) Mikolasch, A.; Niedermeyer, T.H.J.; Lalk, M.; Witt, S.; Seefeld, S.; Hammer, E.; Schauer, F.; Salazar, M. G.; Hessel, S.; Julich, W.D.; Lindequist, U. *Chem. Pharmaceut. Bullet*. **2007**, *55* (3), 412–416.
(16) Mikolasch, A.; Hammer, E.; Jonas, U.; Popowski, K.; Stielow, A.; Schaner, F. *Tetrahedron*. **2002**, *58*, 7589–7593.
(17) Potthest, A.; Rosenanu, T.; Chen, C-L; Gratzl, J.S. *J. Org. Chem*. **1995**, *60*, 4320–4321.
(18) Fritz-Langhals; Kunath, B. *Tetrahedron Lett*. **1998**, *39*, 5955–5956.
(19) Sahay, R.; Yadav, R.S.S.; Yadava, S. *Appl. Biochem. Biotechnol*. **2012**, *166*, 563–575.
(20) Laemmli, U.K.; Favre, M. *J. Mol. Biol*. **1973**, *80*, 575–599.
(21) *Polyacrylamide Gel Electrophoresis: Laboratory Techniques*; Pharmacia, Laboratory Separation Division: Uppsala, Sweden, **1984**.
(22) *Catalogue of strains-2000* (5th edition); Microbial Type Culture Collection and Gene Bank Institute of Microbial Technology: Chandigarh, p 60.
(23) Coll, M.P.; Fernandez-Abalos, J.M.; Villomueva, J.R.; Somtamaria, R.; Perez, P. *Appl. Env. Microbiol*. **1993**, *59*, 2607–2613.
(24) Osborn, M.; Weber, K. *J. Biolog. Chem*. **1969**, *244*, 4406–4412.