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Selective Oxidation and N-Coupling by Purified Laccase of *Xylaria polymorpha* MTCC-1100[1]

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Abstract—The chemical route 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 h. Coupling of amines are another important reactions known for fungal laccases; coupling reactions generally take 3–7 h. We have used the purified laccase of molecular weight 63 kDa obtained from the fungal strain *Xylaria polymorpha* MTCC-1100 with activity of 1.95 IU/mL for selective oxidation of 2-fluorotoluene, 4-fluorotoluene, and 2-chlorotoluene to 2-fluorobenzaldehyde, 4-fluorobenzaldehyde, and 2-chlorobenzaldehyde, respectively, and syntheses of 3-(3, 4-dihydroxyphenyl)-propionic acid derivatives by N-coupling of amines. In each oxidation reactions, ABTS was used as mediator molecule. All the syntheses are ecofriendly and were performed at room temperature.

Keywords: laccase, 2-fluorotoluene, 4-fluorotoluene, 2-chlorotoluene, 3-(3,4-dihydroxyphenyl)-propionic acid derivatives

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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. Laccases are dimeric or tetrameric glycoproteins. To perform their catalytic functions, laccases depend on Cu atoms that are distributed at three different copper centers viz. type-1, or blue copper center, type-2, or normal copper center and type-3, or coupled binuclear copper center. The center types differ in their characteristic electronic paramagnetic resonance (EPR) signals [6, 7]. 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 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 oxidizable 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].

Fungal laccases have been used for the selective oxidation reactions [17–22] and N-coupling reactions [16]. N-coupling reactions have been done by using crude fungal laccases, previously. The objective of this communication was to do the selective oxidations of substituted toluenes, such as 2-fluorotoluene, 4-fluorotoluene, and 2-chlorotoluene to corresponding 2-fluorobenzaldehyde, 4-fluorobenzaldehyde, and 2-chlorobenzaldehyde in the presence of ABTS as mediator molecule, as well as synthesis of 3-(3,4-dihydroxyphenyl)-propionic acid derivatives by N-coupling reactions, using purified laccase. For this purpose, we used purified laccase from *Xylaria polymorpha* MTCC-1100 of molecular weight of 63 kDa having activity 1.95 IU/mL, directly as reported by Pankaj et al. [23].

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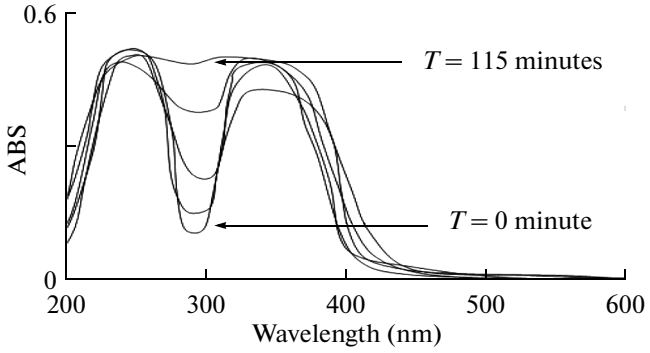
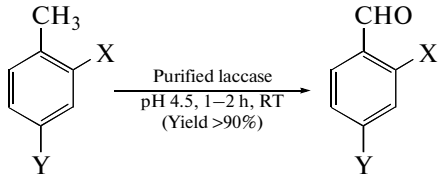


Fig. 1. UV–visible spectra of the biotransformation reaction of 2-fluorotoluene to 2-fluorobenzaldehyde. $T = 0$ shows the spectrum of reaction solution after the addition of laccase at zero time. $T = 115$ shows the spectrum of reaction solution after 115 min from the addition of laccase.

RESULTS AND DISCUSSION

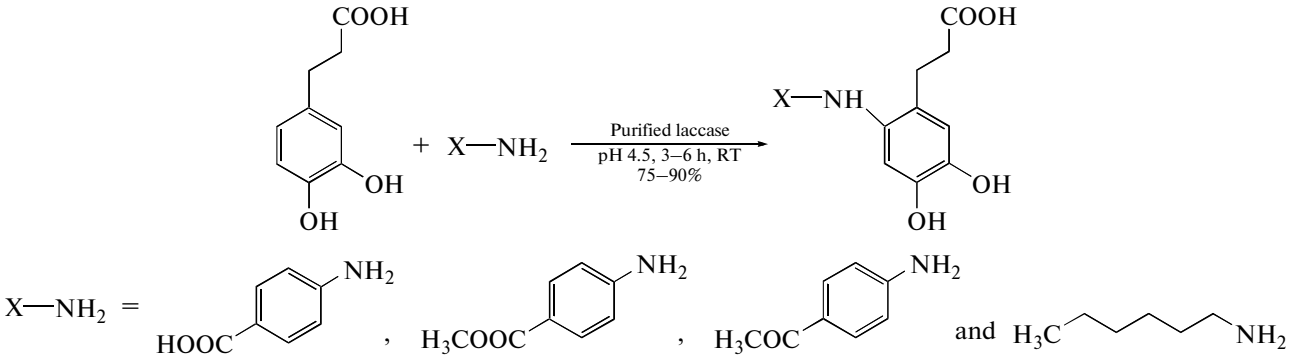
One of the best applications of the laccases in organic synthesis is the selective oxidation of the aromatic methyl group to the corresponding aldehyde. The chemical routes of this conversion are inconvenient because methyl groups are preferably converted into carboxylic acids and it becomes very difficult to stop the reaction at aldehyde stage. Moreover, they require drastic reaction conditions which pollutes the environment. The conversion done with pure laccase occurs under milder conditions, the yield is >90% and the process is ecofriendly. The use of purified laccases for this purpose has been studied [17, 18] in the presence of mediator molecules like 2,2'-azino-*bis*-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) [17]. The potentiality of the pure 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 2-fluorotoluene, 4-fluorotoluene, and 2-chlorotoluene as substrates for this fungal strain (Scheme 1).



(i) X = F/Cl, Y = H
(ii) X = H, Y = F

Scheme 1. Syntheses of substituted benzaldehydes from substituted toluenes.

Other important reactions of laccases are the coupling reactions [16]. These coupling reactions have been done by using crude laccase previously. In this communication, synthesis of 3-(3,4-dihydroxyphenyl)-propionic acid derivatives have been done by N-coupling reactions with purified laccase of molecular weight 63 kDa having activity 1.95 IU/mL purified by Pankaj et al. from the liquid culture growth medium of *Xylaria polymorpha* MTCC-1100 [23] (Scheme 2). All the above synthesized products were characterized and identified by HPLC, IR, and NMR spectroscopy.



Scheme 2. Syntheses of 3-(3,4-dihydroxyphenyl)-propionic acid derivatives by coupling reactions with purified laccase of *X. polymorpha* MTCC-1100.

UV-Visible Spectroscopy

Completions of the reactions were confirmed by spectrophotometry measurements. In all the cases, reaction solution was firstly measured by UV-visible spectrophotometry at zero time of laccase addition and then measured after constant time interval and changes in UV-spectrum were observed. Figure 1 presents an example of a UV-spectrum of an oxidation reaction of 2-fluorotoluene to 2-fluorobenzaldehyde by laccase in which time taken for reaction completion was 115 minutes.

High Performance Liquid Chromatography (HPLC)

In the above mentioned selective 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 profiles of standard aldehyde compounds with the enzymatically transformed compounds. Retention times of the standard samples of 2-fluorotoluene, 4-fluorotoluene, 2-chlorotoluene, 2-fluorobenzaldehyde, 4-fluorobenzaldehyde, and 2-chlorobenzaldehyde were 7.17, 6.97, 7.33, 6.14, 6.11, and 6.25 min, respectively. Retention times of the products of the enzyme catalyzed reaction (6.12, 6.1, and 6.24 min) revealed that they were 2-fluorobenzaldehyde, 4-fluorobenzaldehyde, and 2-chlorobenzaldehyde. Yields of the extracted 2-fluorobenzaldehyde, 4-fluorobenzaldehyde, and 2-chlorobenzaldehyde were 94, 94, and 96%, respectively.

Retention time of major peak obtained for N-coupling products of 3-(3,4-dihydroxyphenyl)-propionic acid with 4-aminobenzoic acid, methyl 4-aminobenzoate, 4-aminoacetophenone, and 1-hexylamine were 5.21 (yield 89%), 5.16, (82%), 5.10 (86%) and 4.82 min (76%), respectively, while retention times for 3-(3,4-dihydroxyphenyl)-propionic acid, 4-aminobenzoic acid, methyl 4-aminobenzoate, and 4-aminoacetophenone were 5.85, 6.21, 5.97, and 6.15, respectively, which demonstrates the formation of coupling products that have been identified and characterized by IR and NMR spectroscopy. HPLC chromatograms of all the coupling products are presented in Fig. 2.

In IR spectra of biotransformed products, a band near 815 cm^{-1} was due to C–Cl stretching. A band at ~1705 cm^{-1} was due to aldehydic carbonyl stretching confirming formation of the products. In IR spectra of coupling products, a single stretching band at ~3441 cm^{-1} was due to the N–H (2-amino group) and showed that these two reactants have been coupled and desired products have been formed.

In ^{1}H NMR spectra of different biotransformed products, a peak at $\delta > 9.00$ was due to the aldehydic proton, confirming formation of expected benzaldehydes. In spectra of different coupling products, peak between 3.73–3.85 ppm (s, 1H) was due to NH hydrogen, which shows that reactants have been coupled to form the product.

The results of HPLC, IR and ^{1}H NMR-spectroscopy demonstrate that oxidation products were 2-fluorobenzaldehyde, 4-fluorobenzaldehyde, and 2-chlorobenzaldehyde (Scheme 1), while coupling products were 3-(3,4-dihydroxyphenyl)-propionic acid derivatives of 4-aminobenzoic acid, methyl 4-aminobenzoate, 4-aminoacetophenone, and 1-hexylamine (Scheme 2). Possibilities of any side reactions ignored since the rate of side reactions is low; also, this manuscript do not include a study on side reactions of dihydroxyphenylpropionic acid in the presence of laccase.

CONCLUSIONS

Thus, this communication reports the successful involvement of a purified laccase in selective oxidation of aromatic methyl group of substituted toluenes to their respective aldehyde group in the presence of ABTS as mediator molecule and synthesis of 3-(3,4-dihydroxyphenyl)-propionic acid derivatives by N-coupling of amines. These above syntheses, easily done by laccase are novel because there is neither need of drastic reaction conditions nor costly reagents and each synthesis has been done at room temperature. One more important thing is that these syntheses are ecofriendly because purified laccase was used instead of synthetic catalysts.

EXPERIMENTAL

Materials

4-Fluorotoluene, 2-fluorotoluene, 2-chlorotoluene, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS), 3-(3,4-dihydroxyphenyl)-propionic acid, and 4-aminobenzoic acid 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.

Selective Oxidation in the Presence of ABTS

The biotransformation [17, 18, 23] of 2-fluorotoluene to 2-fluorobenzaldehyde was done in 7 mL of 100 mM sodium acetate buffer, pH 4.5, containing 20 mM 2-fluorotoluene in 15 mL of dioxane, 0.1 mM ABTS, and 300 μL of purified laccase [23] in a 100 mL conical flask; the mixture was stirred vigorously for 115 minutes. 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 $\lambda = 254$ nm. The oxidation of 2-chlorotoluene and 4-fluorotoluene to 2-chlorobenzaldehyde and 4-fluorobenzaldehyde were also studied using the same method described above except for time of stirring the reaction solutions were 90 and 110 min, respectively.

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 only

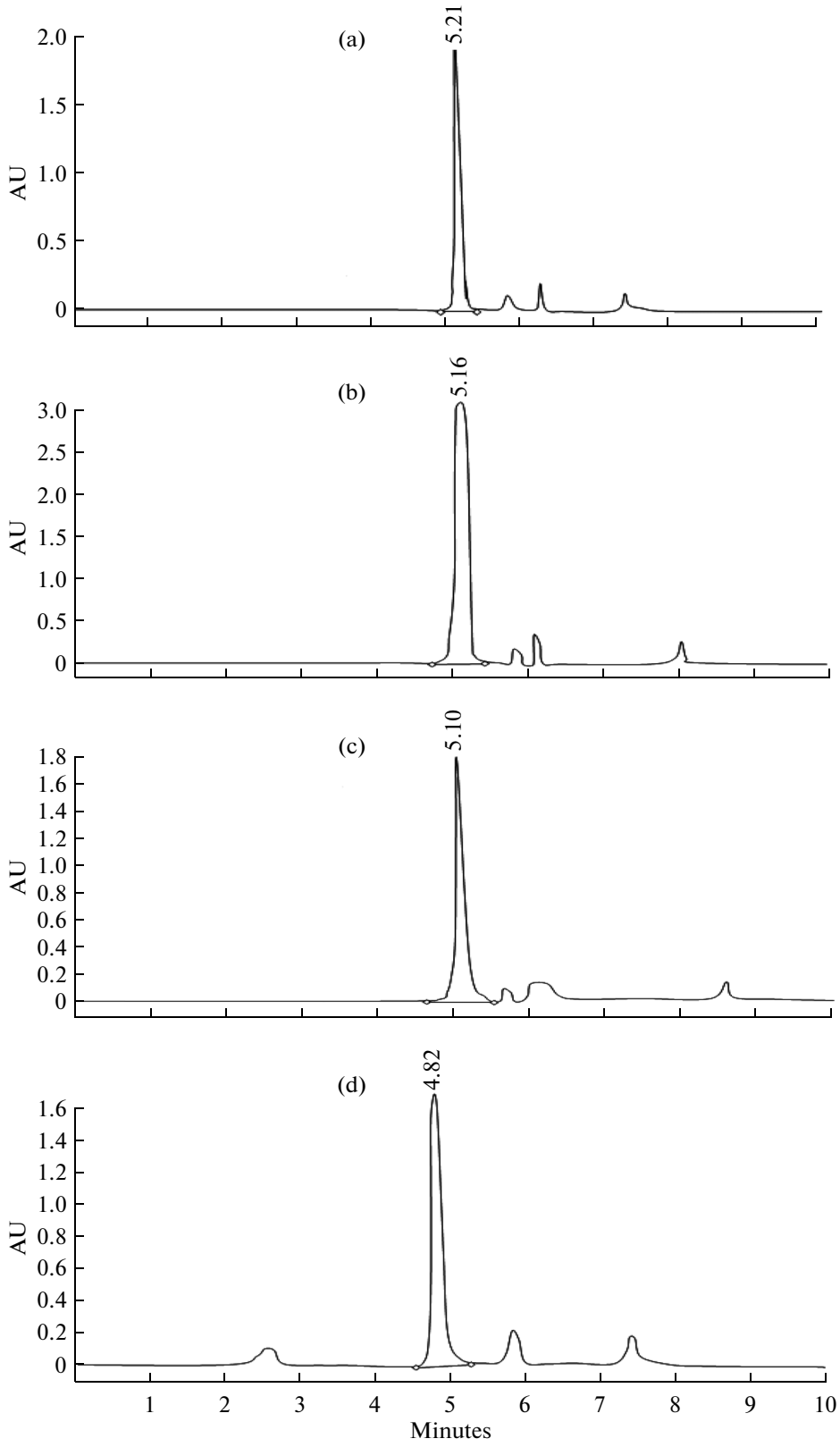


Fig. 2. HPLC profiles of coupling products of 3-(3,4-dihydroxyphenyl)-propionic acid with 4-aminobenzoic acid (a), methyl 4-aminobenzoate (b), 4-aminoacetophenone (c), and 1-hexylamine (d).

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 organic solvent for the extraction of products and found almost pure benzaldehydes and substituted benzaldehydes in high yields (average yield was 95%).

Synthesis of 3-(3,4-dihydroxyphenyl)-propionic Acid Derivatives by N-coupling of Amines

(i) Coupling of 3-(3,4-dihydroxyphenyl)-propionic acid with 4-aminobenzoic acid and 4-aminoacetophenone [16]. The enzyme was diluted with 20 mM sodium acetate buffer, pH 4.5. The substrates, 3-(3,4-dihydroxyphenyl)-propionic acid (1 mM) and 4-aminobenzoic acid (1 mM), were added to 2 mL of this diluted solution and reaction mixture was incubated for 3:45 h at room temperature and stirred vigorously.

Similar method was used for coupling of 3-(3,4-dihydroxyphenyl)-propionic acid with methyl 4-aminobenzoate, and 4-aminoacetophenone.

(ii) Coupling of 3-(3,4-dihydroxyphenyl)-propionic acid and 1-hexylamine [16]. Similar method was applied for this synthesis also; the substrates, 3-(3,4-dihydroxyphenyl)-propionic acid (1 mM), and 1-hexylamine (6 mM), were added to 2 mL of diluted solution and reaction mixture was incubated for 6 h at room temperature and stirred vigorously.

All the synthesized products were extracted from their reaction solutions with ethyl acetate and products were identified and characterized by HPLC, IR, and ^{1}H NMR techniques.

Characterization

Infrared (IR) spectroscopy. IR results obtained for the expected products 2-fluorobenzaldehyde, 4-fluorobenzaldehyde, and 2-chlorobenzaldehyde were ~3010, ~2890, ~1705, ~1355, and ~1250 cm^{-1}.

Important bands appeared in the IR-spectrum for the coupling products of 3-(3,4-dihydroxyphenyl)-propionic acid with 4-aminobenzoic acid, methyl 4-aminobenzoate, 4-aminoacetophenone, and 1-hexylamine; they include ~2940, ~3048, ~1715, ~1695, ~3441, ~2570, ~3610, and ~2950 cm^{-1}.

^{1}H NMR. ^{1}H NMR spectral data obtained for the expected products, i.e. 2-fluorobenzaldehyde and 2-chlorobenzaldehyde, were δ ~ 9.13 (s, 1H), ~7.92 (d, 1H), ~7.53 (d, 1H), ~7.31 (t, 1H), and ~6.85 (t, 1H). ^{1}H NMR spectral data obtained for the expected product, 4–fluorobenzaldehyde were δ ~ 9.26 (s, 1H), 7.78 (d, 1H), and 7.42 (d, 1H).

^{1}H NMR spectral data obtained for the coupling product of 3-(3,4-dihydroxyphenyl)-propionic acid with 4-aminobenzoic acid is described below: δ = 8.15 (d, 2H), 7.03 (d, 2H), 5.87 (s, 1H), 6.27 (s, 1H), 2.59 (t, 2H), 2.41 (t, 2H), and 3.73 ppm (s, 1H).

^{1}H NMR spectral data obtained for the coupling product of 3-(3,4-dihydroxyphenyl)-propionic acid with methyl 4-aminobenzoate, and 4-aminoacetophenone were almost similar to those obtained in the case of 4-aminobenzoic acid, except for the singlets at δ = 4.13 and 2.25 due to the methyl groups, one present as ester and another one, attached to ketonic carbon, respectively.

NMR results obtained for the coupling product of 3-(3,4-dihydroxyphenyl)-propionic acid with 1-hexylamine were δ = 5.95 (s, 1H), 6.18 (s, 1H), 2.67 (t, 2H), 2.31 (t, 2H), 3.21 (t, 2H), 1.61 (q, 2H), 1.52 (q, 2H), 1.33 (q, 2H), 1.32 (sextet, 2H), 1.25 (t, 3H), and 3.85 (s, 1H).

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