IDENTIFICATION, PURIFICATION AND CHARACTERIZATION OF A NOVEL EXTRACELLULAR LACCASE FROM CLADOSPORIUM CLADOSPORIOIDES

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ABSTRACT
Cladosporium cladosporioides was identified as a laccase producer when grown on malt extract media supplemented with 0.0 2% ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid). Extracellular laccase was purified to homogeneity from culture supernatant of Cladosporium cladosporioides by acetone precipitation, gel filtration chromatography and anion exchange chromatography, and was also characterized. Purified laccase (specific activity 1785.71 U·mg⁻¹) is a monomeric protein with molecular weight of 50 kDa on SDS-PAGE. Purified laccase showed maximum activity at pH 5 and optimal temperature was observed 40 °C. The enzyme showed a high relative activity over a wide range of pH from 3 to 6 and was stable in a temperature range of 40 ° to 70 °C. The Kᵫ value of the purified enzyme was found to be 15 µmol·L⁻¹, while the Vmax value was observed to be 4612 µmol·L⁻¹·min⁻¹ with ABTS as a substrate. The catalytic activity of laccase was completely inhibited by diethiothreitol, sodium azide and L-cysteine at a final concentration of 5 mmol·L⁻¹, and slightly inhibited by EDTA and SDS at a final concentration of 10 mmol·L⁻¹. The laccase was activated by Cu²⁺ and Mg²⁺, and certain metal ions such as Ni²⁺, Ba²⁺ and Ag⁺ inhibit the catalytic activity of enzyme at a final concentration of 1.0 mmol·L⁻¹. To the best of our knowledge this is the first report on the purification and characterization of laccase from Cladosporium cladosporioides.

Keywords: Cladosporium cladosporioides, laccase, ABTS, purification

Introduction
Laccases (EC 1.10.3.2) are copper containing oxidases (16) that exist mostly in higher plants and fungi (26, 40). They oxidize different organic substances including monophenols, diphenols, polyphenols, aromatic amines, lignin, and polycyclic aromatic hydrocarbons with reduction of oxygen to water (6). Many laccases have been isolated from fungi such as soft rot, white rot-causing polypores, from geophilous saprophytic and phytopathogenic fungi and also from many edible mushrooms. Laccases have also been purified and characterized from numerous basidiomycetes, such as T. versicolor, L. edodes, A. bisporus, and P. ostreatus (5, 31, 33, 35).

Fungal laccases also occur as isozymes and their molecular weight ranges from about 50 kDa to 110 kDa (10, 40). Most laccases purified from fungi are monomeric proteins but laccases with multiple subunits have also been characterized. Laccases from fungi such as P. ribis (28), P. pulmonarius (9), T. villosa (44), C. cibarius (32) and R. solani (42) consist of two similar subunits. Oligomeric laccases have also been purified from some ascomycetes. Analysis by gel filtration shows the molecular weight of laccase from M. indicum to be 100 kDa, and SDS-PAGE shows its three subunits with molecular weights of 24 kDa, 56 kDa and 72 kDa (39). The G. gramminis fungus produces laccase consisting of three 60 kDa subunits (11), and the laccase from ascomycete such as P. anserina is a tetrameric protein consisting of 80 kDa subunits (29).

The substrates used for detecting and measuring laccase activities are 4-hydroxy-3,5-dimethoxy-benzaldehyde azine (syringaldazine) (17) or 2,2-azino-di(-3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (20). Fungal laccases are present as important proteins which are responsible for the biodegradation of lignocellulosic materials (5). In higher plants, laccases are responsible for the lignification of cell walls and regeneration of the protoplasts (10). The biochemical mode of action of laccases has been studied for many decades for their applications in the textile dye (1) and food industry (8). The natural or synthetic redox mediators such as ABTS are essentially required for measuring the activity of laccase enzymes (5).

Recent studies infer a role of laccases in the pulp paper bleaching and environmental applications (2). The preparation of paper in industry is an energy requiring and multistep process that produces large volumes of pollutants (36). The potential application of laccase in pulp and paper industry describes this enzyme as an alternative to costly and polluting processes (4). Fungal laccases have various other biotechnological applications such as bioremediation (19), biosensors (41), chemical synthesis (23) and immunoassays (21). Laccases also degrade many synthetic dyes including azo, anthraquinone, and tryphenylmethane (1, 3, 31).

One of the major advantages associated with laccases is that they do not require H₂O₂ for substrate oxidation unlike peroxidases and they have broad substrate specificity (37). The removal of synthetic dyes from industrial waste using laccases has increased tremendously (1, 45). Therefore, the search of potential laccases to cope with the demand in the area of dye degradation is an important task (30).
In this study, a novel extracellular laccase was purified to homogeneity from *C. cladosporioides*, by a combination of chromatographic techniques. The enzyme was biochemically characterized and its molecular mass was determined.

**Materials and Methods**

**Microorganism and screening media**

The *C. cladosporioides* strain was obtained from The Institute of Agricultural Sciences, University of the Punjab, Lahore. Fungus was maintained on media containing 0.1 % yeast extract, 0.1 % peptone, 0.1 % starch and 1.5 % agar for 7 days at 30 °C and stored at 4 °C. The laccase activity was identified by supplementing growth media with 1 % peptone, 2 % glucose, 0.0005 % CuSO4, 0.5 % malt extract and 0.02 % ABTS or 0.02 % guaiacol separately after incubating the fungus for 7 days at 30 °C.

**Growth of *C. cladosporioides***

For large-scale production of enzyme, 1 L of growth media supplemented with 1 % peptone, 2 % glucose, 0.0005 % CuSO4, 0.5 % malt extract was inoculated with 10^{10} to 10^{11} *C. cladosporioides* spores and incubated for 14 days at 30 °C on a rotary shaker at 150 rpm. The culture supernatant containing laccase enzyme was processed for the purification and characterization of the enzyme.

**Laccase assay**

The laccase activity from the culture media of *C. cladosporioides* was monitored by using substrate ABTS. One hundred microliters of culture media was mixed with 100 µl of 10 mM ABTS in 800 µl of 50 mM sodium acetate buffer (pH 5) and incubated for 25 min at 30 °C. The appearance of green colour due to oxidation of ABTS was measured spectrophotometrically at A_{420} (ε = 36000 L·mol⁻¹·cm⁻¹). The maximum production of enzymes was checked at different time intervals starting from the 3rd day to the 14th day. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per minute at 30 °C.

**Purification of laccase enzyme**

**Precipitation of laccase enzyme**

After 14 days of growth, the culture was filtered through Whatman No.1 filter paper and centrifuged at 6000 rpm for 15 min at 4 °C. The supernatant was collected and an equal volume of chilled acetone was slowly added with constant stirring on ice and stored further in ice for 2 h. The mixture was centrifuged at 10000 rpm for 10 min at 4 °C. After centrifugation, the supernatant was aspirated and the pellet was dissolved in 1.0 mL of 0.05 mol·L⁻¹ acetate buffer, pH 5.0, and dialyzed against 2 L of 0.05 mol·L⁻¹ acetate buffer, pH 5.0. The total protein concentration was estimated by the Bradford assay, using bovine serum albumin as a standard (7).

**Gel filtration chromatography**

The dialyzed sample was loaded on sephadex G-100 column (1.5 cm × 40 cm) equilibrated with 0.05 mol·L⁻¹ acetate buffer, pH 5.0. The enzyme was eluted with the same buffer and 2 ml fractions were collected at a flow rate of 0.5 mL·min⁻¹. The fractions containing laccase activity were pooled and concentrated by lyophilization. The concentrated protein was analyzed by 10 % SDS-PAGE. The protein concentration was determined by the Bradford assay (7) and the concentration of laccase enzyme was adjusted to 0.1 mg·mL⁻¹.

**DEAE-sephadex chromatography**

The fractions pooled from gel filtration chromatography containing laccase activity were loaded on DEAE-sephadex column (1.5 cm × 20 cm) equilibrated with 0.05 mol·L⁻¹ acetate buffer, pH 5.0. The column was washed with two column volume of the same buffer. The adsorbed proteins were eluted from the column with a stepwise gradient of NaCl (0.1 mol·L⁻¹, 0.2 mol·L⁻¹, 0.3 mol·L⁻¹, 0.4 mol·L⁻¹, and 0.5 mol·L⁻¹) in 0.05 mol·L⁻¹ acetate buffer (pH 5.0) at a flow rate of 1.0 mL·min⁻¹. One milliliter fractions were collected and fractions containing laccase activity were pooled, dialyzed against 2 L of 0.05 mol·L⁻¹ acetate buffer (pH 5.0), and finally concentrated by lyophilization. The concentrated protein fraction was analyzed by 10 % SDS/PAGE and the protein concentration was determined by the Bradford assay (7).

**SDS-PAGE and native-PAGE analysis**

The purified fractions containing laccase activity were analyzed by SDS-PAGE as well as by native-PAGE as described by Laemmli (25). The protein band was visualized by staining the gel with Coomassie Brilliant Blue R-250.

**Characterization of laccase**

**Optimal pH and temperature**

The optimum pH of the laccase enzyme activity was determined by using 10 µmol·L⁻¹ ABTS as the substrate in a 50 mmol·L⁻¹ sodium acetate buffer at different pH (2.0 to 5.0) and 100 µmol·L⁻¹ ABTS in 50 mmol·L⁻¹ sodium phosphate buffer at different pH (6.0 to 8.0). The effect of pH on enzyme activity and stability was measured after 1 h incubation at various pH at 30 °C. The optimum temperature for the laccase activity was determined at different temperatures (20 °C, 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C) by incubating the purified laccase with 10 µmol·L⁻¹ ABTS in a 50 mmol·L⁻¹ sodium acetate buffer (pH 4.5) for 1 h.

**Heat stability**

The heat stability of the purified laccase was determined by incubating the purified enzyme for 15 min at different temperatures from 40 °C to 70 °C. After incubation, the enzyme activity was determined by using ABTS in 0.05 mol·L⁻¹ acetate buffer.

**Determination of kinetic constants**

The kinetic constant *Km* and *Vmax* of the purified laccase were determined by using ABTS as substrate (1 mmol·L⁻¹ to 5 mmol·L⁻¹) at their optimal temperature and pH values. The assays were carried out in triplicate. The *Km* and *Vmax* were calculated by the Lineweaver–Burk plot method.
Metal ions and inhibitors
The effect of metal ions (K⁺, Mg²⁺, Cu²⁺, NH₄⁺, Ni²⁺, Ca²⁺, Ag⁺, and Ba²⁺) on the purified laccase was determined by using different metal ions at a final concentration of 0.5 mmol·L⁻¹ to 1.0 mmol·L⁻¹ separately. The effect of other potential inhibitors (ethylenediaminetetraacetic acid [EDTA], dithiothreitol [DTT], sodium azide [NaN₃], sodium dodecyl sulphate [SDS], and L-cysteine) on laccase activity was also determined separately by adding at a final concentration of 1 mmol·L⁻¹ to 10 mmol·L⁻¹. Control assays were performed in parallel in the absence of the metal ions and inhibitors.

Results and Discussion
Laccase production
The presence of laccase activity in *C. cladosporioides* was identified by adding 0.02 % guaiacol and 0.02 % ABTS in malt extract agar medium as substrates. Laccase oxidizes the guaiacol and ABTS that was identified by formation of reddish brown and green zones respectively (Fig. 1). Laccase activity appeared on the 5th day of incubation and the maximum activity (190 U·mL⁻¹) was achieved on the 14th day (Fig. 2). The cell-free culture supernatant was routinely harvested after 14 days of growth to obtain maximum laccase activity. ABTS was used as a substrate to monitor the laccase activity. The oxidation of C was monitored spectrophotometrically by measuring the increase in the A₄₂₀. The enzyme units were calculated by using molar extinction coefficient (ε = 36000 L·mol⁻¹·cm⁻¹) (27).

![Fig. 1. Oxidation of ABTS (A) and guaiacol (B) by laccase from *C. cladosporioides*. Oxidation of ABTS by laccase produces green zones (A) and oxidation of guaiacol by laccase produces redish brown zones (B).](image)

Purification of laccase
Extracellular laccase was purified from a culture filtrate of *C. cladosporioides* by three simple steps: 1) acetone precipitation, 2) gel filtration chromatography, and 3) anion-exchange chromatography. The elution profile of the enzyme in gel filtration and ion-exchange chromatography is shown (Fig. 3 and Fig. 4, respectively).

![Fig. 3. Elution profile of *C. cladosporioides* laccase from Sephadex G-100 gel filtration column. Fractions 8–9 contained the highest laccase activity. Enzyme activity (red line); shows absorbance at 280 nm (black line). The fractions containing positive laccase activity were pooled and concentrated by lyophilization.](image)

![Fig. 4. Time course of laccase production by *C. cladosporioides* during growth in malt extract media. The maximum laccase activity was observed on the 14th day of incubation. Biomass (red line); enzyme activity (black line).](image)

**TABLE 1**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (U·mL⁻¹)</th>
<th>Total protein (mg·mL⁻¹)</th>
<th>Specific activity (U·mg⁻¹)</th>
<th>Purification fold</th>
<th>Yield (%)</th>
</tr>
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<tbody>
<tr>
<td>Culture supernatant</td>
<td>190</td>
<td>1.4</td>
<td>135.71</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Acetone precipitation</td>
<td>182</td>
<td>0.71</td>
<td>256.33</td>
<td>1.88</td>
<td>95.78</td>
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<tr>
<td>Sephadex G-100</td>
<td>148</td>
<td>0.14</td>
<td>1057.14</td>
<td>7.78</td>
<td>77.89</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>125</td>
<td>0.07</td>
<td>1785.71</td>
<td>13.15</td>
<td>65.78</td>
</tr>
</tbody>
</table>

Data are mean values from triplicate experiments.

Laccase was purified from the culture supernatant of *C. cladosporioides* by a combination of chromatographic techniques. The fractions from Sephadex G-100 with laccase activity were concentrated by lyophilization and analyzed.

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by SDS-PAGE (Fig. 5B). The concentrated fractions from Sephadex G-100 were subjected for dialysis and then loaded into DEAE-Sephadex resin. The enzyme was eluted with a step-wise NaCl gradient. The fractions containing laccase activity were dialyzed, lyophilized and analyzed by SDS-PAGE and native-PAGE (Fig. 5C). The molecular mass of the purified laccase was observed to be 50 kDa by SDS-PAGE (Fig. 5C). The assay of purified enzymes was performed using 10 mmol·L⁻¹ ABTS as a substrate. The purification scheme of laccase from C. cladosporioides is given in Table 1.

The optimal pH and temperature and heat stability
The optimal pH and temperature of purified laccase by using ABTS as a substrate is described in Table 2. The optimal activity of purified enzyme was observed at pH 5 (Fig. 6) and optimal temperature was observed 40 °C (Fig. 7). The purified laccase retained its 50 % activity after heating at 70 °C for 15 min (Fig. 8).

**TABLE 2**

<table>
<thead>
<tr>
<th>Metal ions (1 mmol·L⁻¹)</th>
<th>Relative activity (%)</th>
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<tbody>
<tr>
<td>None</td>
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</tr>
<tr>
<td>KCl</td>
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</tr>
<tr>
<td>MgCl₂</td>
<td>109</td>
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<tr>
<td>CuCl₂</td>
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<tr>
<td>NH₄Cl</td>
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<td>CaCl₂</td>
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</tr>
<tr>
<td>AgNO₃</td>
<td>58</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>54</td>
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</table>

Data are mean values from triplicate experiments.
Kinetic constants and effects of metal ions and inhibitors

The effect of metal ions on laccase activity was tested by using ABTS as the substrate. The laccase activity was inhibited by 1 mmol·L⁻¹ of Ni²⁺, Ba²⁺, Ag⁺ ions and stimulated by 1 mM of Cu²⁺ and Mg²⁺, and essentially unaffected by 1 mM of K⁺, NH₄⁺, Ca²⁺ (Table 2). Other inhibitors such as dithiothreitol and sodium azide also inhibit the laccase activity while L-cysteine inhibits laccase activity at a concentration of 5 mmol·L⁻¹ and were slightly inhibited by EDTA and SDS at a concentration of 10 mmol·L⁻¹ (Table 3). The kinetic constants of the purified laccase using ABTS as a substrate are shown in Table 4.

TABLE 3
Effect of various inhibitors on C. cladosporioides laccase activity

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration (mmol·L⁻¹)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>DTT</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
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<td>0</td>
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<tr>
<td></td>
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</tr>
<tr>
<td>L-cysteine</td>
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<tr>
<td></td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>1</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>5</td>
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<td>5</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>85</td>
</tr>
</tbody>
</table>

Data are mean values from triplicate experiments.

Final remarks

Many fungal laccases have been purified and characterized (12, 14, 19). The fungal laccases are monomeric proteins and their molecular masses range from 50 kDa to 90 kDa (14, 24, 46). The studies related to laccase enzymes were focused on Trametes versicolor, Pycnoporus sanguineus and Phanerochaete chrysosporium. In this study, a new purification scheme for laccase from Cladosporium cladosporioides was standardized and its molecular mass was found to be 50 kDa.

C. cladosporioides produced laccase when it was grown in malt extract media containing 0.0005 % CuSO₄. The laccase activity in the culture media of C. cladosporioides was monitored by using ABTS as a substrate, at different time intervals starting from the 3rd day to the 14th day of incubation. The laccase production started on the 5th day of incubation and the maximum activity (190 U·ml⁻¹) was reached on the 14th day of incubation (Fig. 2). Extracellular laccase was purified to homogeneity from the culture filtrate of C. cladosporioides by acetone precipitation, gel-filtration chromatography and anion-exchange chromatography as shown in Table 1. The purified laccase was found to be monomeric when analyzed by SDS-PAGE and native-PAGE, similar to laccases purified from other fungi (15, 38, 43). The molecular weight of the purified laccase was estimated to be 50 kDa by SDS-PAGE. The molecular weight of the purified laccase is less than that of other laccases from other fungi, which range from 61 kDa to 81 kDa (18, 22, 24, 28, 33, 34, 38, 44).

Temperature stability and pH tolerance are essential for industrial applications of enzymes. The optimum pH of this laccase was found to be 5 with ABTS as a substrate. Most fungal laccases have an optimum pH of about 3 with ABTS (22, 24, 33, 34, 38). The laccase activity also decreases quickly beyond optimum pH (18, 22, 28) but this laccase showed a high relative activity over a wide range of pH from 3 to 6. The optimum temperature for the C. cladosporioides laccase activity was found to be 40 °C with ABTS as a substrate and the enzyme retained its 50 % activity after heating at 70 °C for 15 min. The purified laccase was completely inhibited by DTT, NaN₃, and L-cysteine at a concentration of 5 mol·L⁻¹ and was slightly inhibited by EDTA and SDS at a concentration of 10 mmol·L⁻¹. The enzyme was strongly inhibited by 1 mmol·L⁻¹ of Ni²⁺, Ag⁺, Ba²⁺, slightly stimulated by 1 mmol·L⁻¹ of Mg²⁺, Cu²⁺, and essentially unaffected by 1 mmol·L⁻¹ of K⁺, NH₄⁺, Ca²⁺.

The $K_m$ value of the purified enzyme was found to be 15 µmol·L⁻¹, while the $V_{max}$ value was observed to be 4612 µmol·L⁻¹·min⁻¹ with ABTS as a substrate. The $K_m$ values of other fungal laccases are higher than that of this enzyme, which indicates that this enzyme has higher substrate affinity. For example, Trametes sp. strain AH28-2 laccase has a $K_m$ value of 25 µmol·L⁻¹ for ABTS (43). $K_m$ values of 45 µmol·L⁻¹ and 56.7 µmol·L⁻¹ were observed for ABTS in Trichophyton rubrum LKY-7 and Coriolus hirsutus laccases respectively (22, 30). Ceriporiopsis subvermispora Laccases L1 and L2 have $K_m$ values of 30 µmol·L⁻¹ and 20 µmol·L⁻¹, respectively (13).

Physiochemical properties and kinetic constants of C. cladosporioides laccase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Molecular weight (kDa)</th>
<th>Optimal pH</th>
<th>Optimal temperature (°C)</th>
<th>$K_m$ (µ mol·L⁻¹)</th>
<th>$V_{max}$ (µ mol·L⁻¹·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td>50</td>
<td>5.0</td>
<td>40</td>
<td>15.0</td>
<td>4612</td>
</tr>
</tbody>
</table>
Conclusions
The present study showed that laccases from C. Cladosporioides are new members of a growing family of laccases that possess important properties for industrial applications. C. cladosporioides laccases are consistent with the hypothesis that these phenoloxidases also have a wide range of substrate specificity, as they oxidized guaiacol and ABTS. Moreover, the possibility of converting recalcitrant range of substrate specificity, as they oxidized guaiacol and ABTS. Moreover, the possibility of converting recalcitrant laccases that possess important properties for industrial applications.

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