JOURNAL OF PURE & APPLIED MICROBIOLOGY, Nov. 2014. Vol. **8**(Spl. Edn. 2), p. 727-731

Production and Partial Purification of Laccase from *Pseudomonas aeruginosa* ADN04

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(Received: 06 September 2014; accepted: 13 October 2014)

The efficient extracellular production of laccase in the liquid culture medium of *Pseudomonas aeruginosa* ADN04 and the enzyme extraction, characterization and purification were studied. Purified laccase from the culture filtrate of the bacterium has been attained maximum activity after employing ammonium sulphate precipitation, Gel filtration chromatography (Sephadex) and Affinity chromatography DEAE-Sephadex. The molecular mass of the laccase was determined by SDS-PAGE that showed a relative molecular weight of 43 kDa. The enzymatic stability characteristics at different pH and temperature of the purified laccase have been determined. 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) was used as the substrate and have been found to be Vmax is 12.06 and Km is 56.9at 37°C respectively.

Key words: Laccase, *Pseudomonas aeruginosa* ADN04, Optimization, Purification.

Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) is a multicopper oxidase with four copper atoms per monomer distributed in three redox sites. It is one of the extracellular glycoprotein enzymes expressed by microorganisms that play a vital role in the terrestrial carbon cycle by helping to decompose lignocellulosic substances. (Gianfreda *et al.*, 1999)

Laccases are multinuclear enzymes (Piontek *et al.*, 2002) and the active site of laccase contains four copper atoms which are distributed in three sites, referred to as T1, T2 and T3 (Solomon, 1996). The laccase enzyme accepts electrons from substrates and converts them to free radicals. After receiving four electrons, the enzyme donates them to molecular oxygen to form two water molecules (Bourbonnais and Paice, 1990). Laccases plays very important role in the lignification and delignification. The main application of laccases is in many industrial processes like pulp and paper industry, biobleaching, biosensing and beverage refining (Cuoto and Herrera, 2006). Other than that in the environmental bioremediations it plays very important role in removal of a large number of environmental pollutants, such as alkenes, chlorophenols, dyes, herbicides, polycyclic aromatic hydrocarbons and benzopyrene (Sathishkumar *et al*., 2013). Fungi plays very important role in the production of laccase, but now days other than fungi, bacteria are also the main producers of laccase. The aim of this work was to develop a procedure for the purification of extracellular laccases from *Pseudomonas aeruginosa* ADN04, to determine the individual physicochemical and catalytic properties of these enzymes and to study the kinetics property of the enzyme.

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MATERIALS AND METHODS

Bacterial culture

The bacterial culture was isolated, identified biochemically and 16S rRNA sequencing further it was confirmed as *Pseudomonas aeruginosa* ADN04 (Arunkumar *et al.,* 2014). This purified strain was stored in Nutrient Agar Slants at 4°C in Department of Biotechnology, Sathyabama University. All the analysis was performed using this strains.

Enzyme Production

The organism was inoculated in the medium containing KH_2PO_4 (9 g/L), $NaNO_3$ (6 g/L), NH_4Cl (2g/L), NaCl (5 g/L) and $MgSO_4$ (1.25 g/L). After 3 days of cultivation at 37°C.

Enzyme extraction

The crude laccase was extracted by mixing 10g of fermented materials with distilled water, stirred for 30 minutes in the shaker, filtered and then centrifuged for 30 minutes. The supernatant was used as the crude enzyme and then studied for enzymatic measurements by appropriate method.

Laccase assay

Laccase activity was determined by the oxidation of ABTS method. The non-phenolic dye ABTS (2, 2´-azino-bis- [3 – ethyl benzothiazoline – 6 –sulphonic acid]) is oxidized by laccase to the more stable and preferred state of the cation radical. The concentration of the cation radical responsible for the intense blue-green colour can be correlated to enzyme activity and is read at 420nm. The assay mixture contained 0.5mM ABTS, 0.1M sodium acetate (pH 4.5), and a suitable amount of enzyme. Oxidation of ABTS was monitored by determining the increase in A420 (µ420, $3.6 \times 104\ M^{-1}cm^{-1}$). The reaction mixture contained 0.5mM substrate (ABTS), 2.8 mL of 0.1 M sodium acetate buffer of pH 4.5, and 100 µL of culture supernatant and incubated for 5 min. Absorbance was read at 420 nm in a spectrophotometer against a suitable blank. One unit was expressed as the amount of the laccase that oxidized 1 µmol of ABTS substrate per min. (Majcherczyk *et al.,* 1998). The absorbance was read after 10 min interval using UV/VIS spectrophotometer (Varian Cary® 100 UV-Vis).

Laccase purification

Precipitation of laccase enzyme

Pseudomonas aeruginosa ADN04 was incubated at 35°C on a rotary shaker (150 rpm). After 3 days of growth, the culture was 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 $mol \cdot L^{-1}$ acetate buffer, pH 5.0, and dialyzed against 2 L of 0.05 $mol \cdot L^{-1}$ acetate buffer, pH 5.0. The total protein concentration was estimated by the Lowry's' method, using bovine serum albumin as a standard (Lowry *et al*, 1951).

The redissolved protein was applied to a Sephadex G-100 column, which was equilibrated with buffer. The column was subsequently washed with 785 ml of equilibration buffer, and the enzyme fractions eluted with a linear concentration gradient of 0.5 M NaCl in the same buffer. Fractions containing laccase activity were collected, dialyzed using dialysis membrane, and loaded onto a DEAE-Sephadex A-50. The column was equilibrated and eluted with buffer at a flow rate of 0.7 ml/min. Fractions containing laccase activity were collected and concentrated.

Determination of Molecular Mass

SDS-Polyacrylamide gel electrophoresis

The homogeneity of the enzyme preparation was checked by SDS-PAGE using the method of Weber and Osborn. The separating gel was 12% acrylamide in 0.375 mol/L Tris·HCl buffer pH 8.8 and stacking gel was 5% acrylamide in 0.063 mol/L Tris·HCl buffer 6.8. The molecular weight marker were attained from Bangalore Genei Pvt. Ltd. (Bangalore India) Gel was run at a constant current 20 mA.

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 $mmolL^{-1}$ sodium acetate buffer at different pH (2.0 to 5.0) and 10 $\mu mol \cdot L^{-1}$ ABTS in 50 $mmol \cdot L^{-1}$ sodium phosphate buffer at different pH (6.0 to 10.0). The effect of pH on enzyme activity and stability was measured after 2 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 2 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 puriûed 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 using Hyper32.

RESULT AND DISCUSSION

The organism was isolated from the soil sample (Harur forest, Tamilnadu), was preserved as pure culture in the Department of Biotechnology, Sathyabama University, Chennai. The media which was optimized by Response Surface Methodology by Arunkumar *et al.*, 2014 was used to as Fermentation media. After incubation the culture was centrifuged and the supernatant was used for purification.

Purification

The purified laccase was determined on SDS PAGE that was monomeric protein. The single band corresponding to 43 kDa relative to the standard protein marker shows it as a single polypeptide protein. Many report suggests that the molecular weight of protein ranges from 35 – 90 kDa (Sadhasivam *et al.*, 2008; Park and Park, 2008; Ben Younes and Sayadi, 2011; Rodriguez Couto *et al.*, 2005)

Characterization of Laccase

Effect of pH on Purified laccase

Laccase activity was studied at varying pH (2-10). The stability of the enzyme was maximum at 8.0 pH and further rise in pH deactivated the protein after 2 hours of incubation.

Effect of temperature on Purified laccase

Laccase activity was studied at varying

Table 1. Purification summary of Laccase from the culture filtrate *Pseudomonas aeruginosa* ADN04

Purification step	Total activity (U·mL-1)	Total protein (mg·mL-1)	Specific activity (U·mg-1)	Purification fold	Yield (%)
Culture supernatant	46.7	1.35	34.59	1.00	100
Acetone precipitation	38.4	0.84	45.71	1.32	82.22
Sephadex G-100	32.9	0.49	67.14	1.47	70.44
DEAE-Sephadex	20.2	0.12	168.33	2.51	43.26

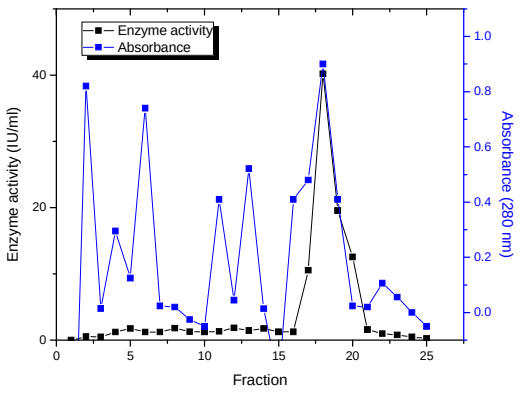
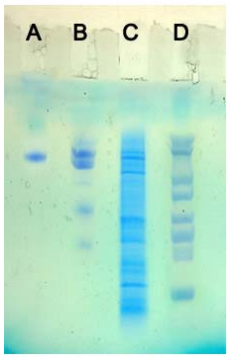


Fig. 1. Gel permeation chromatography of laccase by Pseudomonas aeruginosa ADN04



Lane A, purified enzyme after DEAE Sephadex Ion exchange chromatography. Lane B, partially purified enzyme after Gel permeation chromatography. Lane C, Crude enzyme. Lane D, standard molecular mass marker proteins lane. B, purified enzyme.

Fig. 2. SDS-PAGE of Laccase enzyme

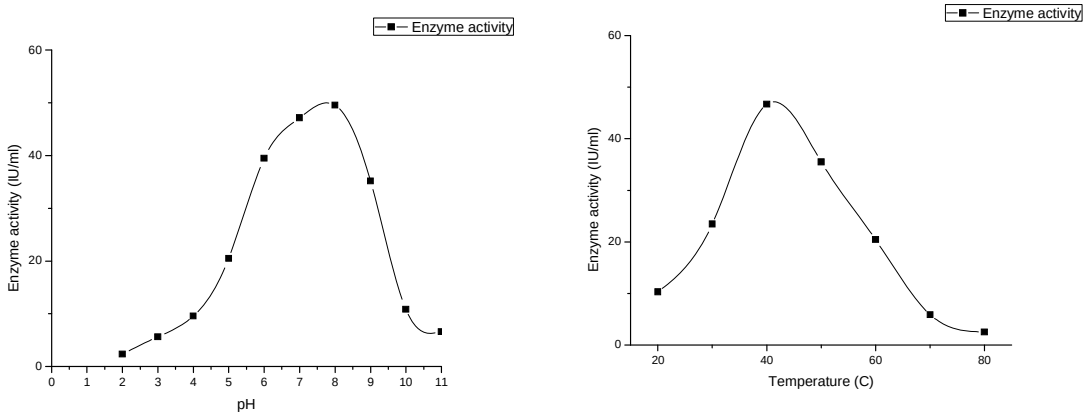


Fig. 3. Effect of pH and temperature on activity and stability of laccase produced by *Pseudomonas aeruginosa* ADN04

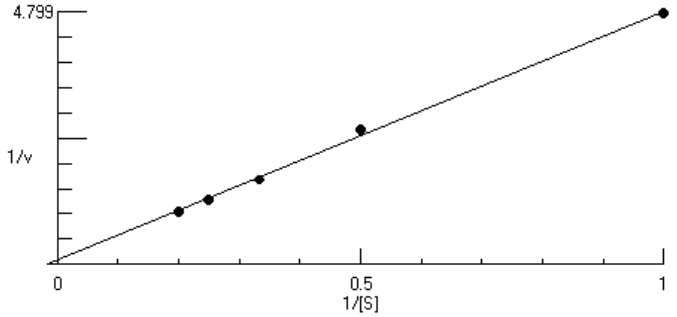


Fig. 4. Reciprocal plot for determining Km and Vmax for laccase using Hyper32(software) Lineweaver-Burk Plot Results Vmax = 12.06 and Km = 56.9

temperature (20-80°C). The stability of the enzyme was maximum at 40°C and further deduction showed inactivity and on rise in temperature above 50°C for two hours showed deactivated the protein after 2 hours of incubation.

Determination of kinetic constants Km and Vmax

The Km value of the purified enzyme was found to be 12.06 µmol·L-1, while the Vmax value was observed to be 56.9µmol·L-1·min-1 with ABTS as a substrate. The Km values of other 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 Km value of 25 µmol·L^{-1} for ABTS (Piontek *et al.*, 2002). Km 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. *Ceriporiopsis subvermispora* Laccases L1 and L2 have Km values of 30 µmol·L-1 and 20 µmol·L-1, respectively (Litthauer, *et al.*, 2007).

CONCLUSION

Laccase from *Pseudomonas aeruginosa* was extracted, purified and showed stability at pH 8 and at temperature of 40°C for 2 hr. The enzyme produced possesses a broad pH working range and shown a better thermo stability. The lower the Km and higher the Vmax value indicated the enzyme has more affinity to its substrate and higher catalytic activity as compared with other laccase reported.

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