



## Purification and partial characterization of laccase from *Lachnocladium* sp.

P. A. WUYEP<sup>1</sup>, O. N. UME<sup>2</sup>, M. T. BAKARE-ODUNOLA<sup>2\*</sup>, A. J. NOK<sup>3</sup>,  
H. M. INUWA<sup>3</sup> and N. B. AFOLABI-BALOGUN<sup>4</sup>

<sup>1</sup>Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria.

<sup>2</sup>Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria, Nigeria.

<sup>3</sup>Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria.

<sup>4</sup>Center for Biotechnology Research and Traininig, Ahmadu Bello University, Zaria, Nigeria.

\* Corresponding author, E-mail: [mojitaibat@yahoo.com](mailto:mojitaibat@yahoo.com); Tel.: +2348035896043

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### ABSTRACT

Laccase, a multicopper oxidase that catalyzes the oxidation of various aromatics, particularly phenolic substrates, e.g. hydroquinones guaiacol, 2,6-dimethoxyphenol or phenylene diamine, was purified and partially characterised from culture filtrates of a white rot fungus, *Lachnocladium* sp. This enzyme was purified by anion exchange and gel filtration chromatography. Laccase activity was determined using ABTS (2, 2'-azino-bis-(3-ethylbenzthiazoline)-6-sulphonic acid) substrate. The culture filtrate had maximum laccase activity of 1.62 U/ml after 14 days of incubation. The purified laccase had an optimum temperature of 50 °C and its optimum pH was 6 for ABTS. The activity of this enzyme was enhanced by Fe<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup> and Ca<sup>2+</sup>, and was inhibited by EDTA and sodium iodide. Laccase from *Lachnocladium* sp. had a K<sub>m</sub> of 0.119 mM and a V<sub>max</sub> of 0.313 U.

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**Keywords:** *Lachnocladium* sp., anion exchange chromatography, gel filtration chromatography, ABTS, DMP.

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### INTRODUCTION

Laccases (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase) belong to the multicopper oxidase family and catalyse the oxidation of various substrates with the simultaneous reduction of molecular oxygen to water (Alcalde, 2007). Laccase was first discovered by Yoshida in 1883 from the latex of Japanese lacquer tree (*Rhus vernicifera*) and are widely distributed in nature; in fungi, in plants, in some insects (Dittmer et al., 2004; Kramer et al., 2001) and in a few bacteria (Claus, 2003). Fungal laccases are considered

as ideal green catalysts of great biotechnological impact due to their few requirements (they only require air, and they produce water as the only by-product) and their broad substrate specificity. Moreover, in the presence of small molecules known as redox mediators e.g. ABTS [2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonate diammonium salt)], laccases enhance their substrate specificity (Kunanmeni et al., 2007). These redox mediators lead to higher rates and yields of laccase substrates and act as diffusible electron carrier enabling the oxidation of high

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molecular weight biopolymers such as lignin, cellulose or starch (Kunamneni *et al.*, 2007; Alcalde, 2007). In addition, very effective phenolic mediators, e.g. 2, 6-dimethoxyphenol (DMP) and succinic acid, enhance the transformation of non-natural laccase substrates like cyprnidil to mixed oligomers through cross coupling (Kang *et al.*, 2002). The application of fungal laccases for troublesome transformations (synthesis of antibiotics, anti-cancer drugs and hormone derivatives; digestion of lignocellulose to use as a carbon source; modifications of lignosulfonates for production of emulsifiers, surfactants and adhesives; synthesis of polymers with properties as redox films for bioelectronic devices and much more) has expanded the need for this biocatalyst (Kunamneni *et al.*, 2007). Also, different laccase applications require particular laccase with properties more suited for those applications. Laccases have been reviewed several times in recent years, generally with emphasis on narrow aspects. Xu (2005), Riva (2006) and Alcalde (2007); provide excellent summaries of the enzymology, electron transfer mechanisms of laccases and their industrial applications

In this work, we reported the purification and partial characterization of one of these laccases from a white-rot fungus *Lachnocladium* sp which is russuloid basidiomycete found mostly in the tropics and have a worldwide distribution of about 94 species (ZipcodeZoo.com).

## MATERIALS AND METHODS

### Microorganism and culture conditions

The fungal strain, *Lachnocladium* sp. was obtained from Gumbi hill along Kaduna – Kano expressway in Kaduna State, Nigeria. The fresh mycelia was dried and stored in a closed Petri dish at 0 °C. This was cultured on malt extract agar medium at 28 °C for 5 days. For laccase production, mycelial plugs from the malt extract medium were used as inoculum for 25 ml of liquid medium

(prepared from ripe pineapple fruits), and was incubated at 32 °C for 14 days.

### Enzyme assays

The filtered homogenate of the liquid culture was assayed for laccase activity using ABTS and DMP as substrates. ABTS and DMP were from Apollo Scientific Ltd, UK. DEAE Cellulose and Sephadex G-100 were obtained from Sigma Chemical Co. St. Louis USA. All other chemicals used in this investigation were of analytical grade and was used without further purification. The assay solutions contained 1 mM DMP in McIlvaine buffer (pH 7.0) and 0.4 mM ABTS in 25 mM sodium acetate buffer (pH 4.5) for DMP and ABTS substrates respectively. In the case of ABTS, the reaction was monitored by measuring the absorbance at 420 nm ( $\epsilon_{\max} = 3.6 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ ) (Bourbonnais and Paice, 1990). For DMP, the reaction was monitored by measuring the absorbance at 477 nm ( $\epsilon_{\max} 14.8 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ ) (Palmieri *et al.*, 1997). Helios Zeta UV-VIS Spectrophotometer (Thermo Scientific Inc.) was used for absorbance measurement. One unit of enzyme activity was defined as the amount of enzyme required for the oxidation of 1 mol of the substrate per minute in the reaction mixtures. The Specific activity, Purification fold and the Percentage yield of the enzyme were estimated from the following formulae:

$$\text{Specific activity} = \frac{\text{Activity (U/ml)}}{\text{mg protein}}$$

$$\text{Purification fold} = \frac{\text{SAEP}}{\text{SACE}}$$

SAEP = Specific activity of enzyme after a purification step.

SACE = Specific activity of the crude enzyme.

$$\text{Percentage yield} = \frac{\text{TAEAP}}{\text{TAECE}} \times 100$$

TAEAP = Total amount of enzyme after a purification step

TAECE = Total amount of enzyme in the crude extract

### **Purification of laccase**

The liquid culture homogenate was used for the purification of enzyme after filtration through Whatman No. 1 filter paper. The filtrate was loaded onto a Sephadex G-100 column pre-equilibrated with 25 mM sodium acetate buffer, pH 4.5. The enzyme was eluted with the same buffer at a flow rate of 1 ml/min and 5 ml fractions were collected. These were assayed for enzyme activity and protein concentration. Protein concentration was determined using Layne's spectrophotometric method (Layne, 1957). The fractions with high enzyme activity were pooled, dialyzed and further purified with DEAE cellulose anion exchange chromatography. The DEAE Cellulose column was pre-equilibrated with 25 mM sodium acetate buffer and the enzyme was eluted with a linear concentration gradient of 0-0.5 M NaCl in the same buffer at a flow rate of 1 ml/min and 5 ml fractions were collected. The fractions containing high laccase activity were collected and pulled together.

### **Characterization of laccase**

#### ***pH and temperature optimum***

The effect of temperature on laccase activity was determined following the laccase-catalyzed oxidation of 1 mM DMP at temperatures ranging from 25 – 100 °C in McIlvaine buffer (pH 7.0).

The pH-dependence of the laccase activity was examined at room temperature (25 °C) in the pH range of 3-9.0 using DMP and ABTS as substrates.

#### ***Kinetics and inhibition studies***

The kinetics studies were performed using different concentrations (0.1-1.0 mM) of ABTS in 25 mM acetate buffer (pH 4.5). These were incubated with 0.5 ml of the enzyme at 37 °C for 10mins and the activity was determined spectrophotometrically. The data obtained were used for Lineweaver-Burk

plot from which the  $K_m$  and  $V_{max}$  were determined.

The inhibitory effects of EDTA and sodium iodide on the activity of the enzyme were studied. The potential inhibitors, EDTA and sodium iodide at concentrations of 25, 50, 75 and 100 mM each were mixed with ABTS and 0.08 ml of the purified enzyme. All assays were performed in duplicate.

#### ***Effects of incubation time and divalent cations***

Laccase from *Lachnocladium* sp. was studied for the effect of incubation time on its activity. The enzyme was incubated with ABTS at 37 °C and the activity was monitored at ten minutes interval for 60 minutes.

The effect of some divalent cations;  $Zn^{2+}$ ,  $Ca^{2+}$ ,  $Fe^{2+}$  and  $Cu^{2+}$  on the enzyme was also studied using ABTS as substrate. The substrate, 0.05 ml of the enzyme and 0.2 ml of 25 mM of each divalent cation were incubated in different test tubes and activity was determined as described earlier.

## **RESULTS**

The elution profile of the gel filtration chromatography and anion exchange chromatography of the enzyme revealed multiple distinct activity peaks Figures 1, 2 and 3. The purification chart for the purification of laccase from the culture filtrate of *Lachnocladium* sp. is shown in Table 1. Gel filtration chromatography gave the highest specificity (34.33 U/mg), purification fold (78.3) and percentage yield (27%).

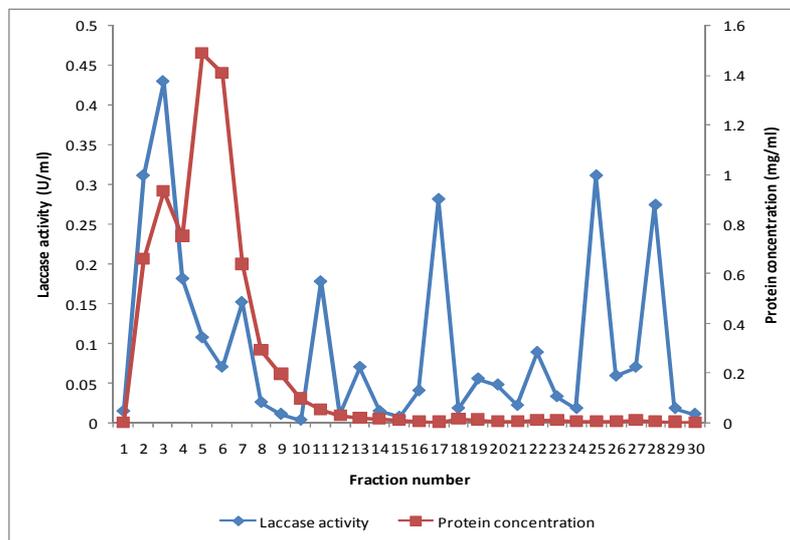
Laccase showed activity over a wide pH range between pH 3 and 7. The optimum pH of the enzyme for DMP was 7 (Figure 4) while that of ABTS was 6 (Figure 5). The dependence of laccase on pH rendered a bell-shaped profile as can be seen with both ABTS and DMP.

The optimum temperature for laccase from *lachnocladium* sp. for DMP oxidation was 50 °C. The enzyme is active over a wide

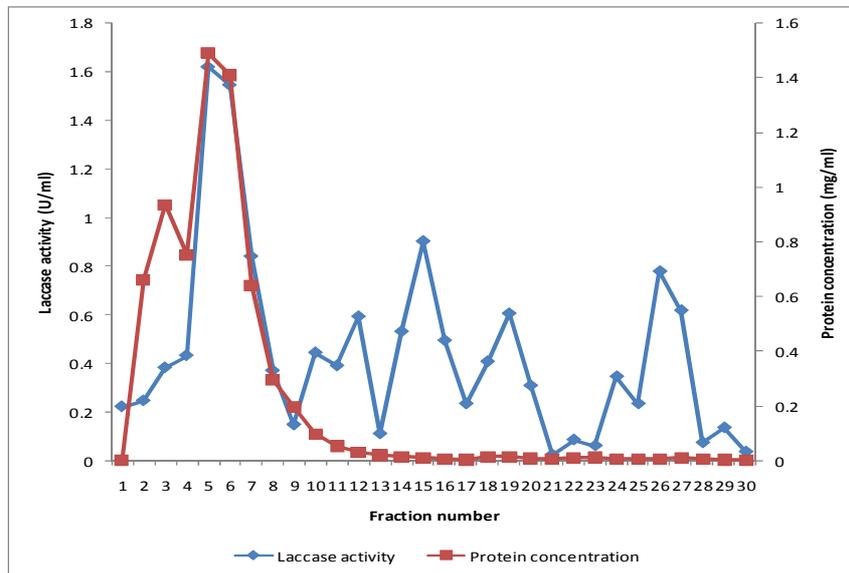
temperature range. Significant activity was detected as low as 25 °C and 60 % of activity was maintained as high as 90 °C (Figure 6).

The inhibition by NaI was only observed at higher concentration (above 75

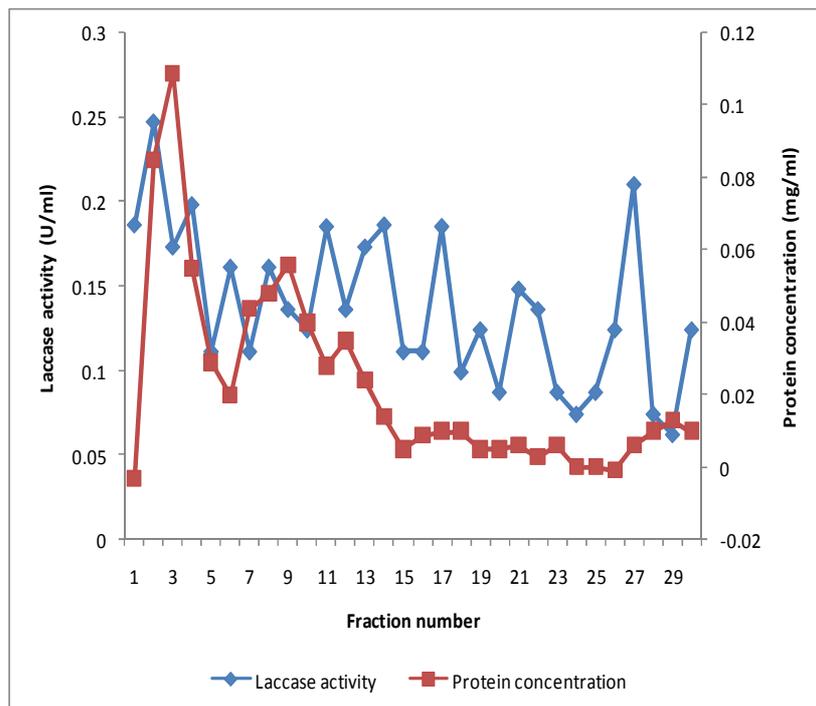
mM) while that of EDTA was observed to increase as its concentration increases (Figure 7); and the laccase activity decreased with longer incubation time (Figure 8).



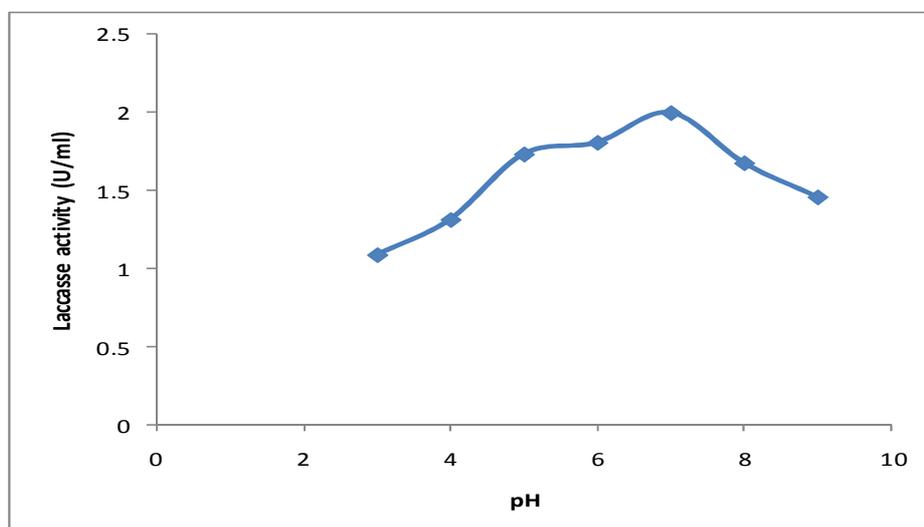
**Figure 1:** The elution profile of laccase on Sephadex G-100 gel filtration chromatography using ABTS as substrate.



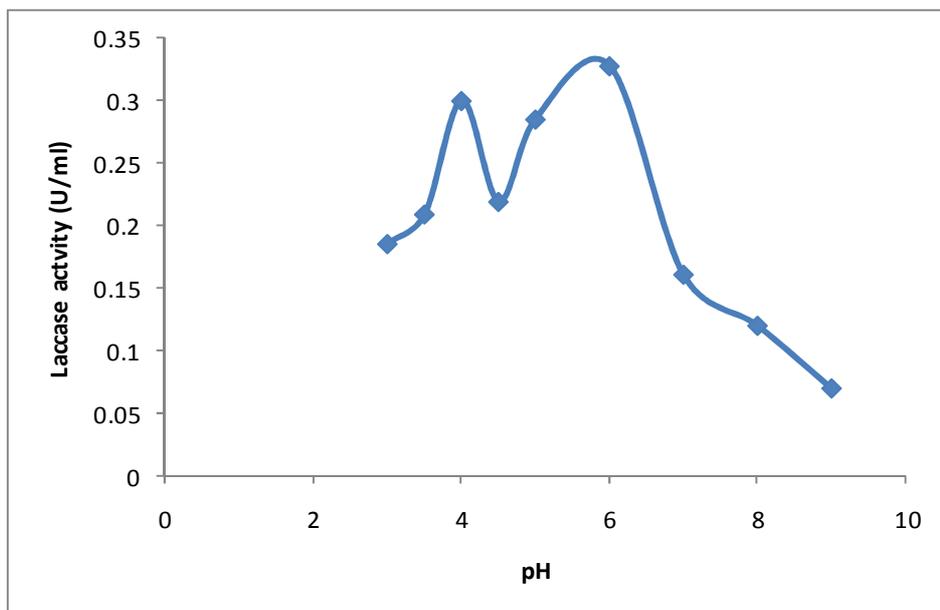
**Figure 2:** The elution profile of laccase on Sephadex G-100 gel filtration chromatography using DMP as substrate.



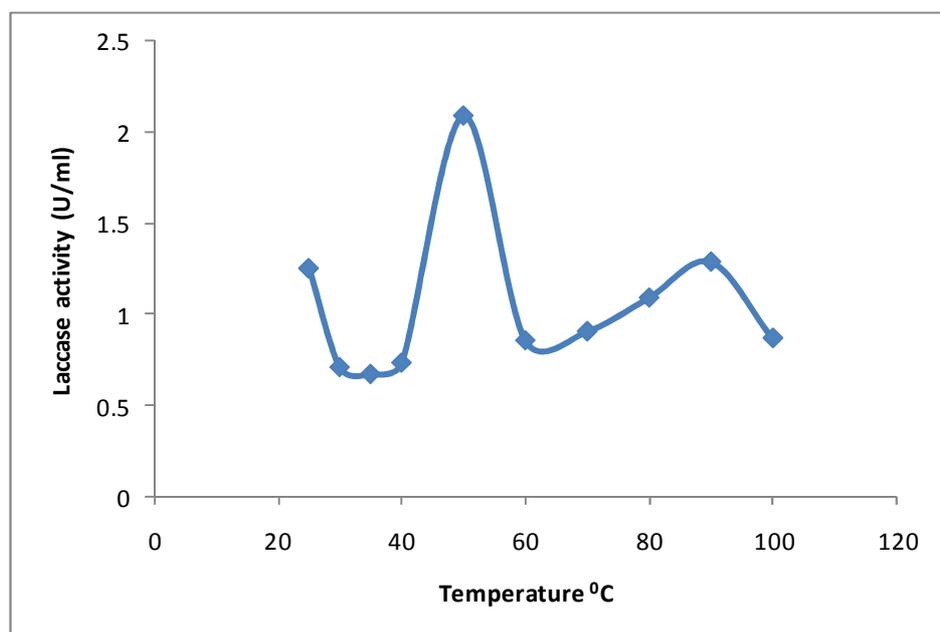
**Figure 3:** The elution profile of laccase on DEAE Cellulose anion exchange chromatography.



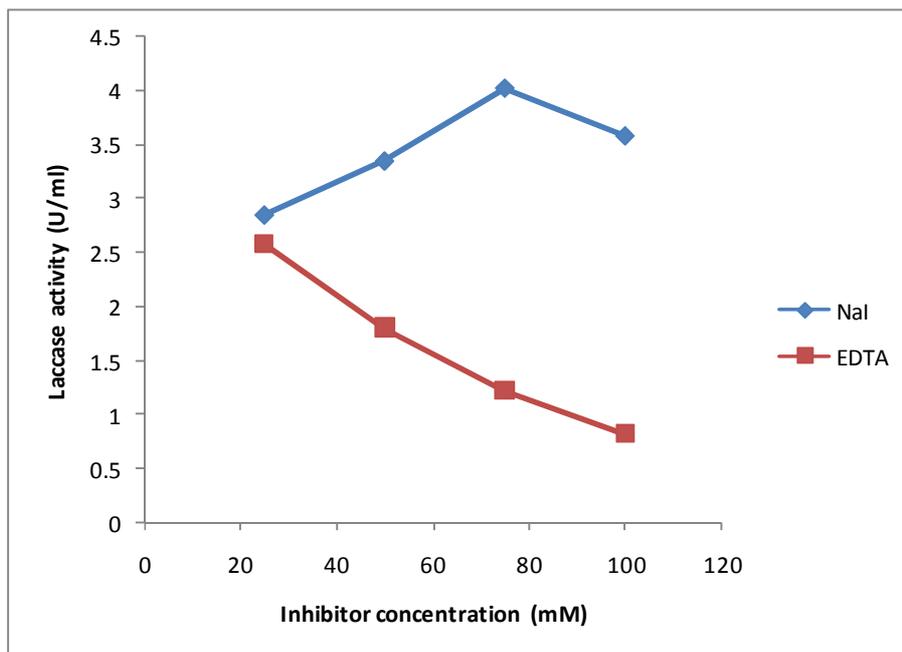
**Figure 4:** Effect of pH on laccase activity using DMP.



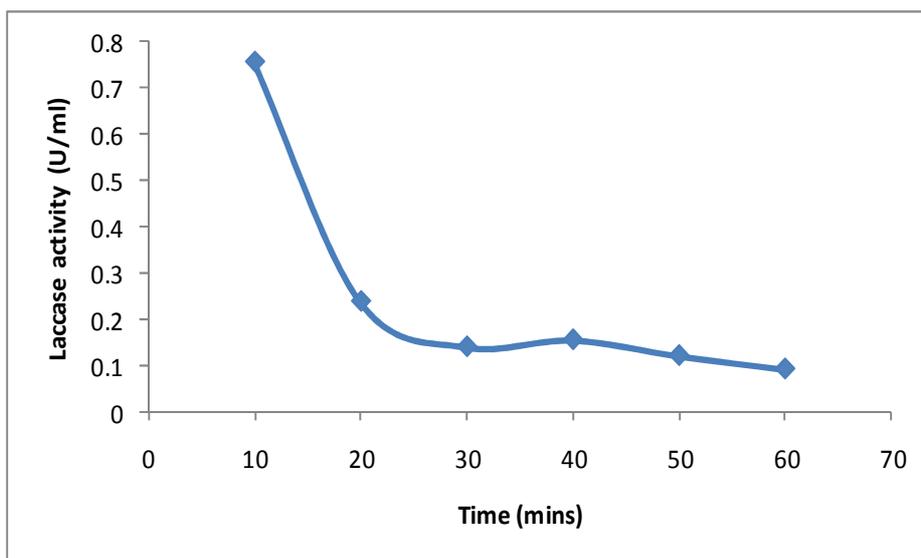
**Figure 5:** Effect of pH on using ABTS.



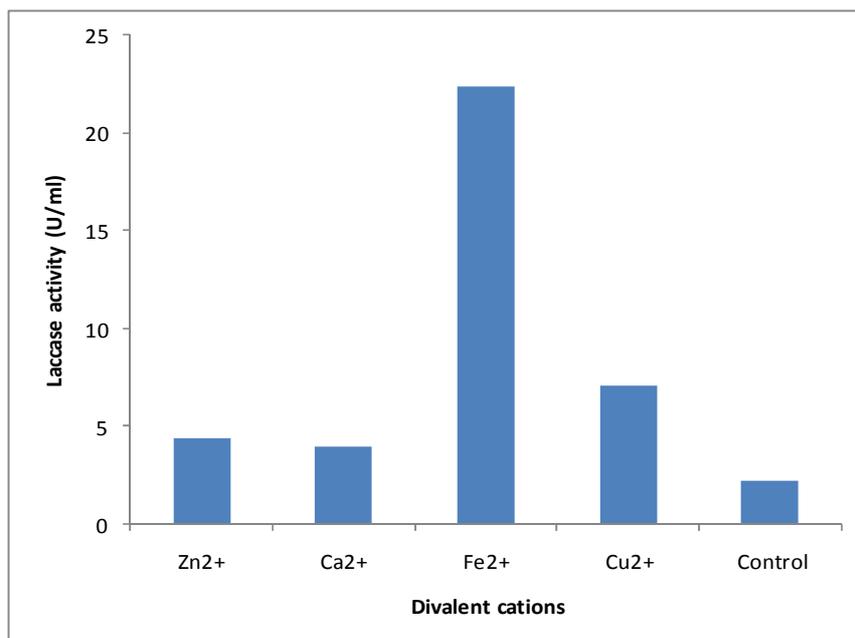
**Figure 6:** Effect of temperature on laccase activity for DMP oxidation.



**Figure 7:** Effect of varying concentration of inhibitors on laccase activity.



**Figure 8:** Effect of incubation time on laccase activity.



**Figure 9:** Effect of divalent cations on laccase activity.

**Table 1:** Purification chart of laccase from *Lachnocladium* sp.

Purification Step	Protein (mg)	Total Activity (U/ml)	Specific Activity (U/mg)	Purification Fold	% Yield
Crude enzyme	2.5	1.11	0.44	1	100
Gel filtration (Sephadex G-100)	0.201	0.30	34.33	78.3	27
Anion exchange (DEAE Cellulose)	0.024	0.19	17.1	38.86	17

## DISCUSSION

### Purification of laccase

Screening for oxidative enzymes or mediators involves the investigation of many samples, as there are many parameters involved. For this reason one usually relies on the use of inexpensive, rapid and sensitive testing methods (Ko *et al.*, 2001). The screening strategy must aim to identify fungal strains and enzymes that will work under

industrial conditions (Alcalde, 2007). Hence the fungal culture for the extraction of laccase in this study was cost effective. The fungal filtrate had a maximum laccase activity of 1.62 U/ml after 14 days of incubation. The multiple activity peaks of the enzyme suggested isozymes. This agrees with the report of Anastasia and Joan (2002); that fungal laccases often occur as multiple isozymes expressed under different cultivation

condition. The purification chart for the purification of laccase indicated the fact that laccases are not specific for their substrate range, as it varies from one organism to another (Trovaslet *et al.*, 2004).

#### **pH and Temperature optimum**

The bell-shaped pH profile was as a result of two opposing effects: the first effect is due to the redox potential difference between a reducing substrate (e.g. phenolic compound) and the type 1 copper center of laccase, where the substrates dock and electron transfer rate was favoured for phenolic substrates at a high pH. The second effect was generated by the binding of a hydroxide anion to the type 2 / type 3 copper centres of laccase, which inhibited the binding of O<sub>2</sub>, the terminal electron acceptor, and therefore inhibited the activity at a higher pH because of the increased amount of OH<sup>-</sup> ions (Martínez-Alvarez *et al.*, 2008).

Significant activity was detected as low as 25 °C and approximately 60% activity was maintained as high as 90 °C; this is in agreement with the temperature profiles of laccase activity which usually do not differ from other extracellular ligninolytic enzymes with optima between 50 °C and 70 °C (Baldrian, 2006). However, few enzymes with optima below 35 °C have been described, e.g. the laccase from *G. lucidum* with the highest activity at 25 °C (Ko *et al.*, 2001).

#### **Kinetic and inhibition studies**

The low K<sub>m</sub> value of 0.119 mM indicates high binding affinity of the enzyme for the substrate ABTS and the V<sub>max</sub> (0.313 U) gave an indication of its efficiency with this substrate. EDTA was found to be more efficient in binding with the copper atoms when compared with NaI. EDTA had a high inhibitory effect towards this enzyme. EDTA exhibits metal chelating properties so could

combine with the copper atoms in the laccase active site and reduce the affinity for the substrate.

#### **Effect of incubation time and divalent cations**

Enzymes are deactivated over a period of time even at moderate temperatures. The divalent cations tested (Fe<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup> and Ca<sup>2+</sup>), were found to be activators of laccase activity as shown in Figure 9. This implies that they were required as cofactors for laccase activity. Most enzymes need factors (cofactors) other than polypeptide molecules for full activity.

This study has successfully purified laccase from *Lachnocladium* sp. chromatographically and partially characterised the enzyme by ion exchange and gel filtration chromatography. The crude culture filtrate had a maximum laccase activity of 1.62 U/ml after 14 days of incubation. The laccase was purified chromatographically and partially characterized. Its optimum pH was found to be 7 for DMP and 6 for ABTS while the optimum temperature for its activity was 50 °C. Future research will be focussed on molecular studies towards large-scale production of the recombinant enzyme for industrial applications.

#### **ACKNOWLEDGEMENTS**

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