KINETIC CHARACTERIZATION OF PURIFIED LACCASE PRODUCED FROM *Trametes versicolor* IBL-04 IN SOLID STATE BIO-PROCESSING OF CORNCOBS

Muhammad Asgher,[a,*] Hafiz M. Nasir Iqbal,[a] and M. Javaid Asad [b]

A locally isolated white rot fungal strain *Trametes versicolor* IBL-04 produced high laccase activities in solid state bio-processing of corn cobs. Addition of glucose and yeast extract (C: N ratio; 25:1) enhanced laccase synthesis. Addition of Tween-80 and $CuSO_4$ enhanced laccase production to 1012 U/mL under optimized process conditions. Laccase was further purified to 2.89-fold (specific activity of 840 U/mg) by ammonium sulfate fractional precipitation, dialysis, and Sephadex G-100 gel filtration chromatography. The purified laccase had a relative molecular mass of 63 kDa as detected by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Best enzyme activity was at pH 5 and 40^{o}C. Using 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate, the enzyme showed maximum activity (V_{max}) of 780 U/mL with a corresponding Michaelis constant (K_m) value of 73µM. Among the different activators/inhibitors, Cu^{2+}, Mn^{2+}, and Fe^{2+} stimulated laccase activity, whereas EDTA and cystein inhibited the enzyme. The higher V_{max} and lower K_m for *T. versicolor* IBL-04 laccase as compared to most of the reported laccases suggests its potential for industrial applications.

Keywords: Trametes versicolor IBL-04; Corn cobs; Extracellular laccase; Purification; Kinetic studies

Contact information: a: Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan; b: Department of Biochemistry, PMAS-University of Arid Agriculture, Rawalpindi, Pakistan;
** Corresponding author: Phone: +92-41-9200161/3312; E-mail:mabajwapk@yahoo.com*

INTRODUCTION

White rot fungi (WRF) are known producers of extracellular ligninolytic enzymes, including lignin peroxidase (LiP), manganese dependent peroxidase (MnP), manganese independent peroxidase (MiP), and laccase (Pointing 2001). WRF have the capability to mineralize lignin and phenolic organic substrates due to the nonspecific and non-stereoselective nature of their enzyme system that functions together with H_2O_2-producing oxidases and secondary metabolites (Wesenberg et al. 2003; Levin et al. 2008; Sadhasivam et al. 2008). Ligninolytic enzymes of WRF find a wide range of commercial applications including degradation of dyes, bioremediation of industrial effluents, lignin hydrolysis for ethanol production (Stoilova et al. 2010), clarification of musts and wines, improving brightness of conventionally bleached cotton (Rodríguez-Couto et al. 2001; Lorenzo et al. 2006), biostoning of denims (Pazarlioglu et al. 2005), and biobleaching and biopulping in the pulp and paper industry (Hakala et al. 2005). Ligninases are also used as additives in food and beverage processing and to assay the total antioxidant concentration (TAC) in human plasma (Mazumder et al. 2008). In view of the extensive

biotechnological applications of these enzymes there is an acute need to characterize ligninolytic enzymes from different WRF strains for diverse industrial applications (Myasoedova et al. 2008; Stoilova et al. 2010).

Laccases are N-glycosylated multicopper blue oxidases having molecular masses in the range of 58 to 90 kDa (Asgher et al. 2008). Different WRF laccases have pH and temperature optima varying from 2-10 and 40-65^{o}C, respectively (Zouari-Mechichi et al. 2006; Asgher et al. 2008). Laccase isozymes with different optimum pH, temperature, and kinetic characteristics have also been identified in *Physisporinus rivulosus* T241i and *Cerrena unicolor* 137 (Cadimaliev et al. 2005; Lorenzo et al. 2006; Mäkelä et al. 2006). Low molecular mass compounds such as syringaldehyde, ABTS (2, 2′-azino-bis (3-ethyl benzthiazoline-6-sulphonate), 2,6-dimethoxyphenol (DMP), violuric acid, and 1-hydroxybenzotriazole (HBT) have been found to perform the role of mediators of laccase and enhance pollutant degradation (Lu et al. 2007; Quaratino et al. 2007). Addition of lower concentrations of cations such as Cu^{2+}, Cd^{2+}, Ni^{2+}, Mo^{3+}, and Mn^{2+} has been reported to increase the activity of WRF laccases in most cases, whereas Ag^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+} were inhibitory. Sodium azide (NaN_3) and H_2O_2 have also been found inhibitory in different concentrations (Ullrich et al. 2005; Murugesan et al. 2006). However, a laccase from *Trametes trogii* was not affected by low concentrations of Cd^{2+}, Al^{3+}, Li^{1+}, and Ca^{2+} at all (Zouari-Mechichi et al. 2006), whereas the activity of *Pleurotus ostreatus* laccase was enhanced by Pb^{2+} and Zn^{2+} (Baldrian 2005).

This manuscript reports the results of a study focused on purification and characterization of an extracellular laccase produced by an indigenous strain of *T. versicolor* IBL-04 to investigate its physicochemical and catalytic properties for possible biotechnological applications.

EXPERIMENTAL

All the experimental and analytical work was executed in the Industrial Biotechnology Laboratory (IBL), Department of Chemistry and Biochemistry, University of Agriculture Faisalabad (UAF), Pakistan. Fermentation experiments and enzyme assays were performed in triplicate. The results have been presented as mean ± S.E. (standard error) in tables and the S.E values have been displayed as Y-error bars in figures.

Chemicals and Lignocellulosic Substrate

All the chemicals were of analytical grade and were purchased mainly from Fluka-Sigma-Alrich (USA), Merck (Germany), and Scharlau (Spain). The corn cobs were collected from CPC-Rafhan Private Ltd, Faisalabad, Pakistan. The substrate was chopped into small pieces, oven dried (60 ^{o}C), ground to 40 mm mesh particle size in a Wiley Mill and stored in moisture free polyethylene bags.

Fungal Culture and Inoculum Development

The pure culture of indigenous strain *T. versicolor* IBL-04 available in Industrial Biotechnology Laboratory, UAF was used as laccase producing organism. Inoculum was developed by growing the fungus in Kirk's basal salt medium (Tien and Kirk 1988). The

medium was sterilized (121 ^{o}C) in a laboratory scale autoclave (Sanyo, Japan) for 15 min. After cooling to room temperature, the spores of *T. versicolor* IBL-04 from PDA slant were transferred into the broth under sterilized conditions in a laminar flow hood (Dalton, Japan). The inoculated flask was incubated for 5 days at 30 ^{o}C in orbital shaker (Sanyo-Gallemkemp, UK) at 120 rpm to get homogeneous spore inoculum containing 10^6-10^8 spores/mL (Kay-Shoemake and Watwood 1996).

Laccase Production in SSF

The production of laccase by *T. versicolor* IBL-04 was carried out in 500 mL Erlenmeyer flasks under some pre-optimized growth conditions. Triplicate flasks contained 5 g substrate moistened (60 % w/w moisture) with Kirk's medium (pH 4.5), unless otherwise stated. The flasks were autoclaved (120 ^{o}C) and inoculated with 5 mL homogeneous fungal spore suspension in laminar air flow. The inoculated flasks were kept at 30 °C in a temperature controlled still culture incubator (EYLA SLI-600ND, Japan) for five days.

Harvesting and Enzyme Extraction

At the end of the selected incubation time, the enzyme was extracted by adding distilled water (100 mL) to the solid-state cultures, followed by shaking at 120 rpm for 30 min (Gomes et al. 2009; Iqbal et al. 2011). The contents were filtered through Whatman No.1 filter paper, and the filtrates were centrifuged at 3,000×g (5415C, Eppendorf, Germany) for 10 min. The supernatants were carefully collected and used as crude enzyme extract for laccase assay and purification.

Optimization of Culture Conditions

A series of experiments was conducted to study the effect of fermentation time, additional carbon sources (glucose, glycerol, starch, wheat bran, and molasses), nitrogen additives (urea, yeast extract, beef extract, peptone, and corn steep liquor), C:N ratio (carbon to nitrogen ratio), 1 % Tween-80 (0.1-0.5 mL), and 1 mM metal salts ($CuSO_4$, $CaCl_2$, $FeSO_4$, $ZnSO_4$, and KCl) on the production of laccase in SSF of corn cobs. The Classical Strategy was adopted, varying a single parameter in triplicate and maintaining the previously optimized parameters at optimal level. The effects of carbon and nitrogen sources were studied in a single experiment using Completely Randomized Design (CRD) to accommodate the corresponding interactions among different carbon and nitrogen additives (Steel et al. 1997).

Laccase Activity Assay

Laccase activities in supernatants collected at after each optimization step were determined at room temperature by the assay of Wolfenden and Wilson (1982) as described earlier (Iqbal et al. 2011). Laccase was expressed in international units (U) as the amount of enzyme forming 1 μmol of $ABTS^{\bullet+}$ per min under the assay conditions.

Determination of Protein Contents

Protein contents of the crude and purified enzyme extracts were determined by the method by Bradford (1976) using bovine serum albumin (BSA) as standard.

Purification of Laccase

Ammonium sulfate precipitation

Crude laccase extract from 5-d-old cultures was centrifuged (3,000×g) for 15min at 4 ^{o}C. The supernatant was placed in an ice bath, crystals of ammonium sulfate were added to attain 50% saturation, and the mixture was kept overnight at 4 ^{o}C. The resulting precipitate was collected by centrifugation at 3000×g for 20 min at 4 ^{o}C. The pellets of precipitated proteins were discarded. In the supernatant, more crystals of ammonium sulfate were added to attain 90% saturation, and the mixture was again kept overnight at 4^{o}C and centrifuged as previous. The pellets were dissolved in minimal volume of 50 mM sodium malonate buffer at pH 4.5 and dialyzed against distilled water with 4 equal changes of water every 6 h to remove residual ammonium sulfate.

Gel filtration chromatography

Laccase was further purified by gel filtration chromatography using a Sephadex G-100 (Sigma, USA) column (120×2cm). Phosphate buffer (100 mM) with 0.15 M NaCl was used as elution buffer at a flow rate of 0.5 mLmin^{-1}. All the active fractions were pooled.

SDS–PAGE for Molecular Mass Estimation

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed on 5% stacking and 12% resolving gel according to the method by Laemmli (1970) using a Minigel Electrophoresis apparatus (V-GES, Wealtec, USA). Electrophoresis was performed at room temperature for 3 h at constant voltage (120 V). The gel was placed in fixing solution, and the protein bands were visualized by staining with Coomassie Brilliant Blue G (Sigma, USA). The molecular weight of the purified laccase was estimated in comparison to standard marker proteins (standard protein markers, 21-116 kDa; Sigma, USA) bands after gel staining.

Characterization of Purified Laccase

Effect of pH on laccase activity

Laccase was incubated for 10 min in buffers of different pH (3-10), followed by standard assay protocol. The buffers of different pH were (0.2 M): tartrate buffer, pH 3.0; sodium malonate buffer, pH 4.0; citrate phosphate, pH 5.0 and pH 6.0; sodium phosphate, pH 7.0 and pH 8.0; carbonate-bicarbonate buffer, pH 9.0 and pH 10.0.

Effect of temperature on laccase activity

Laccase was incubated in sodium malonate buffer (50 mM, pH 4.5) with temperatures ranging from 25 to 60 ºC for 10 min followed by normal assay protocol as previously described.

Determination of kinetic parameters

The Michaelis–Menten kinetic parameters K_M and V_{max} were determined by measuring the laccase activity using various concentrations of ABTS as substrate. The parameter values were obtained by curve fitting of the reciprocal plot of reaction rate

versus substrate concentrations using the Lineweaver-Burk plot transformation of the Michaelis-Menten rate equation.

Effect of activators/inhibitors

The effect of 1mM concentration of putative activators/inhibitors (Cu^{2+}, Mn^{2+}, Fe^{2+}, EDTA, and cystein) on activity of purified laccase was studied. The purified laccase was incubated at 40 °C along with 0.2 M citrate phosphate buffer at pH 5.0 and 100 µL of 1 mM activators/inhibitors for 10 min, followed by the normal laccase assay using ABTS (0.5 mM) as substrate.

RESULTS AND DISCUSSION

Optimization of Laccase Production in SSF

Fermentation Time Period

T. versicolor IBL-04 was grown in SSF medium with corn cobs as the substrate for the production of laccase for 10 days. Fungal growth and laccase production increased with time, and maximum laccase activity (680 U/mL) was observed after 5 days of incubation (Fig.1). In our previous study (Iqbal et al. 2011) the same strain was screened on different substrates, and most suitable substrate for MnP, and LiP production was rice straw, which was selected for further process optimization. However, it produced lower laccase activity in rice straw medium. It also showed lower laccase activities using corncobs in a screening trial, but the production of laccase was not optimized for hyperproduction using this substrate. In the present study laccase production was carried out under pre-optimized pH, temperature, moisture, and inoculum size (not reported in this paper). Addition of glucose and yeast extract, optimization of C:N ratio, and addition of activators and surfactants enhanced the production of laccase many fold using corncobs.

The time taken for ligninolytic enzymes synthesis depends on the length of the lag phase and primary metabolism, which vary with the chemical composition of lignocellulosic substrates (Zadrazil and Punia 1995). Low-cost substrates such as wheat flour, wheat bran, straws, and molasses are suitable for fungal growth and enzyme production (Mehta et al. 2006; Sen et al. 2009). *T. versicolor* IBL-04 produced higher laccase activity in 5 days than those reported earlier (Sack et al. 1997; Levin et al. 2003) for *T. versicolor* and *T. trogii* strains in 10 and 17 days, respectively.

Effect of Carbon and Nitrogen Sources and C: N Ratio

The experiment was carried out using different carbon and nitrogen additives in SSF medium of corn cobs under pre-optimized conditions. Results showed that the combination of glucose and yeast extract yielded maximal laccase activity (709 U/mL) whereas all other combinations inhibited fungal growth and enzyme production (Table 1).

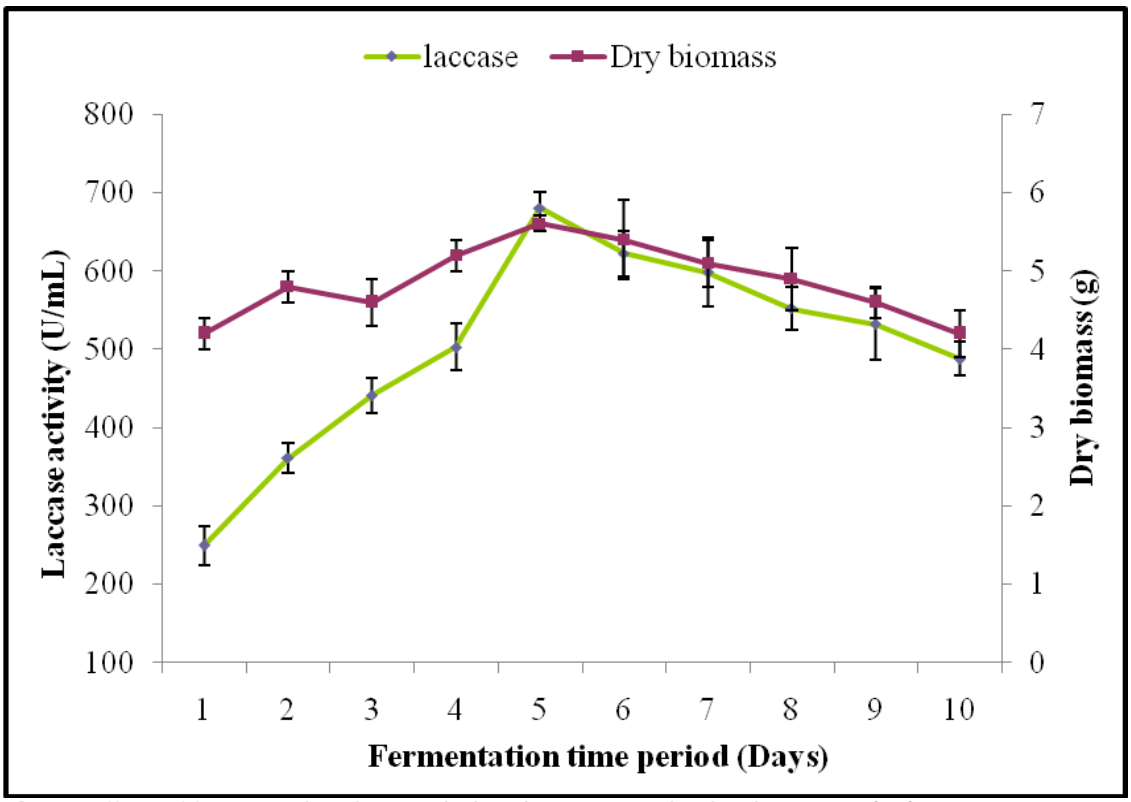


Fig. 1. Effect of fermentation time period on laccase production by *T. versicolor* IBL-04

A subsequent experiment was conducted with 10:1, 15:1, 20:1, 25:1, and 30:1 C: N ratios using glucose and yeast extract as carbon and nitrogen sources, respectively. The maximum laccase production (743 U/mL) was observed in the medium with 25:1 ratio. It was noted that laccase production steadily increased with an increase in C:N ratio from 5:1 to 25:1 (Fig. 2) but further increase in C:N ratio caused a decrease in enzyme synthesis by the fungus.

Table 1. Activities of Laccase Produced by *T. versicolor* IBL-04 with Different Carbon and Nitrogen Sources under Pre-optimized Conditions *

Enzyme Activities (U/mL)					
Nitrogen sources (0.2% w/w)	Carbon sources (1% w/w)				
	Glucose (C_1)	Glycerol (C_2)	Starch (C_3)	Wheat Bran (C_4)	Molasses (C_5)
Urea (N_1)	488±2.5	502±3.3	454±1.9	480±4.8	431±6.7
Yeast extract (N_2)	**709±6.2**	612±4.2	588±3.8	646±4.1	599±3.4
Beef extract(N_3)	577±3.1	521±3.9	543±4.3	519±2.9	532±4.1
Peptone (N_4)	510±4.7	491±3.8	527±4.5	591±4.8	583±6.3
Corn steep liquor (N_5)	629±3.8	579±6.3	599±4.1	617±3.9	603±5.3

*: Substrate 5g; pH 4.0; temperature, 30°C; moisture, 60% (w/w)

Both the nature and concentration of carbon and nitrogen sources are powerful influencing nutritional factors regulating ligninolytic enzyme formation by WRF (Galhaup et al. 2002; Mikiashvili et al. 2005; Elisashvili et al. 2008). Adejoye and Fasidi (2009) also reported enhanced laccase production by *Schizophyllum commune* using yeast extract and glucose as nitrogen and carbon supplements, respectively. The C:N ratios of fermentation medium significantly influence the production of laccase by WRF (Stajic et al. 2006; Kurt and Buyukalaca 2010). *Pleurotus pulmonarius* has been reported (Tychanowicz et al. 2004) to produce maximum laccase activity using a C:N ratio of 30:1 in solid state fermentation of corn cobs.

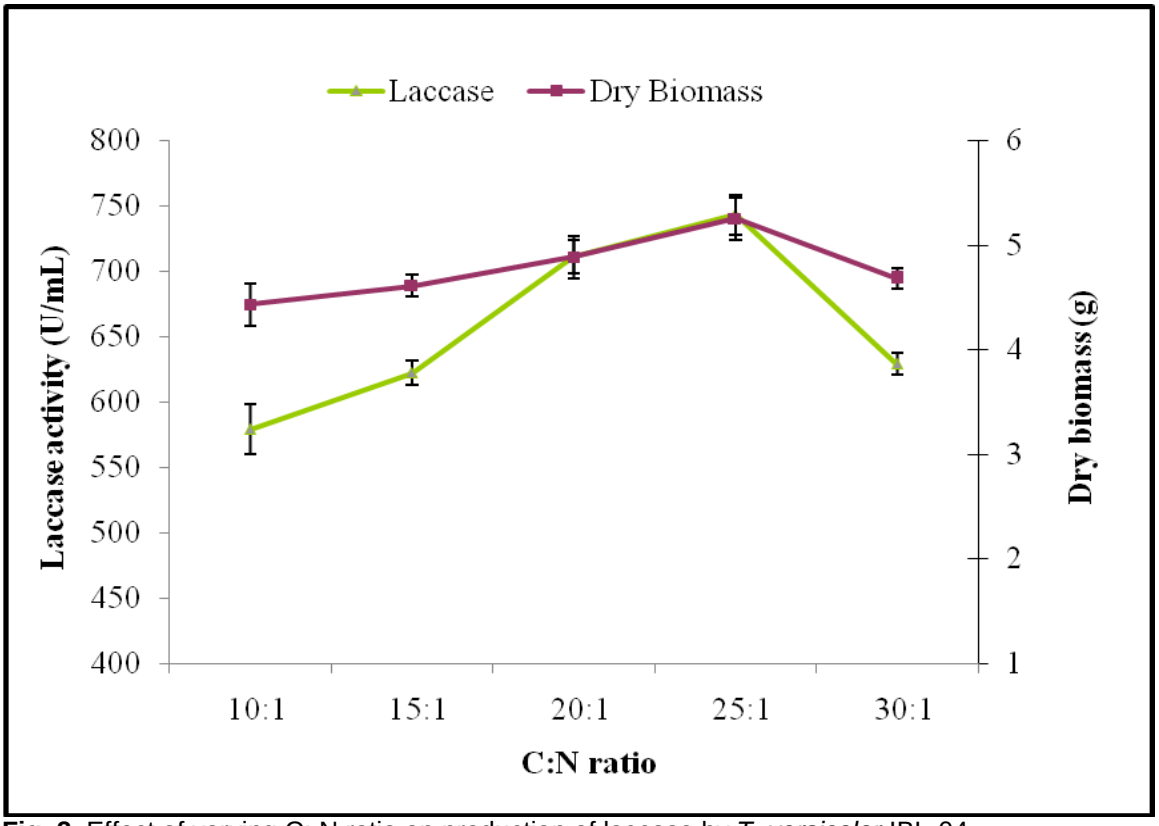


Fig. 2. Effect of varying C: N ratio on production of laccase by *T. versicolor* IBL-04

Effect of surfactant (Tween-80)

The effect of varying loadings of 1% Tween-80 (0.1, 0.2, 0.3, 0.4, and 0.5 mL) on laccase production by *T. versicolor* IBL-04 in SSF of corn cobs was investigated. Lower concentrations of Tween-80 were found to enhance laccase production by *T. versicolor* IBL-04 (Fig. 3), and flasks receiving 0.3 mL of 1 % Tween-80 showed maximum production of laccase (756 U/mL). Surfactants promote the penetration of water into the solid substrate matrix and increase the surface area for microbial growth, thereby enhancing enzyme production and secretion by fungi (Jager et al. 1985). Moreover, Tween-80 has the capability to affect the cell membrane structure and promote the excretion of ligninolytic enzymes from the fungal cells into the medium (Asther et al. 1987; Rodrigues et al. 2008).

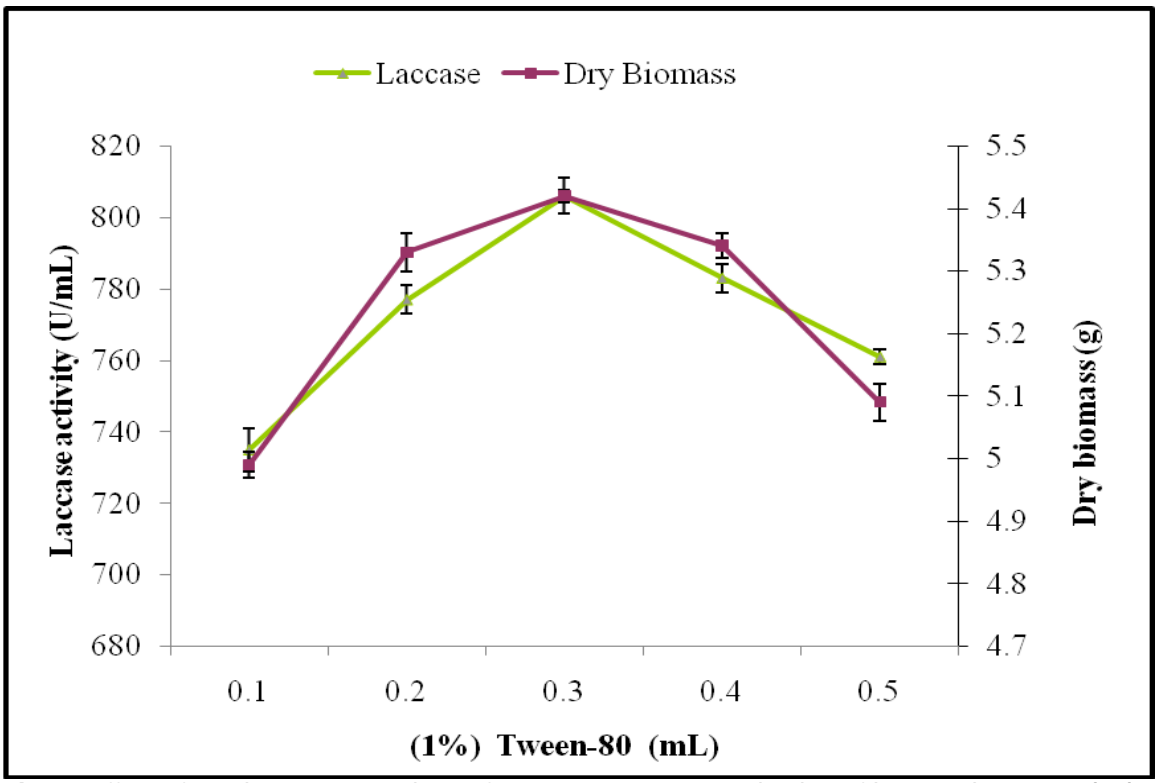


Fig. 3. Effect of varying concentrations of 1% Tween-80 on production of laccase by *T. versicolor* IBL-04

Effect of metal salts

The fermentation medium containing 5g corn cobs moistened with Kirk's medium (60 % w/w) at pH 4.0 was supplemented with different metal salts (1 mM) including $CuSO_4$, $CaCl_2$, $FeSO_4$, $ZnSO_4$, and KCl. It was noted that $CuSO_4$ and $ZnSO_4$ increased laccase production by *T. versicolor* IBL-04, and maximum laccase activity (1012 U/mL) was found in the flasks receiving 1 mM $CuSO_4$ (Fig. 4). $CaCl_2$, $FeSO_4$, and KCl were found inhibitory to fungal growth and laccase synthesis. Metal ions can significantly affect the growth and the extracellular enzymes production capabilities of WRF (Pointing and Vrijmoed 2000; Baldrian 2003). In a previous study (Lorenzo et al. 2006) *T. vesicular* was reported to yield 6342 U/L laccase using 2mM $CuSO_4$. The laccase yield was increased up to 8277 U/L by increasing the concentration of this salt to 3.5mM, suggesting its role as an inducer.

Extraction and Purification of Extracellular Laccase

After filtration of the fermention broth, the filtrate was centrifuged at 3,000×g for 20 minutes at 4 ^{o}C to obtain clear supernatant. The supernatant with a laccase activity of 202400 U/200mL and specific activity of 234.26 U/mg was used as crude enzyme extract. The enzyme was precipitated at 90% ammonium sulfate saturation to obtain a specific activity of 271.16 U/mg, which was equivalent to 1.16 purification fold. The pellets (precipitate) were dissolved in minimal volume of 50 mM sodium malonate buffer at pH 4.5 and dialyzed against distilled water to remove the extra salt.

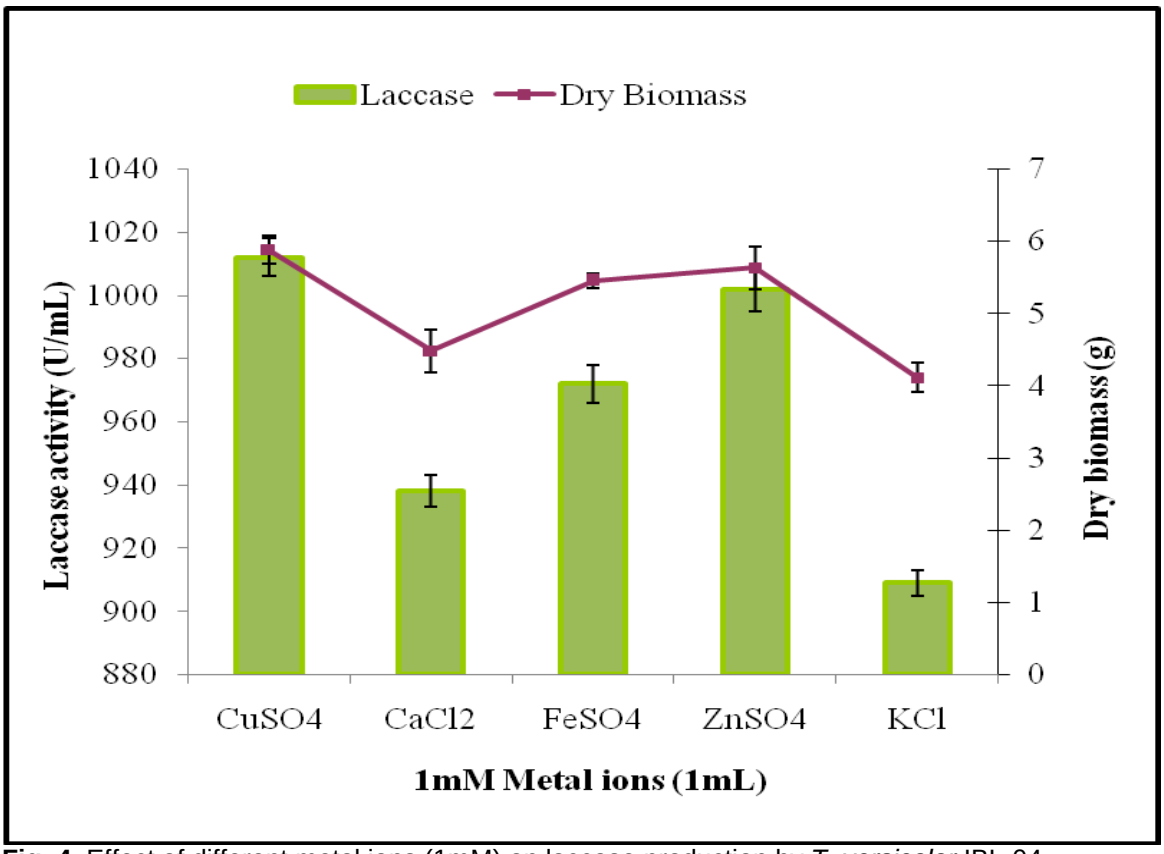


Fig. 4. Effect of different metal ions (1mM) on laccase production by *T. versicolor* IBL-04

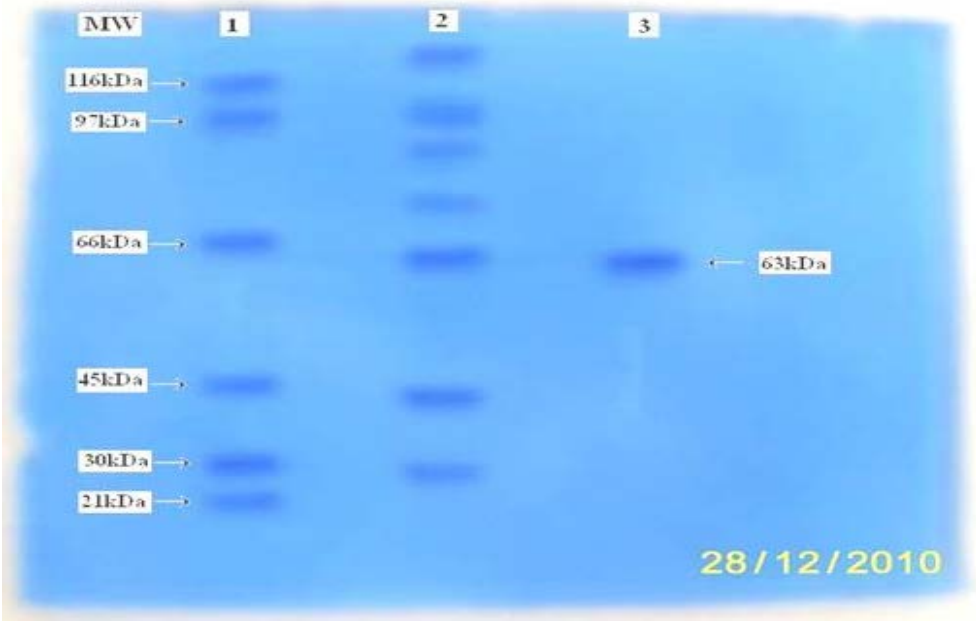
The dialyzed fraction (partially purified laccase) was loaded onto a Sephadex-G-100 gel filtration column. After gel filtration the enzyme was found to be 2.17 fold purified with a specific activity of 508.24 U/mg (Table 2). A-el-Gammal et al. (2001) and Mtui and Nakamura (2008) achieved best ligninolytic enzymes isolation by 50 to 80% $(NH_4)_2SO_4$ saturation, followed by chromatographic purification techniques for the recovery of pure ligninolytic enzymes.

Table 2. Purification Summary for Laccase from *T. versicolor* IBL-04

No.	Purification Steps	Total Volume (mL)	Total Enzyme Activity (U)	Total Protein Content (mg)	Specific Activity (U/mg)	Purification fold	% Yield
1	Crude Enzyme	200	202400	864	234.26	1	100
2	$(NH_4)_2SO_4$ Precipitation	30	27930	103	271.16	1.16	13.80
3	Dialysis	25	26850	67.5	397.77	1.70	13.26
4	Sephadex-G-100	12	8640	17	508.24	2.17	4.27

Molecular Mass Determination through SDS-PAGE

The purified laccase resolved on SDS-PAGE was a homogenous monomeric protein, as indicated by a single band corresponding to 63 kDa compared to the standard molecular weight markers (Fig. 5). The laccase from *T. versicolor* IBL-04 is comparable to those purified from other WRF species such as *Trametes pubescens*, M.W. 65 kDa (Galhaup et al. 2002); *Funalia trogii*, M.W. 58 KDa (Patrick et al. 2009); *Trametes trogii* B6J, M.W. 62 kDa (Zouari-Mechichi et al. 2006), and *Trametes* sp AH28-2, M.W. 72 (Xiao et al. 2004). Most of the WRF laccases have been reported to be extracellular glycosylated proteins with molecular weights ranging from 58 to 90 kDa (Quaratino et al. 2007; Asgher et al. 2008).



[Lane MW, Molecular weights in kDa of standard marker; lane 1, standard protein markers (β-Galactosidase, 116kDa; Phosphorylase B, 97kDa; albumin, 66kDa; ovalbumin, 45kDa; carbonic anhydrase, 30kDa and trypsin inhibitor, 21kDa); lane 2, Crude extract; lane 3, Purified extra cellular laccase (63kDa)]

Fig. 5. Molecular mass determination of purified laccase by SDS-PAGE

Characterization of Purified Extracellular Laccase

Effect of pH on laccase activity

Purified laccase was incubated in buffers with various pH before assay. The pH-activity profile (Fig. 6) showed that laccase activity increased initially with pH rise and it peaked at pH 5. The enzyme had fairly good activity over a wide pH range (pH 5.0 to 8.0), which may be a useful characteristic for various industrial and biotechnological applications. However, further increase in pH caused rapid deactivation of the enzyme. pH optima for different fungal laccases are different, but the enzymes usually have pH optima in the range of pH 2.0 to 10 (Koroleva et al. 2002; Baldrian P 2005; Lu et al. 2005; Zouari-Mechichi et al. 2006; Asgher et al. 2008; Patrick et al. 2009). Minussi et al. (2007) reported two isoenzymes of laccase L1 and L2 from *Trametes versicolor* CCT

4521 with optimum pH 4.0 and 5.0, respectively, whereas laccase from *Trametes* sp. AH28-2 had an optimal pH of 4.7 (Xiao et al. 2004). The optimum pH of laccase from *Coriolus hirsutus* varied in the range of 2.5 to 4.0 and was substrate dependent (Shin and Lee 2000).

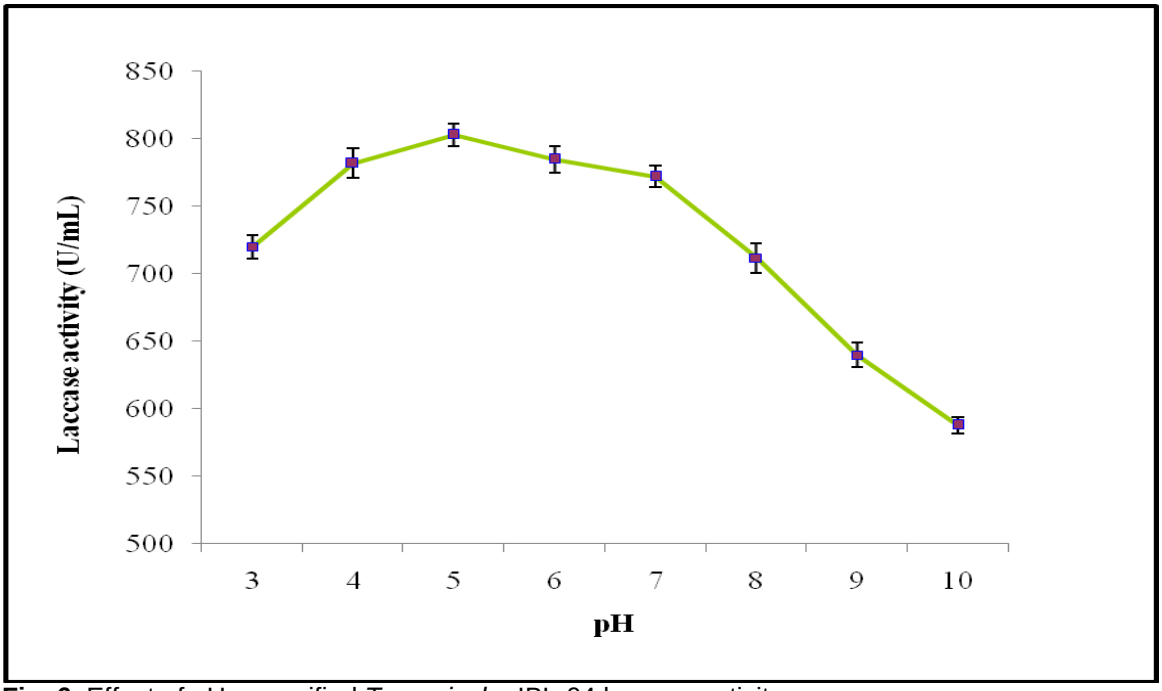


Fig. 6. Effect of pH on purified *T. versicolor* IBL-04 laccase activity

Effect of temperature on laccase activity

Laccase was incubated at various temperatures for 10 min, and laccase assay was then carried out to determine the effect of temperature on enzyme activity. The temperature versus laccase activity curve showed an increase in activity with initial increase in temperature. The maximum activity was displayed at 40 ^{o}C (Fig. 7) with a rapid loss in activity at higher temperatures due to thermal denaturation. For a variety of industrial applications, relatively high thermostability is an attractive and desirable characteristic of an enzyme. However, most of the WRF laccases are stable and almost fully active in a temperature range of 30 to 50 ^{o}C (Nagai et al. 2002), and they start losing their activities rapidly as temperature increase above 60 ^{o}C (Cambria et al. 2000; D'Souza et al. 2006; Zouari-Mechichi et al. 2006). Stoilova et al. (2010) reported maximum activity of crude laccase from *Trametes versicolor* at 45 ^{o}C and the two laccase isozymes of *Trametes versicolor* CCT 4521 L1 and L2 also exhibited optimum activities at 40 ^{o}C (Minussi et al. 2007).

Effect of substrate concentration: Determination of K_M and V_{max}

The K_M and V_{max} values for purified laccase were determined using various concentrations of ABTS as substrate. Enzyme activities were measured under standard assay conditions and results were used to construct reciprocal plot using Line-Weaver and Burk equation.

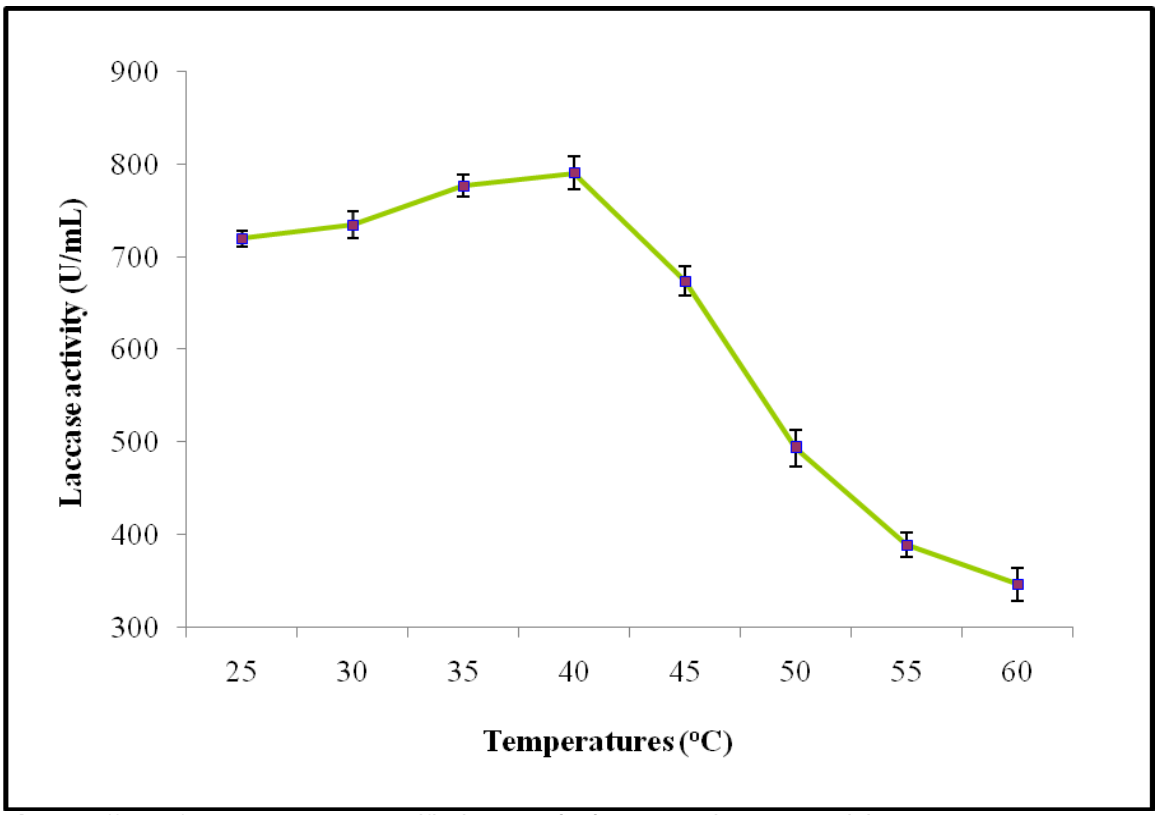


Fig. 7. Effect of temperature on purified *T. versicolor* IBL-04 laccase activity

The reciprocal of laccase activity (1/V) in U/mL was plotted against reciprocal of substrate concentration (1/[S]) in µM (Fig. 8). The K_M and V_{max} values of *T. versicolor* IBL-04 laccase were 73 µM and 780 U/mL, respectively. The relationship between rate of reaction and concentration of substrate depends on the affinity of the enzyme for its substrate expressed as K_M (Michaelis constant) of the enzyme. A lower value of K_M reflected higher affinity of the *T. versicolor* IBL-04 laccase for ABTS as compared to laccase from *Pleurotus pulmonarius* and *Trametes* sp. AH28-2 having apparent K_M values of 210 µM and 177 µM, respectively (De'Souza and Peralta 2003; Xiao et al. 2004). However, a laccase from *Cerrena maxima* had a K_M value of 65 µM for ABTS (Koroleva et al. 2002) and that from *Coriolus hirsutus* had 62.9 µM for guaiacol (Koroljova et al. 1999).

Effect of activators/inhibitors

It was observed that 100 µL of 1 mM Cu^{2+} caused laccase activation, whereas cystein, EDTA, Mn^{2+}, and Fe^{2+} caused enzyme inhibition. Cystein was the most potent laccase inhibitor, followed by EDTA, Fe^{2+}, and Mn^{2+} (Fig. 9). Addition of $Cu^{2+}$and Mn^{2+} have previously been reported to increase the activity of WRF laccases, in most cases (Ullrich et al. 2005; Murugesan et al. 2006). The activation of laccase by Cu^{2+} may be due to the filling of type-2 copper binding sites with copper ions (Nagai et al. 2002; Sadhasivam et al. 2008). However, *Sinorhizobium meliloti* CE52G laccase is inhibited to different extents by Fe^{3+}, Mn^{2+}, and Cu^{2+}, suggesting that these metal ions interfere with the oxidation of the classical organic laccase substrates (Rosconi et al. 2005).

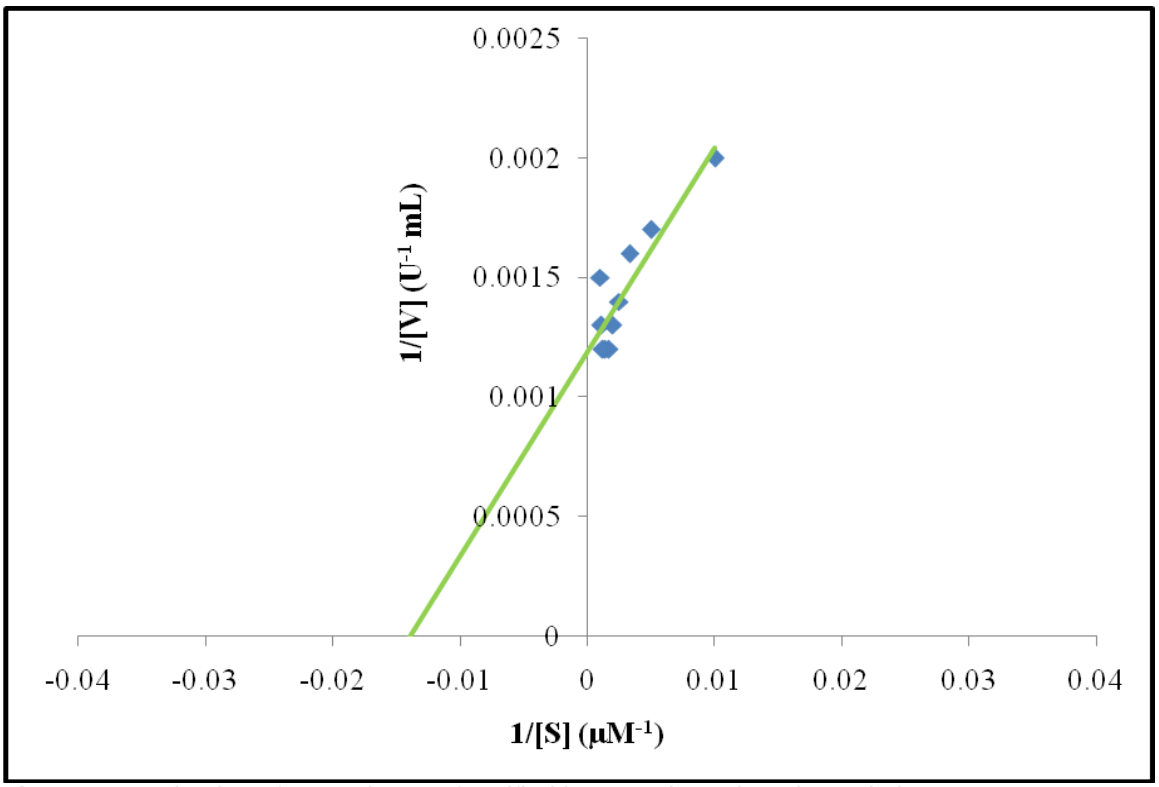


Fig. 8. Determination of K_m and V_{max} of purified laccase through reciprocal plot

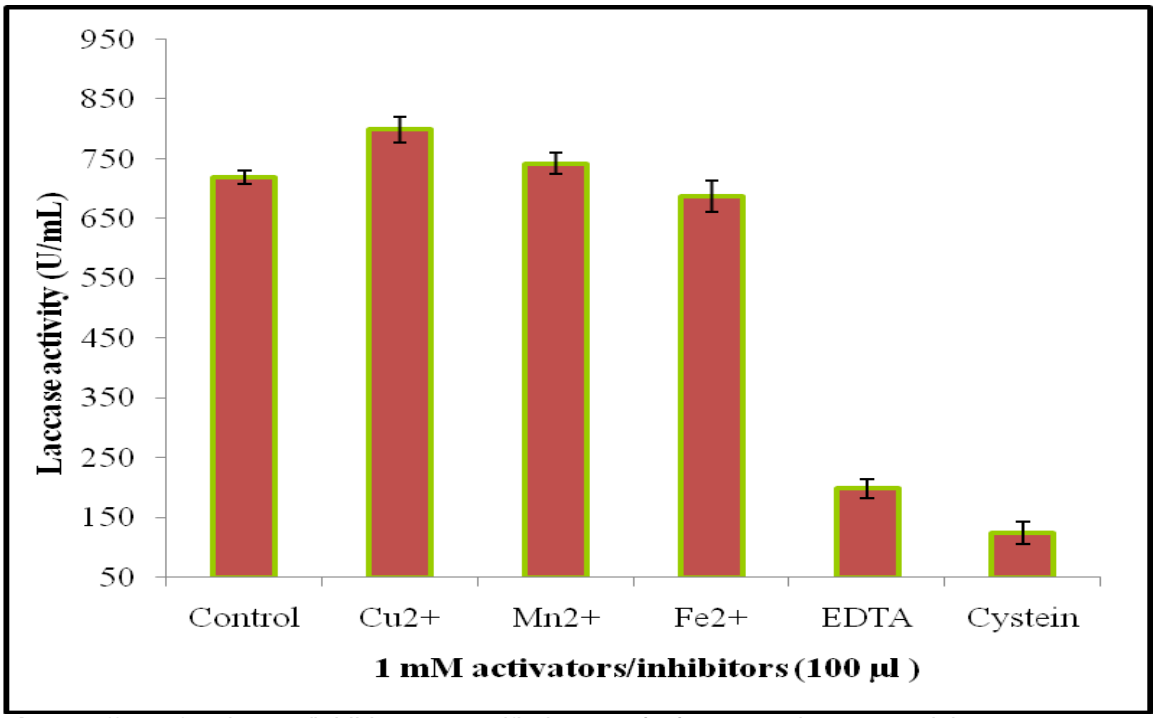


Fig. 9. Effect of activators/inhibitors on purified *T. versicolor* IBL-04 laccase activity

EDTA is an inhibitor of metallo-enzymes including laccases due to its ability to form inactive complexes with inorganic cofactors of the enzyme (Sadhasivam et al. 2008; Asgher and Iqbal 2011). However, this is not true for all laccases; *Marasmius quercophilus* and *Sinorhizobium meliloti* CE52G laccases are not significantly affected by EDTA (Dedeyan et al. 2000; Rosconi et al. 2005). L-cysteine and dithiotheitol have also been reported as effective inhibitors of *Trichophyton rubrum* LKY-7 laccase in the 1 to 5 mM concentration range (Jung et al. 2002).

CONCLUSIONS

T. versicolor IBL-04 showed tremendous potential for laccase synthesis through SSF of corncobs in higher concentration (1012 U/mL) than reported for other *Trametes* (*Coriolus*, *Polylorus*) species. The extracellular laccase from *T. versicolor* IBL-04 had molecular weight of 63 kDa with optimum activity at pH 5 and 40 ^{o}C. The enzyme showed maximum activity (V_{max}) of 780 U/mL with its corresponding K_M value of 73 µM. The catalytic efficiency and substrate affinity of this extracellular laccase from *T. versicolor* IBL-04 is greater than those of other reported *Trametes* species suggesting its usefulness for industrial applications.

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