# PURIFICATION AND CHARACTERIZATION OF LACCASE PRODUCED BY *Schyzophylum commune* IBL-06 IN SOLID STATE CULTURE OF BANANA STALKS

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*Shyzophyllum commune* IBL-06 produced 367 IU/mL of laccase in solid state bioprocess of banana stalk under optimum physical and nutritional parameters. The optimum SSF conditions were pH 4.5; temperature, 35°C; inoculum size, 3mL ($10^6$-$10^8$ $mL^{-1}$); moisture content, 60%; C:N ratio in the medium, 15:1 (glucose and ammonium nitrate as additional carbon and nitrogen sources, respectively), ABTS (1mM), 1 mL, and $CuSO_4$ (1mM), 1mL. Laccase was purified 3.95-fold with specific activity of 158 U/mg by ammonium sulfate precipitation, followed by gel filtration chromatography using Sephadex G-100 column. Molecular weight of the laccase was 63 KDa on SDS-PAGE. Purified laccase had an optimum pH of 6.0 and was stable in the pH range from 6 to 7. The optimum temperature was 40 °C, and it displayed considerable stability within the range 30 to 35 °C with 24 h incubation. *K*m and $V_{max}$ were 0.025 mM and 80 mM/min, respectively, using ABTS as a substrate. Copper sulfate increased the activity of purified laccase when used at low concentration, but silver nitrate exerted the strongest inhibition.

*Keywords: Laccase; Shyzophylum commune IBL-06; Banana stalk; Purification; Characterization*

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## INTRODUCTION

Lignocellulosic plant biomass accounts for more than 60% of the total biomass production on earth and is a renewable resource for food, energy, and chemicals (Couto et al. 2006; Ikehata et al. 2004). Pakistan has an agro-based economy producing wheat straw, corncobs, corn stover, sugarcane bagasse, rice straw, and banana stalk as major agro industrial wastes that contain high levels of lignocellulose. These lignocellulosics are efficient substrates for white rot fungi, which produce industrially important lignino-lytic and cellulolytic enzymes (Reddy et al. 2003).

Many microorganisms that decompose lignocellulosic materials are being studied as producers of enzymes to perform enzymatic hydrolysis of the lignocellulosic material present in residues from the agro-industries. White rot fungi (WRF) are capable of degrading lignin and recalcitrant environmental pollutants such as textile dyes, polyaro-matic hydrocarbons, polychlorophenols, and polychlorinated biphenyls (Asgher et al. 2006). White rot fungi excrete extracellular polyphenol oxidases, particularly lignin peroxidases, manganese peroxidases, and laccases, which are effective in degrading lignin (Revankar and Lele 2006). Laccases and manganese peroxidases also are able to

oxidize phenolic and non-phenolic substrates under certain conditions.

Laccases are dimeric or tetrameric glycoproteins that contain four copper atoms distributed in redox sites. They do not need $H_2O_2$ for substrate oxidation and have broader spectrum than peroxidases (Mishra and Kumar 2007). Laccases oxidize mediator compounds such as phenol, aniline, 4-hydroxybenzoic acid, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), which in turn degrade dyes and a wide variety of organic and inorganic substrates, including mono-, di-, and polyphenols, aminophenols, methoxyphenols, aromatic amines ascorbate, and organic polluntants with concomitant four-electron reduction of oxygen to water (Podgornik et al. 2001; D'Souza et al. 2006; Asgher et al. 2008). Laccases have become important, industrially relevant enzymes because of a number of diverse applications, e.g. for biocatalytic purposes such as delignification of lignocellulosics and cross-linking of polysaccharides, for bioremediation applications such as waste detoxification and textile dye transformation, for food technological uses, for personal and medical care applications, and for biosensor and analytical applications (Hakala et al. 2005; Pazarlioglu et al. 2005; Singh et al. 2010; Wang et al. 2011). The ligninolytic enzymes are generally produced during secondary metabolism under conditions of limited nitrogen (Mishra and Kumar 2007; Liu et al. 2009).

*Schyzophyllum commune* is a common worldwide mushroom. Its gills function to produce basidiospores on their surface. It is a very successful wood decaying fungus that causes a white rot. Researchers have focused for the last few years on production of ligninase enzymes responsible for wood decay by the fungus through bioprocess. The objective of this work was to purify and characterize the extracellular laccase enzyme of *Schyzophyllum commune* IBL-06.

## EXPERIMENTAL

### Lignocellulosic Substrate

Banana stalks collected from the fruit market of Ghulam Muhammad Abad, Faisalabad were chopped into small chips. The chips were sun dried, oven dried (50 $^{o}$C) to constant weight, ground to 40 mm mesh size, and stored in plastic jars to keep the material moisture-free.

### Fungal Strain and Inoculum Development

Pure culture of *Schyzophyllum commune* IBL-06 was obtained from Industrial Biotechnology Laboratory, Department of Chemistry and Biochemistry, University of Agriculture Faisalabad. *The Schyzopxyllum commune* was grown in Kirk's basal liquid medium (Tien and Kirk 1988) at pH 4.5 in a 500 mL Erlenmeyer flask. After inoculation with fungus, incubation was carried out at 35 °C (150 rpm) for 5 days to get homogeneous spore inoculum ($10^6$-$10^8$ spores/mL)

### Laccase Production in Solid State Bioprocess

Triplicate flasks (500 mL) containing 10 gm lignocellulosic substrate banana stalk were adjusted to 60% moisture (w/w) with pre-optimized medium of pH 4.5, containing

glucose as carbon source and $NH_4NO_3$ as nitrogen source in 15:1 C: N ratio; 1mM Tween-80, 1 mL; 1mM ABTS, 1mL; and 1mM $CuSO_4$, 1 mL (Iqbal et al. 2010). The flasks were autoclaved, inoculated with 3 mL of inoculum, and incubated at 35 $^oC$ for 5 days under stationary solid state conditions in a still culture incubator (EYLA SLI-600ND, Japan).

## Enzyme Extraction

The fermented biomass was harvested after five days by adding 100 mL of 50 mM sodium malonate buffer of pH 4.5 and shaking at 150 rpm for 30 min. The contents of the flask were filtered through Whatman No.1 filter paper (125mm) and washed thrice with 10 ml buffer each time. The filtrate was centrifuged at 3,000×g for 5 min at 25 $^oC$ to remove fungal pellets, and clear supernatant was used as enzyme extract for laccase assay and purification.

## Laccase Assay

Laccase was assayed by monitoring the oxidation of 2,2 azinobis (3-ethylbenz-thiazoline)-6 sulphonate (ABTS) by the enzyme extract (Wolfendon and Wilson 1982) at pH 4.5 and 35 $^oC$ temperature. Laccase activity assay was performed in 2.1 mL reaction mixture containing 1 mL of 50 mM malonate buffer (pH 4.5), 1 mL of 1mM ABTS and 0.1 mL of enzyme solution, and ABTS oxidation was followed at 420 nm ($\varepsilon_{420}$ 36000 M $cm^{-1}$). The absorbance of each sample was taken after a 10 min. interval. Control samples contained 0.1 mL of distilled water instead of enzyme solution. One unit laccase activity was defined as change in absorbance of the assay mixture 420 nm in 10 min. The change in absorbance in 10 min. was equivalent to μM of ABTS oxidized in 10min. That result was then converted to uM of ABTS oxidized per min to calculate the IU.

## Determination of Protein Content

Protein contents of the crude and purified enzyme extracts were estimated following the method of Bradford (1976) using Bovine serum albumin as standard.

## Purification of Laccase

Crude extract obtained from *S. Commune* IBL-06 was centrifuged at 3,000×g for 15 min at 4$^oC$ to increase clarity to maximum, and the filtrate was concentrated by freeze drying.

## Fractional Precipitation and Dialysis

After optimizing the concentration of ammonium sulfate, the crude enzyme concentrate was placed in ice bath, and crystals of ammonium sulfate were added to attain 70% saturation at 0$^oC$. The flask was kept overnight at 4$^oC$. Then it was centrifuged at 3,000×g for 30 min. at 4$^oC$. The pellet of precipitated proteins was discarded. In the supernatant, more crystals of ammonium sulfate were added to attain 90% saturation at 0$^oC$. It was again kept for a night at 4$^oC$ and centrifuged as previously described. This time a pellet was collected and supernatant was discarded. The pellet was dissolved in a minimum quantity of buffer and dialyzed against distilled water several

times to remove ammonium sulfate. Total protein content and laccase activity of the pooled/dialyzed fractions were determined

## Gel Filteration Chromatography

A pooled fraction from dialysis was loaded on a Sephadex G-100 gel filtration column (16×2 cm) that was equilibrated with 50mM malonate buffer of pH 4.5. The 200µL/run of sample was applied, 100 mM phosphate buffer (pH 6.0) having 0.15M NaCl was used as elution buffer, and 12 major positive fractions were collected with the flow rate of 0.5 $mLmin^{-1}$. After each purification step, the total protein content and enzyme activity were determined to calculate specific activity and purification factor.

## Molecular Mass Determination through SDS-PAGE

To verify the purity and subunit molecular mass, the enzyme was run on sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by the method described by Laemmli (1970). The purified enzyme sample was dissolved in a minimum amount of distilled water and subjected to SDS-PAGE on 10% polyacrylamide gels using low and medium molecular weight protein markers. A cut was applied on the lower side of the gel to illustrate the direction of electrophoresis. The protein bands were stained with Coomassie Brilliant Blue G-250 stain prepared according to Merril (1990). The standard protein markers (Sigma) were: β-Galactosidase, 116 kDa; Phosphorylase B, 97 kDa; albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa and trypsin inhibitor, 21 kDa.

## Characterization of Laccase

The purified laccase was subjected to characterization through kinetic studies by studying the following:

*Effect of pH on laccase activity*

Laccase was assayed at different pH ranging from 3 to 9. Laccase was assayed at pH 3 to 3.5 in 20 mM succinate buffer, 50 mM sodium tartrate buffer), pH 4 to 5 in 50 mM malonate buffer, pH 6 to 7 in 100 mM phosphate buffer, and pH 8 to 9 in 100 mM sodium phosphate buffer.

*Effect of temperature on laccase activity*

Purified laccase was assayed at different temperature ranging from 25 to 70 ºC at optimum pH 4.5; the enzyme was incubated at varying temperatures for 15 min and assayed by standard assay protocol.

*Effect of substrate concentration: determination of K*m *and V* max

The Michalis-Menten kinetic constants (*K*m, *V*max) were determined by using varying concentrations of ABTS ranging from 0.1 to 0.5 mM following the method described by Farnet et al. (2004).

*Effect of activators / inhibitors*

Effects of different organic compounds including TEMED, mercaptoethanol, EDTA, and metal ions including $Pb^{2+}$, $Ca^{2+}$, $Mg^{2+}$ and $CU^{2+}$ in 1-5 mM concentration range on laccase activity were investigated. The enzyme was incubated at 35°C (assay temperature) in the presence of varying concentration solutions (pH 4.5) of the respective compounds for 30 min. followed by the standard assay procedure.

## RESULTS AND DISCUSSION

### Laccase Production

*S. Commune* IBL-06 was cultivated in SSF medium containing banana stalk as the main lignocellulosic substrate supplemented with glucose, ammonium nitrate, and salts under optimum process conditions. The fungus produced 367 IU/mL laccase in 6 days under optimum conditions of solid state bioprocess. The optimum conditions were: pH 4.5; temperature, 35 °C; inoculums size, 3 mL (per 10g substrate); moisture content, 60% (w/w); glucose, 1g/5g substrate; $NH_4 NO_3$ , 0.2 g/5g substrate; C: N ratio, 15:1, 1mM mediators ABTS, 1mL; 1mM Tween-80, 1mL, and 1mM $CuSO_4$, 1 mL.

### Purification of Laccase

The total laccase activity of crude extract (500 mL) was 183500 U with specific activity of 40 U/mg. The purification steps for laccase are given in Table 1. Laccase was salted out with ammonium sulfate saturation of 50%. After dialysis and gel filtration the enzyme was purified to 3.95-fold with specific activity of 158 U/mg.

A single peak obtained in gel filtration profile (Fig. 1) showed that laccase had a single enzyme activity and there was no isozyme of laccase. A-el-Gammal et al. (2001) and Mtui and Nakamura (2008) also achieved fractionation by 50 and 80% $(NH_4)_2SO_4$ saturation, respectively, followed by chromatographic purification techniques for recovery of pure ligninolytic enzymes. Previously, a ligninolytic MnP from *Phanerochaete sp* was salted out at 65% $(NH_4)_2SO_4$ saturation with 2.68-fold purification and 5.56% yield (Rajan et al. 2010).

**Table 1.** Purification Summary of Laccase from *S. commume* IBL-06

| Sr. No | Purification Steps | Total Volume (mL) | Total Enzyme Activity (U) | Total Protein Content (mg) | Specific Activity (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|---|
| 1 | Crude laccase | 500 | 183500 | 4535 | 40 | 100 | 1 |
| 2 | $(NH_4)_2SO_4$ ppt | 40 | 88750 | 855 | 104 | 48 | 2.6 |
| 3 | Dialysis | 40 | 79166 | 718 | 110 | 43 | 2.75 |
| 4 | Sephadex G100 | 12 | 6031 | 38 | 158 | 3 | 3.95 |

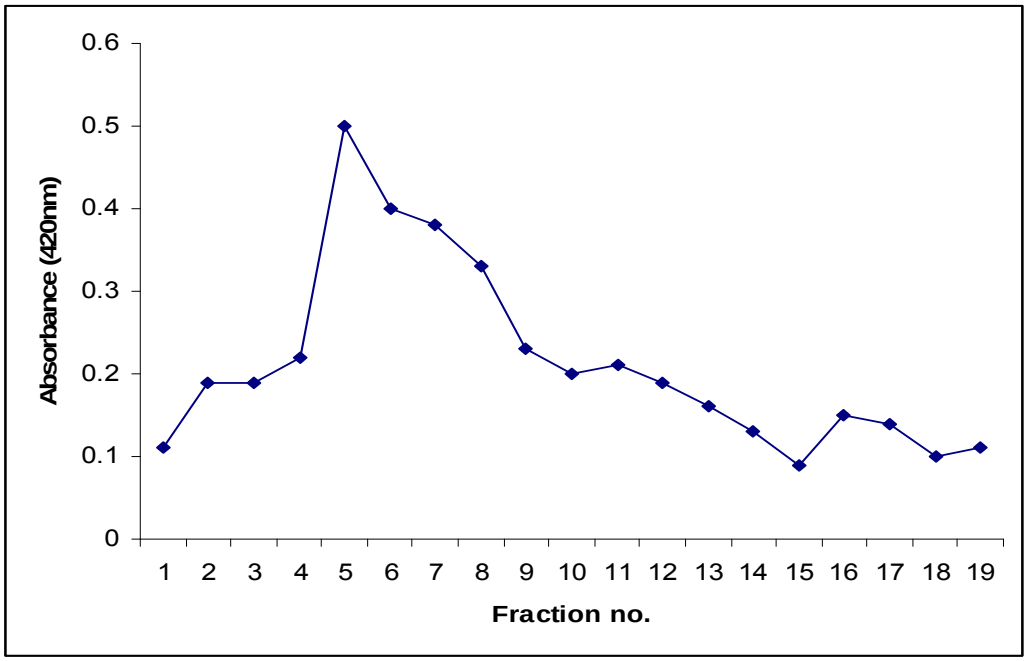



**Fig. 1.** Gel filtration chromatography of laccase produced by *S. commune* IBL-06

## SDS-PAGE

The purified laccase resolved on SDS-PAGE was a homogenous monomeric protein as indicated by a single band corresponding to 63 kDa relative to the standard molecular weight markers (Fig. 2). A single band on SDS-PAGE showed that the enzyme is a single polypeptide protein.

The molecular masses of laccases from different WRF in the 55-90 kDa range have previously been reported (Yaropolov et al. 1994; Ryan et al. 2003; Farnet et al. 2004; Murugesan et al. 2006; Salony et al. 2006; Zouari-Mechichi et al. 2006; Quaratino et al. 2007).

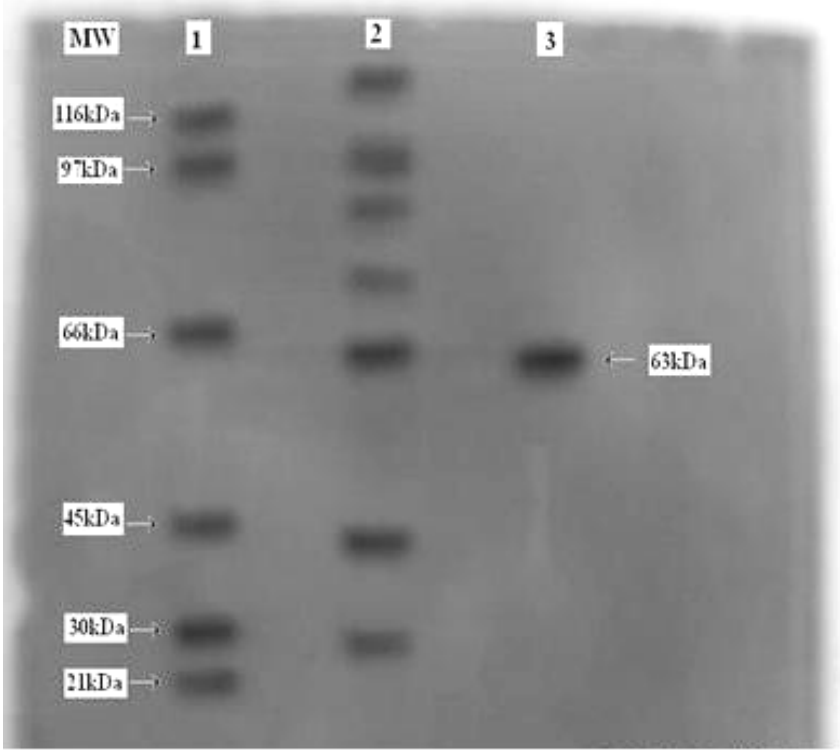
## Characterization of Laccase

*Effect of pH on purified lacasse*

Laccase activity was studied at varying pH (pH 3 to 9). The optimum pH of purified laccase produced by *S. commune* IBL-06 was 6.0 for ABTS oxidation. A further rise in pH was found to deactivate the laccase protein. The purified laccase remained quite stable within the pH range of 5-8 after 1h incubation (Fig. 3).

The enzyme retained only 22% of its activity at pH 9 after 24 h incubation at 35°C. *S. commune* laccase had higher optimum pH as compared to *Lentinula (Lentinus) edodes* laccase that was optimally active at pH 4.5 and was stable in lower pH range (Boer et al. 2004).

A laccase from *Mauginiella sp.* had optimal activity at acidic pH 2.4 and remained reasonably stable within the 4-8 pH range after 24-h incubation (Palonen et al. 2003). Recently a crude laccase produced by *Trametes versicolor* having optimum pH 4.5 has also been reported (Stoilova et al. 2010).




(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. 2.** SDS-PAGE of laccase

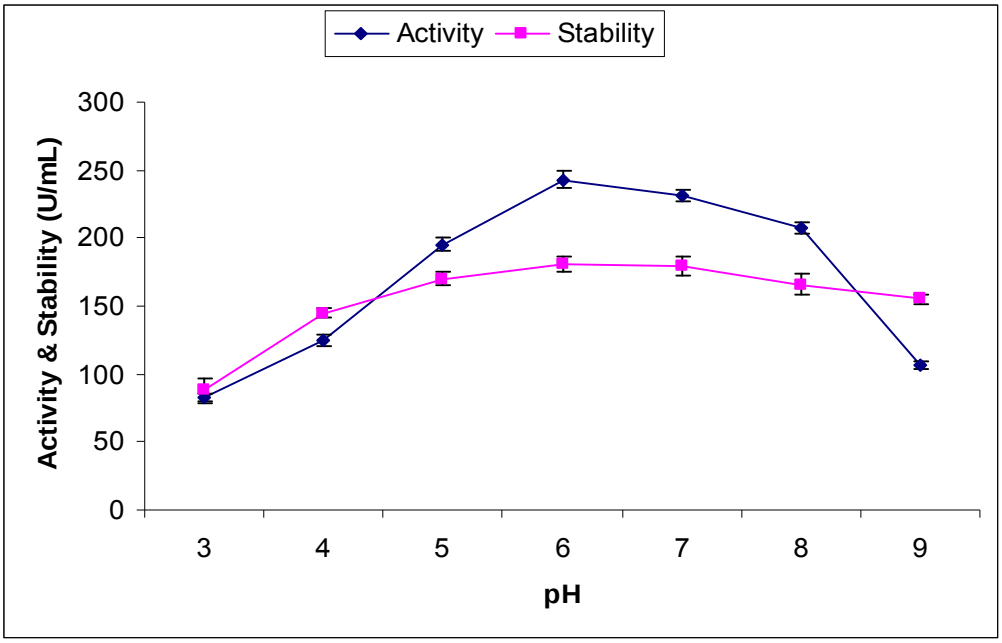



**Fig. 3.** Effect of pH on activity and stability of laccase produced by *S. commune* IBL-06

*Effect of temperature on purified lacasse*

The optimum temperature for purified laccase was 40°C, which is lower as compared to most of the previously reported laccases. An initial increase in temperature increased the enzyme activity, possibly by enhancing the kinetic energy of the molecules and increasing the interaction between enzyme active site and interacting groups of the substrate. The enzyme had very low thermostability, as it retained only 33% of its activity at 60°C after incubation for an hour, as shown in Fig. 4. Laccases from different WRF have been reported to show optimum activities in the temperature range of 40 to 65 °C (Murugesan et al. 2006; Quaratino et al. 2007; Asgher et al. 2008). Two laccase isozymes from *Trametes trogii* had optimum temperature around 50°C. *Cerrena unicolor* 137 laccase displayed optimum activity at 60°C, and *Panus tigrinus* laccase isozymes showed a similar optimum at pH 7.0 and 60 to 65°C (Cadimaliev et al. 2005; Makela et al. 2006; Michniewicz et al. 2006; Zouari-Mechichi et al. 2006). Stoilova et al. (2010) reported that laccase from *Trametes versicolor* had 45ºC as an optimum temperature.

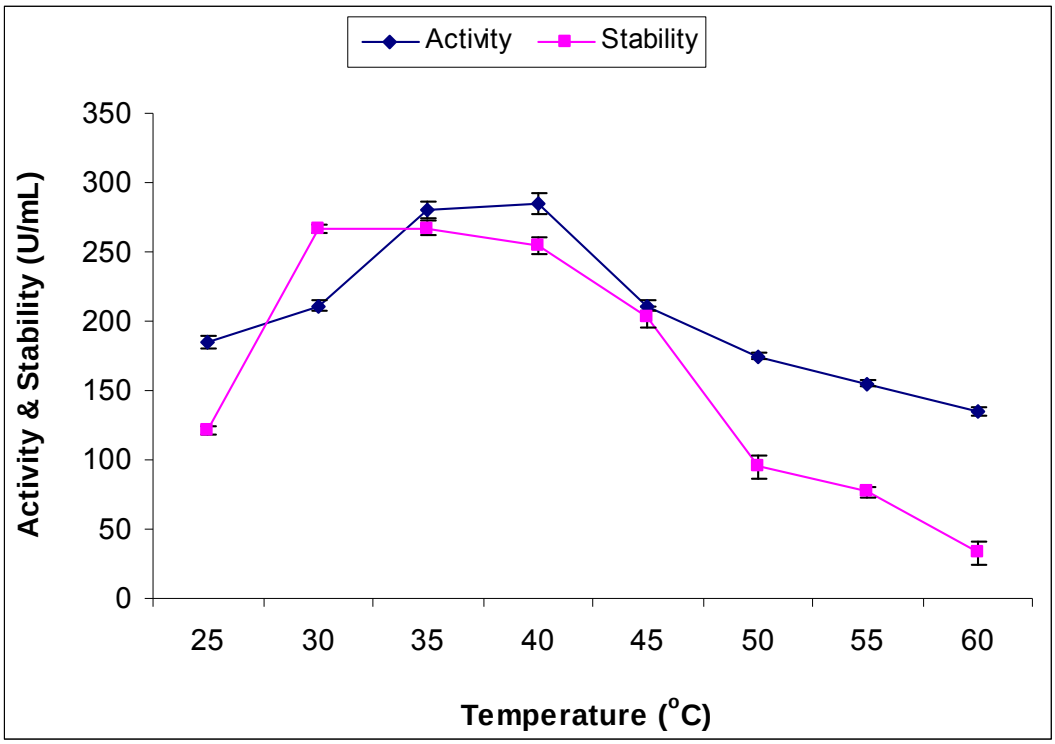



**Fig. 4.** Effect of temperature on activity and stability of laccase produced by *S. commune* IBL-06

*Determination of kinetic constants $K_M$ and $V_{max}$*

The $K_M$ and $V_{max}$ values for purified laccase were determined using varying 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. The reciprocal of laccase activity (1/[V]) in IU/mL was plotted against the reciprocal of substrate concentration (1/[S]) in µM (Fig. 5). The values of kinetic parameters $K_M$ and $V_{max}$ for purified laccase were 0.025 mM and 80 mM/min, respectively, using ABTS as substrate. 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. Very low $K_M$ and high $V_{max}$ values for this novel *S. commune* IBL-06 laccase reflect its higher affinity for ABTS and high catalytic efficiency as compared to a laccases from *Cerrena maxima* (Koroleva et al. 2002) and *Pycnoporus sanguineus* (Litthauer et al. 2007) that have been reported to show higher $K_M$ and lower *Vmax* values.

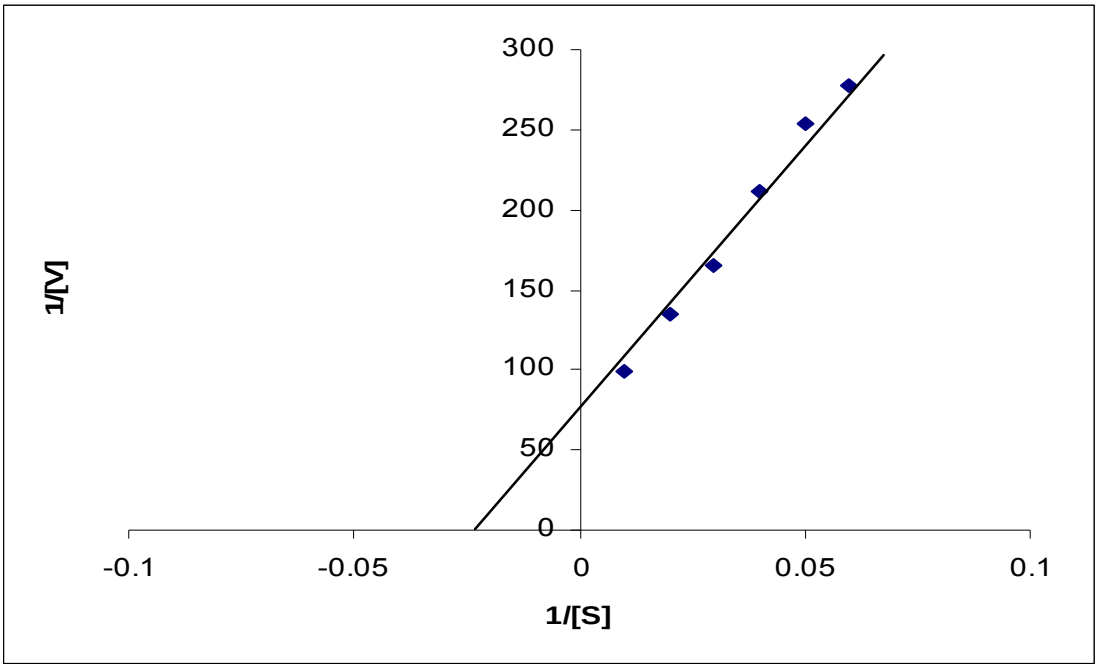



**Fig. 5.** Reciprocal plot for determination of $K_M$ and $V_{max}$ for laccase

*Effect of organic and inorganic activators/inhibitors*

Among the various ions and organic compounds used, only 1 mM $CuSO_4$ increased the activity of laccase. By contrast, with $AgNO_3$, TEMED, and marcapto-ethanol there was almost 25% reduction in laccase activity (Fig. 6), and the inhibitory effect of $MnSO_4$ was non-significant on laccase activity. The activation of laccase by $Cu^{2+}$ suggests that type-2 copper ions fill the copper binding sites (Sadhasivam et al. 2008; Nagai et al. 2002). However, *Sinorhizobium meliloti* CE52G laccase was found to be inhibited by $Cu^{2+}$ ions. EDTA is an inhibitor of metallo-enzymes including laccases due to its property of forming inactive complexes with inorganic prosthetic groups/ cofactors of the enzyme (Sadhasivam et al. 2008). However, this is not true for all laccases; *Marasmius quercophilus* and *Sinorhizobium meliloti* CE52G laccases were not significantly affected by EDTA (Rosconi et al. 2008; Dedeyan et al. 2000). Similar laccase activity inhibition by EDTA (1.0 mM), cystein (1.0 mM), NaCN (1.0 mM), $FeCl_3$ (1.0 mM), $CuCl_2$ (10.0 mM), mercaptoethanol (0.1 mM), reduced glutathione (0.1 mM), and $MnCl_2$ has also previously been reported, whereas strongest inhibition was observed with sodium azide (Stajic et al. 2006; Farnet et al. 2004; Ryan et al. 2003).

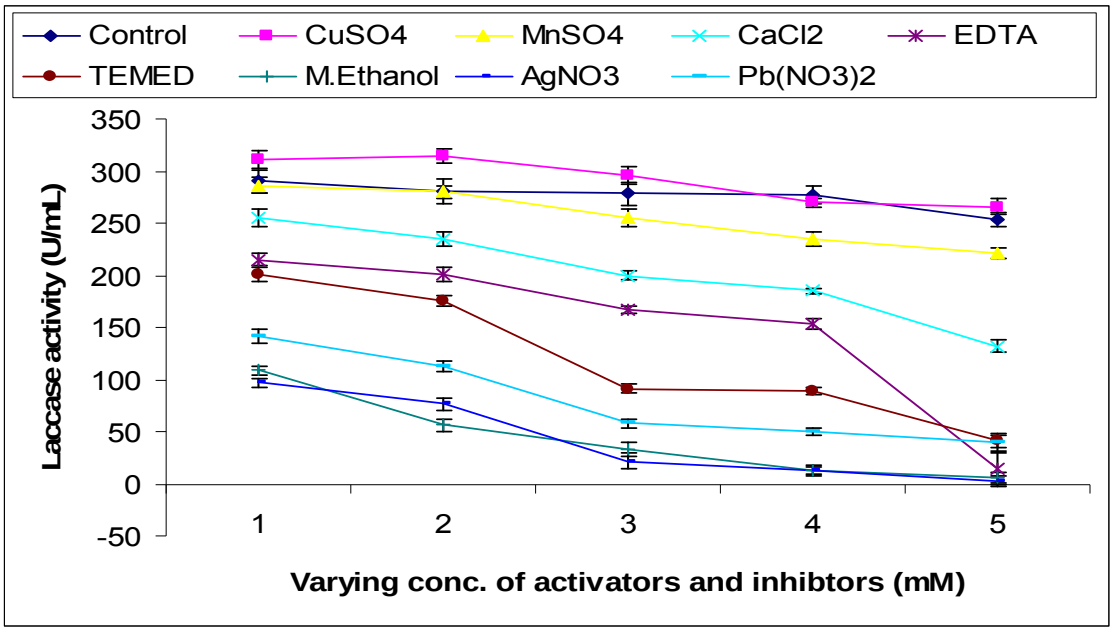



**Fig. 6.** Effect of metals ions and organic compound on laccase produced by *S. commune* IBL-06

## CONCLUSIONS

*S. Commune* IBL-06 produced a substantial amount of laccase in SSF of banana stalk under optimum conditions. The enzyme was stable at neutral pH but lost its activity in alkaline media. The lower $K_M$ and high $V_{max}$ values for purified laccase indicated that the enzyme had high affinity for its substrate ABTS and high catalytic activity as compared to most of the previously reported laccases. However, the enzyme had lower thermostability properties, which could be improved by enzyme engineering or molecular techniques. $CuSO_4$ increased the activity of laccase, but $AgNO_3$, TEMED and marcapto-ethanol caused enzyme inhibition.

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