PURIFICATION AND CHARACTERIZATION OF LiP PRODUCED BY *Schyzophyllum commune* IBL-06 USING BANANA STALK IN SOLID STATE CULTURES

Muhammad Asgher,[a,*] Muhammad Irshad,[a,b] and Hafiz Muhammad Nasir Iqbal [a]

Lignin peroxidase was produced from *Schyzophyllum commune* IBL-06 through solid state fermentation of an abundantly available agro-industrial waste, banana stalk, under pre-optimized conditions. LiP was fractionated by 65% saturation with NH_4SO_4 and dialysis to 1.5-fold purification. The enzyme was further purified by Sephadex G-100 gel filtration chromatography to 2.34 fold with specific activity of 468 U/mg. A single band of 80 kDa was obtained on native gel while on sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE), and two bands having molecular weight of 33 & 47 kDa were obtained, suggesting that LiP was a two polypeptide oligomeric protein. The present LiP from *S. commune* IBL-06 was optimally active at pH 5 and 35^{o}C. The stability assay showed that LiP retained activity in an acidic pH range of 4 to 6 and a temperature of 25 to 45°C after 24 h of incubation. Lignin peroxidase oxidized the vertry alcohol and showed kinetic constants K_M and V_{max} values of 0.46 mM and 388 mM/min, respectively. All organic and inorganic compounds inhibited *S. commune* LiP, but EDTA, β-Marcaptoethanol, and $Pb(NO_3)_2$ were the most inhibitory.

Keywords: Schyzophyllum commune IBL-06; Lignin peroxidase; Purification; Characterization

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

INTRODUCTION

White Rot Fungi (WRF) produces a wide range of hydrolytic enzymes including xylanases, cellulases, and ligninases. Lignin biodegradation by WRF involves the action of ligninolytic enzymes that have a promising potential for various biotechnological applications including biopulping, denim stone washing, bio-leaching, paper industry uses, animal feedstuffs, delignification, bio-ethanol production, and wastewater treatment (Revankar and Lele 2006; Stoilova *et al.* 2010; Asgher and Iqbal 2011; Asgher *et al.* 2012). WRF belong to the class basidiomycota, and together with the Ascomycota, they comprise the subkingdom Dikarya often referred to as the "higher fungi" within the kingdom Fungi. WRF are the most efficient and extensive lignocelluloses degraders due to their capability for the synthesis of hydrolytic and oxidative ligninolytic enzymes (Wesenberg *et al.* 2003; Sadhasivam *et al.* 2008).

Lignin peroxidases (LiPs) are extracellular glycosylated heme proteins secreted during the secondary metabolism and catalyze the H_2O_2-dependent oxidation of a variety

of lignin-related aromatic structures, such as aromatic amines, phenols, ethers, and polycyclic aromatic hydrocarbons (Asgher *et al.* 2007, 2008; Iqbal *et al.* 2011). From the biotechnological point of view, LiP is an important enzyme having potential applications to degrade highly toxic phenolic compounds from bleach plant effluents (Minussi *et al.* 2007). In recent years, a lot of work has been done on the development and optimization of bioremediation processes through ligninolytic enzyme systems (Revankar and Lele 2006; Asgher *et al.* 2008).

Schyzophyllum commune is probably the most widespread fungus found in every continent except Antarctica. It is an efficient wood-decaying fungus that causes white rot of soft woods. Banana is the most consumed fruit in the world, which creates an abundant magnitude of banana wastes that consists of lignocellulosic material. By keeping in mind the extensive industrial applications of LiP, the focus of this work was to purify and characterize LiP from an indigenous strain of *S. commune* IBL-06, which secretes LiP in high titers (Irshad *et al.* 2011) compared to the previously reported *Schyzophyllum* species under optimum physical and nutritional conditions.

EXPERIMENTAL

Chemicals and Lignocellulosic Substrate

Coomassie Blue, SDS, Sephadex G-100, EDTA, and ß-mercaptoethanol were purchased from Fluka and Sigma-Aldrich (USA). Banana stalk used as substrate for LiP production was collected from a local fruit market of Ghulam Muhammad Abad, Faisalabad, Pakistan.

Microorganism and Inoculum Development

S. commune IBL-06 was used as a test organism in the present study, and its pure culture was obtained from Industrial Biotechnology Laboratory, Department of Chemistry and Biochemistry University of Agriculture Faisalabad. For the preparation of inoculum, *S. commune* IBL-06 was grown in a liquid medium (pH 4.5) containing (g/L): glucose 2; $MgSO_4 \cdot 7H_2O$ 0.05; $CaCl_2 \cdot 2H_2O$, 0.1; NH_4Cl, 0.12, and thiamine, 0.001. After inoculation with fungus, incubation was carried out at 35°C (150 rpm) for 5 days to get a homogeneous spore suspension (10^7-10^8 conidia/mL).

Production of LiP in Solid State Fermentation

In triplicate flasks 5 g of banana stalk was moistened with Krik's basal medium of pH 4.5. Each flask was autoclaved, inoculated with 3 mL inoculum, and incubated at 35˚C for 3 days under pre-optimized conditions (Irshad and Asgher 2011). After 3 days, the fermented samples were harvested with the addition of 100 mL of 100mM tartrate buffer (pH 4.5).

Flasks were kept in a shaker (120 rpm) for 30 min followed by filtration and centrifugation (3,000 × g). The clear supernatant was utilized as crude enzyme extract. LiP activity (Tien and Kirk 1988) and protein contents (Bradford 1976) were determined as described previously (Asgher and Iqbal 2011; Irshad and Asgher 2011).

Purification by $(NH_4)_2SO_4$ Precipitation

Crude LiP obtained from *S. commune* IBL-06 was first centrifuged at 5,000 × g for 15 min at 4°C to get clear supernatant and then concentrated by freeze-drying. The supernatant was placed in ice, and crystals of ammonium sulfate were added to attain 60% saturation.

The mixture was allowed to stand overnight at 4°C. The precipitate was collected by centrifugation at 3000 × g for 20 min at 4°C. In the supernatant, more ammonium sulfate was added to attain different levels of saturation, and each time the mixture was kept overnight at 4°C and centrifuged as previously done. The pellets were dissolved in minimal volume of 100 mM sodium tartrate buffer of pH 4.5 and dialyzed against the same buffer to remove ammonium sulfate (Asgher and Iqbal 2011).

Gel Filtration Chromatography

After $(NH_4)_2SO_4$ precipitation, the pooled active fractions (having LiP activity) were passed through the sephadex G-100 column (2×25cm, Sigma, USA). Phosphate buffer (100 mM) with 0.15 M NaCl was used as an elution buffer at a flow rate of 1 mL/min. The active fractions (1 mL each) were pooled and monitored for LiP activity and protein content as described before.

SDS-PAGE

The purified LiP was run on SDS-PAGE for estimation of molecular weight, following the method of Laemmli (1970). The approximate molecular mass of LiP was determined by calibration against broad molecular weight range (21 to 116 kDa) molecular markers (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 LiP

The purified LiP was subjected to characterization through kinetic studies by studying the effect of pH, temperature, substrate concentration, and activator/inhibitors.

Effect of pH

LiP was assayed after incubating the enzyme in varying pH buffers ranging from pH 3 to 9 for 10 min. For stability studies, the enzyme was incubated for 24 h at varying pH. The buffers used were (0.2 M): potassium 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; and carbonate-bicarbonate buffer, pH 9.0.

Effect of temperature

Effect of varying temperature in the range of 25 to 60ºC on purified LiP activity and stability was also investigated. LiP was incubated in sodium tartrate buffer (100 mM, pH 4.5) at temperatures ranging from 25 to 60ºC for 10 min followed by standard LiP assay. For stability studies, the enzyme was incubated for 2 h at varying temperatures without substrate, and then the LiP was assayed.

Effect of substrate concentration: Determination of kinetic constants

The Michalis-Menten kinetic constants K_m and V_{max} were determined by investigating the effect of different concentrations (0.1 to 1 mM) of veratryl alcohol as catalytic substrate. The parameter values were obtained by plotting the reciprocal of reaction rate ($1/V_o$) against substrate concentrations ([S]) using the Lineweaver-Burk plot reciprocal transformation of the Michaelis-Menten rate equation.

Activators/inhibitors

The effects of varying concentrations (1 to 5 mM) of organic compounds (TEMED, Marcaptoethanol, EDTA) and metal ions (Cu^{2+}, Mn^{2+}, Ca^{2+}, Ag^{1+}, and Pb^{2+}) on LiP activity were also investigated. The enzyme was incubated at 45°C in the presence of 100 µL of 1 mM activators/inhibitors for 10 min, followed by routine LiP assay.

RESULTS AND DISCUSSION

Purification of LiP

Lignin peroxidase was produced from *S. commune* IBL-06 on banana stalks solid state cultures under some previously optimized fermentation conditions as described earlier (Irshad and Asgher 2011). The enzyme was salted out at 65% $(NH_4)_2SO_4$ saturation. After dialysis, the enzyme was purified to 1.75 fold with specific activity of 350 U/mg (Table 1). Active dialyzed fractions were pooled and run on Sephadex G-100 column and eluted fractions were monitored at 280 nm as shown in Fig. 1. After gel filtration, LiP was 2.34 fold purified with specific activity and percent yield of 468 U/mg and 5.2%, respectively. Previously, Sugiura *et al.* (2003) also used anion exchange and gel chromatography to purify LiP produced from *Phanerochaete sordida* YK-624. In another study, LiP from *Loweporus lividus* MTCC-1178 was purified by Amicon concentration and DEAE cellulose chromatography (Yadav *et al.* 2009).

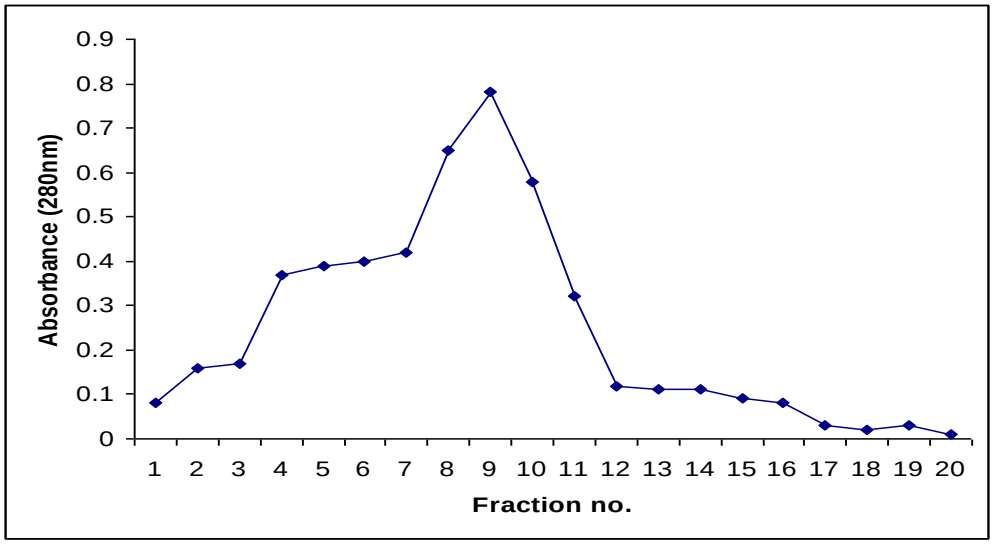


Fig. 1. Gel filtration chromatography of LiP produced by *S. commune* IBL-06

Table 1. Purification Summary for LiP Produced by *S. commune* IBL-06

Sr. No.	Purification Steps	Total Volume (mL)	Total Enzyme Activity (IU)	Total Protein Content (mg)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
1	Crude LiP	500	1372500	6843	200	100	1
2	$(NH_4)_2SO_4$ (65% saturation)	40	998520	3289	303	72	1.5
3	Dialysis	40	886563	2529	350	64	1.75
4	Sephadex-G100 gel filtration	12	69790	149	468	5.2	2.34

SDS-PAGE for LiP

The presence of a single band on Native PAGE (Fig. 2A) and two bands on SDS-PAGE (Fig. 2B) confirmed that the enzyme was an oligomeric protein consisting of two polypeptide chains. The molecular masses of the LiP polypeptides from *S. commune* IBL-06 were 33 and 47 kDa. Contrary to our finding, LiPs purified from most WRF cultures have been reported to be single polypeptides having molecular masses in the range of 37 to 50 kDa (Hirai *et al.* 2005; Asgher *et al.* 2007). However, different isozymes of LiP (38 and 40 kDa) have been reported from *Phanerocheate chryososporium* ATCC 20696 (Wang *et al.* 2008), while LiP isozymes from *Pleurotus sajorcaju* MTCC-141 were of 38 and 40 kDa (Yadav *et al.* 2009).

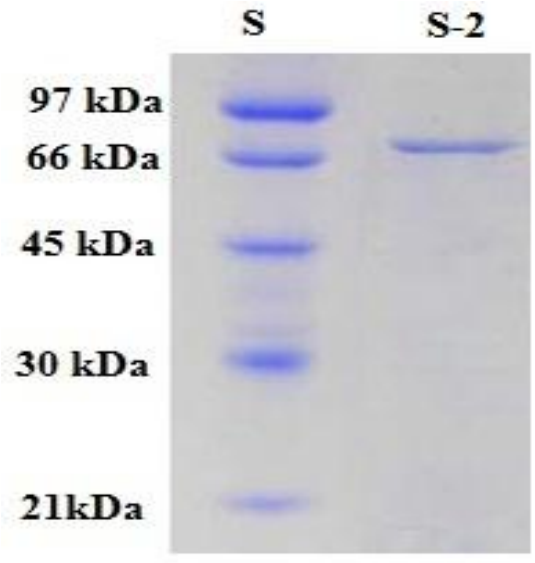
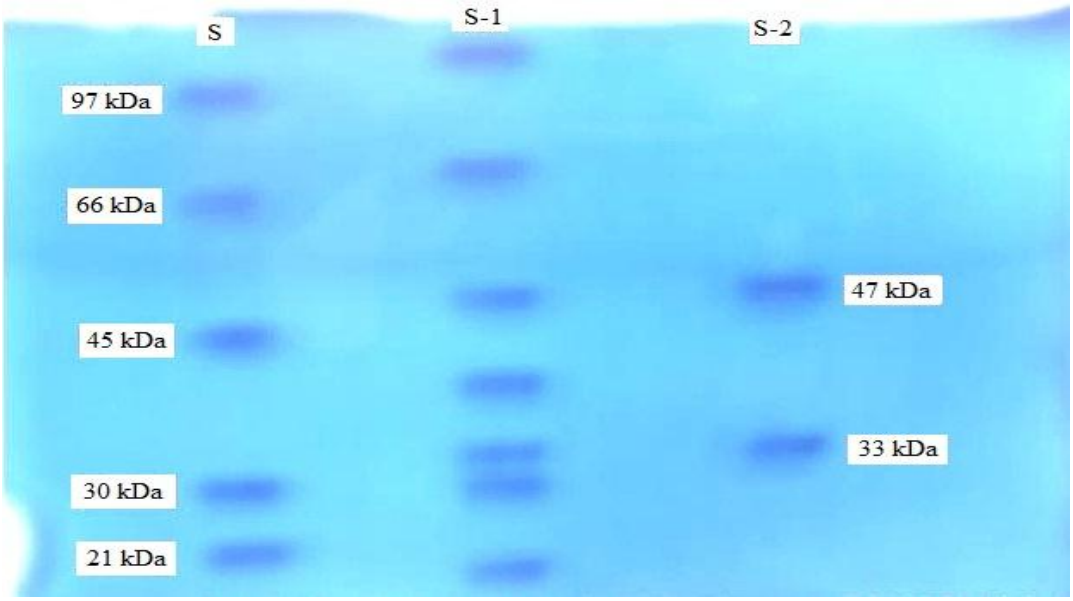


Fig. 2A. Native gel for *S. commune* IBL-06 LiP
[Lane S, Standard Protein Marker; Lane S-1, S-2, Purified Sample]



[Lane S, Standard Protein Marker; Lane S-1, Crude Sample; S-2, Purified Sample]

Fig. 2B SDS-PAGE for *S. commune* IBL-06 LiP

Characterization of LiP

Effect of pH on LiP activity

The pH-activity profile (Fig. 3) shows that activity of LiP increased with a rise in pH and it peaked at pH 5. A further increase in pH caused a gradual deactivation of the enzyme.

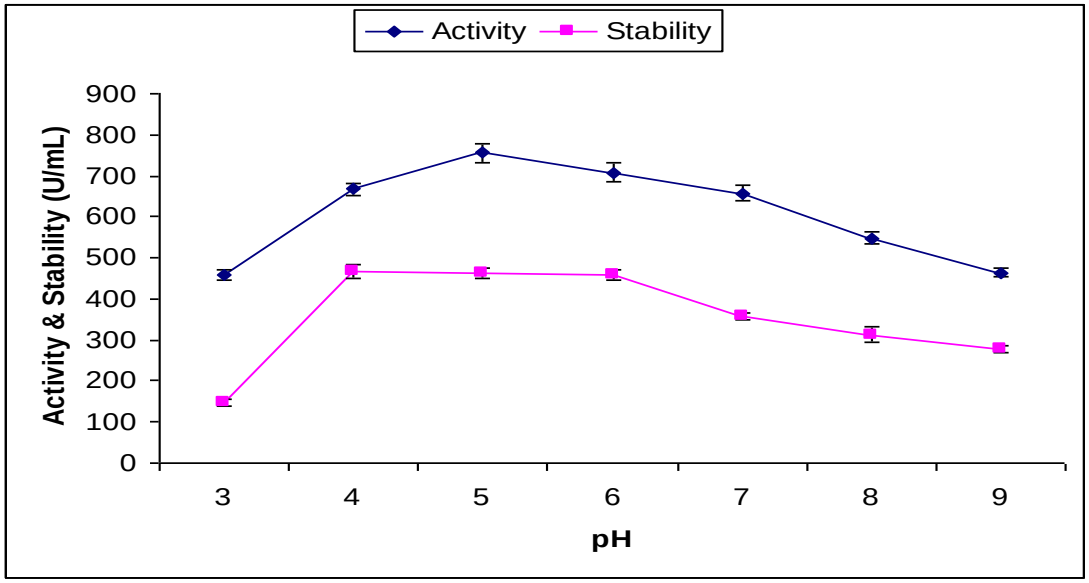


Fig. 3. Effect of pH on activity of LiP from *S. commune* IBL-06

The stability assay showed that LiP retained its activity in the acidic environment with a wider pH range of 4 to 6 after 24 h incubation time. The pH activity profiles of LiPs from different sources vary significantly and most LiPs have been reported to have optimum activities between pH 2 to 5 (Yang *et al.* 2004; Asgher *et al.* 2007; Snajdr and Baldrian 2007).

Effect of temperature on LiP activity

The purified LiP was incubated at varying temperatures, and optimal activity was recorded at 35°C. To investigate the stability of LiP, the enzyme was incubated at varying temperatures for 24 h. The stability profile showed that LiP remained reasonably stable in the temperature range of 25 to 45°C, as shown in Fig. 4. According to Yadav *et al.* (2009), LiP from *Loweporus lividus* MTCC-1178 was optimally active at 24°C, whereas LiP from *P. chrysosporium* showed better thermostability and was stable at 34°C (Rodríguez-Couto and Sanroman 2006).

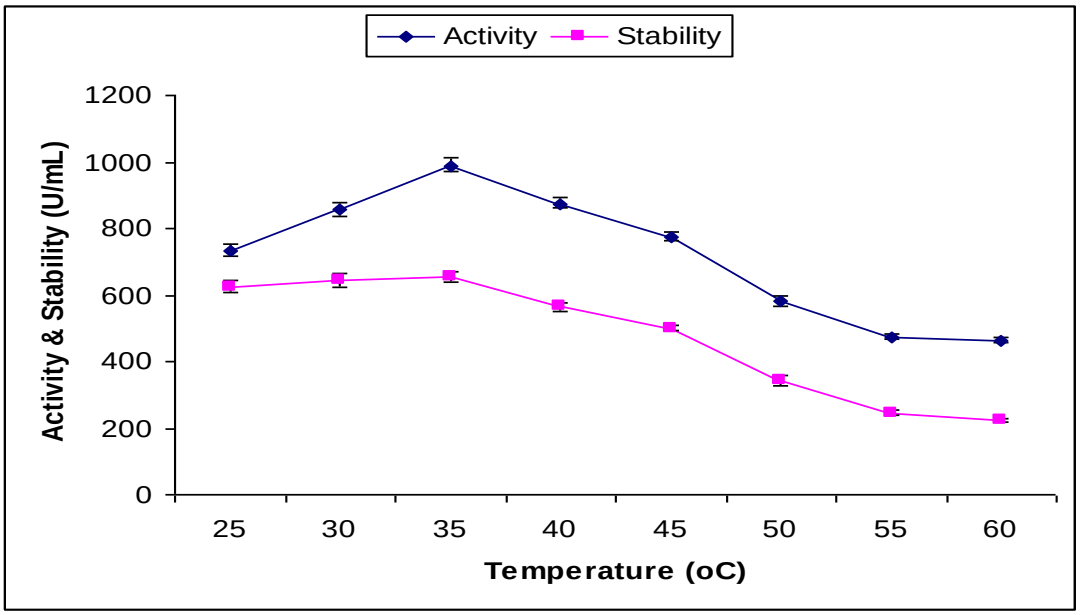


Fig. 4. Effect of temperature on activity of MnP from *S. commune* IBL-06

Determination of kinetic constants K_m and V_{max}

The effect of varying concentrations of varatryl alcohol (VA) on LiP activity was studied, and the data was used to plot a graph between 1/S and $1/V_o$ to determine the values of kinetic parameters. The values of K_m and V_{max} for LiP purified from solid-state culture filtrates of *S. commune* IBL-06 were 0.4 mM (400 µM) and 388 mM/min (Fig. 5). Lower K_m and high V_{max} suggested high affinity of LiP for VA and high catalytic efficiency, respectively. The K_m values for LiP from *Pleurotus sajorcaju* MTCC-141 for veratryl alcohol, n-propanol, and H_2O_2 were 57 µM, 500 µM, and 80 µM, respectively (Yadav *et al.* 2009). In another study, the K_m of LiP was 167 µM using VA as substrate (Hayatsu *et al.*1979). The difference in K_m values may be due to the genetic variability among different WRF species.

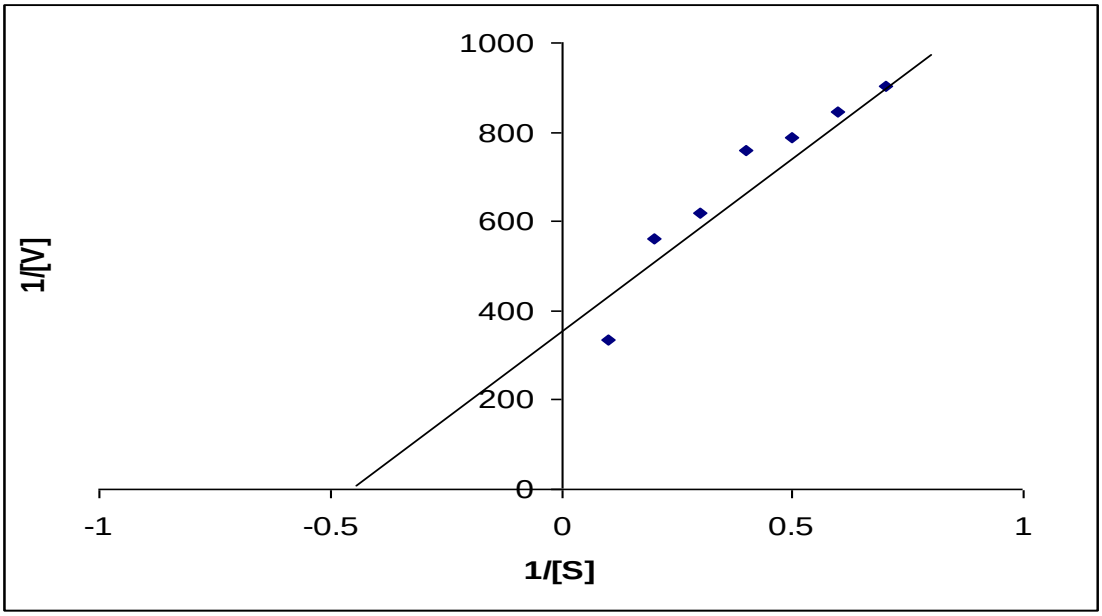


Fig. 5. Reciprocal plot of 1/[S] Vs 1/[V] for determination of *K*m and *V*max of LiP produced by *S. commune* IBL-06

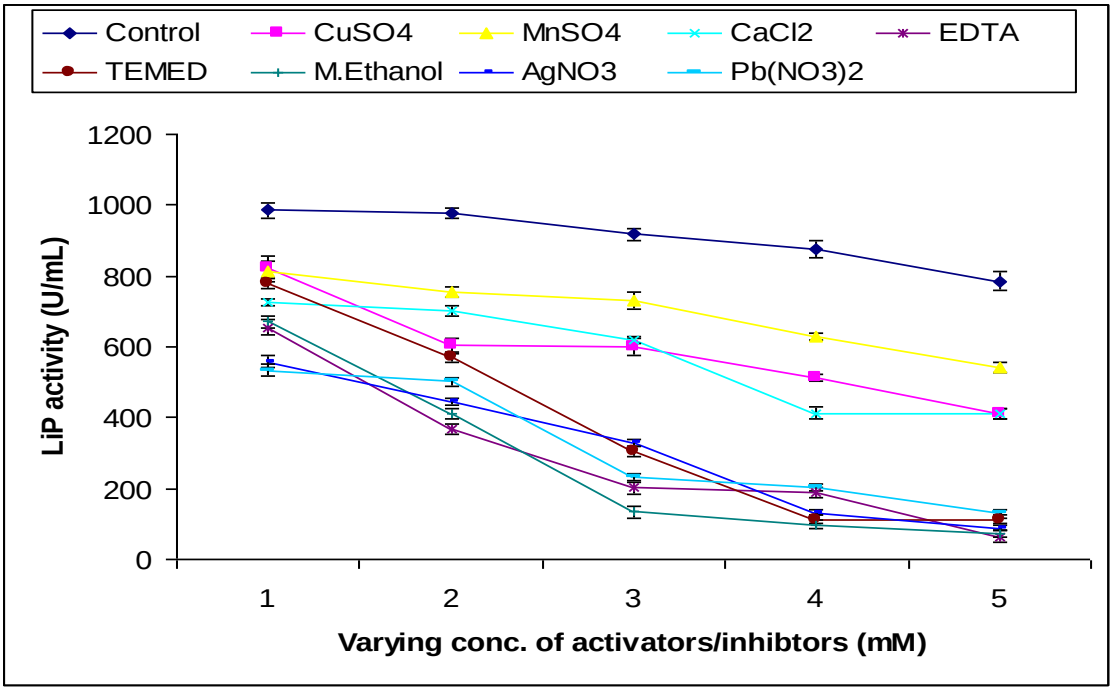


Fig. 6. Effect of activators/inhibitors on purified LiP produced by *S. commune* IBL-06

Effect of activators/inhibitors

The effect of different inorganic and organic compounds (1 mM to 5 mM) on LiP activity was investigated. All organic and inorganic compounds inhibited *S. commune* LiP. EDTA, β-Marcaptoethanol, and $Pb(NO_3)_2$ were found to be strong inhibitors of LiP activity (Fig. 6). EDTA inhibits LiP and the mechanisms of inhibition are different for

different substrates depending on the concentration (Chang and Bumpus 2001). Lignin peroxidase activity was inhibited about 90% by potassium cyanide, sodium azide, and the chelating agent, EDTA (Jeon *et al.* 2002; Asgher and Iqbal 2011). Previously, the addition of 2 mM TEMED and 2 mM EDTA has been reported (Chang and Bumpus, 2001) to cause 79 and 95% inhibition of *P. chrysosporium* LiP.

CONCLUSIONS

Lignin peroxidase from *Schyzophyllum commune* IBL-06 was found to be an oligomeric protein composed of two polypeptide chains. LiP was stable in an acidic pH and within the 25 to 45°C temperature range. A low value of K_m and a high V_{max} of the enzyme for varatryl alcohol suggested its high substrate affinity and catalytic efficiency.

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