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Screening, Production and characterization of lacasse from fusant strain of *Pleurotus species*

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Abstract: *The current work emphasizes on the screening, production and characterization of laccase from improved strain of Pleurotus species. Temperature stabilities of laccases were found to be 70°C. The optimum pH of the laccase differed with the substrate used. The optimum pH for the guaiacol was 7.0. The effect of different metal ions on the activity of laccase enzyme was investigated. Results showed that all the metal ions used exhibited inhibition effects to the enzyme activity but with different degrees. $FeSO_4$ showed complete inhibition effect on laccase enzyme. The metal ions KCl and NaCl have less effect on the enzyme activity where the enzyme retained about 71% of its initial activity. Effect of a range of potent laccase inhibitors on the enzyme activity was tested. The oxidase inhibitors sodium azide and sodium cyanide caused about 77.77 and 51.48% inhibition of laccase activity respectively. However the enzyme retained 69.04% of its activity in the presence of 1 mM SDS. Other inhibitors, EDTA and aniline have low effect on laccase activity. The effect of enzyme concentration on enzyme activity was carried out under standard conditions with varying amounts of enzyme (mg protein) added. The results showed that, the maximum laccase activity was obtained with 0.0036 mg protein of the purified enzyme per reaction mixture. The effect of substrate (guaiacol) concentration on the activity of the purified laccase enzyme was studied. Results indicated that the activity increased linearly up on increasing the guaiacol concentration to reach maximum at 40 mM and higher substrate concentrations than 40 mM resulted in a constant enzyme activity. The kinetic parameters (Km and Vmax values) of the purified laccase enzyme was determined by using guaicol as a substrate and calculated from Lineweaver and Burk plots. The results indicated that the Km value was 71.43mM and Vmax value was 142.86U/ml protein. The laccase from Pleurotus species was purified using DEAE-Cellulose and eluted with NaCl showed the highest laccase activity. The molecular mass of purified laccases was found to be approximately 43 kDa.*

Key Words: *Laccase, Purification, Pleurotus, DEAE- Cellulose, Kinetic parameters*

Introduction

Laccase was first demonstrated in the exudates of *Rhus vernicifera*, the Japanese lacquer tree **(Yoshida, 1883)** belongs to Anacardiaceae family. **(Huttermann** and **Mai *et al.*, 2001)**. Laccase (benzenediol) is an enzyme which belongs to the type oxidoreductase and the enzyme commission number is 1.10.3.2. Laccases are mostly extracellular glyco-proteins **(Archibald *et al.*, 1997; Heinzkill *et al.*, 1998)** and are multinuclear enzymes **(Gayazov** and **Rodakiewicz-Nowak, 1996)** with molecular weights between 60 and 80 kDa **(Heinzkill *et al.*, 1998; Leontievsky *et al.*, 1997; Thurston, 1994)**. Mushrooms constitute a source of important compounds comprising lectins, laccases, proteases, ribonucleases, ribosome inactivating proteins, antibacterial proteins, antifungal proteins, and polysaccharides **(Ng, 2004)**.Besides *Agaricus* spp. and *Lentinula* spp., *Pleurotus* spp. is very commonly cultivated mushroom in the world. It can be naturally found in tropical and subtropical rainforests, and can be artificially cultivated **(Dundar *et al.*, 2009)**. Laccase is responsible for pigment production in mycelia and fruiting bodies, improves cell-cell adhesion assists in the formation of rhizomorphs, and is also responsible for the formation of polyphenolic glue that binds hyphae together. Various plant pathogens also produce extracellular laccase that enable the fungus to overcome the immune response of the host **(Thurston, 1994)**. Laccase are widely distributed in higher plants, bacteria, fungi and insects. Strain improvement generally includes higher yield, better nutritional quality, colour and sporelessness. Strain improvement has been carried out using different techniques viz. Protoplast fusion **(Das** and **Mukheerjee, 1995)**, dikaryon mating **(Larraya *et al.*, 2001)** and interspecific hybridization **(Jaswal *et al.*, 2013)**. Protoplast mediated genetic transformation provides great potential for mushroom strain improvement with desired characters. It was proved potentially useful method for the improvement of desired traits in higher basidiomycetes **(Gold *et al.*, 1983)**.Induced

protoplast fusion can overcome vegetative incompatibility and produce hybrids with the combined properties of both parents **(Zhao** and **Chang, 1995)**. In this study, we preferred *Pleurotus* species for the screening of laccase because of its potentiality.

Materials and methods

Sample collection: The improved strain, fusant of *Pleurotus* species **(Aswini *et al.*, 2014)** was collected from Dr.MGR Janaki College of Arts and Science for Women, Chennai.

Screening for laccase production: The *Pleurotus* culture was inoculated in Czapek dox agar amended with Guaiacol (0.1mM) a substrate and the plates were incubated at 27°C for 5days. The substrate utilized reddish brown color in screening medium indicates the positive strain.

Production of laccase

Media optimization: Various parameters affect the production of enzyme in the medium. The tested parameters include pH, temperature, metal ions and inhibitors.

Enzyme production: The mycelium of improved strain fusant of *Pleurotus species was* separately inoculated into laccase production medium. The medium was incubated at 37°C in a rotary shaker. The amount of enzyme produced in the medium was assayed at regular intervals.

Laccase assay: Laccase activity was determined using guaiacol as a substrate according to the method of **Jhadav *et al.*, 2009** with some modifications. The culture filtrate was centrifuged at 5000rpm for 10 minutes and the supernatant was used as enzyme source. Each test tube containing 3ml of sodium acetate buffer (10mM pH 5), 1ml of enzyme source, and 1ml of guaiacol was taken. The blank contained 1ml of distilled water instead of enzyme source was taken. The mixture was then incubated at 30°C for 15 minutes and the absorbance was read at 450nm using UV Spectrophotometer. Enzyme activity was expressed as IU/ml.1 IU/ml is defined as amount of enzyme is required to oxidize 1 micromole of substrate. Enzyme activity was calculated using the formula,

$$\text{Enzyme activity} = \frac{\text{Absorbance at 450nm (A) x Total volume of reaction mixture (v)}}{\text{Incubation time (t) x Extinction coefficient (e) x Enzyme volume (v)}}$$

(Extinction coefficient of guaiacol = 12, 100 M-1 cm-1)

Purification of laccase

Partial purification was done by Ammonium sulphate precipitation and Dialysis techniques.

DEAE-Cellulose Chromatography (Das *et al.*, 2001): The DEAE- Cellulose was pre-equilibrated with 100mM of sodium phosphate buffer (pH 6). The dialysate was loaded onto the column and washed with 500ml of sodium phosphate buffer to remove unbound sample components. Different concentration of NaCl (0.2-1.0M) was dissolved in 100mM of sodium acetate buffer and it was used to elude the bound protein from the column. Eluted fractions were collected and assayed for laccase activity.

Characterization of laccase

Certain parameters for purified laccase was estimated viz effect of pH, temperature, inhibitors and metal ions. The concentration of protein and carbohydrate was determined by the method of **Lowry *et al.*, (1951)** and **Dubois *et al.*, (1956)** respectively.

Kinetic constants: The Michaelis –Menten kinetic parameters Km and Vmax were determined by measuring the laccase activity using various concentration of guaiacol as substrate. The parameter values were obtained by curve fitting of the reciprocal plot of reaction rate versus substrate concentration using the Lineweaver-Burk plot transformation of Michaelis – Menten rate equation.

SDS-PAGE: The molecular weight of laccase was determined by SDS-PAGE.

Results

Selection of strain: The fusant strain of *Pleurotus* species (Plate 1, 2) was selected for the screening of laccase enzyme

Plate 1: Fruiting bodies of *Pleurotus species* Plate 2: Fusant strain of *Pleurotu speciess*

Screening of *Pleurotus* for laccase production: *Pleurotus* species was screened for the production of laccase on Czapek dox agar amended with substrate guaiacol. The oxidation of guaiacol was observed as reddish brown color in screening medium, which was shown in Plate 3.

Plate 3: Reddish brown color pigmentation showing the presence of laccase on Czapek dox agar.

Production of laccase: *Pleurotus* species was grown on laccase production media for 3 days and activity of laccase was determined by Jhadav *et al*, 2009 method. *Pleurotus* showed laccase activity in the production media and it was monitored at 450 nm. The production media was optimized for various parameters including pH, temperature, inhibitors and metal ions. The results were shown in Figure 1, 2, 3 and 4 respectively. According to the results the activity of laccase is high in pH 7 and it shows thermostability at 70°C.Among the inhibitors tested, the laccase activity is highly inhibited by SDS (90%) followed by Sodium azide and Aniline and EDTA does not shows significance inhibition. Among the metal ions tested, the presence of $FeSO_4$ shows significant result (79%) followed by NaCl and KCl.

Figure 1: Optimization of laccase production medium –pH

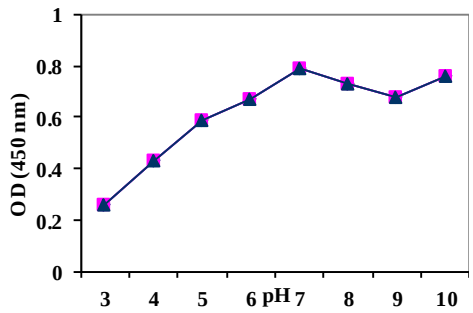


Figure 2: Optimization of laccase production medium-Temperature

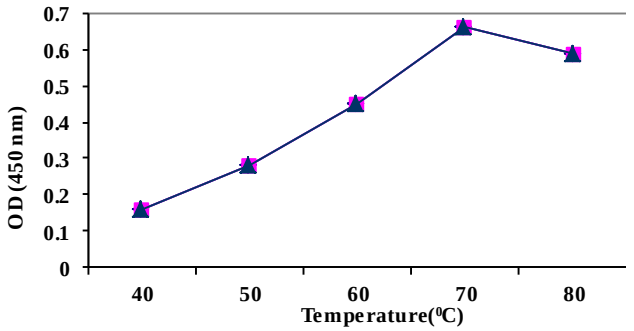


Figure 3: Optimization of laccase production medium-Inhibitors

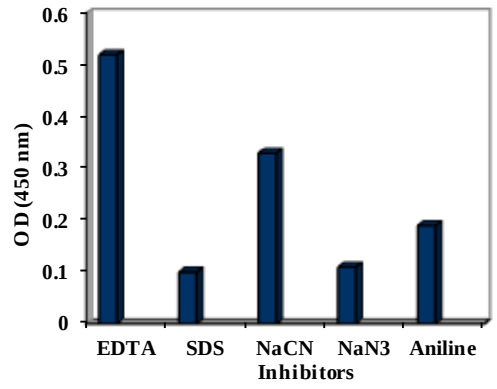
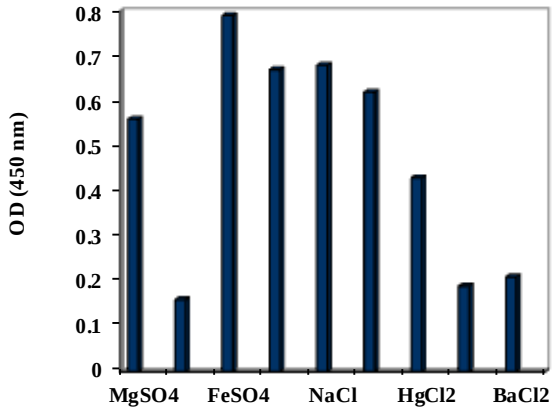


Figure 4: Optimization of laccase production medium-Metal ions



Laccase assay: The laccase enzyme was produced using optimized medium and the laccase activity was measured at regular intervals at 450 nm. It was tabulated in Table 5 and shown in Figure 5 respectively. The laccase activity is high in 3rd day since the rate of utilization of substrate is more followed by day 4. The enzyme activity for laccase was found to be 677.6 IU/ml.

Enzyme purification: The enzyme laccase was partially purified by performing ammonium sulphate precipitation and dialysis method.It was further subjected to DEAE-cellulose chromatography technique for purification. The summary table for enzyme purification was tabulated in Table 1

. **Table 1: Summary and purification procedure of *Pleurotus* species**

Purification steps	Volume (ml)	Total laccase activity (U)	Total protein (µg)	Specific activity of laccase (U/ µg)	Purification Fold	Yield (%)
Crude enzyme (culture filtrate)	1000	850	400	2.12	1.0	100
Ammonium sulfate precipitation (80%)	25	560	160	3.5	1.44	69.4
DEAE-Cellulose	15	430	60	7.16	2.95	48.8

Enzyme characterization: The purified laccase was characterized for various effects which include pH, temperature, inhibitors, and metal ions. The results were shown in Figure 5, 6, 7 and 8. After DEAE-Cellulose purification method, the laccase enzyme shows significant difference in effect of inhibitors where SDS shows 100% inhibition. The other parameters i.e., pH, temperature and effect on metal ions does not show any significant difference.

Figure 5: Effect of pH on Purified laccase

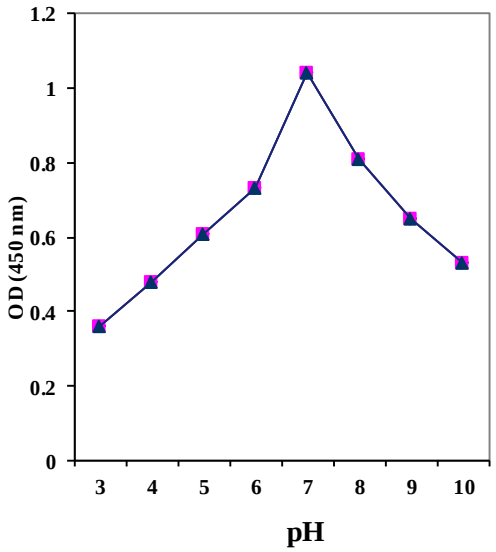


Figure 6: Effect of temperature on Purified laccase

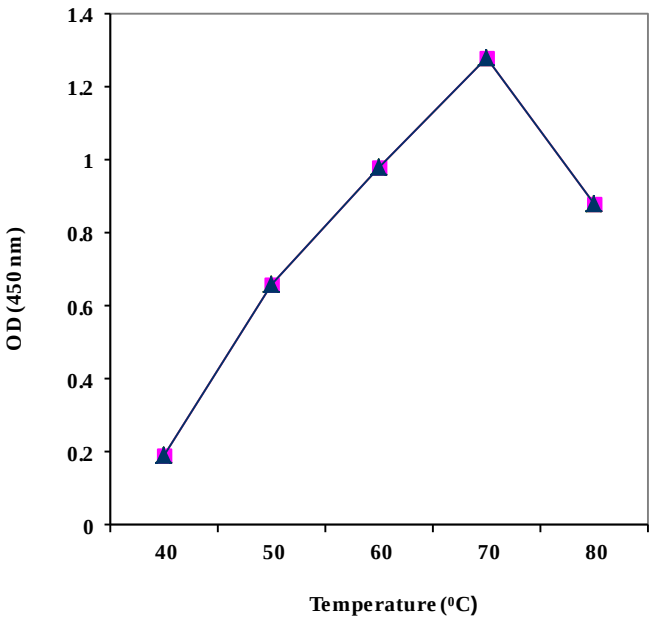


Figure 7: Effect of inhibitors on Purified laccase

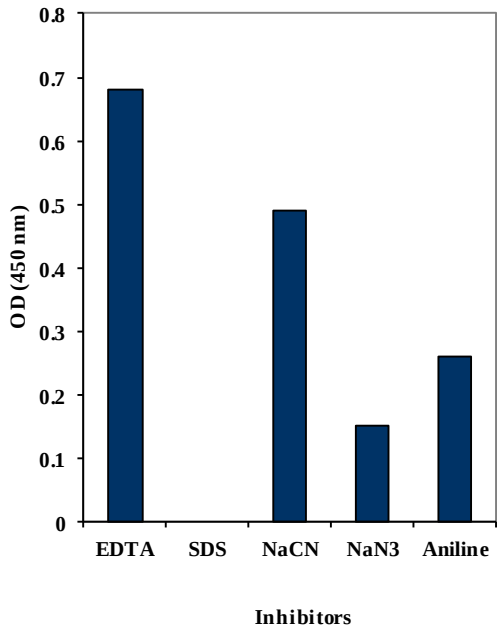
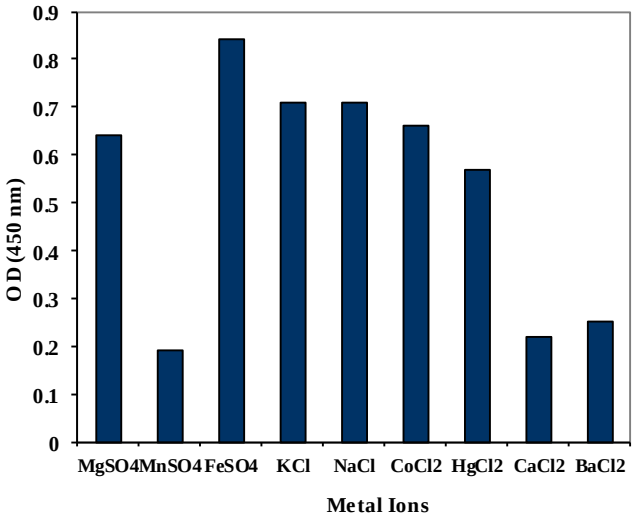


Figure 8: Effect of metal ions on Purified laccase



Estimation of total protein: The total protein concentration of purified laccase was determined by Lowry's *et al.*, method using BSA as standard. The total protein content for the purified laccase was found to be 0.4 mg/ml and 400 µg/ml.

Estimation of total carbohydrate: The total carbohydrate content of purified laccase was determined by Dubois *et al.*, method using mannose as standard. The total carbohydrate content was found to be 2.75mg/ml.

Determination of molecular weight by SDS-PAGE: The molecular weight of purified laccase was determined by SDS-PAGE method and it was found to be 43 KDa .The results were shown in Plate 4.

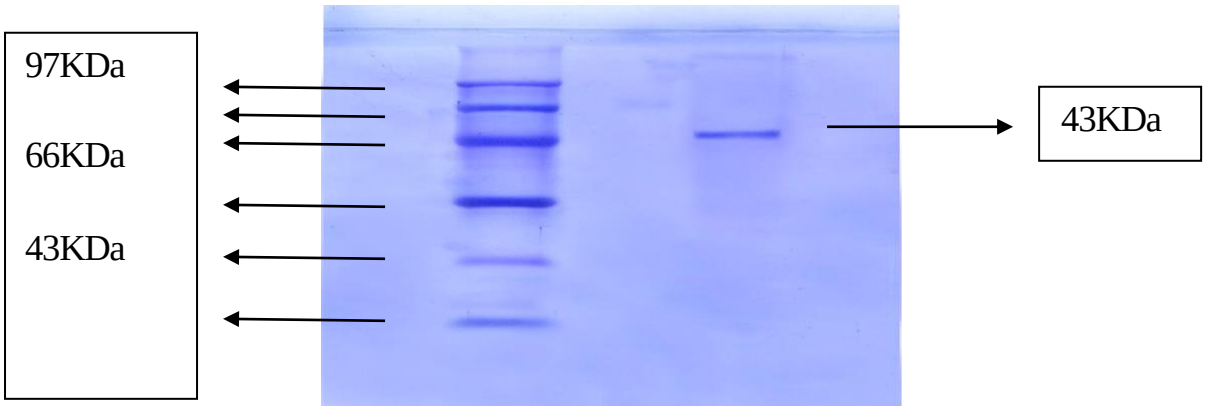
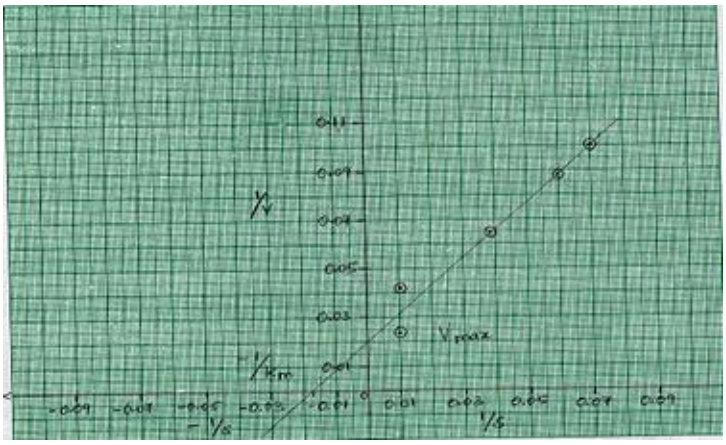


Plate 4: SDS- PAGE gel showing discrete band for laccase enzyme from *Pleurotus species*

Determination of Km and V max value through reciprocal plot: Km and V max value through reciprocal plot for the purified laccase was found to be 71.43 and 142.86 respectively and the graph was shown in Fig. 9.

Figure 9: Determination of Km and V max value through reciprocal plot



-1/Km = -0.014; **Km = 71.43,** Km = Vmax/2, Vmax = 71.43 X 2, **Vmax = 142.86**

Discussion

The general properties of the purified laccase enzyme were studied. Depending on the source of the microorganism, thermal stability varied at different temperatures. *Pleurotus ostreatus* laccase was almost fully active in the temperature range of 40°C- 60°C and showed a half-life of 30 minutes at 60°C. The thermal stability of enzymes may be influenced by the presence of hydrophobic or charged residues, which increase enzyme rigidity and restrict conformational changes during substrate binding **(Fields *et al.*, 2004)**.This result to some extent was similar to that reported by **Liers *et al.*, 2007** who found that the highest activity of *Xylaria polymorpha* laccase enzyme was observed between 55 and 60°C.

The optimum pH of the laccase differed with the substrate used. The optimum pH for the guaiacol and the *o*-dianisidine was 6.0 and 5.5 respectively. The other two substrates 1-napthol and pyrocatechol optimum activity lies at pH 5.0. The variation of pH with respect to the substrate was reported by **Palmieri *et al.*, 1997**. The highest activity of the *Pleurotus ostreatus* laccases with respect to pH profile also varied with the changes of substrate. The variation of optimum pH might due to different role of substrate protonation in the reaction mechanism **(Palmieri *et al.*, 1993; Youn *et al.*, 1995)** .Our study showed that the enzyme laccase have good stability at neutral and slightly alkaline pH values *Pleurotus pulmonarius* **(De souza and Peralta, 2003)** using guaiacol as the substrate.

The effect of different metal ions on the activity of laccase enzyme was investigated. Results showed that all the metal ions used exhibited inhibition effects to the enzyme activity but with different degrees. $FeSO_4$ showed complete inhibition effect on laccase enzyme while in the presence of $CoCl_2$, $MgSO_4$ and $HgCl_2$ the enzyme retained about 66.01, 64.38 and 57.58% of its initial activity, respectively. The metal ions KCl and NaCl have less effect on the enzyme activity where the enzyme retained about 71% of its initial activity, the other metal ions $BaCl_2$, $CaCl_2$ and $MnSO_4$ have little effect on the enzyme activity. **Sadhasivam *et al.*, 2008** showed that the metals such as Co, Hg, Fe, K, Mg, Mn, Na, Ba and Ca at a concentration of 1 mM had no significant effect over *Trichoderma harzianum* WL1 laccase activity except Hg, which caused 17.2% inhibition. On the other hand the purified laccase from the edible mushroom *Lentinula edodes* was inhibited in the presence of 1 mM Sn2+ (99%), Ca2+ (70%), Zn2+ (64%), Hg2+ (55%), K+ (54%) and Cd2+ (45%) **(Nagai *et al.*, 2002)**. The observations indicated that the effect of metal ions on laccase activity was highly dependent on its source and the type of metals used, which had a great influence on the catalytic activity of the enzyme.

Effect of a range of potent laccase inhibitors on the enzyme activity was tested. The oxidase inhibitors sodium azide and sodium cyanide caused about 77.77 and 51.48% inhibition of laccase activity, respectively. However the enzyme retained 69.04% of its activity in the presence of 1 mM Sodium dodecylsulfate (SDS). Other inhibitors, EDTA and aniline have low effect on laccase activity, similar to *T. harzianum* laccase which was mildly inhibited by the metal chelator EDTA 1 mM concentration (16.8% inhibition).*T.atroviride* laccase was strongly inhibited by the typical laccase inhibitor sodium azide, but it was not sensitive to EDTA and SDS**(Chakroun *et al.*, 2010).** Laccase can be inhibited when the inhibitor binds strongly to it stopping further catalysis of the reaction. This occurs when the Cu at the catalytic center is removed/chelated or by competing for O_2, which is the specific co-substrate of laccase.

The effect of enzyme concentration on enzyme activity was carried out under standard conditions with varying amounts of enzyme (mg protein) added. The results showed that, the maximum laccase activity was obtained with 0.0036 mg protein of the purified enzyme per reaction mixture. On the other hand, mg protein of the crude enzyme per reaction mixture was the best concentration for the maximum enzyme activity. This result indicated that the crude sample contains high amount of protein with low laccase activity but the pure sample contains low protein with high enzyme activity.

The kinetic parameters (Km and Vmax values) of the purified *Pleurotus* laccase enzyme was determined by using guaicol as a substrate and calculated from **Lineweaver** and **Burk plots (1934)**. The results indicated that the Km value was 71.43mM and Vmax value was 142.86U/ml protein. This result is lower than that reported by **Chakroun *et al.*, 2010** who found that the Km value of *T. atroviride* laccase enzyme was 2.5 mM towards ABTS. This observation of the different Km values confirms the suggestion of **Banerjee and Vohra, 1991** who attributed these differences to be due to alteration of culture conditions, pH and substrate used.

Carbohydrate content of purified laccase enzyme was estimated to be 23% of the enzyme sample, while it contained 77% protein. This result is similar to that showed by Xiao *et al.*, 2004, who indicated that *Trametes sp.* AH28–2 laccase B was a monomeric glycoprotein with 25% carbohydrate content. This agree with that is known about fungal laccases where laccase typically contains 15–30% carbohydrate, which may contribute to the high stability of the enzyme.

The laccase obtained from *Pleurotus* species was purified using DEAE-Cellulose and eluted with NaCl showed the highest laccase activity. This result shows similarity with the results of laccases from the mushroom *Pleurotus eryngii* [70]. The molecular mass of purified laccases was found to be approximately 43 kDa, as determined by SDS-PAGE. The SDS-PAGE results showed that *Pleurotus species* laccase has a molecular weight of 43KDa close to that of laccase from *P. chrysosporium* having molecular weight of 46.5 KDa which is in the range reported for other Basidiomycetes **(Yaropolov, 1994).** Molecular weight of most fungal laccase protein falls between 43,000 and 110, 00 Dalton **(Xiao, 2003).**

Conclusion:

The laccase from *Pleurotus species* was purified to apparent electrophoretic homogeneity with 43 KDa recoveries and the purification fold was 2.9. The purified enzyme exhibits narrow optimum pH and temperature 7.0 and 70 °C respectively. The thermostable property and its wide range of substrate oxidation, the purified laccase may be considered as a good choice for industrial applications. Because of the high yield and easy purification procedure laccase enzyme could be of interest for the biotechnological applications that have been suggested for laccases from other fungal species. The purified laccase has many desirable characteristics such as abundant production, wide substrate oxidation and reasonable thermostable property.

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