

## PRODUCTION, PURIFICATION AND CHARACTERIZATION OF LACCASE FROM WHITE ROT FUNGUS.

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**ABSTRACT:** Laccases are oxidoreductases enzymes that belong to the sub class copper oxidases. They are mainly produced by white rot fungi which are involved in lignin metabolism. The objective of this research was the effective utilization of agro wastes for the economical production of laccase from, fungi *Pleurotus florida*. Submerged fermentation was employed for the production of enzyme and agro wastes namely rice husk, wheat bran and sugarcane bagasse used as substrates. The activity of enzyme was analyzed spectrophotometrically at 420nm, using ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as a substrate. The maximum production was obtained with sugarcane bagasse 9.9 IU/ml at day 9. The optimum temperature and pH were found to be 30°C and 7. The best nitrogen source optimized was ammonium sulphate which enhanced the production up to 11.57 IU/ml. The large scale batch (1litre) with already optimized conditions produced 13460 IU/L of enzyme. Purification was performed and molecular weight was determined at 90kDa through SDS-PAGE. Characterization of enzyme was done in terms of pH, temperature and effect of inducers and inhibitors.

**Keywords:** Submerged fermentation, ABTS, SDS-PAGE, Substrate, optimum production.\

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### INTRODUCTION

Laccase (oxidoreductase, EC 1.10.3.2) is blue copper dependent oxidases which is a main ligninolytic enzyme produced by white rot fungus. Laccases catalyze the oxidation of large numbers of phenolic compounds (Kunamneniet. al., 2007 and Poonkuzhaliet. al., 2011). These enzymes have a molecular weight of 60-90 kDa and consist of 15-30% carbohydrates. Laccase was initially found by Yoshida in 1883 in the sap of Japanese lacquer tree named as *Rhus vernicifera*. Later in 1896, Bertrand and Laborde determined that laccase is a fungal enzyme. (Shraddha et. al., 2007 and Giardina et. al., 2010).

Laccases are extensively present in nature, originating from plants, bacteria and fungi (Poonkuzhali and Palvannan, 2011). In fungi, laccases are widely distributed in *ascmycetes*, *deuteromycetes* and *basidiomycetes*. The laccase producing fungi include *Trametes versicolor*, *Pleurotostreatus*, *Polyporus*, *Trametespubescens*, *Cerrenaunicolour*, *Phanerochaetechrysosporium* and *Funa liatrogii* etc which have been reported by (Dwivediet. al., 2011). Laccases occur more in fungi, than in the higher plants. These are also present in few bacteria such as *S.lavendulae*, *S.cyaneus*, and *M.mediterranea* (Viswanathet. al., 2008 and Arias et. al., 2003). In vegetables laccases have been recognized in turnips, apples, pears, cabbages, potatoes, beets,

asparagus and various other vegetables (Jhadav et. al., 2007).

Fungal laccases have boundless biotechnological functions across the globe like the decolouration and detoxification of industrial effluent, bleaching of pulp, phenolics elimination from wine, in preparation of biosensors in detergents blocking dye transfer- functions (Yaveret. al., 2001). It is also used in the formation of anticancer drugs, including in few cosmetics for reducing their toxicity (Couto and Herrera 2006). In recent years, laccases have been skillfully used to the field of nanobiotechnology due to their capacity to mobilize electron transfer reactions without further addition of cofactor (Shraddha et. al., 2007).

The advantages of agro-industrial leftovers for the cultivation of media is of immense concern, as agriculture waste cuts down the expenditure of enzyme production and enhances the understanding on energy protection and recycling (Mansur et. al., 2003). These agriculture wastes are comparatively economical and also contain ample nutrients such as lignin, cellulose and hemicellulose. These nutrients serve as inducers to energize the production of enzyme (Vassil et. al., 2000). Due to these properties these agricultural waste can be used as substrate for the production of ligninolytic enzymes during the process of fermentation.

The huge potential of laccases requires advancement in its production (Herrera et. al., 2007). Pakistan being an agricultural country manufactures tons

of agricultural by products every year. These agricultural wastes are accessible in the markets at a very reasonable price (Minussiet. *al.*, 2007). The major purpose of conducting this research was to design an optimized fermentation process through which a large amount of this enzyme can be prepared from agricultural wastes.

## MATERIALS AND METHODS

**Fungus Maintenance and storage:** An unknown fungus was isolated from a papaya and was further processed for its identification and its culture was revived. The media used for this purpose was reported by (Tien and Kirk, 1998). The chemical composition and quantity of the media was  $\text{KH}_2\text{PO}_4$  0.42 gm,  $\text{CaCl}_2$  0.08gm,  $\text{MgSO}_4$  0.06, glucose 1gm,  $(\text{NH}_4)_2\text{SO}_4$  0.4gm, yeast extract 0.8gm, agar 3gm and peptone 0.8 gm. The culture was maintained on both slants and petri plates and stored at 4°C.

### IDENTIFICATION OF THE FUNGUS

**Spawn Preparation:** The spawn was prepared from barley seeds, inoculated with the white rot fungus and incubated at 25°C.

**Preparation of Mushroom substrates bags:** Agro wastes were used for the preparation of substrate bags. After preparation the bags were inoculated with spawn and incubated at 25°C. When the mycelium was fully grown the bags were placed on the shelf of mushroom cultivation room. Water spraying was done thrice a day. The room temperature was recorded on daily basis which varied from 25.5°C to 28°C. Once the pin heads of the mushroom matured, its characteristics were noted down and identified (Narayanasamy *et. al.*, 2008).

### FERMENTATION OF LACCASE

**Inoculum preparation:** The inoculum was prepared using Kirk's and Tein broth. The inoculated flask was placed on shaker at 130 rpm at room temperature. After every 24 hours the absorbance of the media was taken on UV spectrophotometer at 620 nm and simple media was used as control. When the absorbance of the inoculum reached at 1.6 A, 5ml inoculum was used for further inoculation of fermentation batch.

**Fermentation medium for laccase production:** Kirk's medium was used for laccase production having a quantity and composition of 0.21g  $\text{KH}_2\text{PO}_4$ , 0.04 g  $\text{CaCl}_2$ , 0.03 g  $\text{MgSO}_4$ , 1.5 g glucose, 0.2 g  $(\text{NH}_4)_2\text{SO}_4$  and 0.4 g yeast extract respectively (Tien and Kirk, 1998). Each flask was containing 100 ml liquid media. The pH of the above medium was 5.5-5.7. After cooling the media was inoculated with organism under sterilized conditions. The culture flasks were prepared in triplicates and incubated on an orbital shaker at 130 rpm.

**Optimization of Fermentation parameters:** On the basis of experimental data, the parameters for the production of enzyme were optimized. Different quantities of wheat bran, sugar cane bagasse and rice husk were used as 1gm, 2gm, 3gm, 4gm and 5gm respectively, to find the optimum substrate concentration.

The substrates were dried at 50°C in electric oven and then grinded. The substrates were sieved through 425 micron mesh sieve (Retsch Test sieve, No.40) to get the fine particle size for fermentation media. Triplicates of each were prepared.

The incubation time period varied from 2 to 10 days. The process of fermentation was carried on different temperatures ranging from 20 to 50°C. The pH of the medium was altered from 3 to 8 for optimal production of laccase. The pH of the media was adjusted at 0.1 N HCl to attain acidic pH and 2M NaOH to maintain basic pH. Three different nitrogen sources i.e Maize steep liquor, yeast extract and ammonium sulfate were optimized.

**Enzyme Assay:** Enzyme production was monitored using ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). After every 24 hours the activity of enzyme was checked. The enzyme mixture was filtered and 0.1ml of enzyme was used for this purpose. The buffer used was 1mM sodium acetate buffer pH, was 5.5 (1ml) and 1mM of ABTS (1ml). The mixture was spectrophotometrically analyzed at 420 nm. The control used was buffer, ABTS, and distilled water in same amounts as used for mixture of enzyme. The activity of enzyme is explained in IU/ml and one unit was expressed as 1micro mole of substrate oxidized in the provided incubation time (Han *et. al.*, 2005 and Irshadet. *al.*, 2011).

The enzyme activity was calculated by the following formula.

$$\text{Activity (IU/ml)} = \frac{\text{umol of substrate / ml of laccase}}{\text{Time of incubation} \times \text{Mol.wt of ABTS}}$$

### PURIFICATION OF ENZYME

**Ammonium sulfate precipitation:** The enzyme to be purified was first precipitated from the fermentation media by using ammonium sulphate. The best saturation at which high amount of protein precipitated was 70% at 4°C, after following the procedure of (Patrick *et. al.*, 2009).

**Dialysis:** After precipitation the excess salts in the enzyme solution was removed by the dialysis using 1mM sodium acetate buffer at a pH of 5.5 by using 12-14 kDa cut off membrane (Cellu Sep).

**Gel Filtration:** The enzyme thus obtained was further purified by using sephadex G-50 (50x2 cm). The elution buffer was 1mM sodium acetate at 5.5 pH. During the process different fraction were obtained.

**SDS-PAGE:** The molecular mass of the purified enzyme was determined with the help of SDS-PAGE using 10% separating gel and the gel was stained with coomassie brilliant blue .The standard ladder was used to interpret the molecular weight of the enzyme.

**CHARACTERIZATION OF ENZYME**

**Effect of pH:** The optimum pH of purified laccase was confirmed by incubating laccase at different pH ranging from 3 to 9. The buffers used were 1mM citrate buffer for (pH 3 and 4), 1 mM sodium acetate buffer of (pH 5 and 6), and 1Mm phosphate buffer of (pH 7 to 9).

**Effect of temperature:** The optimum temperature of free laccase was confirmed by incubating the enzyme at different temperatures ranging from 20 to 80°C.

**Effect of inhibitors and inducer:** The effect of inhibitors was discovered by incubating the purified laccase with various concentrations of inhibitors. The inhibitor used were EDTA, 2-mercaptoethanol and inducer was copper sulphate. The different concentrations optimized were 0.1mM, 1mM, 10mM and 0.1M. (Sathishkumaret al., 2013).

**RESULTS AND DISCUSSION**

**Sub-culturing of fungus on petri plates and slants:** The tissue picked from the source, when placed on plates containing kirks and tein media under sterilized condition showed little white fur like mycelia after 24 hours of incubation at 24°C in the incubator and complete growth took 5 days.

**Spawning:** Spawn prepared on barley seeds showed little growth after 3 days and complete growth after 17 days, when it was kept in the incubator at 24°C. Substrate bags were prepared from corn cob and wheat straw. The incubation period of corn cob was 26 days and of wheat straw was 21 days at 26°C. When bags were placed on shelf's of mushroom cultivation room it showed fruiting bodies after 9 days at 27°C, similar technique was used by (Narayanasamyet. al., 2008) to identify the unknown fungus(Fig 1).



Figure 1. Mature Fruiting bodies of *pleurotusflorida* on wheat straw

**FERMENTATION PARAMETERS OPTIMIZATION**

**Descriptive statistics of substrates, incubation time and enzyme activity:**

Table 1: Descriptive statistics of substrates, incubation time and enzyme activity.

	Descriptive Statistics				
	N	Minimum	Maximum	Mean	SD
Substrate	125	1.00	3.00	2.0400	0.82696
Quantity	125	1.00	5.00	3.0000	1.41990
Incubation Time	125	2.00	10.00	5.6800	2.42168
Enzyme Activity	125	1.73	9.95	4.1496	1.87803

Table 1 depicts that minimum number of substrate used was 1 and maximum were 3, the minimum quantity of substrate used was 1gm and maximum was 5gm the minimum incubation time was 2 days and

maximum time was 10 days, moreover, the minimum enzyme activity observed was 1.73IU/ml and maximum was 9.95 IU/ml with a mean of 4.14 IU/ml.

Table 2.ANOVA among substrates quantity in grams and enzyme activity.

	ANOVA				
	Substrate_Quantity		Mean Square	F	Sig.
	Sum of Squares	df			
Between Groups	41.632	4	10.408	3.156	.017
Within Groups	395.717	120	3.298		
Total	437.348	124			

Table-2 represents that there was significant difference between the substrate quantity and enzyme activity levels (F=3.156, P=.017 at level of significance  $\alpha \leq 0.05$ ).

Incubation time of substrates

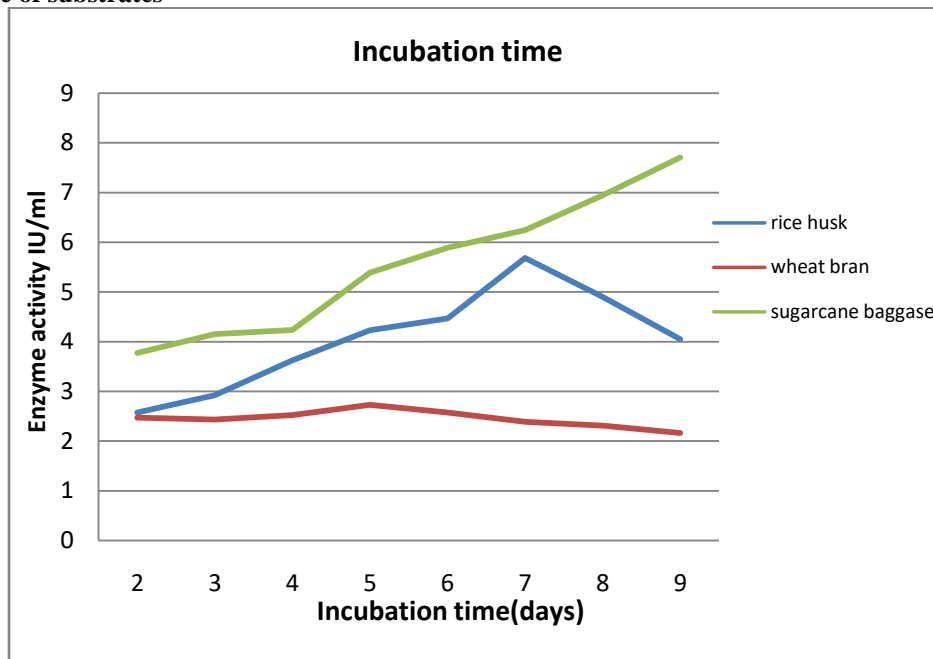


Figure 2. The effect on activity of enzyme produced on various incubation times.

The optimum duration for the production of enzyme was day 7 for rice husk and day 5 and 9 for wheat bran and sugarcane bagasse respectively (Fig.3).

Optimization of nitrogen sources :The change in the quantity of yeast and ammonium sulphate did affect the activity of enzyme as mentioned in (fig 4 and 5). However the increase in concentration of maize steep liquor reduced the activity of enzyme (figure 6).

Optimization of pH:

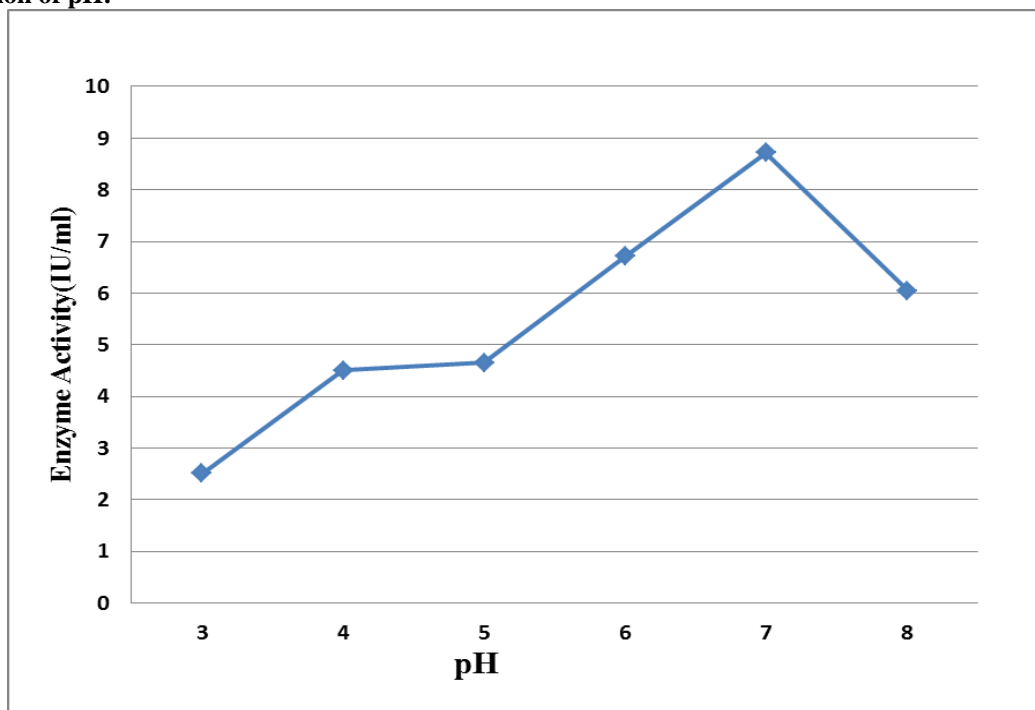


Figure 3: Effect of pH on activity of the enzyme

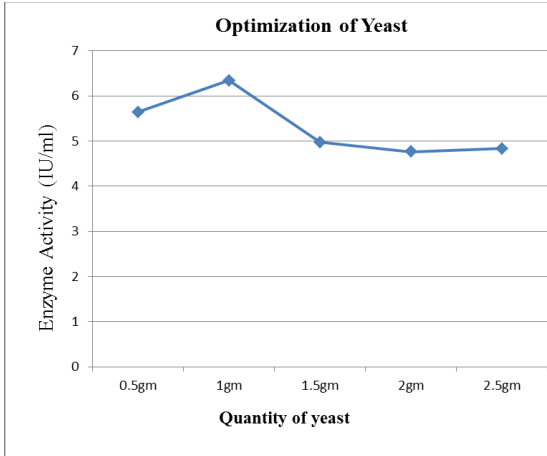


Figure 4: Optimization of yeast.

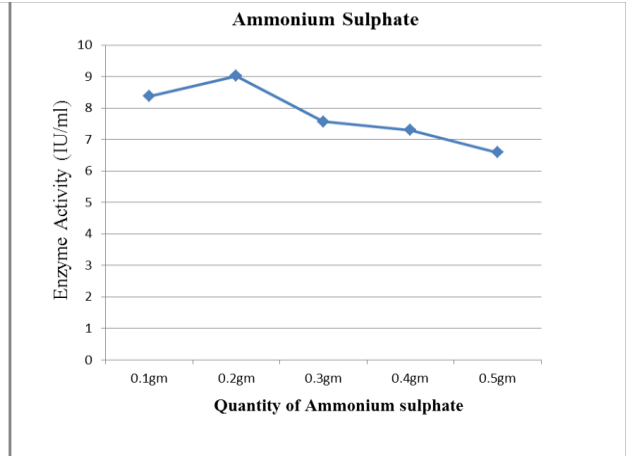


Figure 5: Optimization of ammonium sulphate

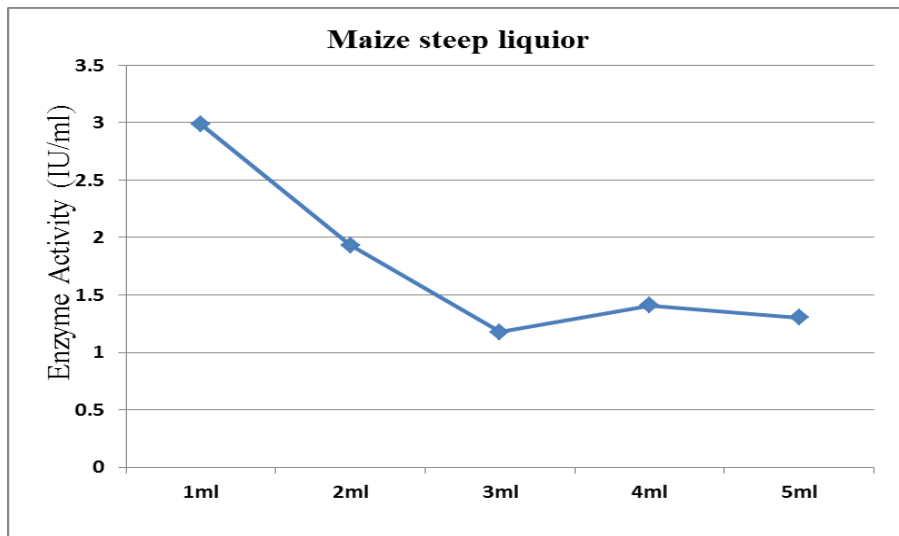


Figure 6: Optimization of maize steep liquor

Characterization of purified enzyme  
Optimum temperature

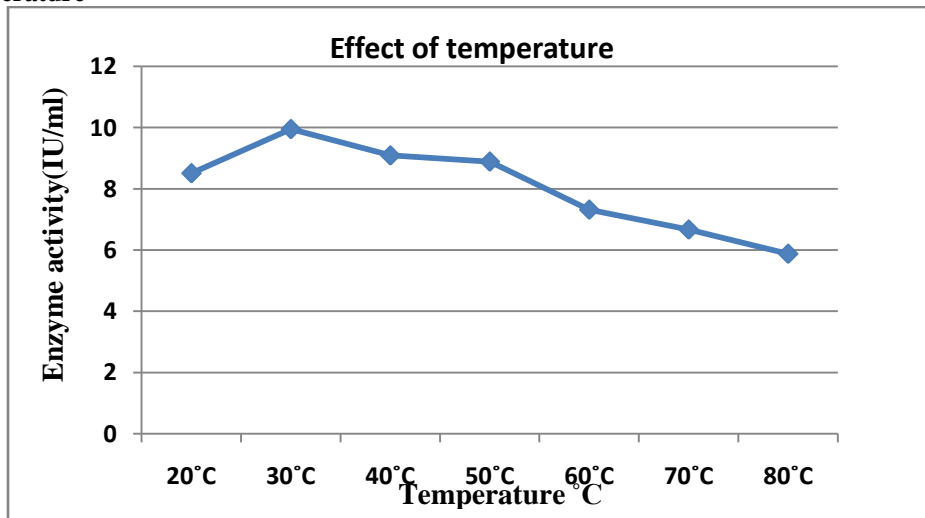


Figure 7: Effect of temperature on purified enzyme

Optimum pH

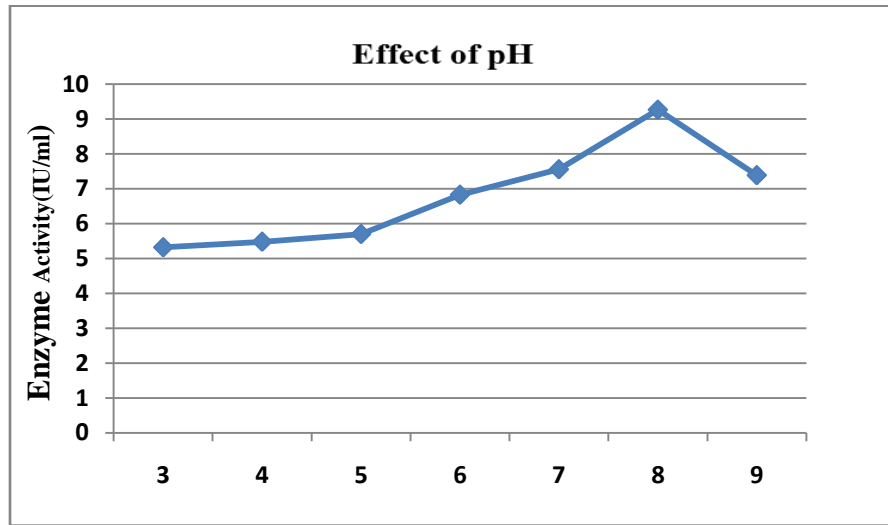


Figure 8: Effect of pH on purified enzyme

Effects of inducer (Copper sulphate)

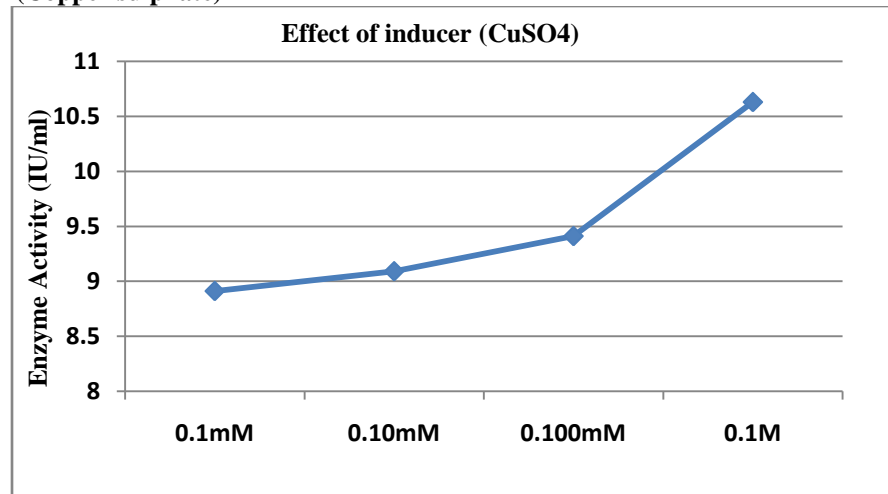


Figure 9: Effect of various concentrations of CuSO<sub>4</sub> on purified enzyme

Effect of Inhibitors

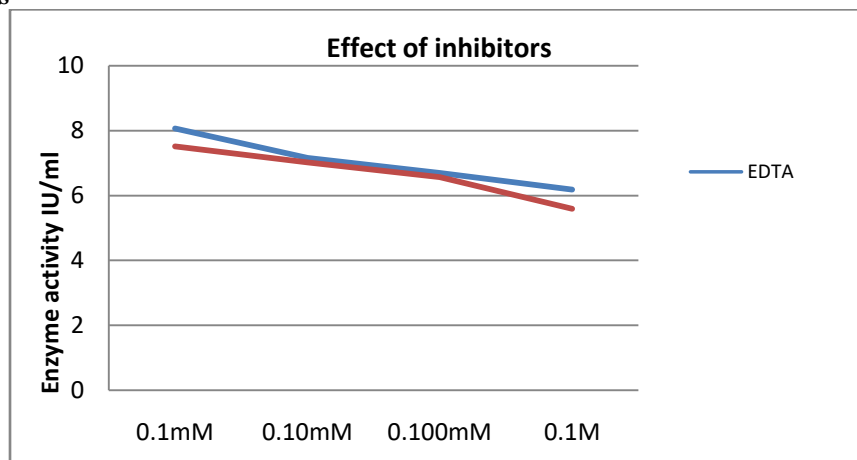


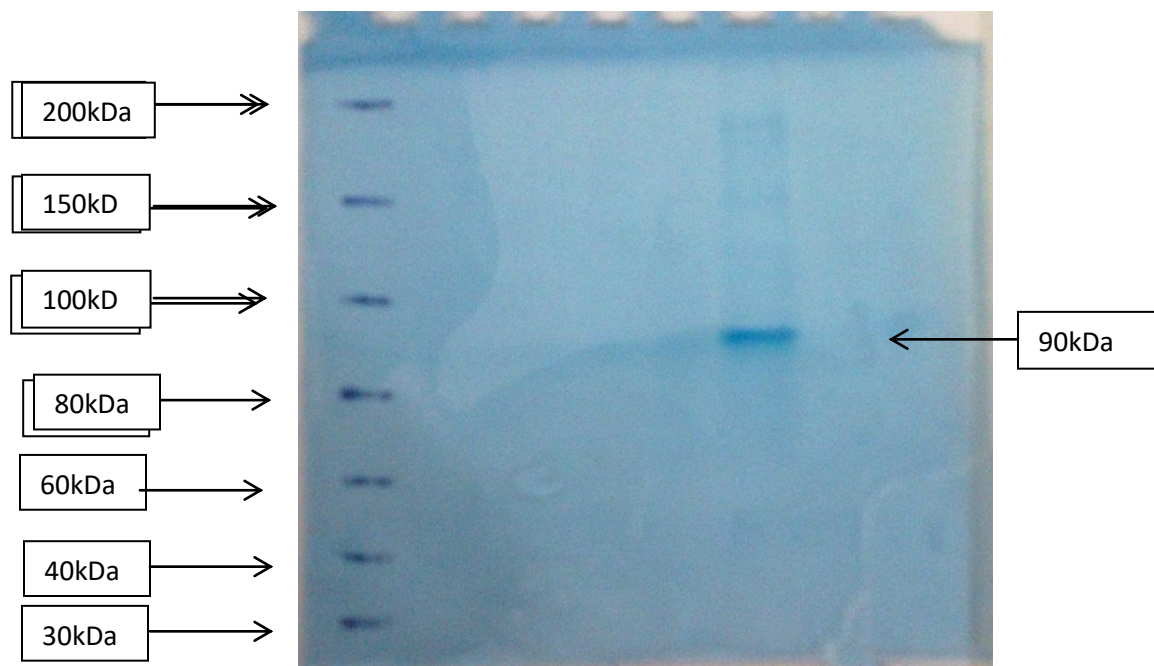
Figure 10: Effect of various concentrations of EDTA & 2-Mercaptoethanol on purified enzyme.

The results showed that the fungus *Pleurotus florida* could be best grown at 24 °C with incubation period of 5 days, which was similar to the studies carried out by (Narayanasamy *et. al.*, 2008). The production of enzyme was carried out using submerged fermentation technique and three agro roughages namely wheat bran, rice husk and sugar cane baggasse were used as substrates. In the present study the optimum production of the enzyme (9.9 IU/ml) was obtained with sugarcane bagasse. The concentration used was 1gm/100ml and the results showed significant difference ( $P=0.017$  at level of significance  $\alpha \leq 0.05$ ) between groups and were in line with previous work reported by (Packiyam *et. al.*, 2014). The enzyme produced with other two substrates were less i.e rice husk produced 6.49 IU/ml and wheat bran 3.48 IU/ml. The results of the present study were similar with previous studies carried out by (Elsayed *et. al.*, 2012). The optimum time for the production of enzyme was 7<sup>th</sup> day for rice husk and 5<sup>th</sup> day for wheat bran and 9<sup>th</sup> day for sugarcane bagasse respectively (Fig. 2). The optimum pH was found to be 7 (Fig. 3) and this was close to the findings of (Ding *et. al.*, 2012) i.e 7.42 for the maximum production of enzyme. The optimum temperature for the enhanced enzyme production was found to be 30 °C, which was slightly different from the studies of (Jegatheesan *et. al.*, 2012) i.e 28°C. However a few studies also reported that further increase in temperature could enhanced the

production of enzyme (Radhika *et. al.*, 2013 and Patrick *et. al.*, 2007).

Three different nitrogen sources were optimized and the enzyme produced with maize steep liquor was 3.89 IU/ml, yeast extract was 7.81 IU/ml and ammonium sulphate had 11.57 IU/ml. Among the three, the best source was ammonium sulphate which was an inorganic salt with highest amount of nitrogen that increased the production from 9.9 IU/ml to 11.57 IU/ml (Fig. 4,5,6) and this was similar to the work done by (Jegatheesan *et. al.*, 2012). The large scale production of one liter was carried out under the optimized conditions showed 13460 IU/L and was higher than other ligninocellulosic substrates like coir pith showing 8820 IU/L for *Pleurotus florida*, as has been reported by (Packiyam *et. al.*, 2014).

The characterization of the purified enzyme was done in terms of pH, temperature effect of inducers and inhibitors. The optimum pH and temperature of the purified enzyme was found to be 8 and 30°C in (Fig 7 and 8) respectively. The inducer enhanced the activity of enzyme up to 10.33 IU/ml and inhibitors reduced activity was 6.19 IU/ml and 5.59 IU/ml (Figure 9, 10) respectively as has been reported by (Sathishkumar *et. al.*, 2013). The molecular mass of the enzyme was evaluated as 90kDa by SDS-PAGE (Fig.11), which was similarly reported by (Sivakami *et. al.*, 2012).



**Figure 11: Molecular weight of laccase resolved on 10% SDS-PAGE.**

**Conclusions:** The maximum production of laccase was achieved through the optimization of fermentation parameters. Effective utilization of agricultural wastes

enhanced the production of enzyme up to many folds. The enzyme produced can be used further for many

biotechnological applications, because it can act on broad range of substrates.

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