

MYCOTECHNOLOGICAL PRODUCTION OF LACCASE BY *PLEUROTUS OSTREATUS*-P1 AND ITS INHIBITION STUDY

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ABSTRACT

Laccase is one such copper protein belonging to the oxidoreductase family that oxidizes a large array of organic substrates i.e phenolic and non-phenolic compounds. An attempt was made to screen *Pleurotus ostreatus*-P1 by plate test on Kirk medium for laccase production by using guaiacol and syringaldazine as indicators and six different liquid culture media having varied composition were work out for laccase production. Hyper Laccase production (5.5 ± 0.33 unit/ml) objective was achieved in medium 09-CBZ6 in combination with promising protein production (3mg/ml) under optimized fermentation conditions (pH: 5.5; Temperature: 30°C; Time: 7th day). Laccase production by a long way intensified by the adding of glucose (1.5%, C/N:15) and inducer (CuSO₄) gave promising results (13.72 ± 0.30 U/ml) and other inducers followed laccase production as Na₂SO₄ > ZnSO₄ > FeSO₄ > CaCl₂. Sodium Azide was found a significant inhibitor (100%) for laccase activity than SDS (87%) and EDTA (83%). Study brings to a close potential of *Pleurotus ostreatus*-P1 for laccase production under optimized conditions for industrial applications.

Key words: Laccase, *Pleurotus ostreatus*-P1, Kinetics, Inhibition, Culture Media.

INTRODUCTION

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2), belong to the family of blue multicopper oxidases containing enzymes which perform simultaneously, one-electron oxidation of various substrates molecules such as diphenols, methoxy-substituted monophenols and aromatic and aliphatic amines concomitant with the four-electron reduction of molecular oxygen to water (Elshafei *et al.*, 2012).

Laccase study was initially started by Yoshida (1883) from the sap of the Japanese laquer tree *Rhus vernicifera*. Laccase is distributed in nature and found in plants, insects and bacteria, but the best deliberate laccase are of fungal origin (Desai and Nityan, 2011). However, most laccases are found and studied in lignin-degrading basidiomycetes. Among the basidiomycetes, predominantly white rot fungi such as *Pleurotus ostreatus* and *Trametes versicolor* have received special attention because of their ability to mineralize lignin by secreting oxidative enzymes, such as peroxidases and laccases. However its biological function is still not totally clarified (Halaburgi *et al.*, 2011).

Laccase are by and large extracellular glycoprotein with molecular weight between 60-80kDa. They contain 15-30% of carbohydrates and usually have acidic isoelectric point. They can catalyze and oxidized many complex substrates, due to their remarkable non-specificity on the subject of reducing substrate, they are receiving increasing attention in various industrial applications such as delignification, bleaching of pulp,

dye decolorization, wastewater treatment, enzymatic removal of phenolic compounds in beverages, food processing, construction of biosensors and bioremediation (Couto and Toca-Herrera, 2007).

Majority of laccase characterized so far have been derived from fungi, especially from white rot basidiomycetes that are efficient lignin degraders. Well known laccase producers includes the fungi such as *Trametes versicolor*, *Pleurotus sajor-caju*, *P. eryngii*, *Cerrena unicolor*, *Panus rigrinus*, *Pycnoporous sanguineous* (Litthauer *et al.*, 2007).

Pleurotus ostreatus is spread all around the world in its natural habitat, mainly in forest environments and produce lignocellulosic enzymes, mainly laccase and Mn-peroxidase, which convert lignocellulosic residues into food (Bernardi *et al.*, 2008).

Laccase expression in *Pl. ostreatus* ARC280 distinctly influenced by the composition of the culture medium, carbon, nitrogen content, C/N ratio, inducer compounds and control by pH of the production medium and other relevant nutrition parameters (Maysa *et al.*, 2012). The nutritive substances employed in the culture medium constitute significantly to the total production costs. Hence, it has been a matter of concern to find environmentally sound and economically feasible media constituents for laccase production.

The carbon sources in the medium play an important role in ligninolytic enzyme production glucose as carbon source in cultivation of laccase producing fungal strains have stimulatory as well as inhibitory effect. It was determined that glucose concentration higher than 20g/l caused catabolic repression of laccase

production by *T. viride* and *T. longibrachiatum* (Krastanov *et al.*, 2007).

Some experimental conditions such as pH, temperature, presence of inducers are restraining factors for increased ligninolytic enzymes laccase production. The most favorable pH for the laccase was between 5.5 and 7.5 at 75°C and on the subject of temperature 80% of the initial enzyme activity was maintained after incubation of the laccase at 70°C for 2 h. So laccase was intrinsically highly thermo stable for precious potential applications (Guo *et al.*, 2011).

The production of laccase can also be stimulated by presence of extensive variety of inducers, primarily aromatic compounds interrelated to lignin and lignin degradation compounds. Enzymatic induction of white-rot fungi is very important since their metabolic activity and growth are dependent on the environmental conditions. The addition of inducers such as xyloidine, phenolic mixture, and copper for the white-rot fungi *T. versicolor* and in *Pleurotus ostreatus* has been reported for augmentation in laccase production. Veratryl alcohol and a number of other aromatic compounds have also demonstrated the enhancement in laccase production (Guo *et al.*, 2011).

Therefore, a good strategy is required to augment the productivity of laccase fermentation process by optimization of fermentation medium.

We speculated that in *Pleurotus ostreatus*-P1 laccase producing tolerance system and laccase activity is influenced by media composition and culture conditions. So the goal of this investigation is to optimize biosynthesis of laccase by *Pleurotus ostreatus*-P1 by utilizing diverse operational strategies such as use of different culture mediums, supplementation and addition of inducers. Furthermore various physicochemical parameters will be attempted to optimize laccase production.

MATERIALS AND METHODS

Collection of Strain: In order to enhance the laccase production for industrial applications, laccase-producing fungi *Pleurotus ostreatus*-P1 (fruit body) was collected from the Horticulture Department, Agriculture University Faisalabad Pakistan. Mycelium of fruit body was transferred, after surface sterilization with hydrogen peroxide into Petri plates contained Kirk medium (Tien and Kirk, 1988) and was incubated at 28°C. Fungal growth reached the edge of Petri plates after seven days of incubation in the dark and cultures was transferred on the same medium, pure colonies were obtained and stored under controlled conditions at 4°C.

Plate test for screening of laccase Producing fungi:

The isolated fungal culture was inveterated for lignolytic enzyme, laccase activity by using guaiacol and

syringaldazine as indicator. guaiacol (0.02%) & Syringaldazine (0.1%) were put into effect for screening of laccase producing fungi, develop an intense reddish brown and purple color in the medium around the fungal colony region respectively as laccase indicators (Ang *et al.*, 2010). In addition chloramphenicol (0.01% W/V) was added in the media in order to inhibit the growth of bacteria and Benomyl (1%) in order to select only wood decaying fungi.

Composition of different Medias: Batch study was used to compare six different liquid Medias for laccase production. These media compositions were

09CBZ1:(NH₄Cl,1.5g/l;MgSO₄,1.5g/l;KH₂PO₄,0.3g/l;Na₂HPO₄,0.3g/l;FeSO₄.7H₂O,5mg/l;MnSO₄,35mg/l;CuSO₄,7mg/l)

09CBZ2:(Peptone,2g/l;Glucose,10g/l;KH₂PO₄,0.6g/l;ZnSO₄,1.0mg/l;K₂HPO₄,0.4g/l;FeSO₄,5mg/l;MnSO₄,0.5mg/l;MgSO₄,0.5g/l)

09CBZ3:(Glucose,10g/l;Glycerol,7.5g/l;L.histidine,0.5g/l; CuSO₄, 7mg/l; NaNO₃,1.8 g/l; NaCl,1.8g/l; KCl,0.5g/l;CaCl₂,0.5g/l;FeSO₄,0.05g/l;KH₂PO₄,1.0 g/l;MgSO₄,0.5g/l)

09-CBZ4: (Glucose, 10g/l; Asparagines,1.0 g/l; Yeast,0.5 g/l; KH₂PO₄, 0.5 g/l; MgSO₄, 1.0g/l; FeSO₄, 0.01g/l; Dextrose, 20.0g/l)

09-CBZ5: (CaCl₂, 0.6 g/l; MgSO₄, 0.4 g/l; Maltose, 0.5 g/l; Peptone,0.6 g/l; (NH₄)₂SO₄, 2.0 g/l; KH₂PO₄, 2.1 g/l)

09-CBZ6: (Ammonium citrate, 2.0g/l; Glucose, 10 g/l; KH₂PO₄, 1.0g/l;Yeast,1g/l;MgSO₄,0.5g/l; KCl, 0.5g/l).

These media were prepared in 250 ml conical flask by using 150 ml distilled water and their initial pH was adjusted at 5.5 with 0.1 M sodium acetate buffer before autoclavation. All these media were then sterilized by autoclaving at 1.5 atmospheres and 121°C for 20 minutes. *Pleurotus ostreatus*-P1 was propagated for laccase production by inoculating the 4mm agar block of 7 days, old culture prepared in Petri Plates. Six different media individually having diverse composition were incubated under static conditions at 30±1°C for seven days of incubation. Liquid cultures broths of each six media were centrifuged at 4000 rpm at 4°C for 20 minutes to get clear supernatant for enzyme assay.

Enzyme assay: Laccase activity was determined by enzymatic oxidation of syringaldazine Spectrophotometrically at 525nm ($\epsilon_{525} = 65000 \text{ M/cm}$). The assay mixture contained 850 μl of 0.1 M sodium citrate (pH: 5) 100 μl syringaldazine (0.1 mM) and 50 μl of supernatant. One unit of enzyme activity was defined as the amount of enzyme that caused an increase of one in the absorbance per mint under assay condition (Li *et al.*, 2008). Similarly Laccase activity was also measured by

guaiacol oxidation at 450 nm ($\epsilon = 12\ 100/M/cm$). The reactive mixture contain 3 ml acetate buffer (10 mM, pH: 5), 1 ml guaiacol (2 mM) and 1 ml supernatant (Li *et al.*, 2008) and U/ml was calculated as

$$U/ml = \frac{\Delta A_{450}/min}{\epsilon \text{ mM}} \times \frac{\text{Reaction volume (ml)}}{\text{Enzyme volume (ml)}}$$

Effect of substrate concentration on laccase activity:

To determine the optimum time for maximum conversion of laccase units in to product by using the fixed quantities of substrate syringaldazine (0.5 ml of 0.2 mM) and guaiacol (0.5 ml of 2mM) were used for a period of 3-21 min at optimized conditions (pH: 5.5; Temperature: 25°C) of laccase assay as earlier defined.

Effect of substrate concentrations of syringaldazine (0.05,0.1,0.15,0.2,0.25,0.30 & 0.35 mM) and guaiacol (0.5,1.0,1.5,2.0,2.5,3.0,3.5,4.0 mM) on laccase activity was determined by incubating the fixed quantity of supernatant enzyme (1ml) for a period of 6 and 12 min respectively under optimized conditions of assay as described earlier. Different inhibitors (EDTA & SDS) were also exercised to determine the inhibition rate/inhibition constant by adding the various concentrations of each inhibitor to fixed quantity of syringaldazine (0.2 mM) in supernatant of culture broth of *P. ostreatus*-P1.

Laccase production at different pH: After selection of Medium (09-CBZ6) for higher laccase production, the pH of medium (09-CBZ6) was alter by adding the different buffer solutions of pH (3.5,4.5, 5.5,6.5,7.5,8.5,9.5) to get the optimum and suitable pH for the higher laccase production. pH (3.5,4.5,5.5) was adjusted by using acetate buffer and for pH (6.5,7.5,8.5,.9.5) phosphate buffer was used to maintain the pH of medium.

Laccase production in different Incubation

Temperature: The previously selected media (09-CBZ6) was incubated at 10,20,30,40 and 50°C for a period of seven days in a static phase to optimize the suitable temperature for laccase production.

Laccase production in different Incubation Period:

The laccase activity was observed in medium (09-CBZ6) for a period of 16 days at the interval of 2 days continuously at optimized condition (pH: 5.5; Temperature: 30°C).

Effect of Glucose and Nitrogen Concentration on

laccase production: Diverse glucose concentrations were applied to the selected production medium (09-CBZ6) in the static flask cultures. The concentrations of carbon source (glucose) in the media were 0.55%,1%, 1.5% , 2%,2.5% , 3%, 3.5%. Further, to determine the impact of C/N ratio, nitrogen concentration was fixed here and seven different C/N ratios (5, 10, 15, 20, 25, 30 and 35) were exercised and determined the results as fellows. The left over ingredients were remained the

same as standard medium. (pH: 5.5; Temperature: 30°C ; Time: 7 days).

Effect of different Inducers on laccase production :

In order to study the effect of inducers on laccase production, different chemical such as CaCl₂, CuSO₄, FeSO₄, ZnSO₄ and Na₂SO₄ of 100µM each were incorporated in the culture medium (09-CBZ6). All these inducers were dissolved in distilled water and sterilized before induction. After the selection of CuSO₄ as best inducer, medium (09-CBZ6) was supplemented with various concentrations of CuSO₄ ranged from 50 to 600 µM, to determine the concentration of CuSO₄ for higher laccase production. . (PH: 5.5; Temperature: 30°C ; Time: 7 days).

Inhibition Study:

The effect of three potential inhibitors (Sodium Dodocyl Sulphate (SDS), Ethylene Diamine Tetra Acetic Acid (EDTA) and Sodium Azide (NaN₃) on laccase activity was evaluated. Three different concentrations (0.5, 1, 1.5 mM) of each inhibitors were added in the reaction mixture having 0 .5 ml of 2 mM syringaldazine and 1 ml of culture supernatant and incubated at pH: 5 (Temp: 30°C; Time: 10 min) and reaction velocity was measured to calculate the inhibition percentage (Mishra and Kumar., 2009).

Analytical Techniques:

The C/N ratio was calculated as the quotient of total C over total nitrogen. During the study, pH was determined by using the pH meter (Jenco). Total extra-cellular protein concentration was determined by Folin Lowry method (Lowery et al., 1951) spectrophotometrically with bovine serum albumin as a standard.

Statistical Analysis:

Each result was an average of three analysis, by using the excel (MS-2007), standard deviation was reported along with the average value. SPSS.19 was used to compare the means of six medium by one-way ANOVA and DMR.

RESULTS AND DISCUSSION

Laccase is a unique enzyme, having considerable value, for its production the screening of fungus followed by the selection of best medium for prominent laccase production using statistical approach. The optimization of various parameters (medium composition, inducers, pH, and temperature and incubation period) for laccase production in static flask under different fermentation conditions is presented.

Screening of laccase producing fungus:

The foremost cause of screening fungus *Pleurotus ostreatus*-P1 for laccase production was due to its important as an efficient source of laccase for industrial application (Ang *et al.*, 2010). In the present study guaiacol and syringaldazine were used as an indicator for confirmation and rapid

visual demonstration of laccase.(Fig-1a) The formation of reddish brown and dark purple zone due to oxidation of guaiacol and syringaldazine confirmed the presence of laccase (Wang *et al.*,2010).The quick color formation with guaiacol is easy and reliable source for laccase screening. According to Kumar and Rapheal (2010) this confirmation is supposed to be a baseline for laccase production before its optimization and application.

Selection of Medium for laccase Production: In white rot fungus Laccase production depends on the composition of the culture medium, particularly carbon, nitrogen content and inducer compounds (Adejoye and Fasidi, 2010).

In the present study the white rot fungus *Pleurotus ostreatus*-P1 was grown on six different liquid culture media having different elemental compositions to stir up laccase production.

The production of laccase varies according to composition of medias which differ due to presence of micro and macro nutrients. In diverse nature of liquid media used for the cultivation of *Pleurotus ostreatus*-P1 the maximum enzyme production was obtained in 09-CBZ6 (5.51±0.33 unit/ml) which was 35 %, 40.6% more than 09-CBZ5 and 09-CBZ4 respectively. Similarly the variation in the laccase production was also presented as 09-CBZ 6 > 09-CBZ 5 > 09-CBZ 4 > 09-CBZ 3 > 09-CBZ 2 > 09- CBZ1 (Fig-1). It was also found the 09-CBZ1 showed the least production due to low nutrients ,while others media had the different quantities of nutrients and 09-CBZ1, 09-CBZ2 and 09-CBZ3 showed no significant difference for laccase production with each other. The analysis of variance (F: 0.00) and DMR test confirmed the results and proven that laccase production all the medias was significantly different among each other (Fig-1c) and media 09-CBZ6 is entirely different from all other medias (Table-1). Similarly protein contents were also maximum (2.11mg/ml) in 09-CBZ6. The present study results are in lined with Elsayed *et al.* (2012).

Effect of Incubation Period: The laccase production by *Pleurotus ostreatus*-P1 in medium at (pH 5.5, 30^oC) 9CBZ06 was monitored regularly for a period of 16 days. Results cited in (Fig-2c), indicate that laccase produce in its late log phase i-e on 6th and 8th day, when laccase activity reached at its maximum value with activity 5.53±0.11-5.68±0.08 U/ml and after that decrease subsequently. Similarly there was a sharp increase in laccase production from 2-6 day and after 10 days its activity decrease slightly. The laccase production on 8th was 61.7, 41.4 fold more than 2nd and 16th days respectively. Present study results are in lined with Kumar, *et al.*, (2011) reported that maximal laccase activity in *Pleurotus ostreatus* was on nine day (570 U/L). Whereas the total extracellular protein content were

maximum (3.14mg/ml) on tenth day and later on decrease gradually. This fact is supported by the fact as protein content increases the laccase activity also increases. Same observations were also noted by Mishra and Kumar, (2009). Similarly Ibrahim, *et al.* (2011) also reported that soluble proteins contents reached a maximum level at 10 days of fermentation in *P. ostreatus* after that the levels reduced steadily

Dasai and Nityan, (2011) also reported that *Pl. ostreatus* was able to produce highest quantity of laccase in the 25th day of culture, whereas the other species (*Pl. florida*, *Pl. flabellatus*, *Pl. sajorcaju* and *P. pulmonarius*) exhibit their optimum laccase activities in the 26th day of culture in some other species like *Lentinus edodes* and *Ganoderma sp.* Sivakumar *et al.* (2010) reported the maximum laccase production on the 7th and 10th day of incubation.

Effect of pH: pH is an imperative and significant factor, influenced the extracellular laccase production in *Pleurotus ostreatus*-P1. It is an important factor of culture medium for fungus to grow, lignolytic enzyme production and xenobiotics degradation (Sivakumar *et al.*, 2010).

In present study after the selection of appropriate medium the fungus was cultivated at a series of pH values ranged from 3.5 to 9.5. The optimum pH for various fungi as reported earlier was 4.5-6, whereas presently pH 5.5 was most suitable for the fungus growth and extracellular laccase production (5.58 ±0.17 U/ml). It was also examined that the laccase activity was raised with the increased of pH up to 5.5, later on it shows the declining trend in its production and make parabolic shape (Fig -2e). This decrease is due to the facts that rise in pH not favorable for fungus growth. The most favorable pH for the laccase was between 5.5 and 7.5 at 75^oC and on the subject of temperature 80% of the initial enzyme activity was maintained after incubation of the laccase at 70^oC for 2 h. So laccase was intrinsically highly thermo stable for precious potential applications (Guo *et al.*, 2011). Hadibarata *et al.* (2012) reported the highest laccase production at pH 4 decreases speedily when the pH level rises. It is pronounced effect of pH that laccase production changes the medium pH with time. The results obtained are in agreement with Elsayed *et al.* (2012), that most of fungal cultures preferred a slightly acidic pH of medium for growth and enzyme production. Patel *et al.* (2009); Adejoye and Fasidi. (2010); Sivakumar *et al.* (2010) have also reported that *Pleurotus ostreatus* HP-1, *Schizophyllum commune* and *Ganoderma sp.* gave the best possible laccase production at pH 5.0, 5.5 and 6.0, likewise. pH significantly influence the extra cellular protein production along with laccase activity. The fungus *Pleurotus ostreatus*-P1 released maximum extra cellular protein contents 2.45mg/ml at pH 5.5.

Effect of Incubation Temperature: It is an evident that in fungus *Pl. ostreatus*, temperature significantly influenced the production of mycelia biomass protein and laccase. The optimum cultivation temperature effect the growth kinetics of the microorganism employed rather than on the enzyme produced (Poojary and Mugeraya, 2012). In present study the *Pl. ostreatus*-P1 was incubated at different degrees of temperatures ranged from 10-50°C. At 30 °C the fungus show the maximum growth and laccase production 5.62±0.24 U/ml but at temperature more than 30°C the fungus growth decreased and consequently laccase units drop because high temperature cause the cell membrane composition alteration and stimulation of protein catabolism (Fig-2b). It was also noted in this experiment at 30°C the laccase production was 62.1%, 98.25 % was more than at 10 and 40°C respectively. At the end of study at 50°C, no laccase activity was found due to rise of temperature. The laccase production also showed the increasing trend at low temperature but its fall as the temperature rise. The present findings are in according with the observations found by the Elsayed *et al.* (2012). Many authors also reported that 28°C was the best temperature for laccase production by *Schizophyllum commune* (Adejoye and Fasidi, 2010) and *Pleurotus ostreatus* HP-1 (Patel *et al.*, 2009). On the other hand, reported that temperatures higher than 30°C caused reduction in ligninolytic enzymes production. In case of protein the maximum extra cellular protein was at 30°C when 2.67mg/ml was produced. Temperature more than 30 does not favor the more growth of *Pleurotus ostreatus* and subsequently lesser protein produced.

Effect of Carbon Concentration: The optimum organic carbon concentration in the growth medium of fungus plays an imperative role in laccase production. Many previous studies indicated that basidiomycetes have a wide diversity in their response to carbon sources and its concentration in nutrient media for laccase production (Wang *et al.*, 2008). Significant laccase secretion by *trametes pubescens* started, when glucose concentration in the growth medium reached to certain low critical level, whereas according to Krastanov *et al.* (2007) the use of excessive concentration of glucose as a carbon source in the cultivation of laccase producing fungal strain has an inhibitory effect on laccase production.

In present study seven different glucose concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 & 3.5%) were selected to compare and note the production of laccase (Fig 2d) and an increasing trend was found up to 1.5% but shown the decline as the concentration raised. The high production of laccase was at 1.5% (6.81±0.26 unit/ml), which was 38.22% & 62.8% than 3 and 3.5 % concentrations. So it was considered being the best concentration for maximum laccase production. The present study results are in accordance to Elsayed *et al.*

(2012), used the different concentrations of starch and found the 15g/l best for laccase production. Patel *et al.* (2009) reported that highest laccase production by *pleurotus ostreatus* HP-1 was acquired with 1% glucose containing medium and increase in the quantity of glucose in the media more than 2% consequences in decline laccase activity. After the seven days of cultivation, there was no significant variation in laccase production was observed between the concentration 2% and 2.5%, where as 1.5% was more (Fig-2d). The surfeit amount of glucose inhibits the concentration of laccase but enhance the biomass of fungus. It was also evident that synthesis of laccase is inhibited by higher concentration of glucose (≥3.50 g/l), as per the Ping *et al.*, (2011) guidelines. It was also determined that glucose concentration higher than 20g/l caused catabolic repression of laccase production by *T. viride* and *T. longibrachiatum* (Krastanov *et al.*, 2007).

Effect of C/N Ratio: Fungus *Pleurotus ostreatus*-P1 show diverse behavior for laccase production at different C/N ratio. Carbon and nitrogen contents if mixed in appropriate ratio, serve as a complete source of nutrients for fungus growth and no need of additional nutrients are required for propagation and production of enzyme. The general trend during optimization of medium, the laccase production is inversely proportional to C/N ratio. In present study the same observation has been found. The seven different C/N (5, 10, 15, 20, 25, 30 & 35) were exercised and found in the initial phase the laccase production raised as the C/N ratio increased and at C/N (15) the maximum laccase production was observed, which later on decrease with the rise of C/N (Fig. 2e). Kannaiyan *et al.* (2012) results reveled that the C/N was the important factor for the optimization and selection of inducers also. In particular case the 64 % increased was found at C/N (15) as compared to C/N (5). It was also found Irshad and Asgher. (2011) results finding are in accordance to present study.

Effects of Inducers: Extracellular laccase production can be enhanced and activated in liquid fungal culture by the induction of inducers, because these are the substrates of enzyme to be produced or similar for natural growth substrates of wild strains of the fungus. Liu *et al.* (2009) have taken many attempts to recognize the inducer for laccase synthesis in various funguses. In present study the enzyme production in *Pleurotus ostreatus*-P1 culture media was monitored by induction of inducers (CaCl₂, CuSO₄, FeSO₄, ZnSO₄, Na₂SO₄) were exercised in the synthetic medium to determine the effect of different inducers on laccase production. The results showed that inducers have significant effects on laccase productions. Some inducers are capable of enhancing the lignolytic enzyme activity as compared to control. Among these inducers CuSO₄ (9.1 ± 0.13U/ml) is the most efficient inducer followed by FeSO₄ (7.3±0.44 U/ml) Na₂SO₄

(7.1 ± 0.11 U/ml), ZnSO_4 (6.7 ± 0.32 U/ml) and CaCl_2 failed to induce the laccase production and showed no significant activity (3.5 ± 0.31 U/ml) than control (5.6 ± 0.13 U/ml) (Fig-2f). The positive inductive effect of ZnSO_4 and CuSO_4 has earlier been reported in *Shiraia bambusicola* Henn. strain GZ11K2 by Du *et al.* (2012). The use of these sources for enhanced ligninolytic enzyme production plays an important role in improving the cultivation aspects in morels.

Effect of Copper Sulphate: Copper has been designated a strong laccase producing inducer in fungal species *Pleurotus ostreatus*-P1. Many scientists reported a considerable raise in laccase activity in copper incremented *Pleurotus ostreatus* broth cultures. In current study the augmentation in laccase activity is relative to the amount of copper added and promising results (13.72 ± 0.30 U/ml) were obtained at 250 μM (Fig-2g), which was 61 fold increase after seven day of growth was obtained compared to the basal medium without copper induction (control).

The present study also verified dual effects of copper on the specific laccase activity: inducing and suppressing. The copper effect on the *Pleurotus ostreatus*-P1 growth was studied in control and medias supplemented with different concentration of copper, in order to understand whether the tolerance to copper is a function of media composition.

For all tested conditions, copper sulfate addition to the media significantly effect *Pleurotus ostreatus*-P1 laccase activity. The lowest copper sulfate concentration needed to stimulate the laccase activity under all experienced conditions was 50 μM (Fig-2g). The increase in copper sulfate concentration resulted in increased laccase activity until it reached a maximum values at certain copper sulfate levels that was 13.50 ± 0.30 units/ml (250 μM). In addition the increase in copper sulfate concentration is likely to trim down the laccase activity to lower level (4.63 ± 0.41 u/ml at 600 μM) than that observed under copper-free conditions (5.25 ± 0.05 u/ml). These results are also supported by Kannaiyan *et al.* (2012).

This animated upshot of CuSO_4 supplementation on fungal laccase production is prop up by the fact that Cu is laccase co-factor where four copper is linked with one single polypeptide chain. It has been statement that laccase mRNA levels in numerous white rot species such as *trametes versicolor* and *Pleurotus ostreatus* were considerably raised in media containing high concentration of Cu^{2+} Karp *et al.* (2012).

Copper also play an important role in the regulation of laccase genes transcription and post-transcription modification of enzyme, while copper toxicity is attributed to the interaction of copper ions with proteins, enzymes, nucleic acids, and metabolites associated with cell functions and viability, and due to

oxidative stress (Abo-Stat *et al.*, 2011). In the present study at seven days growth the results revealed that highest protein 3.35mg/ml was secreted by *P. ostreatus*-P1 on the medium 6 supplemented with 250 μM CuSO_4 as indicated in (Fig-2g). Whereas Abo-Stat *et al.* (2011) reported that in *P. ostreatus* the highest protein secretion was ($357 \mu\text{g}/\text{ml}$) by supplementation of 150 μM CuSO_4 and ($302 \mu\text{g}/\text{ml}$) with the supplementation of 200 μM CuSO_4 . Maximal laccase activity and protein were obtained with 300 μM CuSO_4 . The elemental analysis of crude enzyme was evaluated by using the Elemental analyser, Elementar (CHNS) and found the Carbon (46.76%), Nitrogen (5.09%), Hydrogen (6.07%) and Sulpher (1.02%) (Table-2).

Effect of Substrate Concentrations: The effect of substrate guaiacol and syringaldazine concentrations on the rate of oxidation has been noted at 25 °C temperature and optimum pH 5.0 for 15min. laccase activity $\mu\text{mole}/\text{min}$ was when plotted against the both substrates Guaiacol and Syringaldazine concentrations (mM) rectangular hyperbola was obtained as (Fig-3c).

These results are in line with Giardina *et al.* (1999), they reported that Syringaldazine has highest binding affinity for laccase produced by *Pleurotus ostreatus*. However the binding efficiency of different substrates differs significantly between laccases produces by different fungus. Mishra and kumar, (2009) study about laccase by *coriolus versicolor* when it oxidised substrate ABTS.

Effect of Inhibitors: In present study potential of three inhibitors (NaN_3 , SDS & EDTA) on laccase activity inhibition was evaluated. All the inhibitors behave with same mode but their inhibition percentages varied as per their applied concentrations. Sodium Azide (NaN_3) makes complexes with active site of laccase copper, resulting of 100% inhibition of laccase activity during the oxidation of the Syringaldazine as substrate at three different concentrations (0.5, 1.0 & 1.5%). Adegoke *et al.* (2012) used Sodium Azide (0.1 ml/0.1 mM) as an inhibitor and noted the sharp inhibition during their study; these results are in accordance with present observations. In this context (SDS), very interesting information was also found; the inhibition percentage was decrease with the increase of application dose. The inhibition percentage at 0.5% and 1.5% was 87% and 65% was observed respectively. These finding were also supported by Adegoke *et al.* (2012), reported that higher reactivating concentration of SDS is due to limited conformational changes that induce the activation of latent enzymatic form. EDTA is a chelating and complex forming compound and is well known inhibitors to inhibit the laccase at type 2/3 trinuclear copper site. It decreases the redox potential difference between the two copper sites. In present study the maximum inhibition was found at 1.5% (83%). It was also noted there was no

significant difference in results was found between SDS and EDTA.

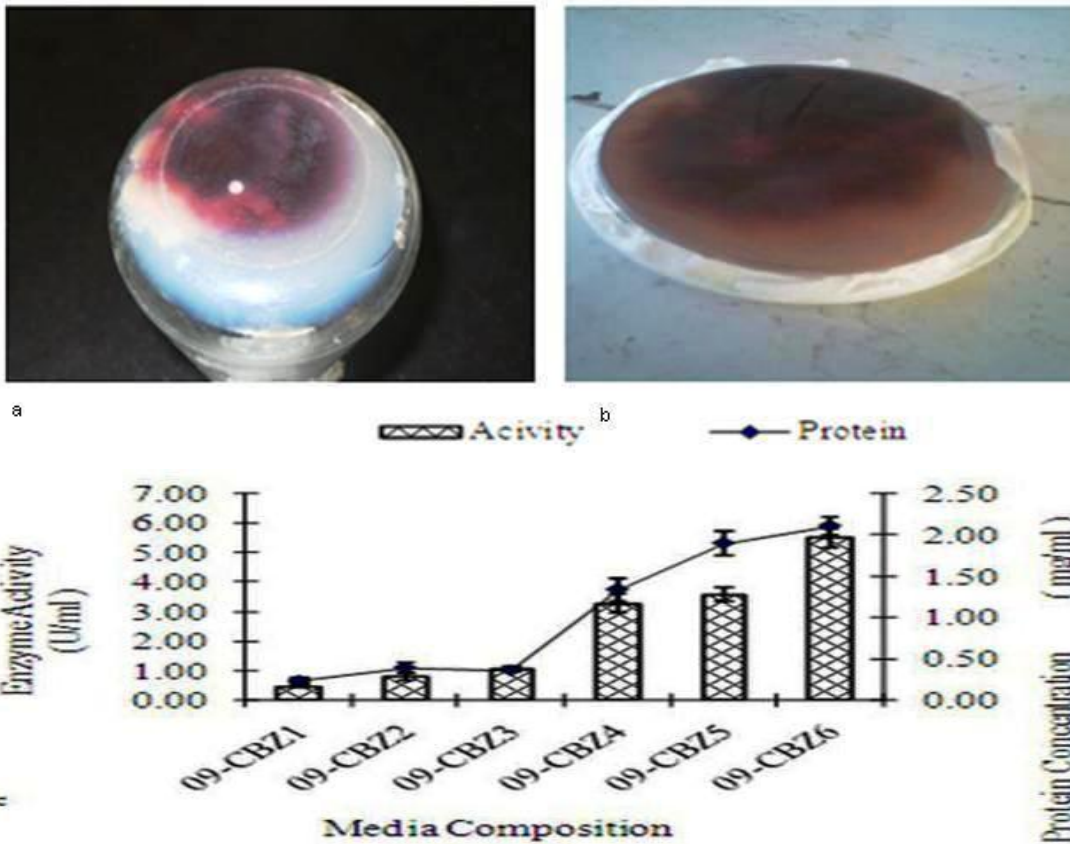
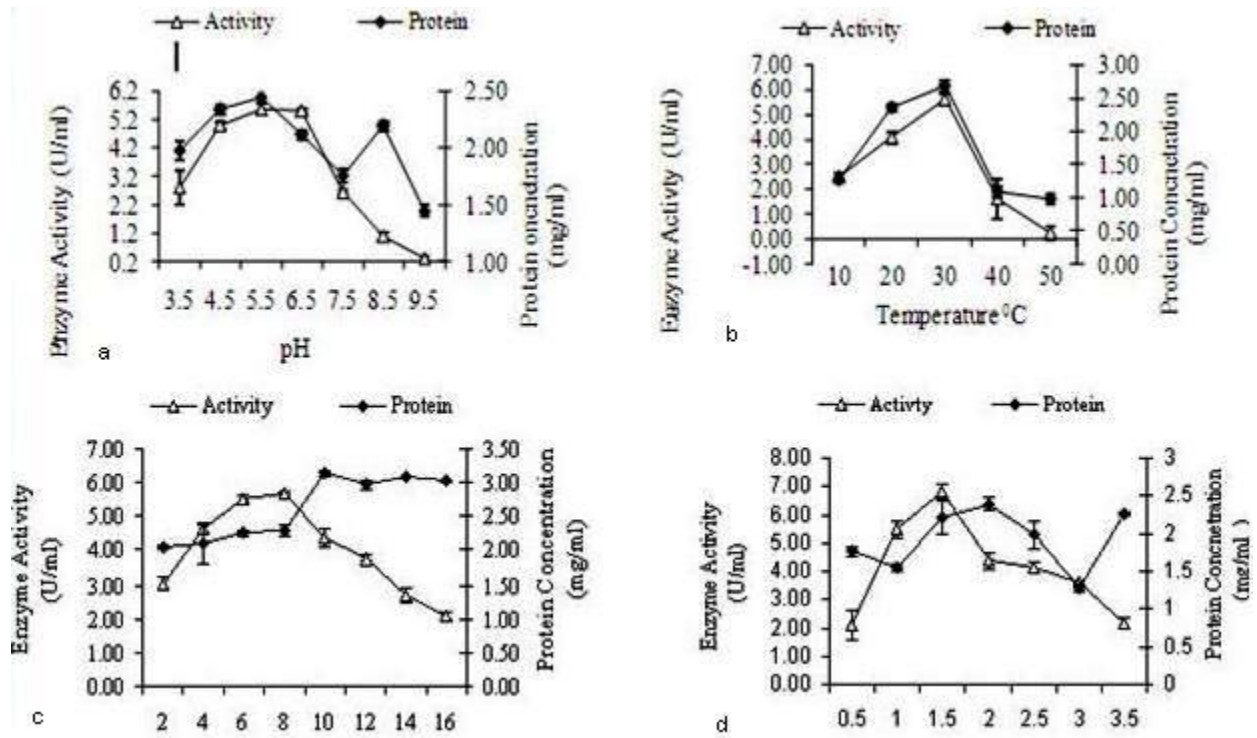


Fig:1 Identification and Production of Laccase (a) Syringaldazine (b) Guaiacol (c) Media Composition



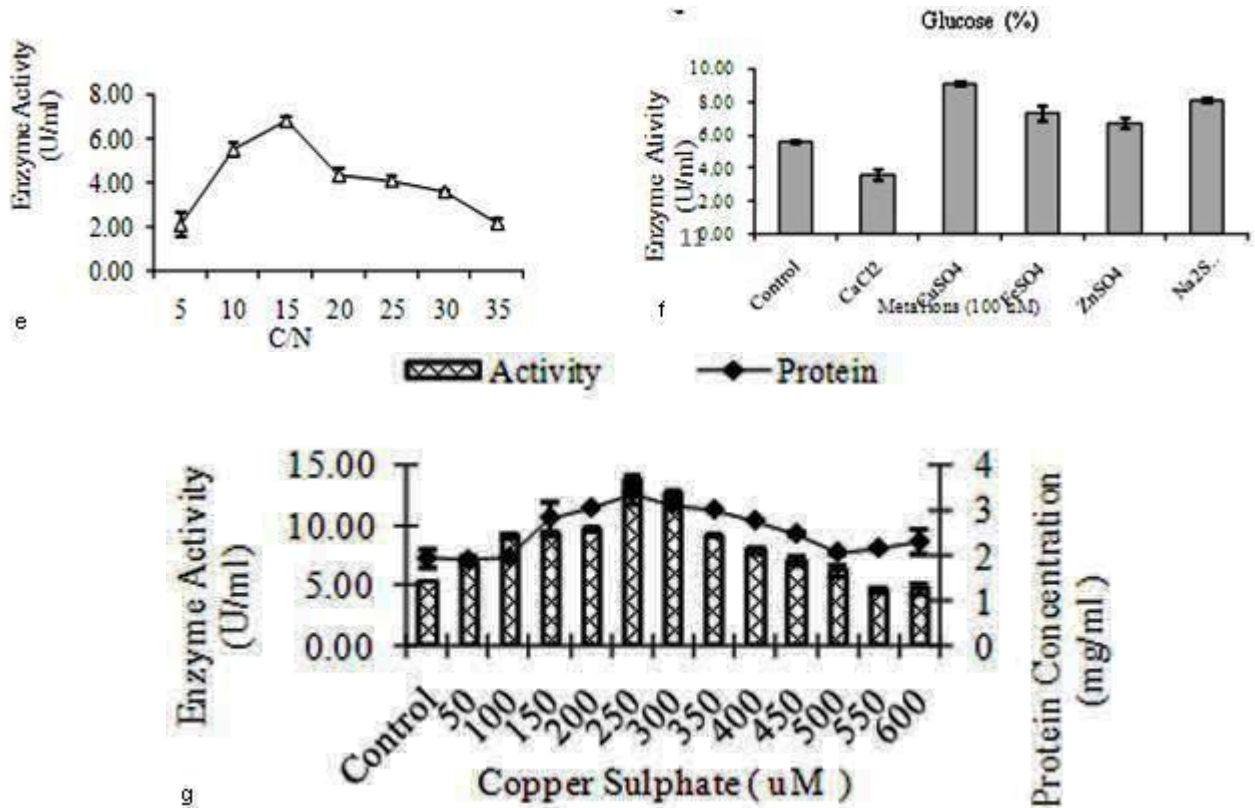


Fig:2 Effect of Physico-Chemical Parameters on Laccase Production (a) pH (b) Temperature °C (c) Incubation Time (d) Glucose (e) C/N (f) Inducers (g) Copper Sulphate

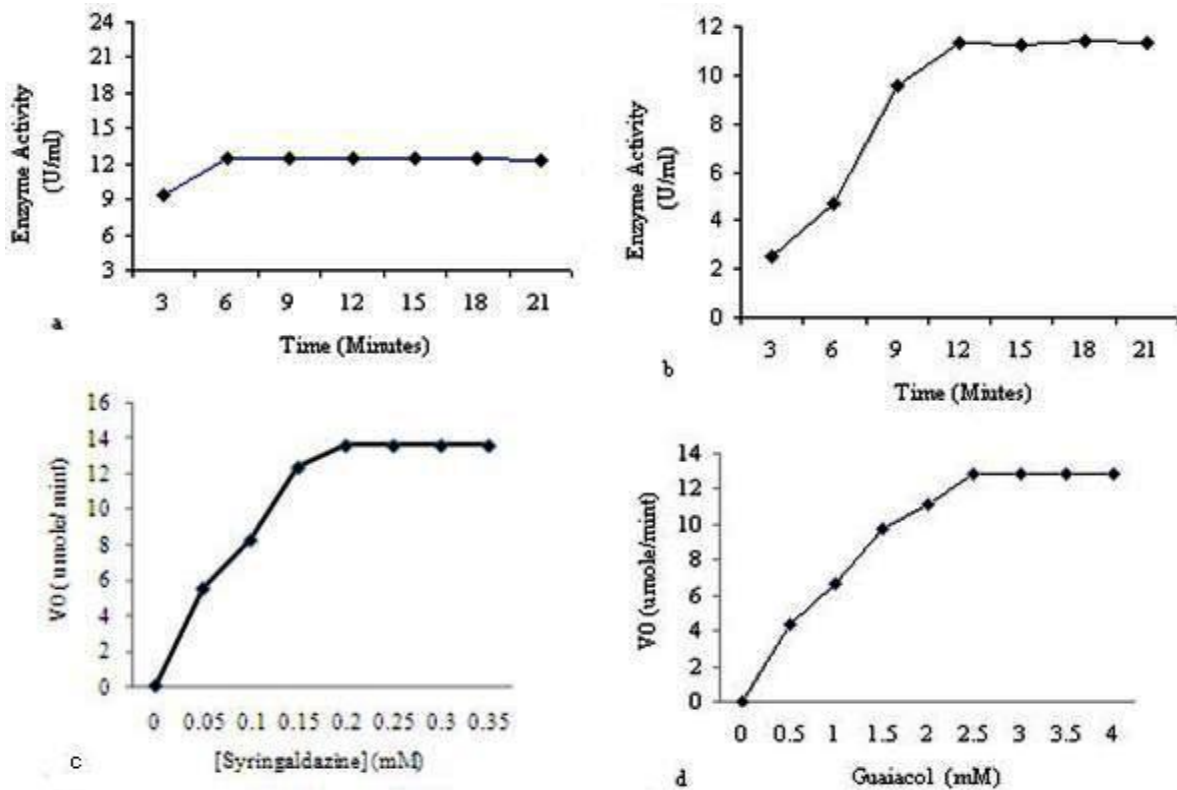


Fig:3 Effect of different concentration of syringaldazine(a,c) and Guaiacol(b,d) on laccase activity.

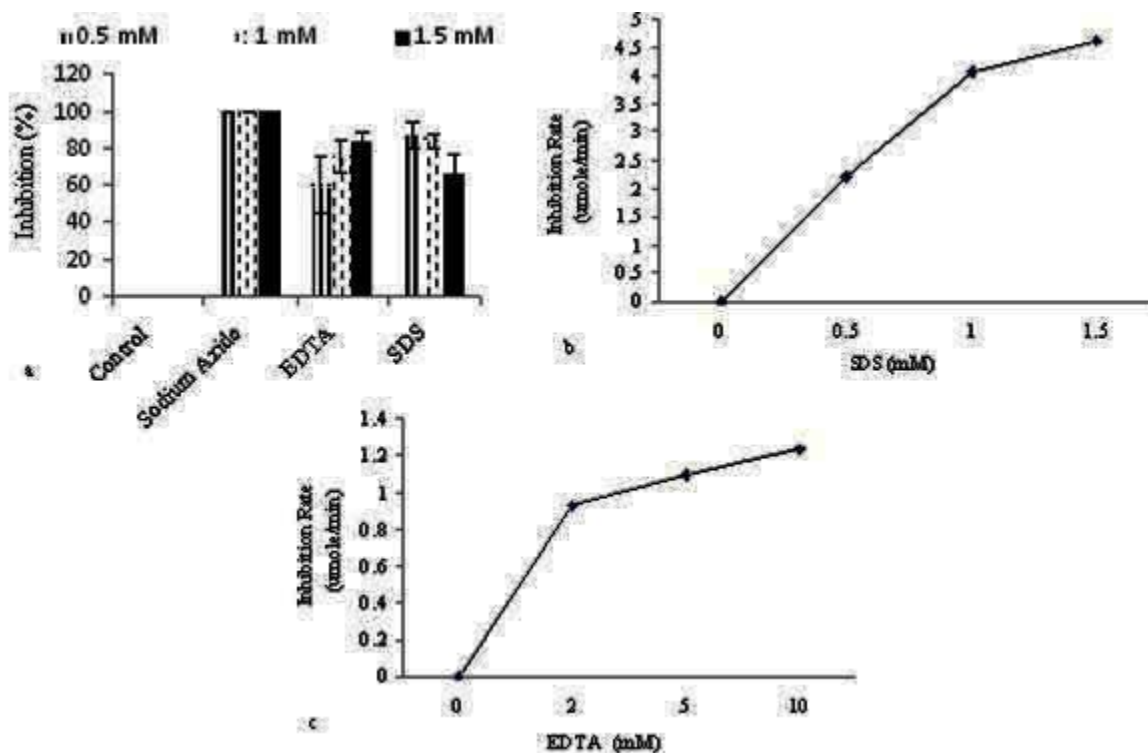


Fig: 4 Inhibition of Laccase (a) Different Inhibitors (b,) SDS (c,) EDTA

Table. 1 Effect of Medium on Laccase Production

Parameters	09-CBZ1	09-CBZ2	09-CBZ3	09-CBZ4	09-CBZ5	09-CBZ6
Enzyme activity (U/ml)	0.444 a	0.768 ab	1.110 b	3.450 c	3.780 c	6.29 d

Values having same letters in column do not differ significantly at $P < 0.05$, according to Duncan's Multiple Range Test.

Conclusion: Main objective of present study was achieved by higher production of laccase under optimized fermentation and nutritional conditions. Results revealed that yield of enzyme largely varied under different temperature, C/N, carbon, inducers and pH. Enzyme study showed that laccase oxidized syringaldazine more efficiently than guaiacol due to its affinity with non-phenolic compounds. The susceptibility of laccase towards inhibitors was evaluated and sodium azide gave maximum inhibitions than other. In future this study will be prospective and auxiliary for biotechnological operation.

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