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Laccase production using mixed substrates containing lignocellulosic materials by *Pleurotus ostreatus* in submerged liquid culture

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Abstract: Enhanced production of the industrially important enzyme laccase from the white rot fungus *Pleurotus ostreatus* MTCC1804 was performed in the submerged liquid culture. The preliminary media component indispensable to increase the laccase production were screened by the classical or one factor at a time technique. The consequence of different lignocellulosic substrates on laccase production in single and combined or mixed substrate mode were investigated under controlled fermentation conditions. From the experimental study it was revealed that, the mixed substrates including rice bran, sugarcane bagasse and glucose rendered maximum laccase activity of 6471U/l than the productivities of individual substrates. Screening of the appropriate nitrogen source permitted to substantial improvement in the laccase production of 8433 U/l by the combined nitrogen sources viz, yeast extract and L-aspargine monohydrate of organic nitrogen sources. The stimulatory effect of various aromatic inducers on the production of extracellular laccase yield was observed. Out of eleven aromatic inducers explored, 2,5 xylidine enhanced the laccase production to 17542 U/l. The statistical analysis for screening of essential media components by Plackett-Burman design was implemented and maximum laccase production of 37452 U/l was obtained in the 9th day of fermentation process.
KeywordsLaccase, *Pleurotus ostreatus*, Lignocellulosic substrates, 2,5 xylidine, Plackett-burmann design.

1.0 Introduction

Laccases (*p*-diphenol:dioxygen oxidoreductases; EC 1.10.3.2) are one of the blue multi copper enzymes (BMCE), which belong to the ligninolytic enzymatic system of fungi, particularly from the white rot fungi. They catalyze the oxidation of number of organic and inorganic compounds including monophenols, diphenols, polyphenols, methoxyphenols and aromatic amines, with the concomitant four electron reduction of oxygen to water[1,2]. Laccases are widely distributed among fungi[3,4,5], higher plants[6,7,8], in some bacteria[9,10] and in several insects[11,12]. Laccases from various sources plays several physiological functions in different manners. In fungi, they are involved in various biological functions viz, morphogenesis, fungal-plant pathogen/host interaction, stress defense, and lignin degradation[2,13]. Plant laccases are participated in the lignification process[14], Bacterial laccases involved in pigment synthesis[15] and in insects they are involved in cuticle sclerotization process[11,12].

Among the microbial populations, fungi are recognized as the prominent source by the researchers, due to their extreme facility in producing a large variety of extracellular enzymes and easy handling. They are the specific organisms responsible for lignocellulose degradation; among them wood-rotting *Basidiomycetous* fungi are identified as the best degrader of lignocellulosic materials[16]. The outstanding ability of secreting the varieties of hydrolytic and oxidative enzymes by the white rot fugal group makes them to grow efficiently on the lignocellulosic substrates and they are very indispensable for lignin, cellulose and hemicellulose degradation. The hydrolytic enzymes often identified from the cultures of white rot fungal broth are endo-1, 4-ß- D-glucanase (EC 3.2.1.4), exo-1,4-ß-D-glucanase (EC 3.2.1.91), and xylanase (EC 3.2.1.8). The lignin modifying enzymes such as lignin peroxidase (EC 1.11.1.14), manganese-dependent peroxidase (EC 1.11.1.13),

and laccase (EC 1.10.3.2) were the common oxidative enzymes. Moreover, their secretion pattern and number enzymes will be depending upon the nature of the substrates, organisms employed and nature of the cultivation mode. The genus *Pleurotus* includes edible and medicinal species that belong to group of white rot fungi. Also they have the ability to produce extracellular ligninolytic enzymes: laccase, peroxidases namely Mn dependent peroxidase (MnP), versatile peroxidase (VP) and one of the ligninolytic auxillery enzymatic systems of hydrogen peroxide synthesing enzyme aryl-alcohol oxidase (AAO), and they can modify and degrade the lignin, the recalcitrant biopolymer[17].

Laccases are typically secreted as multiple isoenzymes by the white rot fungi under different nutritional conditions[1,18]. In general, several culturing parameters influences the laccases production by the white rot fungi such as medium composition, carbon and nitrogen ratio, temperature, pH and aeration ratio[19]. In general, the wild strains of *Basidiomycete* fungi secrete low amount of extracellular laccase, however its production is increased considerably by the addition of wide variety inducers especially aromatic or phenolic compounds related to lignin or lignin derivatives such as 1-hydroxy benzotriazole (HBT), catechol, galic acid, ferulic acid, veratrylalcohol and 2, 5 xylidine[1,20]. Due to the low substrate specificity, capability of catalyzing the vast range of different kinds of substrates and higher stability of this enzyme urges it quite desirable to achieve many industrial and biotechnological applications including Construction of biosensors for analysis of drug and phenolic contents in tea[21,22], synthesis of polymer[7], bleaching of textile dye[23], bioremediation[24,25], fungicidals[26] pulp bleaching[27], clarification of juices and wines[28] and digestibility enhancement of lignocellulosic substrates for animal feed[29].

The huge availability of agro-industrial wastes from crop cultivation and food processing sectors, offers a convenient way to cultivate the microorganisms and convert them into different value-added products. Production of lignocellulolytic enzymes using different plant raw materials by white rot fungi have successfully been performed in Submerged Fermentation (SmF) and Solid-State Fermentation (SSF)[30,31,32,33,34,35,36]. In order to increase the production of laccase it is very important to optimize all the fermentation parameters which lead to the economical fermentation process with increasing yield. However the optimization of significant media components by direct practical experiments is not to be considered as economical approach. To solve these kinds of problems, statistical tools and experimental designs are mandatory to optimize all the process parameters with minimum production cost and reduction of number of experiments.

The objective of the present study is to examine the productivity of the extracellular enzyme, laccase from one of the *Pleurotus* species namely *Pleurotus ostreatus* MTCC 1804 in submerged fermentation. Optimization of preliminary media components such as carbon (natural and synthetic) and nitrogen sources (organic/inorganic models) were achieved by one factor at time (OFAT). The effect of several aromatic inducers on laccase production was experimented to enhance the laccase yield. In alternative to the traditional media optimization method of OFTA, the statistical tool Plakett-Burmann design was applied to simultaneously achieve the screening of significant media components influences the laccase production[37].

2.0 Material and methods

2.1 Chemicals

All the chemicals used in this experimentation were purchased from Hi-Media Limited, Mumbai, India, and were of the highest purity available. 2, 2'-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) was obtained from Sigma, Bangalore.

2.2 Microorganism

The white rot fungus *Pleurotus ostreatus* MTCC 1804 was purchased from Microbial Type Culture Collection (MTCC), Chandigarh and maintained in Potato Dextrose Agar (PDA) at 4^{o}C and sub cultured every 30 days.

2.3 Inoculum preparation

The actively grown slant was used to prepare spore suspension by the addition of sterile water, and 10 ml of the spore suspension was transferred into 100 ml of potato dextrose Broth (PDB) and incubated at 25^{o}C on a rotary shaker at 150 rpm. The three days old culture of *Pleurotus ostreatus* was used as inoculum for laccase production.

2.4 Neutralization of lignocellulosic material

Ligninocellulosic materials such as lemon peelings and orange peelings were chopped into small pieces and soaked in 30 ml of 81.17 mM of potassium hydroxide for 1 h in order to reduce the organic acid content, and washed repeatedly with distilled water and dried at moderate temperature.

2.5 Growth medium and Culture conditions

Experiments were conducted in 250 ml Erlenmeyer flask containing 100 ml of the basal media with the following composition: glucose 20 g/l, yeast extract 1.5 g/l, urea 0.75 g/l, KH_2PO_4 0.25 g/l and 10 ml of salt solution containing $MgSO_4.7H_2O$ 0.5 g, $CaCl_2.2H_2O$ 0.1 g, KCl 0.5 g, $CuSO_4.7H_2O$ 1.0 mM designed by Krishna Prasad et al. (2005)[38] with small modification for laccase production by *Pleurotus ostreatus* MTCC 1804 and then pH was adjusted to 5.6 and sterilized at 121^oC for 15 min. The basal media was inoculated with 3.0 ml of inoculum and incubated at 25^oC on a rotary shaker at 120 rpm for 9 days.

2.6 Experimentation

The white rot fungus *Pleurotus ostreatus* MTCC1804 was used to perform the laccase production in a shaking liquid culture under controlled fermentation conditions. The formation of laccase from *P.ostreatus* is achieved by screening of the suitable carbon, nitrogen and inducers by OFAT. The natural model substrates (lignocelluloses) such as Rice Bran (RB), Wheat Bran (WB), Bannana Leaves (BL), Sugarcane Bagasse (SB), Corn Stalcs (CS), Lemon Peelings (LP), Orange Peelings (OP), Water Hyacinth (WH), Waste Paper (WP) and Elephant Grass (EG) were examined for laccase production by *P. ostreatus*. They were collected from the local market and milled after drying. The substrates such as lemon peelings and orange peelings were neutralized to suppress organic acid contents. Among the lignocellulosic substrates examined for laccase production, higher laccase yielding promising substrates were identified and were utilized for combined or mixed substrate fermentation.

The influence of organic and inorganic nitrogen sources on laccase production was investigated in single and combined nitrogen mode. The nitrogen sources such as Yeast Extract (YE), Malt Extract (ME), Peptone, Soy Bean Meal (SBM), L-asparagine monohydrate (L-asp), Ammonium Tartarate (AT), NH_4SO_4, NH_4NO_3 and Urea were used to screen the ideal nitrogen source for enhancing the laccase production. The impact of various aromatic inducers on laccase production was examined. The inducers such as 2,5xylidine, P-anisidine, Guaiacol, Catechol, 2,6 dimethoxy phenol (DMP), Hydroxybenzotryazole (HBT) 2495, Veratryl alcolhol, Vanilic acid, Galic acid, 1-Napthol and Ferulic acid were investigated with 1.0 mM concentration. Except few compounds (guaiacol and catechol) all other compounds were dissolved in 50% ethanol and sterilized by filtration. These inducers were added at the 3rd day of the fermentation process. The use of statistical method plackett-burman design permitted to identify the most significant media component influences the laccase production as well as to increase yield.

2.7 Biomass determination

The biomass measurement of fungal mycelium was determined by the dry weight method. The culture broth was filtered using Whatman No.1 filter paper. The biomass retained was washed with distilled water and dried at 100^oC to a constant weight.

2.8 Laccase assay

Laccase activity was determined using 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate at room temperature. The reaction mixture contained 5 mM ABTS, 0.1 M sodium acetate buffer (pH 5.0) and 0.17 ml of appropriately diluted culture fluid. Oxidation of ABTS was monitored by following the increase in absorbance at 420 nm (ε = 36000 M^{-1} cm^{-1}). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1.0 µmol of ABTS per minute. The laccase activities were expressed in U/l. Heat inactivated enzyme was employed as control[39].

3.0 Results and Discussion

3.1 Time course of laccase production using *Pleurotus ostreatus* in submerged fermentation

In order to predict the optimum fermentation period for laccase production by *P. ostreatus* was studied with respect to the cell mass concentration using glucose, yeast extract basal media under controlled

fermentation conditions. Samples were withdrawn every 24 hr for analysis in order to evaluate the amount of laccase production and cell mass concentration.

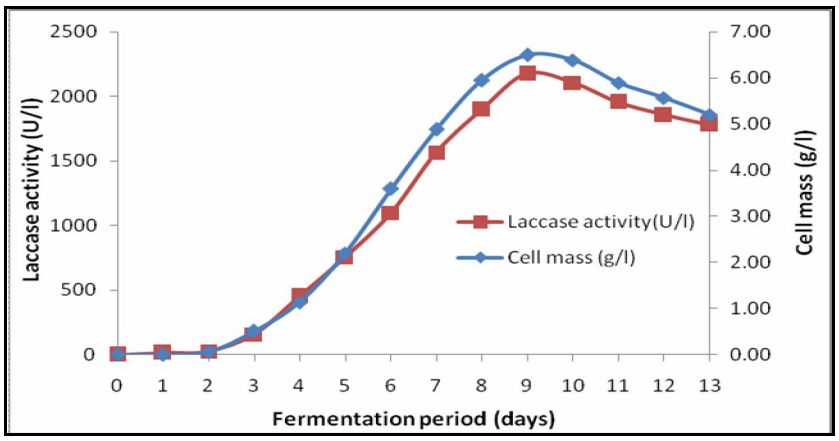

Figure 1 Time course of laccase production and cell mass yield by *Pleurotus ostreatus* in submerged fermentation

From the period of inoculation until 3rd day both cell mass and laccase activity seems to be very low level. The morphology of cell was observed to be pellet, dense as well as small in size. Figure 1 illustrates the laccase production in relation to cell mass concentrations by *P. ostreatus* in submerged fermentation. Maximum laccase production was obtained during the stationary phase on 9th day of fermentation with the laccase activity of 2176.6 U/l and cell mass of 8.29 g/l. Previously, it has been mentioned that expression of laccase and other ligninolytic enzymes from different fungi have been associated with the stationary phase of growth and they have been triggered under nutrition starvation conditions[16].

After 9th day of fermentation, reduction in the production of laccase was observed; in addition the broth characteristics were changed as watery and decline in the cell mass was also observed. This might be due to the digestion of cell pellets by autolysis caused by the protealytic enzymes in the native cells.

3.2 Screening of best lignocellulosic substrates for laccase production

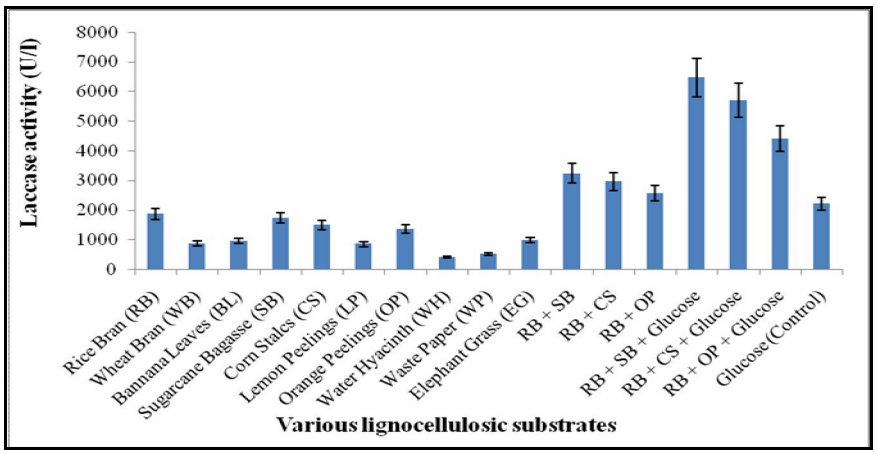

Figure 2 Influence of various lignocellulosic substrates and its mixtures on laccase production using *Pleurotus ostreatus* in submerged fermentation

Screening of the suitable carbon source for laccase production is an essential step to achieve the better laccase production by the wood rotting fungi. In the present study various lignocellulosic substrates with different structural compositions were exploited for achieving higher laccase production as well as to diminish the production cost of fermentation process. Each lignocellulosic material were taken as 2.0 g per 100 ml in yeast extract and urea contained basal medium with 10 ml of mineral salt solution contains: $MgSO_4.7H_2O$ 0.5g; $CuSO_4.7H_2O$ 0.24g; CaCl 0.1g; KCl 0.5g. The culture supplemented with glucose was considered as reference culture. Figure 2 depicts the effect of laccase production by *P. ostreatus* using various lignocellulosic materials. Among the various lignocellulosic substrates tested for laccase production, rice bran showed maximum laccase

activity of 1860 U/l followed by sugarcane bagasse (1732 U/l), orange peelings (1358 U/l) and corn stalks (1490.4 U/l). Very low activities were detected from the substrates such as water hyacinth (407.6 U/l) and waste paper (509.6 U/l). The lignocellulosic substrates such as elephant grass 985 U/l, bannana leaves 955.4U/l, wheat bran 856.2 U/l and lemon peelings 853.4 U/l were produced moderate level of laccase activities. The control culture with glucose produced laccase activity of 2204.6 U/l.

The identified next three best laccase producing lignocellulosic substrates were equally combined with rice bran viz, RB+SB, RB+CS and RB+OP of 1.0 g each to enhance the laccase production. The combinations RB + SB, RB + CS and RB + OP showed laccase activity of 3236 U/l, 2963.1 U/l and 2569 U/l respectively. Further to increase the laccase production, small amount of glucose (0.2 g) was incorporated to all these three combinations. The lignocellulosic mixtures along with glucose resulted in increased procution of laccase activity such as RB+SB+Glucose (6471 U/l), RB+CS+Glucose (4408 U/l) and RB+OP+Glucose (5707 U/l). The performed experiments with mixed lignocellulosic substrates and mixed lignocellulosic substrates along with glucose gave better laccase yield. The reason for improvement in laccase production is may be due to the higher sugar concentration liberated from the lignocellulosic substrate and externally added small amount of glucose which supported the high laccase production in *P. ostreatus*.

3.3 Screening of best nitrogen source for laccase production in mixed carbon source

The proper selection of the type of nitrogen source in the production medium offers the best, easiest and economical way to enhance the laccase production by the white rot fungi. In general laccases are secreted in secondary metabolism under nitrogen depleted conditions[1,40]. In some white rot fungi laccase production was supported by higher nitrogen concentrations[13].

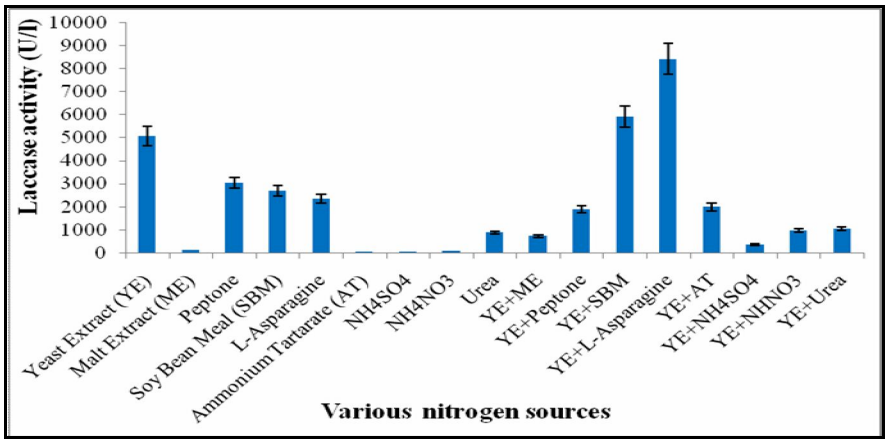

Figure 3 Comparision of various nitrogen source and its combinations in mixed carbon source for laccase production

According to OFAT method the superior nitrogen source was screened using RB + SB + Glucose as the carbon source. The influence of organic and inorganic nitrogen sources in single and combined manner for laccase production by *P. ostreatus* was studied in submerged fermentation. The nitrogen sources as single factor were examined with concentration of 2.25 g per 100 ml, and the combined nitrogen sources such as YE + ME, YE + Peptone, YE + SBM, YE + L-asp, YE + AT, YE + $NH4SO_4$, YE + $NHNO_3$ and YE + Urea were studied with concentration of each 1.125 g per 100 ml. Figure 3 illustrates the comparision of various nitrogen sources and its combinations in mixed carbon source (RB+SB+Glucose) for laccase production. The results obtained for laccase production by single nitrogen sources by *P. ostreatus* showed that peak laccase activity of 5070.5 U/l was detected from the organic nitrogen source namely YE. The other organic nitrogen sources viz, Peptone, SBM and L-asp produces the considerable amount of laccase activity of 3042 U/l, 2698 U/l and 2341 U/l respectively. Among the various organic nitrogen sources examined for laccase production, very low activity of 119.7 U/l was obtained from ME.

The inorganic nitrogen sources investigated for laccase production showed maximum laccase yield of 873.1 U/l from urea. Also laccase production from the cultures of NH_4NO_3, NH_4SO_4 and AT was found to be low activity of 79.38 U/l, 52.92 U/l and 39.69 U/l respectively. Productivity of laccase from inorganic nitrogen sources was found to be very low compared to that of organic nitrogen sources; in addition the cell growth of these cultures was also found to be suppressed. In order to further increase the laccase production, the effect of

combined nitrogen sources was investigated using higher laccase yielding nitrogen source, yeast extract as the pair competitor for both organic and inorganic nitrogen sources. The following combinations were made using yeast extract along with organic and inorganic nitrogen sources such as YE + ME, YE + Peptone, YE + SBM, YE + L-asp, YE + AT and YE + NH_4SO_4. Among the different combinations examined, maximum laccase activity of 8433 U/l was obtained from the organic + organic nitrogen source combination of YE + L-aspargine. A moderate level of laccase activity was observed from the combinations of YE + SBM (5913U/l). YE + Peptone (1885.4 U/l) and YE + ME (713.8 U/l) produced low laccase yields. The maximum laccase yield for organic + inorganic nitrogen source combination was obtained from YE + AT (1984 U/l), however it is lesser amount when compared to that of organic nitrogen source combination (YE+L-asp). The other combinations such as YE + Urea 1047 U/l, YE + $NHNO_3$ (965.7 U/l) and YE + NH_4SO_4 (357.2 U/l) yielded less laccase production. Laccase production by combined nitrogen source mode of YE+ L-asp and YE+SBM gave better results than the single nitrogen mode, but not in all combinations. The organic nitrogen source peptone without combination yielded high laccase activity, nevertheless they did not produce higher amount in the combined mode of nitrogen sources.

It was observed that according to the nature of the nitrogen source laccase production clearly vary and expressed different laccase yield for different nitrogen sources. This clearly indicates that laccase production by *P. ostreatus* depends on the nature of the nitrogen source in the culture medium. The organic nitrogen source peptone usage in *Lentinus edodes* and *P.ostreatus* enhanced the laccase production[41]. Stajic et al., (2005)[42] and Mikiashvili et al., (2006)[43] strongly emphasize that organic nitrogen sources have the ability to increase the laccase yield than the inorganic nitrogen sources.

3.4 Study of copper sulphate addition on laccase production in mixed substrates

a. Effect of copper sulphate concentration on laccase production

Laccase production can also be increased by the supplementation of sufficient concentration of copper sulphate to the culture medium; Copper sulphate is not an inducer, but it is micronutrient that has the potential to increase the laccase production significantly. Effect of different concentrations of copper sulphate on laccase production using *P.ostreatus* was studied with different concentration of copper sulphate such as 0.25, 0.5, 0.75, 1.0, 1.5, 1.75 and 2.0 mM in RB + SB+ Glucose and YE + L-aspargine containing basal media. Figure 4 illustrates the effect of various concentrations of copper sulphate on laccase production by *P.ostreatus* in submerged fermentation. Among the various concentrations of copper sulphate tested, maximum laccase activity of 15247 U/l was obtained from 1.5 mM copper sulphate during the 9th day of fermentation, and lower laccase activity of 6563 U/l was obtained from the 0.25 mM concentration. The fermentation medium without copper sulphate maintained as control culture and produced the laccase activity of 5128 U/l. Both cell mass as well as laccase yields were suppressed due to the addition of copper sulphate at concentrations above 1.5 mM.

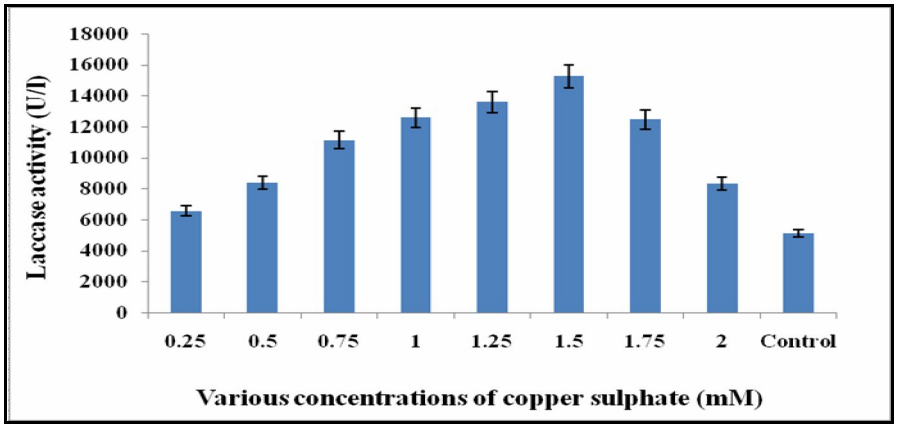

Figure 4 Effect of various concentrations of copper sulphate on laccase production

b. Time course of copper sulphate addition for laccase induction

In order to optimize the exact time period to incorporate the optimized concentration of copper sulphate (1.5 mM) in the culture medium was studied by supplementing the copper sulphate at different interval of time period in *P.ostreatus* using RB + SB + Glucose and YE + L-aspargine containing basal medium for enhancing the laccase production. Addition of 1.5 mM concentration of copper sulphate to the culture medium containing

different flasks was commenced from the period of inoculation to 5th day of the fermentation. Figure 5 illustrates the time course of 1.5 mM copper sulphate addition in *P.ostreatus* for laccase induction in submerged fermentation. Gradual increment in laccase production was identified from the period of inoculation until 3rd day of copper sulphate incorporated fermentation samples, on the other hand it can be said that retardation in cell growth and laccase secretion was observed during those periods. Laccase production was found to be slowly decreased at 4th and 5th day of the fermentation process. Cultures supplemented with 1.5 mM copper sulphate incorporated on 3rd day produced a maximum laccase activity of 15247 U/l in the 9th day of fermentation. A minimum laccase production of 8319 U/l was obtained from copper sulphate addition during the period of inoculation of the fermentation process.

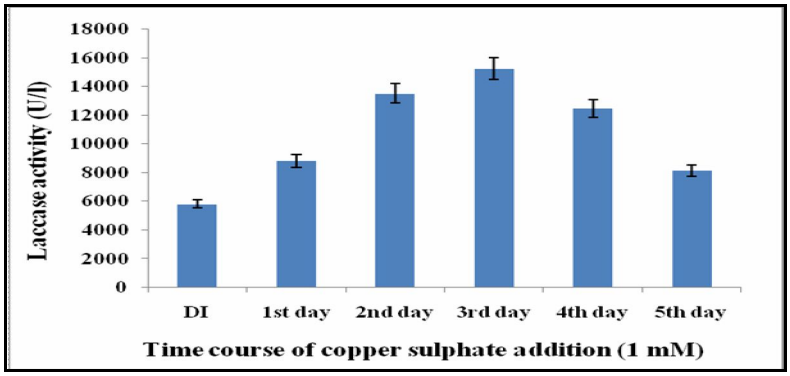

Figure 5 Time course of 1.5 mM copper sulphate addition for laccase induction

3.5 Study of inducers on laccase production in mixed substrates

a. Effect of different inducers on laccase production

The majority of the wild strains belonging to the white rot fungal family used for laccase production showed lower amount of laccase yield; however its production can be enhanced by addition of inducers[18]. To enhance the laccase production, various forms of inducers viz, aromatic compounds, metal ions, organic solvents, vitamins, xenobiotics and surfactants were used in the production medium.

Nevertheless, aromatic inducers have been proved their inducing ability and they have elaborately been used for stimulating the laccase yield in many fungal species by variety of inducing substrates such as, 2,5 xylidine[44], Ferulic acid[45] and Veratryl alcohol[46]. Based on the studies of previous literatures regarding the time course of addition of inducers, suggests that the optimum time period to add the inducers in the production medium is 3rd day of the fermentation process. Accordingly all the inducers were incorporated on 3rd day of the fermentation to avoid the repression of cell growth as well as to acclimatize the environment by the organism in relation to laccase production. In the present study several aromatic inducers such as 2,5xylidine, P-anisidine, Guaiacol, Catechol, 2,6 dimethoxy phenol (DMP), Hydroxybenzotryazole (HBT), Veratryl alcolhol, Vanilic acid, Galic acid, 1-Napthol and Ferulic acid were examined to induce the laccase production by *P. ostreatus* in submerged fermentation. The culture without any inducers was maintained as control. Except guaiacol and catechol, all other inducers were prepared with 50% ethanol, and sterilized by filtration. They were added into the medium with the concentration of 1.0 mM on 3rd day of the fermentation. Figure 6 depicts the effect of different aromatic inducers in mixed carbon source for laccase induction by *Pleurotus ostreatus* in submerged fermentation. The inducers used for laccase induction exhibited considerable variations in relation to cell morphology and laccase production.

Out of eleven inducers investigated for the stimulation of laccase by *P. ostreatus*, maximum laccase activity of 28542 U/l was obtained from 2,5 xylidine. It was observed that culture supplemented with 2,5 xylidine exhibited the cell morphology of dense and small beads and also fermentation broth was found to be viscous and deep pinkish color. Galic acid (12495 U/l), 1-Napthol (12145 U/l), Ferulic acid (11295 U/l) and 2,6 Dimethoxy phenol (10795 U/l) stimulated appreciable amount of laccase activity but lower than the productivity obtained from 2,5 xylidine. The aromatic inducers such as p-anisidine and Vertryl alcolhol were expressed moderate level activities of 9696 U/l and 8746 U/l respectively.

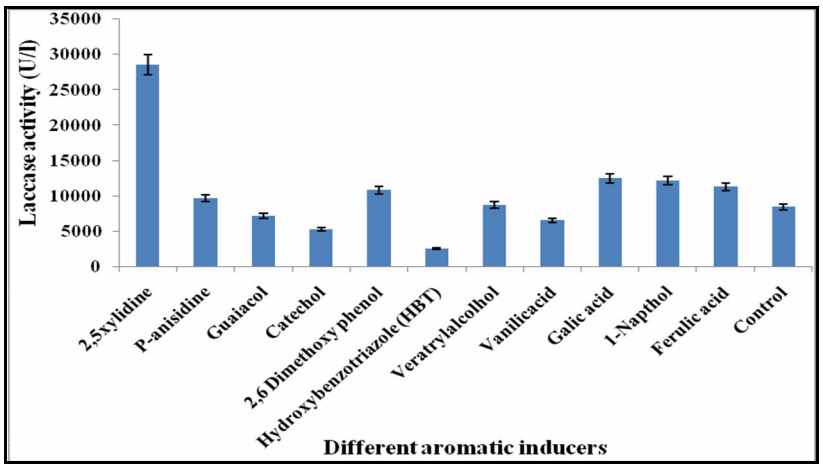

Figure 6 Effect of different aromatic inducers in mixed carbon sources for laccase production

On the contrary, cultures incorporated by several other inducers to stimulate the laccase levels such as, Guaiacol (7147 U/l), vanilic acid (6547 U/l), catechol (5247 U/l) and HBT (2495 U/l) were disclosed their incapability of inducing the high amount of laccase titres, when compared to the control culture (8433 U/l), and also yielded very low cell mass concentration. Laccase production by the lignolytic fungus *Panus tigrinus* 8/18 was enhanced by the addition of aromatic inducer like 2,4 dimethylphenol with $CuSO_4.7H_2O$ and showed ten fold increase in laccase yield[47].

The findings observed were identical with the former reports of laccase induction by the aromatic inducers, for instance the white rot fungus *Trametes versicolor* has showed increased laccase production of 70125 U/l by the addition of 1.0 mM concentration of 2,5 xylidine on 3rd of the fermentation process[20]. In addition, supplementation of 2,5 xylidine to the cultures of *Pycnoporus cinnabarinus*[1] and *Trametes versicolor*[48] lead to higher laccase production.

b) Effect of different concentrations of 2,5 xylidine on laccase production

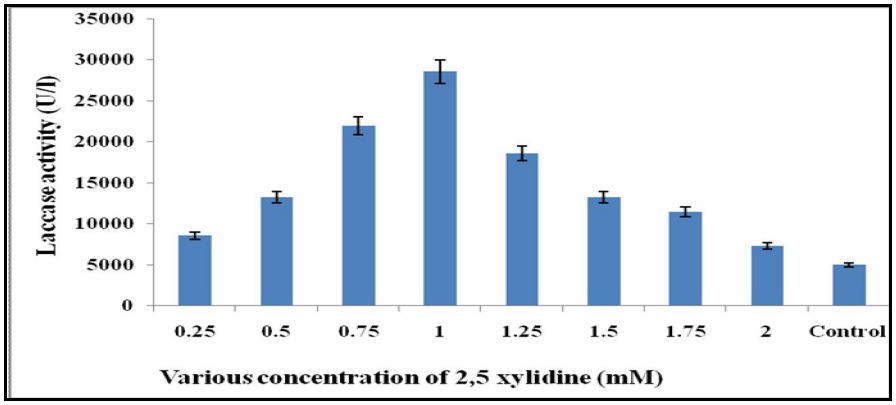

Figure 7 Effect various concentrations of 2,5 xylidine on laccase production

Even though, addition of aromatic inducer (2,5 xylidine) has the ability to increase the laccase production, however which is not a satisfactory one unless to find the impact of optimum concentration on laccase production to achieve the better yield. A series of concentrations of 2,5 xylidine of 0.25 mM to 2.0 mM were analyzed in order to find the impact of the relevant concentration on laccase production by *P.ostreatus* in submerged fermentation. Figure 7 illustrates the Effect various concentrations of 2,5 xylidine on laccase production by *Pleurotus ostreatus* in submerged fermentation. The laccase production was gradually increased by the concentrations of 0.25 mM to 1.0 mM, followed by reduction in laccase production was observed for 1.25 mM to 2 mM of 2,5 xylidine.

The maximum laccase production was obtained from the concentration of 1.0 mM of 2,5 xylidine with the maximum laccase activity of around 28542 U/l. Laccase production of 7325 U/l was obtained from 0.25

mM, as found to be the minimum laccase yield among the concentrations investigated. Control culture incubated without copper sulphate produced a laccase yield of 5012 U/l.

c) Time course of 2, 5 xylidine addition for laccase production

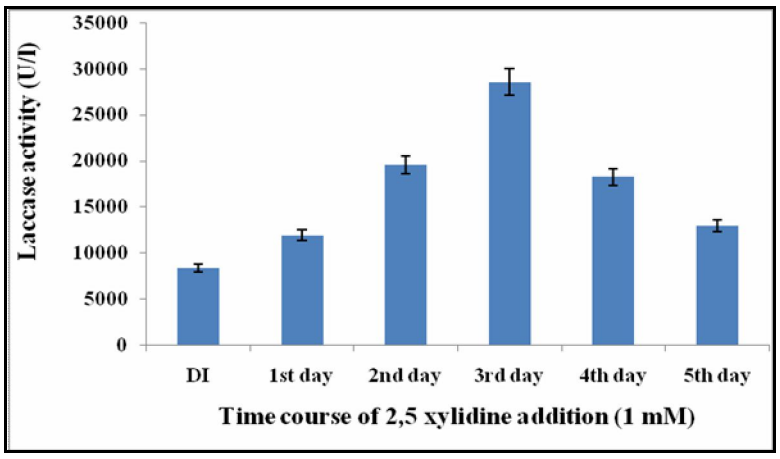

Figure 8 Time course of 2,5 xylidine addition for laccase induction

To investigate the time at which the incorporation of 2,5 xylidine influences the laccase production was examined in submerged fermentation using *P.ostreatus*. However their supplementation into the production medium at accurate time period plays important role. The optimized concentration of 1.0 mM of 2,5 xylidine was supplemented with different time intervals, including the time of inoculation to 5th day of fermentation process. Figure 8 illustrates the time course of 2,5 xylidine addition for laccase induction in submerged fermentation by *P.ostreatus*. Laccase production was gradually increased from the day of inoculation to 3rd day addition of 1.0 mM of 2,5 xylidine in the fermentation process. The high laccase activity of 28542 U/l was obtained from the 3rd day 2,5 xylidine added culture, and was found to be the optimum time period to add 2,5 xylidine. Tavares et al. (2006)[49] have stated that the addition of copper and 2,5 xylidine, phenolic mixtures on 3rd day of fermentation by *Trametes versicolor* increased the maximum laccase activity of 5500Udm^{-3}. Production of laccase was gradually decreased on 4th and 5th day of 2,5 xylidine addition in the fermentation process**.**

3.6 Plackett-Burman design for screening of significant media components for laccase production

To obtain a desire response for the multivariable system, each variable has to be investigated from their lower concentrations to higher concentrations. The influence of significant media components on laccase production by *P.ostreatus* was determined using Plakett-Purman (PB) design. PB design is a fractional factorial design and the main effect (the contrast coefficient) of such a design may be simply calculated as the difference between the average of measurements made at the low level (-1) of the factor and the average of measurements made at the high level (+1) of the following variables: A Rice bran (5.0; 20); B Sugarcane Bagasse (5.0; 20); C Glucose (2.0; 4.0); D Yeast extract (0.5; 5.0); E L-aspargine monohydrate (0.5; 5.0); F $MgSO_4.7H_2O$ (0.05 0.5); G KCl (0.05; 0.5); H KH_2PO_4 (0.25; 2.0); I $CaCl_2$ (0.1; 0.5); J $CuSO_4.7H_2O$ (0.12; 0.24); K 2,5xylidine (0.06; 0.121) and L $MnSO_4.7H_2O$ (0.001; 0.005). Contrast coefficients allow the determination of the effect of each constituent. A large contrast coefficient (CC) either positive or negative show that a factor has a large impact on titre; while a coefficient close to zero means that a factor has little or no effect.

Table 1 Various medium components with lower (-1) and higher (+1) levels of independent variables for laccase production

Variables	Media components	Minimal value (-1) (g/l)	Maximal value (+1) (g/l)
A	Rice bran	5	20
B	Sugarcane Bagasse	5	20
C	Glucose	2	4
D	Yeast extract	0.5	5
E	L-aspargine monohydrate	0.5	5
F	$MgSO_4.7H_2O$	0.05	0.5
G	KCl	0.05	0.5
H	KH_2PO_4	0.25	2.0
I	$CaCl_2.2H_2O$	0.1	0.5
J	$CuSO_4.7H_2O$	0.12	0.24
K	2,5 -xylidine	0.06	0.121
L	$MnSO_4.7H_2O$	0.001	0.005

Table 2 Plackett-Burman design generated for 12 medium components with coded values along with the experimental values of laccase activity

Run	A	B	C	D	E	F	G	H	I	J	K	L	Laccase activity (U/l)
1	+	+	+	+	-	+	-	+	+	-	-	+	35478
2	+	+	+	-	+	-	+	+	-	-	+	-	37452
3	+	+	-	+	-	+	+	-	-	+	-	-	29863
4	+	-	+	-	+	+	-	-	+	-	-	-	31258
5	-	+	-	+	+	-	-	+	-	-	-	+	33522
6	+	-	+	+	-	-	+	-	-	-	+	+	35283
7	-	+	+	-	-	+	-	-	-	+	+	+	22149
8	+	+	-	-	+	-	-	-	+	+	+	+	26321
9	+	-	-	+	-	-	-	+	+	+	+	-	22584
10	-	-	+	-	-	-	+	+	+	+	-	+	13251
11	-	+	-	-	-	+	+	+	+	-	+	-	25364
12	+	-	-	-	+	+	+	+	-	+	-	+	27458
13	-	-	-	+	+	+	+	-	+	-	+	+	25413
14	-	-	+	+	+	+	-	+	-	+	+	-	24178
15	-	+	+	+	+	-	+	-	+	+	-	-	20147
16	-	-	-	-	-	-	-	-	-	-	-	-	19325

A-Rice bran; B-Bagasse; C-Glucose; D-Yeast extract; E-L-aspargine monohydrate; F-$MgSO_4.7H_2O$; G-KCl; H-KH_2PO_4; I-$CaCl_2.2H_2O$; J-$CuSO_4.7H_2O$; K-2,5 xylidine; L-$MnSO_4.7H_2O$

Table 1 shows the 12 medium components with lower (-) and higher (+) concentrations of two levels of each variables for laccase production by *P. ostreatus*. In Table 2, the coded levels of the entire media components were given for 16 trials along with the experimental value of laccase activity. The column represents the different variables selected for the design with coded levels and rows indicate the 16 trials for laccase production. This experimental analysis of design simultaneously measures both impact of most significant media component and improvement in laccase production with the possible interactions of two levels concentrations of each variables. Based on the design experimentation, maximum laccase activity of 37452 U/l and minimum activity of 13251U/l was obtained on 9^{th} day of the fermentation.

The main effect plot (Figure 9) illustrates the positive and negative effect of the media components for laccase production. The components viz, Rice bran, Sugarcane bagasse, Yeast extract, L-aspargine monohydrate, $MgSO_4.7H_2O$, Glucose, 2,5xylidine and $CuSO_4.7H_2O$ were found to be as positive effect, similarly the components such as, $MnSO_4.7H_2O$, KCl, $KH_2PO_4.7H_2O$ and $CaCl_2.2H_2O$ showed negative effect

as evident from the result of PB design analysis. The components identified as the positive effects indicate that the amount utilized for the production is higher than the required concentration; likewise the negative effects emphasize that the medium component utilized is lower than the required concentration.

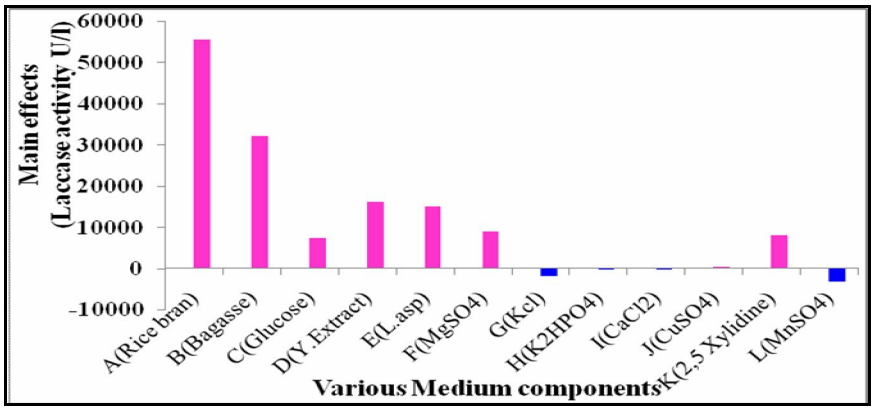

Figure 9 Main effects plot illustrates the positive and negative effect of the media components for laccase production

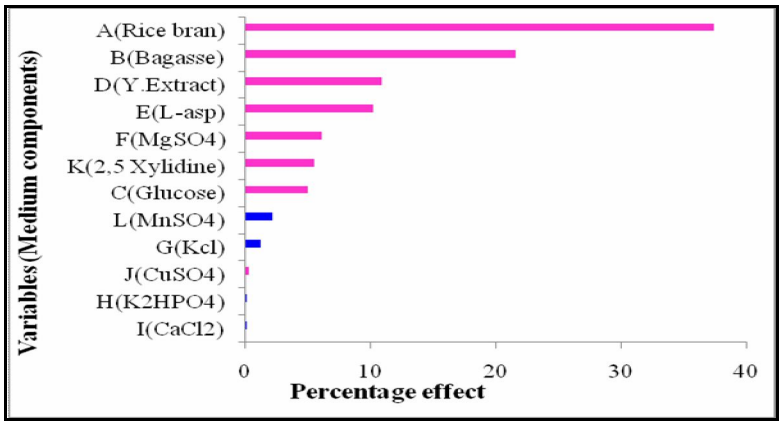

Figure 10 Pareto plot for PB parameter estimates of laccase production

The Pareto plot (Figure 10) offers a convenient view of the findings obtained from the PB design. Representing the significance of the media components based on their effect in laccase production in submerged fermentation. The following components such as Rice bran, Sugarcane bagasse, Yeast extract, L-aspargine monohydrate and $MgSO_4.7H_2O$ were found to be the most significant media components which strongly influence the laccase production.

Conclusion

A detailed study of laccase production by the wood rotting fungus *P. ostreatus* MTCC 1804 was performed using the mixed carbon sources containing natural (agricultural wastes) and synthetic (glucose) substrates in the batch submerged fermentation. In addition, this study accentuates the necessity of screening of the powerful lignocellulosic substrates from diverse lignocellulosic substrates having different structural constituents to disclose the actual potential of the white rot fungus expressing the ligninolytic enzyme laccase. The influence of carbon source on laccase yield gained the importance while using the mixed substrates such as Rice bran, Sugarcane baggase and Glucose with formation of an increased laccase activity of 6471 U/l than the individual substrates utilization. The screening of appropriate nitrogen sources is adequate to enhance the laccase production was confirmed by obtaining the improved laccase yield of 9433 U/l from the combined action of yeast extract and L-aspargine monohydrate. The inducing ability of the different aromatic inducers on laccase production was explored and 2,5 xylidine found to be the most excellent inducer with the stimulated laccase activity of 28542 U/l. Plackett-burman design was adapted to identify the prominent variables responsible for regulating the laccase production. The statistical analysis revealed that the components Rice bran, Sugarcane bagasse, Yeast extract, L-aspargine monohydrate and $MgSO_4.7H_2O$ were highly significant for

laccase production. Moreover the improved laccase productivity of 37452 U/l was attained by the implementation of the PB design of experimental analysis.

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