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RESEARCH ARTICLE

ENHANCED PRODUCTION OF LACCASE ENZYME BY THE WHITE-ROT MUSHROOM FUNGUS *PLEUROTUS FLORIDA* USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Laccase is the chief ligninolytic enzyme produced by the white-rot mushroom fungi and it has several biotechnological applications including removal of toxic organo pollutants, dye degradation and in melanin synthesis. There is a growing need for isolation and identification of new laccase producing organisms to be used in industries. This paper reports that the increase in laccase production and activity in *Pleurotus florida,* an oyster mushroom species, in the presence of PAH (Anthracene) by using media engineering techniques and Statistical design experiments involving Response Surface Methodology. Central composite design was applied to optimize the media components and to evaluate the effect of peptone, malt extract and $CaCl_2$ on laccase activity. This statistical design experiment aimed to reduce the number of experiments and to obtain more information on the mutual interactions between the variables. Central composite design led to 20 sets of experiments. When the optimized amounts of nutrients (Peptone, Malt Extract and $CaCl_2$ was 1, 1, 0.1g respectively) were used as supplements to the basal salt medium, laccase activity obtained was increased ten fold (from 2.20 IU/ml to 22.6 IU/ml) over the control treatment using basal salt medium. The interaction between the three supplements as revealed by the RSM indicates that laccase activity was promoted when basal salt medium was supplemented with peptone, malt extract and $CaCl_2$were in the ratio 1:1:0.1. The significant increase in laccase activity using response surface methodology demonstrates the bioprospecting potential of this isolate as a good source of laccase enzyme for industrial applications.

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INTRODUCTION

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belongs to the small group of enzymes called the blue copper proteins or the blue copper oxidases along with the plant ascorbate oxidase and the mammalian plasma protein ceruloplasmin (Thurston, 1994) among others. Laccase is widely distributed in higher plants and fungi (Messerschmidt and Huber, 1990) and has been found also in insects and bacteria. Recently a novel polyphenol oxidase with laccase like activity was mined from a metagenome expression library from bovine rumen microflora (Beloqui *et al*., 2006). Laccase is aboundant in many white- rot fungi that are involved in lignin metabolism (Bourbonnais *et al*., 1995, Leontievsky *et al*., 1997). Fungal laccases have higher redox potential than bacteria or plant laccases (up to +800 mV) and their action seems to be relevant in nature, finding also important applications in biotechnology. Fungal laccases are involved in the degradation of lignin or in the removal of potentially toxic phenols arising during lignin degradation (Thurston, 1994). The white – rot fungi are a physiological rather than taxonomic grouping, comprising those fungi that are capable of extensively degrading lignin (heterogenous polyphenolic polymer) within lignocellulose substrates (Eaton and Hale, 1993). The ability to catabolize cellulose and hemi cellulose, the polysaccharides forming the other main components of ligno cellulose, is fairly common as a primary metabolic process among fungi and other organisms and occurs under a range of environmental conditions. The oxidation of lignin yields no net energy gain and so lignin is degraded during secondary metabolism in order to access wood polysaccharides locked in lignin - carbohydrate complexes, providing an energy source to which other organisms do not have access (Jeffrics, 1990). White – rot fungi variously secrete one or more of three extra cellular enzymes that are essential for lignin degradation which combine with other processes to effect lignin mineralization.

They are often referred to as Lignin Modifying Enzymes or LMEs. Laccase activity has also been demonstrated in fungi like *Coriolopsis gallica* (Pickard *et al.,* 1999), *Pycnoporus cinnabarinus* (Bourbonnais *et al*., 1997), *Phaenerochaete chrysosporium* (Bumpus , 1989), *Trametes versicolor* (Morgan *et al*., 1991) and *Pleurotus* sp (Bezalel *et al*., 1996). These enzymes catalyze one-electron oxidation of four reducing-substrate molecules concomitant with the four- electron reduction of molecular oxygen to water (Piontek *et al.,* 2002). Laccase are capable of mineralization of a wide range of highly recalcitrant organopollutants with structural similarities to lignin (Pointing, 2001). Currently the degrading properties of

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laccases are being exploited for a range of biotechnological applications and hence studies on laccase producing organisms have been intensified in the recent years. Concerning their use in the biotechnology area, fungal laccases have widespread applications, ranging from effluent decolouration to pulp bleaching, removal of phenolics from wines, organic synthesis, biosensor, synthesis of complex medical compounds and dye transfer blocking functions in detergents and washing powders, many of which have been patented (Yaver *et al*., 2001). The poly aromatic hydrocarbons are classes of compounds consisting of fused aromatic rings in various structural configurations (Stoker *et al*., 1997). Poly aromatic hydrocarbons can be formed as products of the incomplete pyrolysis of organic materials and present in considerable quantities in fossil fuels (2-3%) and are released in the environmental directly. These organic chemicals will be able to enter the soil and cause serious pollution problems. The hydrocarbons are highly hydrophobic materials, which are commonly found in environmental contaminants. Though they are not usually classified as hazardous, these wastes can be hardly degraded or decomposed. In recent years the petroleum exploitation and production activities have increased resulting increased discharge of petroleum hydrocarbons into our environment. (Chhatre *et al*., 1996). The biotechnological use of laccase has been expanded by the introduction of laccase-mediator systems, which are able to oxidize non-phenolic compounds that are otherwise hardly or not oxidized by the enzyme alone. Laccase can be applied for the treatment of soils containing phenolic pollutants as well as other polluted systems due to the broad substrate range of the enzyme (Filazzola *et al*., 1999).

Industrial production

Industrial fermentation processes are based on strong metabolic engineering and process control principles. Optimization studies involving different factors controlling the fermentation process viz. media components, pH, temperature, aeration and agitation need to be designed keeping in mind the type of microorganism involved and the biotechnological product to be produced. Modern statistical tools like Plackett Burman design, Central Composite design and Response Surface Methodology and mathematical modeling programmes are used for optimizing conditions in several industrial fermentations (Vaidya *et al*., 2003, Praveen *et al*., 2008). The conventional method of optimization involves varying one parameter at a time and keeping the others at a fixed level. It is time consuming and expensive when a large number of variables are evaluated at different levels. Statistical methods like, Response Surface Methodology (RSM) fairly reduces the total number of experiments required (only 20) and also manifests any possible interaction effect between the medium constituents (Chhatpar *et al*., 2003). The aim of present work was to apply Central Composite Design (CCD) and Response Surface Methodology (RSM) to examine and to study the effect of supplemented nutrients or the laccase inducer compound Anthracene on laccase production and activity in *Pleurotus florida.*

MATERIALS AND METHODS

Microorganism and its maintenance

Pleurotus florida, the pure culture of a basidiomycetes white-rot mushroom fungus was procured from the Tamil Nadu Agricultural University, Coimbatore, India. The culture was maintained on potato dextrose agar plates containing (g/l) potato 200, dextrose 20 and agar 20 in distilled water with pH adjusted (using 1N HCl / NaOH) to 6.5 – 7 before sterilization. The cultures were stored under refrigeration and were sub-cultured once a month.

Medium and culture conditions

Basal salt medium was used as the substrate for biomass and laccase production. Basal salt medium contains medium ingredients (g/l) *viz*. glucose 10, yeast extract 3, ammonium sulphate 2 and potassium dihydrogen phosphate 5 in distilled water at pH 5. Medium optimization was carried out by varying the nitrogen source in the medium with peptone or yeast extract or ammonium sulphate or by varying the carbon source in the medium with glucose or malt extract or potato extract or by varying the other salt additives in the medium *viz*. KH_2PO_4, $MgSO_4$ and $CaCl_2$. The effect of anthracene, a PAH compound to the medium at 0.5% (w/v) on the biomass and laccase activity was assessed.

Biomass

P. florida was grown by inoculating 7mm diameter of mycelial agar plug of actively growing *P. florida* culture into 50 ml of sterilized Basal salt broth taken in Erlenmeyer flask (100ml). The flasks were incubated statically at room temperature (28 ± 2^0C) and were arranged in randomized complete block design. Biomass fresh weight was measured by weighing the mycelial mat after blotting it dry on coarse filter paper.

Enzyme assay

Laccase activity was assayed based on the oxidation of guiacol (Ramakrishna *et al*., 2004). The assay mixture containing 900µl of sodium acetate buffer (50 mM , pH 4.5), 2mM guiacol and 100µl of enzyme source was incubated at room temperature for 5 minute and the absorbance was measured at 440nm using UV-Visible spectrophotometer (Jasco V-530, Japan). Enzyme activity was expressed in International Units (IU/ml). One IU is defined as the change in absorbance at 440nm brought about by one ml enzyme in one minute. Blanks were maintained with sterile water.

Optimization of Laccase production

Optimization of Laccase production was carried out using Response Surface Methodology in two steps. The first step involved the screening of variables for selecting the independent variables and the second step involved the optimization of independent variables to study their interaction. The screening step with twenty four combinations of basal salt media used to measure biomass production and laccase activity on 12th day by *P. florida* was designed by varying one medium component at a time and keeping others at a fixed level (Table. Ia & Ib). All the experiments were carried out in triplicates. Basal salt medium components or the selected supplement or replacement compounds *viz*., glucose, malt extract, potato extract, peptone, yeast extract, ammonium sulphate, potassium dihydrogen phosphate, magnesium sulphate and calcium chloride were analyzed at three levels of concentration – low, medium and high, to identify the independent variables and the dummy variables. In another set of experiments, anthracene (0.1% w/v and 0.1ml of Tween 80 as surfactant) was added to each of the 24 combination of basal

Table. Ia. Composition of basal salt medium used for Screening of component variables

Media variants	Compositions										
	Glucose	Malt Extract	Potato Extract	Peptone	Yeast Extract	$(NH_4)_2SO_4$	KH_2PO_4	$MgSO_4$	$CaCl_2$	Water (l)	pH
B	10	-	-	-	3	2	5	-	-	1	4.5
B1	20	-	-	-	3	2	5	-	-	1	4.5
B2	1	-	-	-	3	2	5	-	-	1	4.5
M2	-	1	-	-	3	2	5	-	-	1	4.5
M	-	10	-	-	3	2	5	-	-	1	4.5
M1	-	20	-	-	3	2	5	-	-	1	4.5
BP2	10	-	-	1	3	2	5	-	-	1	4.5
BP	10	-	-	10	3	2	5	-	-	1	4.5
BP1	10	-	-	20	3	2	5	-	-	1	4.5
BY	10	-	-	-	0.5	2	5	-	-	1	4.5
BY1	10	-	-	-	1.5	2	5	-	-	1	4.5
BA	10	-	-	-	3	0.5	5	-	-	1	4.5

B- Basal salt Medium Glucose-10g/l, B1- Basal salt Medium Glucose-20g/l, B2- Basal salt Medium Glucose-1g/l, M2- Basal salt Medium Malt extract-1g/l, M- Basal salt Medium Malt extract-10g/l, M1- Basal salt Medium Malt extract -20g/l, BP2- Basal salt Medium +Peptone-1g/l, BP- Basal salt Medium +Peptone-10g/l, BP1- Basal salt Medium+ Peptone-20g/l, BY- Basal salt Medium Yeast extract-0.5g/l, BY1- Basal salt Medium Yeast extract-1.5g/l, BA- Basal salt Medium $(NH_4)_2SO_4$- 0.5g/l,

Table. Ib. Composition of basal salt medium used for Screening of component variables

Media variants	Compositions										
	Glucose	Malt Extract	Potato Extract	Peptone	Yeast Extract	$(NH_4)_2SO_4$	KH_2PO_4	$MgSO_4$	$CaCl_2$	Water (l)	pH
BA1	10	-	-	-	3	1	5	-	-	1	4.5
T2	-	-	50	-	3	2	5	-	-	1	4.5
T	-	-	100	-	3	2	5	-	-	1	4.5
T1	-	-	200	-	3	2	5	-	-	1	4.5
BM2	10	-	-	-	3	2	5	0.5	-	1	4.5
BM	10	-	-	-	3	2	5	1	-	1	4.5
BM1	10	-	-	-	3	2	5	2	-	1	4.5
BC2	10	-	-	-	3	2	5	-	0.1	1	4.5
BC	10	-	-	-	3	2	5	-	0.5	1	4.5
BC1	10	-	-	-	3	2	5	-	1	1	4.5
BD1	10	-	-	-	3	2	2.5	-	-	1	4.5
BD	10	-	-	-	3	2	1	-	-	1	4.5

BA1- Basal salt Medium $(NH_4)_2SO_4$-1g/l, T2- Basal salt Medium Potato extract – 50g/l, T- Basal salt Medium Potato extract – 100g/l, T1- Basal salt Medium Potato extract-200g/l, BM2- Basal salt Medium $MgSO_4$ -0.5g/l, BM- Basal salt Medium $MgSO_4$ -1g/l, BM1- Basal salt Medium $MgSO_4$-2g/l, BC2- Basal salt Medium $CaCl_2$-0.1g/l, BC- Basal salt Medium $CaCl_2$-0.5g/l, BC1- Basal salt Medium $CaCl_2$-1g/l, BD1- Basal salt Medium KH_2PO_4-2.5g/l, BD- Basal salt Medium KH_2PO_4-1g/l

Table – II: Screening of variables for laccase production and their responses in terms of biomass and enzyme yield

S. No.	Type of medium	Basal salt medium		Medium + 0.5% Anthracene	
		Biomass (g)	Laccase (x 10_{-5})	Biomass (g)	Laccase (x 10_{-5})
1.	B	0.30	2.20	0.35	2.30
2.	B1	0.46	5.77	0.58	5.86
3.	B2	0.25	1.97	0.32	1.70
4.	M2	0.20	4.60	0.68	4.93
5.	M	1.58	13.7	1.72	15.4
6.	M1	2.25	15.2	2.15	17.5
7.	BP2	0.20	2.44	0.38	3.17
8.	BP	1.25	8.35	1.36	10.8
9.	BP1	0.80	5.35	0.90	4.78
10.	BY	0.32	5.25	0.20	5.75
11.	BY1	0.37	2.25	0.20	1.84
12.	BA	0.85	2.62	2.12	5.11
13.	BA1	0.76	14.5	0.26	6.89
14.	T2	1.30	4.55	1.42	9.85
15.	T	0.51	8.73	0.30	2.53
16.	T1	0.81	3.84	0.27	1.07
17.	BM2	1.21	0.93	1.47	9.95
18.	BM	1.78	8.77	2.19	13.1
19.	BM1	0.75	13.2	0.92	8.59
20.	BC2	0.42	5.96	0.61	3.00
21.	BC	0.36	1.78	0.22	2.25
22.	BC1	2.61	14.6	2.82	14.5
23.	BD1	0.57	4.78	0.85	5.07
24.	BD	0.38	3.80	0.61	4.01

Table – III: Range of the values for response surface methodology

Original Factors	Uncoded levels				
	-2	-1	0	1	2
Peptone (g)	40	20	10	1	0.1
Malt extract (g)	40	20	10	1	0.1
$CaCl_2$ (g)	2	1	0.5	0.1	0.05

Table- IV: Central composite design consisting of 20 experiments for the study of experimental factors in uncoded units. Observed responses and predicted values

S. No.	Basal salt medium	Peptone (g)	Malt extract (g)	$CaCl_2$ (g)	Laccase activity (IU/ml)		Residual values
					Observed Response	Predicted Value	
1	,,	1	1	0.1	18.54	13.23031	5.309693
2	,,	20	1	0.1	5.91	10.54694	-4.63694
3	,,	1	20	0.1	4.03	8.454222	-4.42422
4	,,	20	20	0.1	8.26	10.96086	-2.70086
5	,,	1	1	1	2.58	3.515095	-0.9351
6	,,	20	1	1	10.8	10.01173	0.788267
7	,,	1	20	1	7.88	6.879011	1.000989
8	,,	20	20	1	9.62	18.56565	-8.94565
9	,,	0.1	10	0.5	5.25	7.56858	-2.31858
10	,,	40	10	0.5	22.6	15.1394	7.460597
11	,,	10	0.1	0.5	6.85	8.915609	-2.06561
12	,,	10	40	0.5	19.3	12.09237	7.207626
13	,,	10	10	0.05	13.1	11.01632	2.083685
14	,,	10	10	2	12.3	9.241668	3.058332
15	,,	10	10	0.5	12.0	17.12204	-5.12204
16	,,	10	10	0.5	12.91	17.12204	-4.21204
17	,,	10	10	0.5	19.95	17.12204	2.827962
18	,,	10	10	0.5	19.82	17.12204	2.697962
19	,,	10	10	0.5	19.29	17.12204	2.167962
20	,,	10	10	0.5	17.88	17.12204	0.757962

salt media broth on the 6th day after inoculation of the fungus (Boonchan *et al.,* 2000). The effect of anthracene on biomass production and laccase activity by *P. florida* was assayed on the 12th day after inoculation. CCD and RSM were applied to study the interaction effect of the independent variables at five coded levels (-2, -1, 0, +1, +2). The coded levels and the values of the corresponding variables used in the CCD design were shown in the Table II. The statistical software Design Expert (Version 7.01, Stat-Ease, Inc) was used for the experimental design of 20 trials for optimization and data analysis. This design was used to evaluate the main effects, interaction effects and quadratic effects and to optimize the levels of parameters for enhancing laccase activity. For this study, 3 fractional factorial designs with six star points and six replicates at the centre points were employed to fit the second order polynomial model which indicated that 20 experiments were required for this procedure. The mathematical relationship of response Y (laccase activity IU/ml) to these three variables can be approximated by quadratic / (Second degree) polynomial equation as shown below:

$$Y = b_o+b_1X_1+b_2X_2+b_3X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3 \quad \text{---- Eq.1}$$

Where, Y is the predicted response, b_0 is the constant, b_1, b_2 &b_3 are the linear coefficients, b_{11}, b_{22} & b_{33} are the quadratic coefficients and b_{12}, b_{13} & b_{23} are the cross-product coefficients.

RESULTS

Biomass production and laccase activity of *P. florida* in the different combination of basal salt medium are given in Table III. Varying the concentrations of malt extract, peptone, ammonium sulphate, magnesium sulphate and calcium chloride in the composition of basal salt broth medium significantly influenced the biomass production (2.25, 1.25, 0.85, 1.78, 2.61g respectively) and laccase activity (15.2, 8.35, 14.5, 13.2, 14.6 IU/ml) of *P. florida.* In contrast varying the concentrations of glucose, yeast extract, potato extract and KH_2PO_4 in the composition of basal salt medium does not have any effect on the biomass production and laccase activity.

Malt extract as the carbon source replacing glucose in the medium induced a significant variation in response in terms of biomass production (0.20, 1.58 and 2.25g respectively) and laccase activity (4.60, 13.7 and 15.2 IU/ml respectively) in the range of concentration used viz., 1, 10and 20 g/l respectively. Similarly peptone (0.20, 1.25, 0.80 g biomass and 2.44, 8.35, 5.35 IU/ml Laccase activity) and $CaCl_2$ (0.42, 0.36, 2.61 g biomass and 5.96, 1.78, 14.6 IU/ml laccase activity) as supplements to basal salt medium were found to elicite significant variation in the response parameters studied (Four fold and seven fold increase in biomass, laccase activity respectively). But potato extract and ammonium sulphate did not have any effect on the biomass yield. Table 3 showed that addition of anthracene to all the twenty four combinations of basal salt medium induced the biomass production and laccase activity except in the treatments which received higher concentrations of potato extract, malt extract or ammonium sulphate

Medium optimization

The selected independent variables were optimized using Central Composite Design and RSM. Contour plots were obtained from the data of laccase activity fed into Design Expert software and analyzed by them. The software has the function by which we can predict the laccase activity within studied range of all three medium components. Each contour plot represents the effect of two medium components at their studied concentration range and at the fixed concentration of the third medium component. The value of the third medium component was varied for that situation with the software and the optimum value was found out. Significantly higher laccase activity (18.54 IU/ml) was observed, when peptone, malt extract and $CaCl_2$ were supplemented to the medium in the ratio 1:1:0.1. Further increase of the carbon or nitrogen source or $Cacl_2$ did not significantly increase either the biomass production or laccase activity.

RSM regression equation and Model analysis

The experimental responses (Laccase activity) along with the predicted values are shown in Table IV. By applying multiple

Fig.1.Response surface plots of interaction between process variables in laccase production by *P. florida*

Fig 1a
Peptone
vs
Malt extract

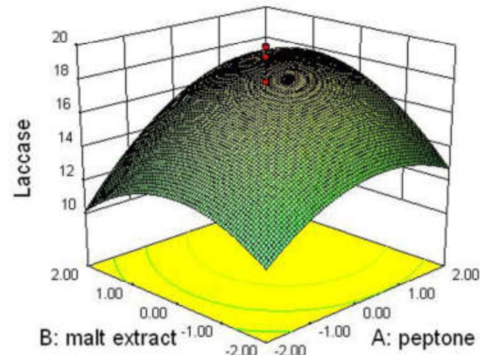


Fig 1b
Malt extract
vs
CaCl2

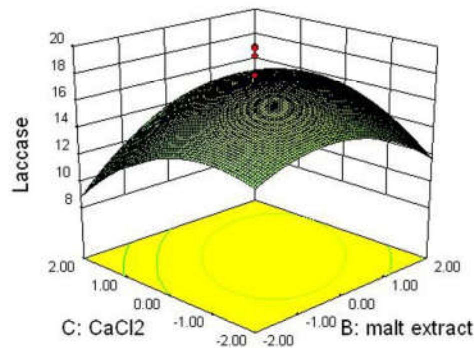
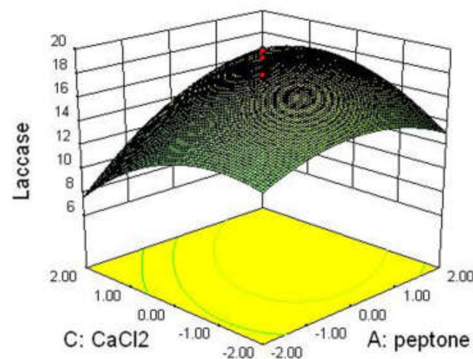


Fig 1c
Peptone
vs
CaCl2



regression analysis, the following quadratic / (Second degree) polynomial equation was obtained as Eq. 2.

Laccaseactivity(Y1)(IU/ml)

$$=17.12+2.25*X_1+0.94*X_2-0.53*X_3+1.30*X_1*X_2+2.30*X_1*X_3+2.04*X_2*X_3-2.04*X_1^2-2.34*X_2^2-2.47*X_3^2 \quad \text{----- Eq.2}$$

Where Y1 is the predicted response (Laccase activity), and X_1, X_2 and X_3 are the coded values of the independent variables, peptone, malt extract and $CaCl_2$ respectively. An ANOVA for response surface quadratic model as shown in Eq.2 was checked by F test. The regression co efficient (R^2) of 0.51 indicated a fairly good fitness of the model. Figure 1a shows the interaction effect of peptone and malt extract on laccase activity. It is clear from the figure that, higher laccase activity was obtained in 1-10g/l concentration of the peptone and malt extract and 0.1-0.5g/l of $CaCl_2$. Figure 1b and 1c show that malt extract, peptone and $CaCl_2$ induce high biomass production and laccase activity at the optimum ratio 1:1:0.1 respectively. The maximum laccase activity (22.6 x 10^{-5} IU/ml) was obtained in Trial No. 10 which had peptone 40 g/l, malt extract 10 g/l and $CaCl_2$ 0.5 g/l as supplement to the other basal salt medium components. But Trail No. 1 which received 1 g/l peptone and malt extract and 0.1 g/l $CaCl_2$ induced

significantly high laccase activity (18.54 g/l) equally that of the average range of concentrations used (Trails 15-20).

DISCUSSION

Results of the preliminary experiment which used classical optimization revealed malt extract, peptone and $CaCl_2$ concentration in the medium directly influenced the laccase activity and they were chosen as the independent variables. Similar to this study, Praveen *et al*., (2008) observed that calcium supplementation had a significant influence on biomass and ligninolytic enzyme production by *P. florida.* Addition of 0.5% of anthracene (a three ringed PAH compound) to the medium significantly increased the biomass and laccase production potential of the mushroom. This may be due to the detoxification mechanism initiated in the mushroom fungus. Bezalel *et al*. (1996) reported that Phenanthrene and Anthracene containing three aromatic rings increased laccase production. Laccase activity is positively correlated with the growth of the fungus. Maximal laccase activity is observed at the beginning of the stationary phase where biomass production reaches the maximum (Tlecuitl-Beristain *et al*., 2008).

Optimization of medium components or supplemented nutrients for maximizing ligninolytic enzyme production by *P. florida* showed that peptone as nitrogen source and malt extract as carbon source were preferred over yeast extract and glucose respectively. Statistical tools like Plackett – Burman design, Central Composite design and Response Surface methodology have been used by several scientists for identifying the factors which have significant influence on the production of several biotechnological products (Vadiya *et al.,* 2003, Praveen *et al.,* 2008). Similarly Central composite design and Response surface methodology used in this study showed that the trial No. 1 with the broth containing peptone, malt extract and $CaCl_2$ in the concentration of 1, 1 and 0.1 g/l, respectively showed high laccase titer value (18.54 IU/ml) comparable to the average central trails (Trail 15-20).

Increasing carbon or nitrogen source did not significantly increase the laccase activity over the value. Buswell *et al.,* (1995) found that higher laccase activity at high nitrogen concentrations, although it is generally accepted that a high carbon to nitrogen ratio is required for laccase production. Laccase activity of twelve days - old *P. florida* was 2.20×10^{-5} IU/ml in basal salt medium and the activity increased to a ten fold higher 22.6×10^{-5} IU/ml in statistically optimized medium (Trail No. 1) . Increasing the carbon or nitrogen or calcium supplements beyond this level did not significantly influence the laccase activity (Trail No. 10 & 12). The model was able to describe the effect of the nutrients studied on biomass production and laccase activity within the range of concentrations fixed for the three independent variables that were the limits of applicability of the model. It is possible to predict the response to any combination of independent variables. The methodologies of CCD and the tools RSM have proved to be very effective for optimization of industrial processes for microbial biomass and enzyme production.

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