Enhancement of Laccase Production from *Pleurotus ostreatus* PVCRSP-7 by altering the Nutritional Conditions using Response Surface Methodology

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Submerged culture conditions for laccase production by *Pleurotus ostreatus* were optimized by response surface methodology (RSM). A total of six factors, carbon (glucose), nitrogen sources (urea and peptone), 2,5-xylidine (inducer), wheat bran (lignocellulosic material), and medium pH, were optimized. A total of 50 experiments were conducted, and the obtained data were modeled using a second-order polynomial. The optimized conditions show significant improvement in laccase expression, by approximately 3.5-fold (12,124 *U*/L).

Keywords: Lignolytic enzymes; Response surface methodology; Laccase; Optimization; Submerged fermentation; Bioprocess; Pleurotus ostreatus

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INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductase [EC1.10.3.2]) belong to a family of multi copper blue oxidases. They catalyze the oxidation of a broad range of organic and inorganic substrates, such as phenols, non-phenols, aromatic amines, and their derivatives. These enzymes have the ability to reduce molecular oxygen to water (Bhattacharya *et al.* 2011; Neifar *et al*. 2011; Riva 2006)

Laccases have great biotechnological market potential due to their broad substrate specificity in diverse fields of industrial applications, such as in pulp delignification (Sathishkumar *et al* 2012; 2014; Sathishkumar & Palvannan 2013; Minussi *et al*. 2007), textile dye bleaching (Lantto *et al.* 2004; Pazarloglu *et al.* 2005; Rodríguez-Couto 2012), wastewater detoxification (Tavares *et al.* 2009), xenobiotic detoxification (Coelho *et al.* 2010; Torres *et al.* 2003), food industry applications (Alper and Acar 2004; Tannoven and Eksi 2005), biosensor applications (Vianello *et al.* 2004), and green chemistry (Riva 2006).

Laccases are obtained from various sources, such as bacteria, fungi, and higher plants. Among all sources, fungal laccases have gained the most attention due to their efficiency in the detoxification of pollutants. Many research reports reveal that white-rot fungi such as *Pleurotus* sp., *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Coriolus versicolor* are the predominant laccase producers. Okamoto *et al.* (2003) reported that *Pleurotus ostreatus* showed strong laccase activity and is relatively easy to culture in a medium.

The industrial production of enzymes is primarily achieved by submerged cultivation. In *Basidiomycetes*, extra-cellular laccases are constitutively produced in

small amounts. Laccase expression by fungi is influenced by culture conditions such as the carbon source, carbon-to-nitrogen ratio, pH of the fermentation broth, presence of inducers, and presence of lignocellulose (Bettin *et al*. 2009; Kachlishvili *et al*. 2006; Prasad *et al*. 2004; Revankar and Lele 2006a, b).

For effective laccase expression, it is highly essential to optimize all the culture conditions as well as composition of production media, which further facilitates economic design of the full-scale fermentation operation system. The current focus on laccase research is therefore oriented toward the optimization of medium components by statistical methods. Conventional optimization procedures involve altering one parameter at a time while keeping all other parameters constant, which enables an assessment of the impact of those particular parameters on the process performance. These procedures are time-consuming, laborious, require many experimental data sets, and cannot provide information about the mutual interactions of the parameters (Desai and Nityananda 2011). The present work was directed toward the optimization of *P. ostreatus* PVCRSP-7 utilization of lignocellulosic substrates for the hyper-production of laccase by semi-solid state fermentation.

EXPERIMENTAL

Chemicals

Glucose, Urea, Peptone, KH_2PO_4, $MgSO_4$, $CaCl_2$, KCl, $CuSO_4$, and 2,5-xylidine were purchased from Sigma-Aldrich, whereas HCl was purchased from Merck chemicals. Wheat bran of good quality was purchased from the local market.

Microorganism

The white rot fungus *P. ostreatus* PVCRSP-7 (Genebank accession KF700247), a hyper-laccase producing strain, was isolated from the Seshachalam forest located at Tirupathi, Andhra Pradesh, India. The isolate was used in the present study to improve the production of laccase. The fungi was maintained on potato dextrose agar (PDA) plates and stored at 4 °C. Sub-culturing was done once every month.

Laccase Production in SMF

A 250-mL Erlenmeyer flask containing 100 mL of production medium was used. The production medium consisted of (in 90 mL of distilled water) a carbon source (glucose: 1.0, 1.5, 2.0, and 2.5 g); a nitrogen source (urea: 0.5, 0.75, 1.0, and 1.25 g); peptone: 1.5, 2.0, 2.5, and 3.0 g; and 10 mL of salt solution. The salt solution consisted of 2.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2$, 0.5 g of KCl, and 2 mM $CuSO_4 \cdot 5H_2O$ dissolved in 100 mL of distilled water. The medium pH was adjusted to 4.5, 5.0, 5.5, 6.0, and 6.5 using 3 M HCl prior to sterilization. Sterilization was carried out at 121 °C at 15 lbs for 15 min. After 96 h, the experiment inducer was added to the culture medium (2,5-xylidine: 0.5, 0.75, and 1.0 µm).

Seven pieces of 5-mm-diameter fungal disc growing on the edge of the mycelium on PDA plates (8-day-old culture) were inoculated in the production media under sterile conditions and the flasks were incubated at 28 °C on a rotary shaker at 100 rpm. The experiment was conducted for up to 12 days.

Lignocellulosic Material

Wheat bran was used as the lignocellulosic material in this study. Prior to use, these materials were thoroughly washed repeatedly with distilled water, dried in a hot air oven (50 °C for 2 days), and subsequently sieved using a metal mesh with a size of 0.43 cm. The sieved portion was used in the fermentation experiments.

Analytical Assay

Samples were withdrawn periodically at intervals of 48 h for up to 288 h of incubation during the fermentation of submerged agitated cultures. After collection, samples were filtered through a 0.2-µm filter, and the clear filtrate was used for the determination of laccase activity. Laccase activity was determined by the oxidation of ABTS at room temperature. The reaction mixture consisted of 5 mM ABTS and 0.1 M sodium acetate buffer (pH 5.5). A suitable amount of enzyme was added to the reaction mixture, and the absorbance increase at 418 nm and 420 nm was measured at 25 °C for 5 to 10 min. Enzyme activity was expressed in the following units (*U*/L), where *U* denotes the amount of enzyme that oxidizes 1 µM of ABTS per min (Prasad *et al.* 2005).

Optimization of Laccase Production by Response Surface Methodology (RSM)

Based on our preliminary studies, six factors were selected for the production of laccase by *P. ostreatus*, and these are shown in Table 1. The factors studied were pH (P); glucose concentration (G); amount of wheat bran (W); and peptone (Pe), urea (U), and 2,5-xylidene (X) concentrations. The variable levels X_i were coded as x_i according to the following equation, such that X_0 corresponded to the central value:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \qquad i = 1, 2, 3.....k \tag{1}$$

where x_i is the dimensionless value of an independent variable, X_i is the the real value of an independent variable, X_0 is the the real value of an independent variable at the central point, and ΔXi is the step change.

The experimental plan and levels of independent variables are shown in Table 1. The response variable was fitted by a second-order model to correlate the response variable to the independent variables. The general form of the second-degree polynomial equation is,

$$Y_i = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i}^{i<} \sum_{j}^{j} \beta_{ij} x_i x_j + e \tag{2}$$

where Y_i is the predicted response (laccase activity), *xi* and *xj* are input variables that influence the response variable *Y*, β_0 is the offset term, β_i is the i^{th} linear coefficient, β_{ij} is the i^{th} quadratic coefficient, and *e* is the error. The statistical analysis of the model was performed in the form of an analysis of variance (ANOVA). The R^2 value indicates the percentage of the variability of the optimization parameter that is explained by the model. Three-dimensional surface plots were drawn to illustrate the main and interactive effects of the independent variable on the dependent variables.

A central composite design (CCD) with total 50 experiments consisting of 32 factorial points (-1 and +1), 10 star points (-2 and +2), and 8 central points (0,0) was performed by varying the 6 selected parameters that had an influence on the laccase production. The design was analyzed and the coefficients of the model were tested for their significance by linear regression analysis using Statistica 6.0. All experiments were conducted in triplicates and mean values are used for analysis.

RESULTS AND DISCUSSION

All experiments were conducted up to 12 days. However 10 days incubated cultures yielded the highest laccase activity. The peak laccase activity corresponding to the experimental run was used as a response for further analysis. Table 1 reveals that the production of laccase varied from 3,918 to 11,985 (*U*/L), indicating the importance of selected factors and their levels on the enzyme production. It was noticed that the 30th run only had 11.35% error, whereas in all other runs it was nearer to the 5% of variation in between the predicted and observed values. The low % of variation between the observed and predicted values indicates the accuracy of the experimentation.

To the obtained data a multiple regression analysis was performed and the accuracy of the data was tested by the regression coefficient (R^2). The regression coefficient (R^2) was 0.9817, indicating that only 1.83% of the variability in the response could not be explained by the model. Further, Fig. 1 depicts the correlation of the observed and predicted values. In this figure, all values near to the line indicate the best correlation between the observed and predicted values.

The observed value of the adjusted R^2 (0.9594) suggested a high significance of the model (Hymavathi *et al.* 2010). The coefficient of variance (CV) is a measure of the precision and accuracy of experiments. A lower CV value indicates better accuracy. In the present experiment, the CV was 4.22%, which indicates good precision and reliability. The application of RSM yielded the following regression equation, which is an empirical relationship between the studied parameters and laccase production:

$$\text{Laccase yield (U/L)} = 11517.78 + 58.35P - 501.55G - 215.10W + 309.35U - 558.8Pe + 486.95X - 617.363P^2 - 539.73G^2 - 468.48W^2 - 396.48U^2 - 444.48Pe^2 - 1047.11X^2 - 113.75PG + 214.43PW + 58.37PU + 220.81PPe + 349.6875PX - 258.00GW - 50.43GU - 508.87GPe + 583.5GX - 295.12WU - 431.31WPe + 195.31WX - 414.87UPe - 137.5UX - 252.438PeX \quad (3)$$

The coefficients were selected based on their corresponding t, F, and p-values (Table 2). Coefficients that have a low p-value and high F-value are considered significant terms. Based on this linear term of pH, interaction terms of pH with glucose, & urea, and glucose *vs.* urea are insignificant terms. The overall p-value is 0.0001 and the F-value is 43.89 (model F-value > p-value), implying that the model is significant. There is only a 0.1% chance that a model F-value this large could occur due to noise. Further, to confirm acceptance of the model, an adequate precision test was performed, which measures signal to noise ratio. Adequate precision value greater than 4 is desirable. In the present experiment, a ratio of 26.939 was observed, indicating an adequate signal. The correlation coefficient value (R^2 = 0.9594), model p and F values, and the adequate precision value (26.939) suggest that the proposed model could be used to navigate the design space.

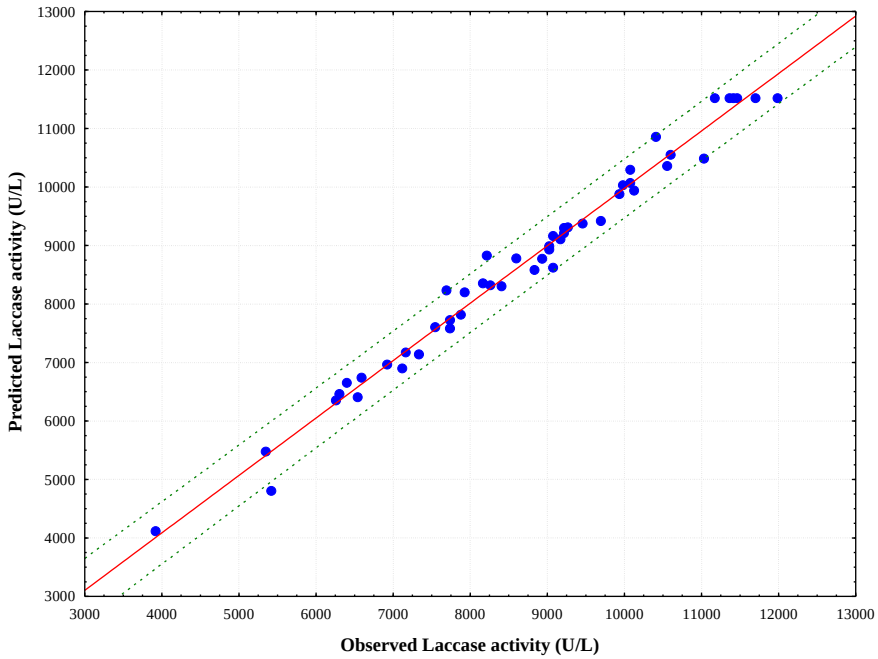

Fig. 1. Correlation graph between the observed and predicted response

Evaluation of Response Surface

The regression model developed (Eq. 3) was used to generate 3D surface plots to evaluate the interaction of selected parameters at different conditions. The laccase yield for different conditions of variables could be predicted from the surface plots shown in Figs. 2a-f. Each surface plot represents an infinite number of combinations of two test variables with the other variables maintained at their respective central level. The highest values of production could be estimated with combinations of the variables that were close to the central points.

Figures 2a and 2b represent the interaction of pH with urea and glucose. The contours were circular in nature, which indicates that there was no interaction between pH with these two parameters on laccase production. Figure 2c indicates the interaction of two nitrogen sources (urea and peptone). It can be seen that the contours were elliptical in nature and slightly inclined toward the peptone concentration, indicating that urea concentration was influenced by the peptone concentration.

From Fig. 2c, it was observed that urea at 0.8 to 1.2 (% w/v) and peptone at 1.0 to 1.8 (% w/v) was optimum for laccase production by isolated *P. ostreatus* PVCRSP-7. The interaction of the carbon source with the nitrogen source was observed in Fig. 2d, and from this it is apparent that the glucose concentration was slightly governed by the peptone concentration. Figures 2e and 2f represent the interaction of wheat bran with urea and the inducer, respectively. The amount of wheat bran is evidently independent of the urea and inducer concentrations.

Verification and Validation of Model

To verify the model (Eq. 3), the laccase yield values were calculated using the coefficients in Table 2. A correlation plot between the observed and predicted values was plotted. A linear correlation coefficient of 0.9818 (R^2=0.9594) suggested a good agreement between the observed and predicted values.

Table 1. Experimental Design with Observed and Predicted Laccase Activity (*U*/L)

S. No	P	G (%,w/v)	W (%,w/v)	U (%,w/v)	Pe (%,w/v)	X (mM)	Laccase activity (U/L)		
							Observed	Predicted	Error
1	5 (-1)	1.5 (-1)	2 (-1)	0.5 (-1)	1.5 (-1)	0.5 (-1)	7736± 240	7584.72	151.28
2	6 (1)	1.5 (-1)	2 (-1)	0.5 (-1)	1.5 (-1)	1 (1)	7334±147	7137.82	196.18
3	5 (-1)	1.5 (-1)	2 (-1)	1 (1)	1.5 (-1)	1 (1)	8213±172	8829.32	-616.32
4	6 (1)	1.5 (-1)	2 (-1)	1 (1)	1.5 (-1)	0.5 (-1)	8931±98	8773.62	157.38
5	5 (-1)	1.5 (-1)	4 (1)	0.5 (-1)	1.5 (-1)	1 (1)	8835±203	8581.92	253.08
6	6 (1)	1.5 (-1)	4 (-1)	0.5 (-1)	1.5 (-1)	0.5 (-1)	7879±268	7819.22	59.78
7	5 (-1)	1.5 (-1)	4 (1)	1 (1)	1.5 (-1)	0.5 (-1)	9695±349	9421.22	273.78
8	6 (1)	1.5 (-1)	4 (1)	1 (1)	1.5 (-1)	1 (1)	10076±181	10296.82	-220.82
9	5 (-1)	2.5 (1)	2 (-1)	0.5 (-1)	1.5 (-1)	1 (1)	9169±413	9107.52	61.48
10	6 (1)	2.5 (1)	2 (-1)	0.5 (-1)	1.5 (-1)	0.5 (-1)	5347±278	5479.32	-132.32
11	5 (-1)	2.5 (1)	2 (-1)	1 (1)	1.5 (-1)	0.5 (-1)	9457±142	9372.82	84.18
12	6 (1)	2.5 (1)	2 (-1)	1 (1)	1.5 (-1)	1 (1)	11032±243	10488.42	543.58
13	5 (-1)	2.5 (1)	4 (1)	0.5 (-1)	1.5 (-1)	0.5 (-1)	6925±125	6963.92	-38.92
14	6 (1)	2.5 (1)	4 (1)	0.5 (-1)	1.5 (-1)	1 (1)	9980±289	10035.02	-55.02
15	5 (-1)	2.5 (1)	4 (1)	1 (1)	1.5 (-1)	1 (1)	10125±213	9941.52	183.48
16	6 (1)	2.5 (1)	4 (1)	1 (1)	1.5 (-1)	0.5 (-1)	7164±129	7173.32	-9.32
17	5 (-1)	1.5(-1)	2 (-1)	0.5 (-1)	2.5 (1)	1 (1)	7736±108	7727.52	8.48
18	6 (1)	1.5 (-1)	2 (-1)	0.5 (-1)	2.5 (1)	0.5 (-1)	8597±155	8781.32	-184.32
19	5 (-1)	1.5 (-1)	2 (-1)	1 (1)	2.5 (1)	0.5 (-1)	9933±228	9878.82	54.18
20	6 (1)	1.5 (-1)	2 (-1)	1 (1)	2.5 (1)	1 (1)	9027±190	8988.92	38.08
21	5 (-1)	1.5 (-1)	4 (1)	0.5 (-1)	2.5 (1)	0.5 (-1)	7690±261	8234.42	-544.42
22	6 (1)	1.5 (-1)	4 (1)	0.5 (-1)	2.5 (1)	1 (1)	9215±378	9300.02	-85.02
23	5 (-1)	1.5 (-1)	4 (1)	1 (1)	2.5 (1)	1 (1)	6542±236	6410.52	131.48
24	6 (1)	1.5 (-1)	4 (1)	1 (1)	2.5 (1)	0.5 (-1)	8262±322	8324.32	-62.32
25	5 (-1)	2.5 (1)	2 (-1)	0.5 (-1)	2.5 (1)	0.5 (-1)	7117±292	6897.02	219.98
26	6 (1)	2.5 (1)	2 (-1)	0.5 (-1)	2.5 (1)	1 (1)	7928±357	8202.62	-274.62
27	5 (-1)	2.5 (1)	2 (-1)	1 (1)	2.5 (1)	1 (1)	7544±234	7604.62	-60.62
28	6 (1)	2.5 (1)	2 (-1)	1 (1)	2.5 (1)	0.5 (-1)	6399±230	6652.92	-253.92

29	5 (-1)	2.5 (1)	4 (1)	0.5 (-1)	2.5 (1)	1 (1)	6303±183	6461.22	-158.22
30	6 (1)	2.5 (1)	4 (1)	0.5 (-1)	2.5 (1)	0.5 (-1)	5418±70	4802.52	615.48
31	5 (-1)	2.5 (1)	4 (1)	1 (1)	2.5 (1)	0.5 (-1)	3918±67	4115.02	-197.02
32	6 (1)	2.5 (1)	4 (1)	1 (1)	2.5 (1)	1 (1)	6591±99	6743.12	-152.12
33	5.5(0)	2 (0)	3 (0)	0.75 (0)	1(-2)	0.75 (0)	10410±125	10857.43	-447.43
34	5.5(0)	2 (0)	3 (0)	0.75 (0)	3 (2)	0.75 (0)	9073±136	8622.23	450.77
35	5.5(0)	1 (-2)	3 (0)	0.75 (0)	2 (0)	0.75 (0)	10555±296	10361.93	193.07
36	5.5(0)	3 (2)	3 (0)	0.75 (0)	2 (0)	0.75 (0)	8166±135	8355.73	-189.73
37	5.5(0)	2 (0)	1(-2)	0.75 (0)	2 (0)	0.75 (0)	10076±124	10074.03	1.97
38	5.5(0)	2 (0)	5 (2)	0.75 (0)	2 (0)	0.75 (0)	9215±122	9213.63	1.37
39	5.5(0)	2 (0)	3 (0)	0.25 (-2)	2 (0)	0.75 (0)	9265±143	9313.13	-48.13
40	5.5(0)	2 (0)	3 (0)	1.25 (2)	2 (0)	0.75 (0)	10602±129	10550.53	51.47
41	4.5(-2)	2 (0)	3 (0)	0.75 (0)	2 (0)	0.75 (0)	9027±170	8931.63	95.37
42	6.5(2)	2 (0)	3 (0)	0.75 (0)	2 (0)	0.75 (0)	9073±212	9165.03	-92.03
43	5.5(0)	2 (0)	3 (0)	0.75 (0)	2 (0)	0.25 (-2)	6257±152	6355.43	-98.43
44	5.5(0)	2 (0)	3 (0)	0.75 (0)	2 (0)	1.25 (2)	8405±213	8303.23	101.77
45	5.5(0)	2 (0)	3 (0)	0.75 (0)	2 (0)	0.75 (0)	11700±284	11517.78	182.22
46	5.5(0)	2 (0)	3 (0)	0.75 (0)	2 (0)	0.75 (0)	11366±480	11517.78	-151.78
47	5.5(0)	2 (0)	3 (0)	0.75 (0)	2 (0)	0.75 (0)	11174±198	11517.78	-343.78
48	5.5(0)	2 (0)	3 (0)	0.75 (0)	2 (0)	0.75 (0)	11462±253	11517.78	-55.78
49	5.5(0)	2 (0)	3 (0)	0.75 (0)	2 (0)	0.75 (0)	11985±278	11517.78	467.22
50	5.5(0)	2 (0)	3 (0)	0.75 (0)	2 (0)	0.75 (0)	11413±253	11517.78	-104.78

Values in parentheses are coded values of the corresponding cell value. P = pH, G = glucose, W = wheat bran, U = Urea, Pe = Peptone, X = 2,5-xylidene

A numerical method given by Myers and Montgomery (Hymavathi *et al*. 2009) was used to solve the regression equation. The optimal values of the test variables were as follows: pH 5.5, glucose concentration 2.0 (% w/v), wheat bran 3.47 (% w/v), urea concentration 0.98 (% w/v), peptone concentration 1.24 (% w/v), and 2,5-xylidene concentration 0.86 (mM), with corresponding laccase production at 12,124 (*U*/L). Conducting the experiments at the predicted conditions, 12,100 (*U*/L) laccase was obtained.

Table 2. Main Effects, Coefficients, and ANOVA

	Effect	Coefficients	SS	df	MS	F-value	t-value	p-value
Mean/ Intercept	11517.78	11517.78	-	-	-		83.83	0.000000
P	116.70	58.35	136188.90	1	136188.90	1.01	1.00	0.325832*
G	-1003.10	-501.55	10062096.10	1	10062096.10	74.62	-8.64	0.000000
W	-430.20	-215.10	1850720.40	1	1850720.40	13.73	-3.70	0.001236
U	618.70	309.35	3827896.90	1	3827896.90	28.39	5.33	0.000024
Pe	-1117.60	-558.80	12490297.60	1	12490297.60	92.63	-9.62	0.000000
X	973.90	486.95	9484812.10	1	9484812.10	70.34	8.39	0.000000
P * P	-1234.73	-617.36	12196366.61	1	12196366.61	90.45	-9.51	0.000000
G * G	-1079.48	-539.74	9322130.21	1	9322130.21	69.13	-8.31	0.000000
W * W	-936.98	-468.49	7023377.21	1	7023377.21	52.09	-7.22	0.000000
U * U	-792.98	-396.49	5030474.81	1	5030474.81	37.31	-6.11	0.000004
Pe * Pe	-888.98	-444.49	6322212.41	1	6322212.41	46.89	-6.85	0.000001
X * X	-2094.23	-1047.11	3586226.81	1	35086226.81	260.20	-16.13	0.000000
P * G	-227.50	-113.75	414050.00	1	414050.00	3.07	-1.75	0.093646*
P * W	428.88	214.44	1471470.13	1	1471470.13	10.91	3.30	0.003236
P * U	116.75	58.38	109044.50	1	109044.50	0.81	0.90	0.378247
P * Pe	441.63	220.81	1560261.13	1	1560261.13	11.57	3.40	0.002561
P * X	699.38	349.69	3913003.13	1	3913003.13	29.02	5.39	0.000021
G * W	-516.00	-258.00	2130048.00	1	2130048.00	15.80	-3.97	0.000642
G * U	-100.88	-50.44	81406.13	1	81406.13	0.60	-0.78	0.445439*
G * Pe	-1017.75	-508.88	8286520.50	1	8286520.50	61.45	-7.84	0.000000
G * X	1167.00	583.50	10895112.00	1	10895112.00	80.80	8.99	0.000000
W * U	-590.25	-295.13	2787160.50	1	2787160.50	20.67	-4.55	0.000159
W * Pe	-862.63	-431.31	5952975.13	1	5952975.13	44.15	-6.64	0.000001
W * X	390.63	195.31	1220703.13	1	1220703.13	9.05	3.01	0.006461
U * Pe	-829.75	-414.88	5507880.50	1	5507880.50	40.85	-6.39	0.000002
U * X	-275.00	-137.50	605000.00	1	605000.00	4.49	-2.12	0.045691
Pe * X	-504.88	-252.44	2039190.13	1	2039190.13	15.12	-3.88	0.000791
Lack of fit			2559716.48	17	150571.55	1.85		0.256800*
Error			2966519.82 406803.33	5	81360.66			
Total SS			162773144.72	49				

SS = sum of squares; df = degrees of freedom; MS = mean square error; * = not significant

Based on preliminary studies (data not shown) with wheat brawn as a lignocellulose material, glucose, urea, and peptone were chosen as additional carbon and nitrogen sources, xylidine as an inducer, and pH were chosen as important parameters on laccase production by isolated *P. ostreatus*. In the literature, culture pH is one of the important parameters in fungal cultivations and optimization of any fermentation process (Janusz *et al*. 2007; Patel *et al.* 2009; Periyasamy and Palvannan 2010; Prasad *et al.* 2005). Prasad *et al.* (2005) and Ardon *et al.* (1996) reported that the maximum laccase produced by *P. ostreatus* was observed at a pH of 5.5 in submerged cultures. Johnsy and

Kaviyarasan (2011) reported that 5.5 is the optimal pH for laccase production by *Lentinus kauffmanii* under submerged culture. Patel *et al.* (2009) also reported that pH 5.0 is an optimal pH for laccase production by *P. ostreatus* under submerged culture. Increasing the pH above 5.5 may alter the three-dimensional structure of the enzymes (Shulter and Kargi 2000).

Production of industrial enzymes in a cost-effective manner is important (Sathish and Prakasham 2010). Production of laccase using a synthetic medium increase the production cost. To reduce the price of raw materials, natural materials having high lignocellulosic content can be substituted. Along with the carbon and nitrogen sources of natural materials, the presence of phenolic compounds is one of the major considerations in the selection of natural lignocellulosic materials for effective laccase production. In the preliminary studies, it was noticed that (data not shown) wheat bran yielded the highest lignolytic enzyme concentration by *P. ostreatus* PVCRSP-7.

Furthermore, wheat bran is a cheap and abundantly available material in India. Murugesan *et al.* (2007) and Li *et al.* (2007) reported that wheat bran is a cheap and natural lignocellulosic solid substrate, contains phenolic compounds. In the present study, the concentration of wheat bran is one of the important parameters. Figures 2e and 2f depict the interaction of wheat bran with urea and the inducer. From these graphs, it was observed that wheat bran at 2.5 to 3.5 (%, w/v) is optimum for laccase production by *P. ostreatus* PVCRSP-7.

Galhaup *et al.* (2002) and Sethuraman *et al*. (1999) reported that the composition of nitrogen and glucose in a production medium has a considerable effect on the laccase production by microorganisms. Glucose is a readily utilizable substrate that can enhance biomass production. It has already been established that substrates that are efficiently and rapidly utilized by the organism result in high levels of laccase activity (Galhaup and Haltrich 2001; Patel *et al*. 2009; Prasad *et al.* 2005). The present results were in accordance with the literature reports. In the present study, it was observed that glucose between 1.6 and 2.4% (w/v) showed the highest activity (Fig. 2b and 2d). The optimum glucose concentration was identified as 2% for higher titers of laccase by *P. ostreatus* PVCRSP-7. Above or below this level, the production decreased. Increasing the glucose concentration above the optimum level inhibits the synthesis of laccase (Hao *et al.* 2007; Periasamy and Palvannan 2010). This catabolic repression is already well-established in fungi and is believed to be an energy-conserving process (Ronne 1995).

The nature and the concentration of nitrogen in the culture media play a vital role in fungus growth and laccase production (Galhaup *et al*. 2002; Patel *et al*. 2009). Galhaup *et al*. (2002) observed that among different complex organic nitrogen sources, peptone from meat stimulated higher laccase secretion by *Trametes pubescens.* Mikiashvili *et al.* (2006) reported that among all nitrogen sources, peptone leads to the greatest increases in laccase production by *P. ostreatus* under submerged culture. In the current research, a combination of both urea and peptone was shown to enhance laccase activity. Peptone at 1.24% and urea at 0.98% was found to be the best suitable combination for enhanced laccase productivity. Patel *et al*. (2009) reported that the combination of nitrogen source organic and inorganic supplemented media enhances the laccase production by *P. ostreatus.*

It has been reported that laccase activity in fungal cultures can be increased by the addition of different aromatic compounds as inducers to the media (Iqbal *et al.* 2011; Patel *et al*. 2009; Prasad *et al.* 2005). The addition of 2,5-xylidine to the culture media after 96 h resulted in a several-fold increase in laccase production.

Figure 2f shows the interaction of the inducer with the substrate; from this, it is inferred that a concentration of 0.7 to 0.9 mM is optimum for higher laccase production by *P. ostreatus* PVCRSP-7. A higher concentration of inducer would negatively influence the lignolytic enzyme production.

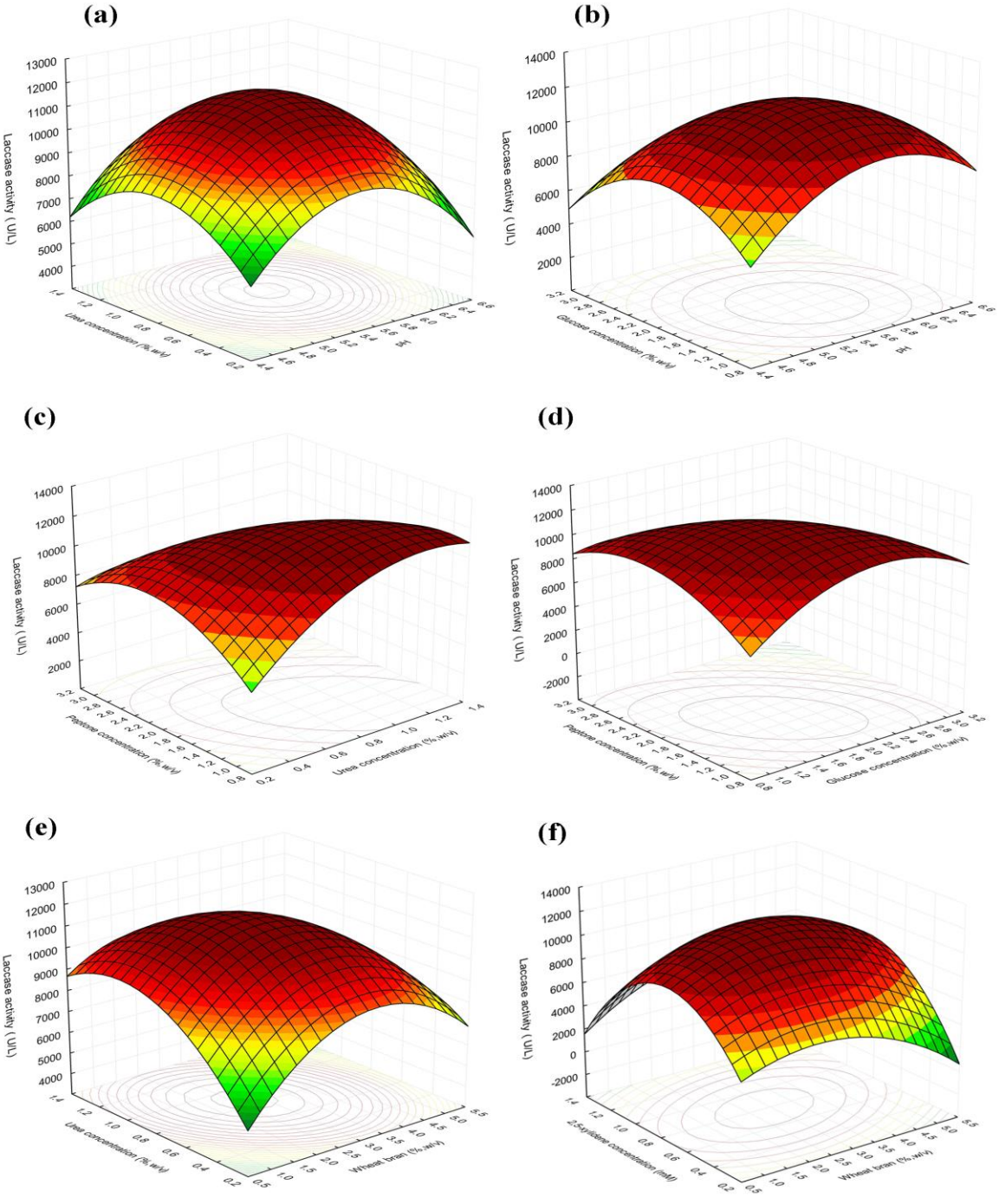

Fig. 2. Surface plots of the selected parameters: a) pH *vs.* urea concentration, b) pH *vs.* glucose concentration, c) urea *vs.* peptone concentration, d) glucose *vs.* peptone concentration, e) wheat bran *vs.* peptone concentration, and f) wheat bran *vs.* inducer concentration

At very high concentrations, 2,5-xylidine could be toxic to organisms, leading to the reduction in cell growth and enzyme production (Sathishkumar *et al.* 2013; Janusz *et*

al. 2006; Prasad *et al.* 2005). The obtained results were in accordance with Prasad *et al*. (2005); they suggested that 1.0 mM 2,5-xylidine is optimum for lignolytic enzyme production. Further, they observed decreased enzyme production by *P. ostreatus* with increasing inducer concentration from 1 mM to 1.5 mM. This may be toxic to organisms, leading to the reduction of cell growth and enzyme production (Patrick *et al.* 2011).

The one-at-a-time method approach of optimization is cumbersome, time consuming and also ignores the important interactions of various parameters. Statistical approaches of DOE such as RSM, Taguchi, *viz*., allow evaluation of the main and interaction effects of the factors individually and in combination. Different statistical methods for medium optimization have been employed to improve laccase production from white rot fungi. Galai *et al.* (2012) and Li *et al.* (2013) employed the RSM for enhancement of laccase production from *Stenotrophomonas maltophilia* and recombinant *Pichia pastoris* GS115-LCCA. Palvannan and Sathishkumar (2010) used the Plackett-Burman design and RSM to enhance the laccase secretion from *Pleurotus florida* NCIM 1243. Mishra and Kumar (2007) used a factorial design for screening various nitrogen sources for hyper laccase secretion from *Pleurotus ostreatus*. Levin *et al.* (2008) with the help of Doehlert experimental design achieved an enhanced lignocellulolytic enzyme production by *Trametes trogii*.

In this study the obtained laccase yield by *P. ostreatus* PVCRSP-7 was higher than the other *P. ostreatus* species reported in the literature (Diaz *et al.* 2011; Prasad *et al*. 2005; Mikiashvili *et al.* 2006).

CONCLUSIONS

1. Optimization of the fermentation conditions by a statistical method resulted in higher laccase production.
2. The lignolytic enzyme production ability of the isolate *P. ostreatus* PVCRSP-7 was enhanced by altering the basal medium and various inducers (carbon, nitrogen, and metal ions) concentrations.
3. Using RSM, an overall 2.5-fold increase in laccase production was attained compared with the conventional optimization method.
4. The solid substrate wheat bran is cheap, safe, and abundantly available in India and can be recommended for the prospective scale-up of enzyme production at the industrial level.

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