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Enhanced Production of Laccase from *Coriolus versicolor* NCIM 996 by Nutrient Optimization Using Response Surface Methodology

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Abstract Plackett and Burman design criterion and central composite design were applied successfully for enhanced production of laccase by *Coriolus versicolor* NCIM 996 for the first time. Plackett and Burman design criterion was applied to screen the significance of ten nutrients on laccase production by *C. versicolor* NCIM 996. Out of the ten nutrients tested, starch, yeast extract, $MnSO_4$, $MgSO_4{\cdot}7H_2O$, and phenol were found to have significant effect on laccase production. A central composite design was applied to determine the optimum concentrations of the significant variables obtained from Plackett–Burman design. The optimized medium composition for production of laccase was (g/l): starch, 30.0; yeast extract, 4.53; $MnSO_4$, 0.002; $MgSO_4{\cdot}7H_2O$, 0.755; and phenol, 0.026, and the optimum laccase production was 6,590.26 (U/l), which was 7.6 times greater than the control.

Keywords Laccase · Plackett and Burman design · Central composite design · Enzyme activity · *Coriolus versicolor*

Introduction

Laccases (*p*-benzenediol:oxygen oxidoreductase; EC 1.10.3.2) are multicopper oxidases present mainly in plants, bacteria, insects, and fungi. Laccase is a glycoprotein of 500–600 amino acids (60 to 80 kDa), and the carbohydrate fraction constitutes 10–40% of the molecular weight [1]. The functional unit of laccase comprises a set of one type 1 Cu (electron mediator), one type 2 Cu, and a pair of type 3 Cu (trinuclear center), one of which gives characteristic blue color. Similar enzymes lacking the Cu atom responsible for the blue color are called 'yellow' or 'white' laccases [2]. Laccases are of particular interest with

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regard to potential industrial applications because of their capability to oxidize a wide range of industrially relevant substrates such as substituted phenolic compounds, aromatic amines and even certain inorganic compounds by using molecular oxygen as the electron acceptor. Their substrate versatility makes laccases highly interesting for various applications, including textile dye bleaching [3], pulp bleaching, and bioremediation where enzymatic catalysis could serve as a more environmentally benign alternative than the currently used chemical processes [4]. Its potential application extends in biosensors also [5].

Laccase is one of the important commercial enzymes and is necessary to improve the fermentative production to reduce the production cost. Studies have been attempted to enhance laccase production using heterologous expression [6–8], inducers [9, 10], xenobiotics [11], antibiotics [12], and various nutritional effects [13, 14]. The white rot fungus *Coriolus versicolor* has been identified as an excellent producer of laccase [15]. There is no published report about the screening of nutrients for laccase production by *C. versicolor* NCIM 996 using Plackett and Burman design criterion and subsequent optimization by applying central composite design. Response surface methodology is a versatile method applied in the optimization of medium constituents and other critical variables responsible for the production of biomolecules [16]. Statistical optimization has the advantage of taking into account the interaction between the nutrients, is less time-consuming, and avoids the erroneous interpretation occurring in one factor at a time optimization [17]. In this present study, response surface methodology was applied to screen the nutrients and to optimize the significant nutrients for enhanced production of laccase by *C. versicolor* NCIM996.

Materials and Methods

Media and Culture Conditions

Laccase production was done by using the fungal strain *C. versicolor* NCIM 996 obtained from National Chemical Laboratory, Pune, India. The organism was maintained on slant culture prepared by using potato dextrose agar medium. The strains were subcultured periodically, and fresh subcultures (5 days at 25 to 30 °C) were prepared and used for each experiment.

Laccase Production Confirmation Test

C. versicolor NCIM 996 was plated on potato dextrose agar (PDA) medium with 0.5% catechol. After 72 h, the plates were examined for the presence of brown color zone around the organism, which is an indication of the production of laccase [18].

Production of Laccase

Production of laccase was carried out in 250-ml Erlenmeyer flask with 50 ml of the production medium having the following composition (g/l): glucose, 10; NH_4Cl_2, 1, $MgSO_4 \cdot 7H_2O$, 0.05; $CaCl_2$, 0.01; yeast extract, 0.025 (pH 5.0) [19]. The inoculum development was carried out in 50 ml of malt extract broth in 250-ml Erlenmeyer flask. Three pieces of fungal mat (each 3-mm diameter) were taken from the PDA plate (fifth day) and were inoculated in malt extract broth for inoculum development at 25 to 30 °C in a

rotary shaker (120 rpm) for 3 days [20]. The pellets from the third day culture of malt extract broth were used to inoculate the production medium at 25 to 30 °C in a rotary shaker (120 rpm). After the incubation period, the broth was centrifuged at 9,000 rpm for 15 min and the supernatant was analyzed for enzyme activity.

Laccase Activity Assay

Laccase activity was determined using continuous spectrophotometric method [21] by measuring the brown color resulted from the oxidation of catechol at 450 nm (2.2×10^3 cm^{-1} M^{-1}). The reaction mixture contained 3 ml of 1% catechol in acetate buffer (pH 4.4, 100 mM), with 3 ml of acetate buffer as blank and 3 ml of 1% *w*/*v* catechol with 3 ml of enzyme as test solution. Absorbance was taken for every 2 min up to 20 min using UV–visible spectrophotometer at 25 °C. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of catechol per minute.

Response Surface Methodology

Response surface methodology was applied in two stages, first to identify the significant nutrients for production of laccase using Plackett and Burman design criterion, and later, the significant nutrients resulted from Plackett and Burman design were optimized by using a central composite design. The experimental design and statistical analysis of the data were done by using Minitab statistical software package.

Plackett–Burman Design

Plackett–Burman design criterion was applied to study the significant variables responsible for laccase production. Each variable was tested at two levels namely a high level (+) and a low level (−) as listed in Table 1. Ten variables were screened by conducting 12 experiments, and the experimental design is given in the Table 2. The variables which were significant at 5% level ($P<0.05$) from the regression analysis were considered to have greater impact on laccase production and were further optimized by central composite design.

Table 1 Level of nutrients used for the production of laccase using Plackett–Burman design criterion.

Nutrient code	Nutrients	Low level (g/l)	High level (g/l)
A	Glucose	5.000	15.000
B	Starch	5.000	15.000
C	Sucrose	5.000	20.000
D	Yeast extract	1.000	3.000
E	$CuSO_4$	0.001	0.004
F	$CaCl_2$	0.050	0.150
G	KH_2PO_4	0.300	1.500
H	$MnSO_4$	0.001	0.004
I	$MgSO_4{\cdot}7H_2O$	0.500	1.000
J	Phenol	0.005	0.010

Table 2 Laccase activity by *C. versicolor* NCIM 996 for Plackett–Burman design.

Run	A	B	C	D	E	F	G	H	I	J	Laccase activity (U/l)
1	–	–	–	+	+	+	+	+	+	–	216.67
2	+	–	+	+	–	+	+	–	–	+	133.33
3	–	–	–	–	–	–	+	–	–	–	33.33
4	+	+	–	+	–	–	+	+	+	+	600.00
5	–	+	–	–	–	+	–	+	–	+	350.00
6	–	–	+	+	+	–	–	+	–	+	333.33
7	+	–	+	–	–	–	–	+	+	–	300.00
8	–	+	+	–	+	–	+	–	+	+	366.67
9	+	+	+	–	+	+	+	+	–	–	316.67
10	–	+	+	+	–	+	–	–	+	–	266.67
11	+	+	–	+	+	–	–	–	–	–	283.33
12	+	–	–	–	+	+	–	–	+	+	116.67

Central Composite Design

Central composite design was applied to determine the optimum concentration of five significant nutrients selected from Plackett–Burman design criterion. The effect of five nutrients (starch, yeast extract, $MgSO_4$, $MnSO_4$, and phenol) on the production of laccase was studied at five experimental levels: $-a$, -1, 0, $+1$, $+a$ where $a=2^{n/4}$; here, n is the number of variables and 0 corresponds to the central point. Laccase activity was analyzed by using a second-order polynomial equation, and the data were fitted into the equation by multiple regression procedure. The model equation for analysis is given below:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_{11} X_{12} + \beta_{22} X_{22} + \beta_{33} X_{32} + \beta_{44} X_{42} + \beta_{55} X_{52} + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{15} X_1 X_5 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{25} X_2 X_5 + \beta_{34} X_3 X_4 + \beta_{35} X_3 X_5 + \beta_{45} X_4 X_5$$

where X_1, $X_2 \ldots X_5$ are the levels of the factors and β_1, $\beta_2 \ldots \beta_5$ are linear coefficients, β_{11}, $\beta_{22} \ldots \beta_{55}$ are quadratic coefficients, and β_{12}, $\beta_{13} \ldots \beta_{45}$ are interactive coefficient estimates with β_0 having a role of a scaling constant. Analysis of variance (ANOVA) and regression analysis were done, and contour plots were drawn using Minitab statistical software package.

Result and Discussion

Laccase Confirmation Test

Plates containing potato dextrose agar medium incubated with *C. versicolor* NCIM 996 showed brown colored ring like zone as in Fig. 1 around the colonies, indicating the production of laccase by the organism. The zones were formed due to the oxidation of catechol present in the agar medium. The diameter of the ring depends on the amount of laccase diffused over the surface of the medium.

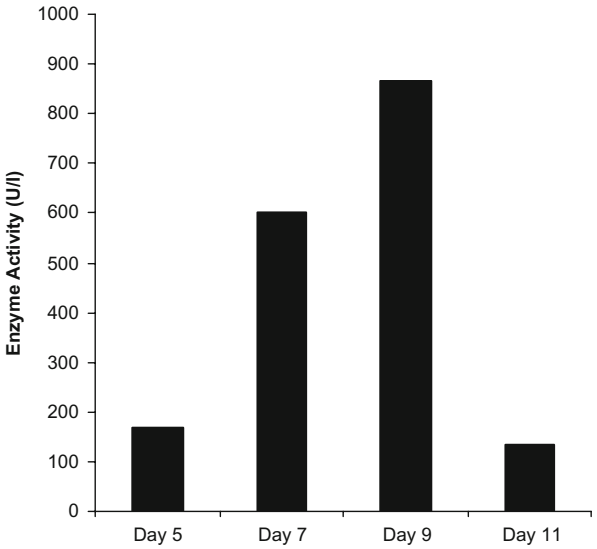

Fig. 1 Production of laccase by *C. versicolor* NCIM 996

Production of Laccase

Laccase activity obtained from the fermentation of *C. versicolor* NCIM 996 in the production media is given in Fig. 2. The enzyme activity was 167 U/l on fifth day, increased to 600 U/l on seventh day, and furthered increased to 866 U/l on ninth day and decreased to 133 U/l after ninth day. Thus, the maximum laccase production was observed on ninth day of incubation, and the enzyme activity in further studies was estimated on the ninth day of incubation. pH of the culture changed from acidic to near neutral at the end of the incubation period (ninth day).

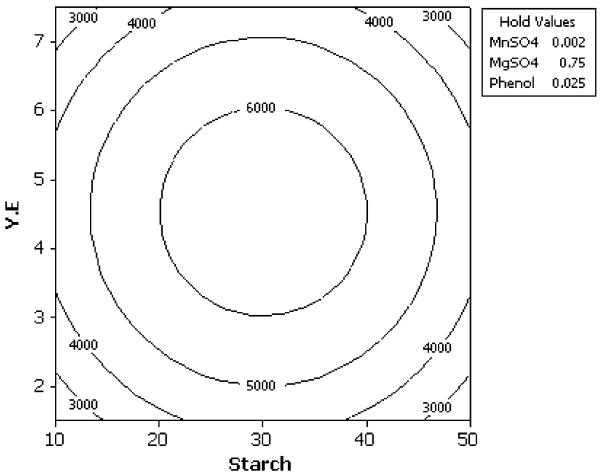

Fig. 2 Contour plot for laccase production at varying concentration of starch and yeast extract

Screening of Nutrient Components Using Plackett–Burman Design

The Plackett Burman design was performed, and the laccase activity was estimated on the ninth day. It was analyzed that among the carbon sources, starch showed significance at low level, and others had no significance on laccase production. Phenol showed significant effect on enhancement of laccase production as an inducer. Among the metallic salts studied, $MnSO_4$ and $MgSO_4 \cdot 7H_2O$ increased laccase production significantly than the other salts studied. Yeast extract showed significant laccase production than other nitrogen sources. Out of the ten nutrient supplements studied, five nutrients (starch, yeast extract, $MnSO_4$, $MgSO_4 \cdot 7H_2O$, and phenol) were found to have significant influence on laccase production as evidenced by their P value (less than 0.05, significant at 5% level) obtained from regression analysis. The coefficient of determination (R^2) of the model was 0.985, which indicates that the model can explain up to 98.5% variation of the data. Laccase yield obtained from

Table 3 Laccase production by *C. versicolor* using significant nutrients based on central composite design.

Run order	Starch (g/l)	Y.E (g/l)	MnSO4 (g/l)	MgSO4 (g/l)	Phenol (g/l)	Laccase Activity (U/l)	
						Experimental	Predicted
1	30	4.5	0.002	0.75	0.025	6,563.22	6,548.98
2	20	6.0	0.001	0.50	0.010	3,616.59	3,592.05
3	20	6.0	0.003	0.50	0.040	3,775.10	3,737.51
4	40	3.0	0.003	0.50	0.040	3,800.90	3,792.46
5	20	6.0	0.003	1.00	0.010	3,582.88	3,567.97
6	20	3.0	0.003	0.50	0.010	3,600.40	3,600.77
7	30	7.5	0.002	0.75	0.025	4,425.28	4,384.86
8	40	6.0	0.001	1.00	0.010	3,674.01	3,656.31
9	40	6.0	0.003	1.00	0.040	3,840.58	3,841.75
10	40	3.0	0.001	1.00	0.040	3,690.03	3,712.48
11	20	3.0	0.003	1.00	0.040	3,763.31	3,768.95
12	40	6.0	0.001	0.50	0.040	3,781.56	3,788.42
13	30	4.5	0.004	0.75	0.025	4,330.25	4,317.72
14	20	3.0	0.001	0.50	0.040	3,696.26	3,685.64
15	30	4.5	0.001	0.75	0.025	4,492.34	4,483.48
16	40	3.0	0.001	0.50	0.010	3,577.16	3,581.73
17	40	6.0	0.003	0.50	0.010	3,611.00	3,628.92
18	30	4.5	0.002	0.75	0.025	6,560.20	6,548.98
19	30	4.5	0.002	0.25	0.025	4,341.31	4,349.74
20	20	6.0	0.001	1.00	0.040	3,877.03	3,842.30
21	20	3.0	0.001	1.00	0.010	3,611.47	3,598.18
22	30	4.5	0.002	0.75	0.025	6,567.25	6,548.98
23	30	1.5	0.002	0.75	0.025	4,323.21	4,341.71
24	10	4.5	0.002	0.75	0.025	4,318.00	4,296.65
25	30	4.5	0.002	0.75	0.025	6,558.80	6,548.98
26	30	4.5	0.002	1.25	0.025	4,460.80	4,430.46
27	30	4.5	0.002	0.75	0.025	6,531.20	6,548.98
28	30	4.5	0.002	0.75	0.055	4,135.47	4,099.91
29	50	4.5	0.002	0.75	0.025	4,385.00	4,384.43
30	40	3.0	0.003	1.00	0.010	3,700.23	3,742.43
31	30	4.5	0.002	0.75	0.025	6,545.00	6,548.98
32	30	4.5	0.002	0.75	0.001	4,750.22	4,765.36

Table 4 Analysis of variance for laccase production by *C. versicolor* using central composite design.

Source	*df*	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	20	35,962,955	35,962,955	1,798,148	6,214.51	0.001
Linear	5	28,165	174,631	34,926	120.71	0.001
Square	5	35,906,935	35,906,935	7,181,387	24,819.33	0.002
Interaction	10	27,854	27,854	2,785	9.63	0.001
Residual error	11	3,183	3,183	289	–	–
Lack of fit	6	2,260	2,260	377	2.04	0.225
Pure error	5	922	922	184	–	–
Total	31	35,966,138	–	–	–	–

Plackett–Burman design experiments showed wide variation (33.33–600 U/l) (Table 2), which indicated that further optimization is necessary to get a maximum response.

Optimization of Significant Nutrients Using Central Composite Design

Response surface methodology using central composite design was applied to optimize the concentration of significant nutrients resulted from Plackett–Burman design experiments. Thirty-two experiments were carried out from the design, and the experimental values are given in Table 3 along with predicted values of the model. By applying multiple regression analysis on the experimental data, the following second-order polynomial equation was found to explain laccase production by *C. versicolor* NCIM 996.

$$Y = -12,560.5 + 331.15X_1 + 2,205.88X_2 + 2,295,678X_3 + 13,034X_4 + 147,173X_5 - 5.5211X_{12} - 242.855X_{22} - 564,803,336X_{32} - 8,635.52X_{42} - 2,888,017X_{52} + 1,935X_1X_3 - 62.3775X_1X_5 - 17,897.5X_2X_3 + 912.644X_2X_5$$

Regression analysis of the experimental data showed that starch, yeast extract, MnSO4, MgSO4·7H2O, and phenol had positive linear effect on laccase production ($P<0.05$). Among the five nutrients, phenol was found to have highest impact on laccase production,

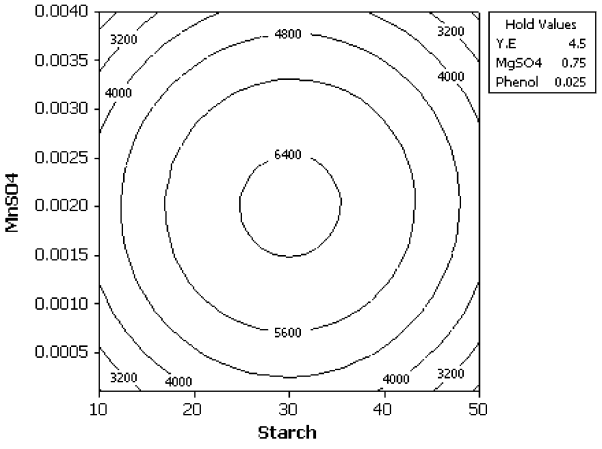

Fig. 3 Contour plot for laccase production at varying concentration of starch and MnSO4

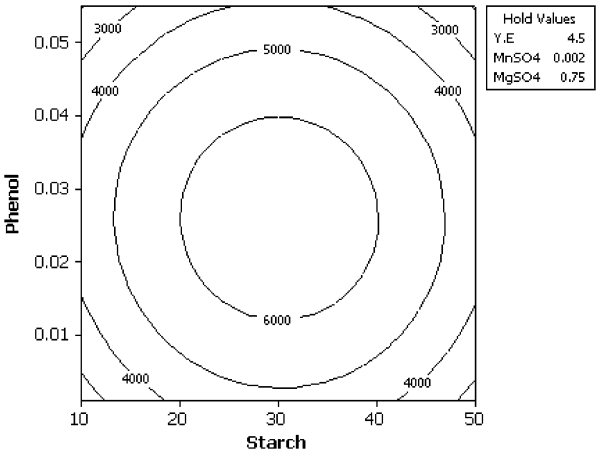

Fig. 4 Contour plot for laccase production at varying concentration of starch and MgSO4

as given by highest linear coefficient (81.40), followed by yeast extract (21.8), magnesium sulfate (21.65), starch (11.94), and MnSO4 (11.19). These nutrients also showed significant negative quadratic effects on laccase production, indicating that laccase production increased as the level of these factors increased and decreased as the level of these parameters increased above certain values. Interaction between these parameters was also significant. The interactions between starch and yeast extract, starch and MnSO4, starch and magnesium sulfate, starch and phenol, yeast extract and MnSO4, yeast extract and magnesium sulfate, yeast extract and phenol, MnSO4 and magnesium sulfate, and MnSO4 and phenol were significant as shown by low P values (<0.05) for interactive terms. The interaction between magnesium sulfate and phenol was found to be insignificant as given by P value above 0.05. So, this term was excluded from the regression equation used for this model.

Analysis of variance for the laccase production obtained from this design is given in Table 4. ANOVA gives the value of the model and can explain whether this model adequately fits the variation observed in laccase production with the designed nutrients level. If the F test for the model is significant at the 5% level (P<0.05), then the model is fit

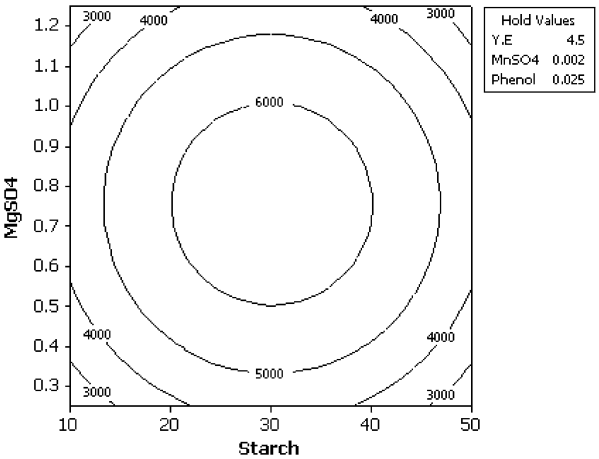

Fig. 5 Contour plot for laccase production at varying concentration of starch and phenol

and can adequately explain the variation observed. If the *F* test for lack of fit is significant ($P<0.05$), then a more complicated model is required to fit the data. The closer the value of *R* (multiple correlation coefficient) to 1, the better the correlation between the observed and predicted values. Here, the value of *R* (0.9901) revealed that the model can explain up to 99.01% variation of laccase production. The *P* value for lack of fit (0.225; Table 4) indicated that the experimental data obtained fit well with the model and explained the effect of starch, yeast extract, $MnSO_4$, $MgSO_4{\cdot}7H_2O$, and phenol on laccase production by *C. versicolor* NCIM 996. Figures 3, 4 and 5 show the contour plots of laccase production for each pair of nutrient concentration by keeping the other three nutrients constant at its middle level. Maximum laccase was produced at middle level of each pair of nutrients at a constant middle level of the other three nutrients. The optimized concentration obtained from response optimizer was (g/l): starch, 30.0; yeast extract, 4.53; $MnSO_4$, 0.002; $MgSO_4{\cdot}7H_2O$, 0.755; and phenol, 0.026. Laccase activity in the optimized concentration of the nutrients was 6,590.26 U/l, which was significantly greater than the reported values for this strain and was close to the predicted value 6,581.98 U/l.

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