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Optimization for Laccase Production by *Pseudomonas putida* LUA15.1 using Response Surface Methodology

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***Pseudomonas putida* LUA15.1 producing laccase was isolated from rice rhizospheric soil samples of paddy field of Una district, Himachal Pradesh (India). The objective of this study was to optimize its culture conditions using a statistical analysis of its laccase production. The interactions between different cultural and nutritional parameters for laccase production were characterized using a Plackett-Burman design and the response surface methodology. The different cultural and nutritional parameters were initially optimized using the conventional one-factor-at-a-time method and a Plackett-Burman experiment was then performed to evaluate the effects on laccase production. Incubation temperature, time, pH, tween-20 and $CuSO_4$ were found to have a significant influence on laccase production, and the optimal concentrations of these five factors were then sequentially investigated using response surface methodology with a central composite design. The resulting optimal medium components for laccase production were: Incubation temperature 28 °C, time 24 hrs, pH 7.0, tryptone 0.5%, yeast 0.3%, $CuSO_4$ 50mg/l, $CaCl_2$ 10 ml/l, tween-20 0.2 ml% and Guaiacol 5 mM. Using these optimized conditions, the yield of laccase was increased 5.52 times to 58 U/l as compared with laccase production with an unoptimized conditions. This is the first report on the statistical optimization of laccase production by *Pseudomonas putida* LUA15.1.**

Key words: *Pseudomonas putida*, optimization, laccase production, Plackett-Burman design, response surface methodology.

Laccases (benzenediol: oxygen oxidoreduceses; EC 1.10.3.2) are glycoproteins, grouped under blue multicopper oxidases in the Enzyme Commission (EC) nomenclature which oxidize diphenols by using molecular oxygen from the air as an electron acceptor[1]. Laccase was first discovered in the sap of the Japanese lacquer tree *Rhus vernicifera,* and its characteristic as a metal containing oxidase was discovered by Bertrand in 1985[2]. Laccases are produced by four type of living organisms including bacteria, insects, higher plants and fungi[3]. Laccase in bacteria is present intracellularly and as periplasmic protoplast[4,5]. The first bacterial laccase was found in the plant root associated bacterium, *Azospirillum lipoferum*[6-8]. Laccase activity has also been demonstated in a number of bacteria including *Bacillus subtilis, Bacillus vallismortis, Bacillus pumilus, Bordetella compestris, Caulobacter crescentus, Escherichia coli, Mycobacterium tuberculosum, Pseudomonas putida, Pseudomonas syringae, Pseudomonas aeruginosa, Yersinia pestis* and *Geobacillus thermocatenulatus*[9-15].

Laccases catalyze the oxidation of a large variety of reducing phenolic and aromatic compounds, which makes them useful for biotechnological purposes[16]. Consequently, it is possible to use the laccase enzyme in many industrial areas such as the removal of textile dyes,

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phenols removal and waste detoxification, since it does not have the substrate specificity. The optimization of physico-chemical conditions is inevitable in any fermentation process and in order to improve the nutrient medium for enhancing laccase production, conventional methods based on the "change-one-factor-at-a time" in which one independent variable is studied while fixing all others at a specific level, may lead to unreliable results and inaccurate conclusion. This experimental procedure is not only expensive but also time consuming and often interaction effects are over looked, which demands the need for a more powerful technique by which multiple variables can be optimized in relatively few experiments. Statistical experimental designs are powerful tools for searching the key factors rapidly from a multi variable system. Plackett-Burman design[17] is one such method that has been frequently used for screening multiple factors at a time. This experimental design is particularly useful for initial screening as it is used for the estimation of only the main effects. The significant factors obtained from the screening experiments could be further optimized by employing response surface methodology that enables the study of interaction effects among different variables. Therefore, mathematical design i.e RSM finds wide application in nutrient media optimization for microbial enzyme production. The aim of this study is to obtain mathematical models showing the dependence of the enzyme activity on independent variables. Optimization of media components for the production of laccase by response surface methodology has been reported in the case of different fungal strains[18-21]. Niladevi *et al.* (2009)[22] used response surface methodology for the optimization of different nutritional and physical parameters for the production of laccase by the filamentous bacteria *Streptomyces psammoticus* MTCC 7334 in submerged fermentation, for which incubation temperature, incubation period, agitation rate, concentrations of yeast extract, $MgSO_4.7H_2O$, and trace elements were found to influence laccase production significantly. Therefore, to enhance laccase production, this study optimized different physical parameters and media components using the conventional one-factor-at-a-time method, and then further optimization of the critical parameters to obtain high levels of laccase was carried out using Central Composite design (CCD) following RSM.

MATERIALS AND METHODS

Microorganism

The microbial culture used in study was *Pseudomonas putida* LUA15.1, which was isolated from rice rhizospheric soil samples of paddy fields of Una district, Himachal Pradesh (India) and maintained on slants of nutrient agar medium at 4°C.

Culture Conditions and Enzyme Extraction

Pseudomonas putida LUA15.1 was inoculated into the Tryptone Yeast (TY) broth and mixed thoroughly by keeping the flasks on a rotary shaker at 150 rpm for 24-48 hours at 28°C. The culture supernatant was obtained by centrifugation of overnight culture of *Pseudomonas putida* LUA15.1 at 10,000 rpm, for 10 minutes at 4°C and used for the enzyme assay.

Laccase Activity Assay

Laccase activity was measured by monitoring the oxidation of ABTS[23]. Catalase was added to the assay solution and incubated for 1 hour at 37 °C to remove the possible effect of H_2O_2 produced by the bacteria. Laccase activity was determined spectrophotometrically at 420 nm with ABTS as a substrate. The reaction mixture contained 200 µl aliquots of crude extracellular enzyme preparation and 0.2 mM ABTS in 0.1 M sodium acetate buffer (pH 4.5) making final volume to 1.0 ml. The reaction was held at 32°C for 10 mins followed by addition of 0.5 ml of 80% trichloroacetic acid to stop the reaction. One unit of enzyme was defined as the amount of enzyme required to oxidize 1.0 µmol of ABTS per min. The molar extinction coefficient of ABTS was found to be 36,000 M^{-1} cm^{-1}.

Medium Optimization Using One-Factor-at-a-Time

The production of laccase was optimized based on varying the physical factors such as incubation temperature, incubation period, incubation pH and inoculum size, and nutritional factors such as Carbon sources, nitrogen sources (different combinations of tryptone and yeast extract), $CuSO_4$, $CaCl_2$, guaiacol and surfactants. The pH range of 4-10, temperature range of 25-

50°C, inoculums size of 1-5% and incubation period of 0-120 hrs were studied for laccase activity. Five carbon sources (glucose, maltose, sucrose, mannitol and lactose), different concentrations of tryptone and yeast extract (0.1, 0.2, 0.3, 0.4 and 0.5% of yeast extract and 0.3, 0.4, 0.5, 0.6 and 0.7% of tryptone), $CuSO_4$ (0.01 g/l to 0.07 g/l of 1mM concentration), $CaCl_2$ (5 ml/l to 15 ml/l of 1M concentration) Guaiacol (1.0 mM-10 mM) and surfactants (Tween-20, Tween-80 and Gallic acid) were used.

Plackett-Burman Experimental Design

Plackett–Burman design, a widely used fractional factorial method was adopted for the screening of cultural and nutritional parameters influencing laccase production. Based on the results of a preliminary study on the production of laccase by *Pseudomonas putida* LUA15.1, a Plackett-Burman (PB) experiment was performed to identify the significant variables affecting enzyme production. A set of twelve experiments was conducted. Each variable was set at two levels, high and low, denoted by (+1) and (-1), respectively and the response value represented the laccase activity.

Central Composite Design and Response Surface Methodology

A central composite design was adopted to optimize the effect of the major variables (temperature, time, pH, tween-20 and $CuSO_4$) on laccase production. The effect of each variable was studied at five different levels, namely – ±,1,0,+1,+±, and the set of 50 experiments was performed in triplicate. The central coded value of all variables was set at zero. The data obtained from the RSM were subjected to analysis of variance. The coefficient of determination (R^2) for laccase production as a function of the independent variables was found to be 98%, which showed that the model correlated well with measured data and was statistically significant at $P \leq 0.05$. The results of the RSM were used to fit a second-order polynomial, Eq. (1), to represent the behavior of the system:

$$Y = b_o + b_1A + b_2B + b_3C + b_4D + b_5E + b_1b_1A^2 + b_2b_2B^2 + b_3b_3C^2 + b_4b_4D^2 + b_5b_5E^2 + b_1b_2AB + b_1b_3AC + b_1b_4AD + b_1b_5AE + b_2b_3BC + b_2b_4BD + b_2b_5BE + b_3b_4CD + b_3b_5CE + b_4b_5DE \quad ...(1)$$

Where, Y is the response variable representing laccase activity, b_o is the intercept, b_1, b_2, b_3, b_4, b_5 are the linear coefficients, $b_1\,b_1$, $b_2\,b_2$, $b_3\,b_3$, $b_4\,b_4$, $b_5\,b_5$ are squared coefficients, b_1b_2, b_1b_3, b_1b_4, b_1b_5, b_2b_3, b_2b_4, b_2b_5, b_3b_4, b_3b_5, b_4b_5 are the interaction coefficients, and A, B, C, D, E, A^2, B^2, C^2, D^2, E^2, AB, AC, AD, AE, BC, BD, BE, CD, CE, DE are the levels of independent variables. The data analysis and generation of the response surface graphs were conducted using the statistical software Design Expert (Stat-Ease 9.0).

Statistical analysis

All the experiments were conducted in triplicate along with equal number of controls. The statistical software package Design-Expert 9.0 (Stat-Ease, Minneapolis, MN) was used for regression analysis of experimental data to obtain working parameters and to generate response surface graphs. ANOVA was used to estimate statistical parameters.

RESULTS

Effect of Physical and Nutritional Factors on Laccase Production

The effect of temperature range of 25-50°C for laccase production was observed and maximum extracellular laccase activity of 20.70 U/l was found at 28°C. Maximum extracellular laccase activity of 19.80 U/l was observed after 24 hrs of incubation period. However, pH range of 4.0-10.00 of the TY medium was examined for laccase production and it was found that maximum extracellular laccase activity of 20.30 U/l was observed at pH 7.0. Whereas, the optimization of inoculum size revealed that an inoculum size of 1% was optimum for laccase production with maximum extracellular laccase activity of 22.40 U/l. To determine whether the surfactants in the medium would affect the laccase activity, the medium was supplemented with, Tween-20, Tween-80 and Gallic acid and it was found that Tween-20 showed maximum laccase activity of 16.54 U/l, whereas Tween-80 and Gallic acid had no obvious effect on laccase production. Maximum extracellular laccase activity of 20.60 U/l was observed using 5mM Guaiacol. Among the nitrogen sources, Tryptone (0.5%) and (0.3%) of yeast extract gave maximum production of laccase with 22.94 U/l extracellular activity. To determine whether the copper concentration in the medium would affect the laccase activity, the

Table 1. Optimization of extracellular laccase production from *Pseudomonas putida* LUA15.1 using one variable at a time

Parameters	Range Tested	Optimum	Laccase activity U/l
Temperature	25-50°C	28	20.70
Time	0-120 hrs	24	19.80
pH	4-10	7	20.30
Inoculum Size	1-5%	1%	22.40
Surfactants	Tween-20, Tween-80, Gallic acid	Tween-20	16.54
Guaiacol	1.0 mM-10 mM	5 mM	20.60
Nitrogen source	Tryptone (0.3, 0.4, 0.5, 0.6 and 0.7% of tryptone)	0.5% Tryptone	
	Yeast extract (0.1, 0.2, 0.3, 0.4 and 0.5% of yeast extract)	0.3% Yeast Extract	22.94
$CuSO_4$	(0.01 g/l to 0.07 g/l of 1mM concentration)	0.05 g/l	22.77
Carbon Source	Glucose, maltose ,sucrose,manittol, lactose (1%)	Sucrose (1%)	10.30
$CaCl_2$	5 ml/l to 15 ml/l of 1M concentration	10 ml	15.60

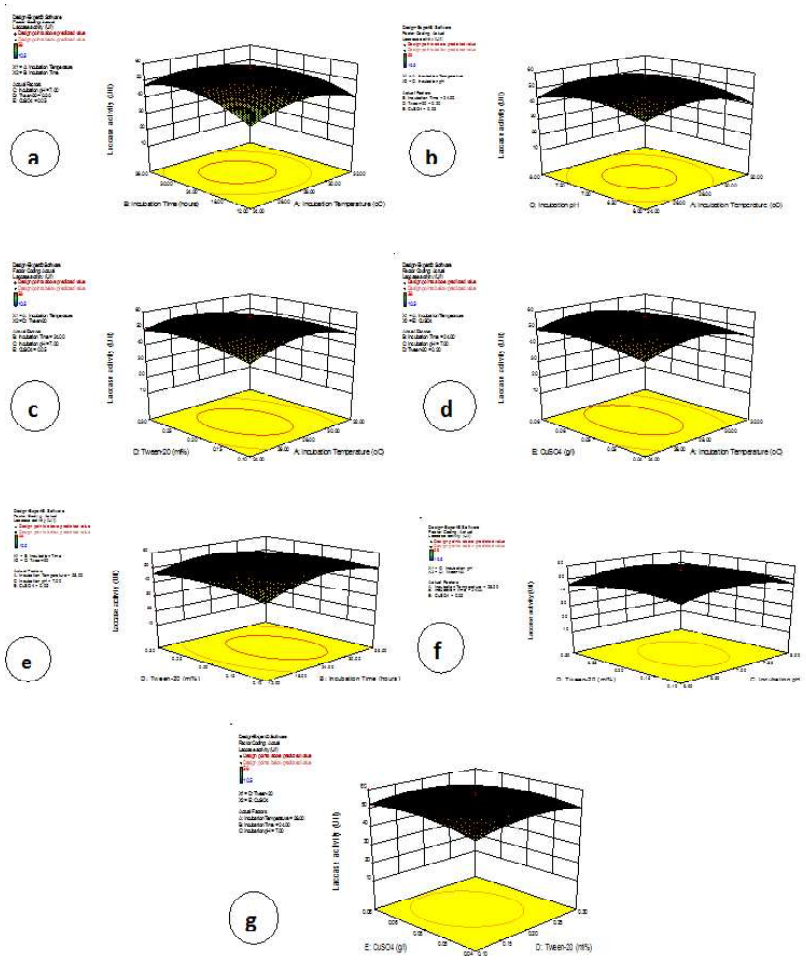

Fig. 1. Three dimensional (3D) response surface plot of the CCD experiment for laccase production by *Pseudomonas putida* LUA15.1. The interactions between, Incubation temperature and Incubation time **(a);** Incubation temperature and Incubation pH **(b);** Incubation temperature and Tween-20 **(c);** Incubation temperature and $CuSO_4$ **(d);** Incubation time and Tween-20 **(e);** Incubation pH and Tween-20 **(f);** Tween-20 and $CuSO_4$ **(g)** are shown.

Table 2. Experimental conditions and response values of Plackett-Burman test for laccase production by Pseudomonas putida LUA15.1

Run	Factor 1 AIncubation Temperature (°C)	Factor 2 BIncubation Time (hours)	Factor 3 CIncubation pH	Factor 4D Inoculum Size(ml%)	Factor 5E Tween-20(ml%)	Factor 6F Guaiacol (mM)	Factor 7G Tryptone (g%)	Factor 8H Yeast (g%)	Factor 9I $CuSO_4$ (g/l)	Factor 10J Sucrose (g%)	Factor 11K $CaCl_2$ (ml/l)	Response Laccase activity (U/l)
1	1.000	1.000	-1.000	1.000	-1.000	-1.000	-1.000	1.000	1.000	1.000	-1.000	32.03
2	1.000	-1.000	1.000	1.000	-1.000	1.000	-1.000	-1.000	-1.000	1.000	1.000	25.13
3	1.000	1.000	-1.000	1.000	1.000	-1.000	1.000	-1.000	-1.000	-1.000	1.000	20.10
4	1.000	-1.000	1.000	-1.000	-1.000	-1.000	1.000	1.000	1.000	-1.000	1.000	30.23
5	-1.000	1.000	1.000	-1.000	1.000	-1.000	-1.000	-1.000	1.000	1.000	1.000	53.15
6	-1.000	-1.000	1.000	1.000	1.000	-1.000	1.000	1.000	-1.000	1.000	-1.000	35.12
7	-1.000	-1.000	-1.000	1.000	1.000	1.000	-1.000	1.000	1.000	-1.000	1.000	32.13
8	-1.000	1.000	-1.000	-1.000	-1.000	1.000	1.000	1.000	-1.000	1.000	1.000	49.35
9	-1.000	1.000	1.000	1.000	-1.000	1.000	1.000	-1.000	1.000	-1.000	-1.000	55.15
10	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	36.05
11	1.000	1.000	1.000	-1.000	1.000	1.000	-1.000	1.000	-1.000	-1.000	-1.000	25.13
12	1.000	-1.000	-1.000	-1.000	1.000	1.000	1.000	-1.000	1.000	1.000	-1.000	28.25

medium was supplemented with various concentrations of $CuSO_4$. Whereas the lower and higher concentrations both partially inhibited laccase activity and it was found that 50 mg/l of $CuSO_4$ was most effective in stimulating laccase production showing maximum extracellular activity of 22.77 U/l. Among the carbon sources, maximum extracellular laccase activity of 10.30 U/l was obtained when sucrose was used as a carbon source, whereas glucose, maltose, mannitol and lactose produced the lowest laccase levels. Thus, sucrose was used as a carbon source for next experiments. The effect of $CaCl_2$ on laccase production was investigated by supplementing the medium with various concentrations of $CaCl_2$ and 10 ml/l of $CaCl_2$ was found to possess maximum laccase activity of 15.60 U/l (Table-1).

Identification of Significant Variables Using Plackett-Burman Design

The first optimization step identified the significant factors for laccase production using 12-run Plackett-Burman design. Variations ranging from 20.1 to 55.15 U/l in the production of laccase were observed which indicated that optimization is important to attain higher productivity (Table-2). Statistical analyses of the responses were performed and the results are presented in Table-3. A value of $Pr < 0.05$ indicates that the model term is significant. As a result, five factors were found to have significant effect on laccase production: Incubation temperature (A), Incubation time (B), Incubation pH (C), Tween-20 (E) and $CuSO_4$ (I). Incubation temperature (A) showed the highest significance and a negative effect on laccase production. Meanwhile, Incubation time (B), Incubation pH (C) and $CuSO_4$ (I), had a positive effect on laccase production, where Incubation time had a stronger effect than $CuSO_4$ followed by Incubation pH and Tween-20 had no significant effect on laccase production. The observed responses were used to compute the model coefficients using the least-square method. The model (Eq.(2)) for laccase production was calculated as:

$$Y=35.0-8.33A+4.0B+2.17C-2.83E+3.33I \quad ...(2)$$

Where A, Incubation temperature; B, Incubation time; C, Incubation pH; E, Tween-20; I, $CuSO_4$. Since the statistical model was able to

Table 3. Analysis of variables for Plackett-Burman test

Source	Sum of Squares	df	MeanSquare	FValue	p-valueProb > F	
Model	1437.67	10	143.77	431.30	0.0375	significant
A-Incubation Temperature	833.33	1	833.33	2500.00	0.0127	significant
B-Incubation Time	192.00	1	192.00	576.00	0.0265	significant
C-Incubation pH	56.33	1	56.33	169.00	0.0489	significant
D-Inoculum Size	40.33	1	40.33	121.00	0.0577	
E-Tween-20	96.33	1	96.33	289.00	0.0374	significant
F-Gauiacol	5.33	1	5.33	16.00	0.1560	
G-Tryptone	16.33	1	16.33	49.00	0.0903	
H-Yeast	16.33	1	16.33	49.00	0.0903	
I-CuSO4	133.33	1	133.33	400.00	0.0318	significant
J-Sucrose	48.00	1	48.00	144.00	0.0529	
Residual	0.33	1	0.33			
Cor Total	1438.00	11				

Statistically significant at 95% of confidence level (Pr < 0.05)

explain 96.66% of the variability in the response, it was concluded that the model fitted well with the measured data.

Statistical Optimization Using Central Composite Design

Based on the PB tests, statistical optimization was used to determine the optimal values of Incubation temperature, Incubation time, Incubation pH, Tween-20 and $CuSO_4$ concentration. The effect of each variable on enzyme production was characterized at five different levels, namely -2.378, 1, 0, +1, +2.378. Thus, a set of five factors with five levels and a total of 50 treatments were performed. The results of the response surface experiments to determine the effects of Incubation temperature, Incubation time, Incubation pH, Tween-20 and $CuSO_4$ concentration are presented in Table-4.

The determination coefficient (R^2) value of 0.9931 indicates that the statistical model was able to expain 99.31% of the variability in the response. For a good statistical model R^2 value should be close to 1.0. Also the model indicated that the predicted R^2 value of 0.9773 was in reasonable agreement with the adjusted R^2 value of 0.9883. In addition, adjusted determination coefficient (adjusted R^2 = 0.9883) was also high, indicating the high significance of the model.

The significance of each term in the model is presented in Table-5. The ANOVA for the selected quadratic model showed that the model was significant with a model F = 208.67 and P > F- value < 0.0001.

The results indicated that the linear effects of $CuSO_4$ (E) and interaction effect of AC, AE. CD, DE were significant ($P < 0.05$). Moreover, the linear effects of Incubation temperature (A), Incubation time (B), the squared effects of A^2, B^2, C^2, D^2, E^2 and interaction effects of AB, AD, BD were more significant than the other factors ($P <. 0.01$). Thus, the response of laccase production (Y) by *Pseudomonas putida* LUA15.1 could be expressed in terms of the following regression equation (Eq. 3):

Y= 56.69 - 1.64A + 1.70B + 0.078C - 0.22D + 0.68E - 7.40A2 - 7.24B2 - 5.82C2 - 3.51D2 - 2.88E2 -1.83AB + 0.58AC - 1.91AD - 0.62AE - 0.30BC - 1.64BD - 0.21BE + 0.66CD - 0.30CE - 0.93DE ...(3)

Where A, Incubation temperature; B, Incubation time; C, Incubation pH; D, Tween-20; E, $CuSO_4$.

A 3D response surface was drawn based on the model equation to investigate the interaction among variables and determine the optimum value of each factor for maximum laccase production by *Pseudomonas putida* LUA15.1. Also, significant interaction (*P d" 0.05*) was observed between different combinations of five independent variables and this synergy was indicated by three dimensional response surfaces (Fig.1(a-g)). Design Expert predicted the maximum laccase yield to be 56.69 U/l in the optimal medium

Table 4. Experimental plan for central composite design performed for selected parameters

Run	Factor 1A Incubation Temperature (°C)	Factor 2B Incubation Time (hours)	Factor 3C Incubation pH	Factor 4D Tween-20 (ml%)	Factor 5E $CuSO_4$ (g/l)	ResponseLaccase activity (U/l)
1	1.000	-1.000	-1.000	1.000	1.000	25.5
2	0.000	0.000	0.000	0.000	0.000	57.5
3	0.000	0.000	0.000	0.000	0.000	57.0
4	1.000	1.000	1.000	1.000	-1.000	25.5
5	1.000	-1.000	1.000	1.000	1.000	27.15
6	-1.000	1.000	-1.000	1.000	1.000	36.5
7	1.000	-1.000	-1.000	-1.000	1.000	31.5
8	1.000	-1.000	1.000	1.000	-1.000	33.15
9	0.000	0.000	0.000	0.000	0.000	56.0
10	-1.000	-1.000	1.000	-1.000	1.000	25.5
11	1.000	1.000	1.000	1.000	1.000	26.0
12	1.000	1.000	-1.000	-1.000	1.000	32.12
13	-1.000	1.000	1.000	1.000	1.000	32.6
14	1.000	1.000	1.000	-1.000	-1.000	31.0
15	-1.000	-1.000	1.000	1.000	-1.000	30.0
16	-1.000	-1.000	1.000	1.000	1.000	33.5
17	0.000	0.000	0.000	-2.378	0.000	38.7
18	-1.000	1.000	1.000	-1.000	-1.000	31.5
19	0.000	0.000	0.000	0.000	0.000	58.0
20	-1.000	1.000	1.000	-1.000	1.000	35.15
21	1.000	1.000	-1.000	-1.000	-1.000	32.0
22	-1.000	1.000	1.000	1.000	-1.000	36.0
23	0.000	0.000	0.000	0.000	0.000	56.0
24	0.000	0.000	2.378	0.000	0.000	24.5
25	0.000	0.000	0.000	0.000	-2.378	38.0
26	0.000	0.000	0.000	2.378	0.000	35.0
27	1.000	-1.000	-1.000	1.000	-1.000	27.15
28	-1.000	1.000	-1.000	1.000	-1.000	36.0
29	1.000	1.000	1.000	-1.000	1.000	32.0
30	0.000	2.378	0.000	0.000	0.000	20.0
31	-1.000	-1.000	-1.000	-1.000	1.000	27.5
32	0.000	0.000	0.000	0.000	2.378	42.8
33	0.000	0.000	0.000	0.000	0.000	57.5
34	-1.000	1.000	-1.000	-1.000	1.000	38.15
35	-1.000	-1.000	-1.000	1.000	1.000	32.0
36	0.000	0.000	0.000	0.000	0.000	56.0
37	0.000	-2.378	0.000	0.000	0.000	11.5
38	1.000	1.000	-1.000	1.000	1.000	22.5
39	0.000	0.000	0.000	0.000	0.000	55.5
40	-2.378	0.000	0.000	0.000	0.000	19.12
41	1.000	1.000	-1.000	1.000	-1.000	23.8
42	-1.000	-1.000	-1.000	-1.000	-1.000	22.5
43	0.000	0.000	-2.378	0.000	0.000	23.0
44	-1.000	1.000	-1.000	-1.000	-1.000	33.5
45	-1.000	-1.000	1.000	-1.000	-1.000	22.5
46	1.000	-1.000	-1.000	-1.000	-1.000	27.0
47	1.000	-1.000	1.000	-1.000	1.000	29.0
48	-1.000	-1.000	-1.000	1.000	-1.000	30.0
49	2.378	0.000	0.000	0.000	0.000	10.5
50	1.000	-1.000	1.000	-1.000	-1.000	27.0

Table 5. ANOVA for Response Surface Quadratic model

Source	Sum of Squares	df	MeanSquare	FValue	p-valueProb > F	
Model	7008.59	20	350.43	208.67	< 0.0001	significant
A-Incubation Temperature	116.49	1	116.49	69.37	< 0.0001	significant
B-Incubation Time	125.02	1	125.02	74.44	< 0.0001	significant
C-Incubation pH	0.27	1	0.27	0.16	0.6933	
D-Tween-20	2.03	1	2.03	1.21	0.2810	
E-CuSO4	20.07	1	20.07	11.95	0.0017	significant
AB	106.69	1	106.69	63.53	< 0.0001	significant
AC	10.85	1	10.85	6.46	0.0166	significant
AD	116.93	1	116.93	69.63	< 0.0001	significant
AE	12.16	1	12.16	7.24	0.0117	significant
BC	2.80	1	2.80	1.67	0.2066	
BD	86.03	1	86.03	51.23	< 0.0001	significant
BE	1.37	1	1.37	0.82	0.3732	
CD	13.87	1	13.87	8.26	0.0075	significant
CE	2.86	1	2.86	1.70	0.2020	
DE	27.70	1	27.70	16.49	0.0003	significant
A^2	3044.13	1	3044.13	1812.70	< 0.0001	significant
B^2	2908.97	1	2908.97	1732.22	< 0.0001	significant
C^2	1882.93	1	1882.93	1121.24	< 0.0001	significant
D^2	682.77	1	682.77	406.57	< 0.0001	significant
E^2	460.18	1	460.18	274.02	< 0.0001	significant
Residual	48.70	29	1.68			
Lack of Fit	42.73	22	1.94	2.28	0.1331	not significant
Pure Error	5.97	7	0.85			
Cor Total	7057.29	49				

which is very close to the actual level of laccase produced i.e. 58 U/l. As a result of optimizing physical and nutritional conditions, a 5.52-fold increase in laccase activity was achieved as compared with that (10.5 U/l) previously obtained under unoptimized conditions. The results showed a good agreement between the predicted and experimental values, thereby validating the model.

DISCUSSION

The response surface methodology (RSM) was first described by Box and Wilson in 1951[24]. RSM is an effective strategy for seeking the optimum conditions for a multivariable system. Traditional optimization methods change one independent variable, while keeping the other variables fixed at a certain level. However, this single-dimentional search is laborious, time consuming, and incapable of reaching a true optimum owing to the interactions among the variables. This statistical method for media optimization has already been successfully utilized to improve various industrially important enzymes by their source organisms like bacteria, fungi, actinomycetes etc. Optimization of media components for the production of laccase by response surface methodology has been reported in the case of different fungal strains[18-21]. Niladevi *et al.* (2009)[22] used response surface methodology for the optimization of different nutritional and physical parameters for the production of laccase by the filamentous bacteria *Streptomyces psammoticus* MTCC 7334 in submerged fermentation. But there are no reports on the optimization of laccase production from *Pseudomonas putida*. Therefore, this study applied RSM to optimize the physical and nutritional conditions for laccase production by *Pseudomonas putida* LUA15.1. The obvious important factors (Incubation temperature, Incubation time, Incubation pH, Tween-20, and

$CuSO_4$) that influenced the production of laccase were obtained using a Plackett-Burman experimental design, and the optimal concentrations of these five factors were then sequentially investigated using the response surface methodology with a central composite design. The final optimal medium components included 50 mg/l $CuSO_4$ in the TY medium containing 5 mM Gauiacol and incubated at final optimal cultural conditions *viz*., incubation temperature of 28°C, incubation time of 24 hrs and incubation pH of 7.0. Moreover the organism produced laccases within a short incubation period of 24 hrs. Being a man grove isolate, this organism exhibited some unusual characteristics such as the expression of laccase activity in the neutral to alkaline pH as compared to the laccases from other bacterial sources such as *Bacillus subtilis*[11] and *Streptomyces cyaneus*[25] that are reported to be active in acidic pHs. These remarkable properties make this organism a better candidate for biotechnological applications. When using the optimal medium along with optimal cultural parameters, the yield of laccase was increased 5.52-fold as compared with the laccase production in an unoptimized medium. Among the important factors affecting laccase production, $CuSO_4$ supplementation at 50 mg/l was effective in improving the laccase production by *Pseudomonas putida* LUA15.1. This expressive effect is also supported by the fact that Cu^{2+} is a laccase cofactor, where every four Cu^{2+} are associated with one single polypeptide chain.

In conclusion, this research is the first systematic medium optimization for laccase production by *Pseudomonas putida* LUA15.1 and showed the effect of each factor. The optimized conditions will be helpful for further studies of laccase production by *Pseudomonas putida* LUA15.1.

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