ScienSage

Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN
0976-9595
Research Article

OPTIMIZATION OF LACCASE ENZYMES PRODUCTION THROUGH RESPONSE SURFACE METHODOLOGY BY *TRAMETES ELEGANS H6*

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ABSTRACT

Laccase are multicopper oxidases containing four copper atoms per monomer distributed in three redox sites. Laccase is significant in light of the fact that it oxidizes both the harmful and nontoxic substrates. It has tremendous applications in different areas like material industry, food preparing industry, wood processing industry, drug industry, and synthetic industry. So isolation of efficient laccase producing microorganism and optimization of the process parameters for laccase production is important. Response surface methodology was employed for the optimization of different nutritional parameters for the production of laccase by the filamentous fungus Trametes *elegans H6* in solid state fermentation. Initial screening of production parameters was performed using a Plackett-Burman design and the variables with statistically significant effects on laccase production were identified. Mannitol, urea and copper sulphate were found to influence laccase production significantly. These variables were selected for further optimization studies using a central composite design (CCD). The statistical optimization by response surface methodology resulted in a 1.65 fold increase in the production of laccase by *Trametes elegans* H6. *Trametes elegans H6* can be a good source of Laccase and it can be use for industrial application.

Keywords: Laccase, *Trametes elegans,* Plackett-Burman, Response surface methodology.

1. INTRODUCTION

Laccases are typically monomeric, dimeric or tetrameric glycoproteins containing four copper atoms positioned at the catalytic site. The distribution of laccase is widespread in nature among higher plants [1], bacteria [2], fungi [3], insects [4], lichens [5] and crustacean [6]. White-rot fungi are considered to be the most promising group of microorganisms as they produce extracellular lignin modifying enzymes, *i.e.* laccase (E.C. 1.10.3.2), manganese peroxidase (MnP; E.C. 1.11.1.13), lignin peroxidase (LiP; E.C. 1.11.1.14), and versatile peroxidase (VP; E.C. 1.11.1.16) along with heme-containing peroxidases, which are highly effective for lignin and lignocellulose biodegradation [3]. White-rot fungi (WRF) are eukaryotic microorganisms that grow on decayed wood by degrading wood lignocelluloses and undergo fascinating fruiting body formation [7]. They have evolved complex enzyme machinery for the decomposition of wood cellulose and lignin with an excellent ability to degrade and mineralize the lignin polymer [8].

Due to large substrate specificity, laccase are known to have many applications in various area including bioremediation, food technology, green chemistry etc. In view of the various applications of laccase, production of large amount of enzyme under low-cost process is the current focus of research towards the development of different biochemical processes. As Solid-state fermentation (SSF) usually involves the use of agricultural or food wastes or other by-products containing natural laccase inducers as a solid support that reduce the cost of raw materials [9]. Optimization of the laccase production can be achieved through classical one-factor-at-a-time method but it has certain limitations. However, optimizing various process parameters using statistical experimental design, Plackett-Burman and response surface methodology can eliminate the limitations of one-factor-at-a-time method [10].

This statistical optimization allows screening a large experimental domain and to determine the impact of each variable on the target response as well as the possible occurrence of interactions among tested variable [11]. A Plackett-Burman design helps to find out which factors in an experiment are important. This design screens out unimportant factors (noise), which means that you avoid collecting large amounts of data on

relatively unimportant factors. Response surface method (RSM) is based on the fundamental principles of statistics; randomization, replication and duplication, which simplifies the optimization by studying the mutual interactions among the variables over a range of values in a statistically valid manner. Basically, this is a three-step optimization process involving the estimation of coefficients in a mathematical model, prediction of the response and subsequent validation of the model [12].

The present investigation deals with the Placket Burman and RSM study of the production medium for laccase production by *Trametes elegans H6* in solid state fermentation. In a previous study, the influence of a large number of experimental variables on laccase production by *Trametes elegans H6* was investigated and their optimum values were determined by one factor at one time method [13]. On the basis of these results, here the attention was focused on factors which influence the laccase production by statistical methods.

2. MATERIAL AND METHODS

2.1. Chemicals

2, 2-Azino-bis (3ethylbenzthiozoline-6-sulphonic acid) (ABTS) and Malt extract agar were purchased from Hi-Media (Mumbai, India). Thiamine HCl,NH_4NO_3, $CuSO_4 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$, $MnSO_4 \cdot H_2O$, ammonium ferric citrate, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $ZnSO_4 \cdot 7H_2O$, KH_2PO_4, Tween 80 Mannitol, Urea were purchased from Rankem Chemicals, (Mumbai, India). Rice straw was collected locally and used as alignocellulosic substrate.

2.2. Maintenance of Culture and Inoculum Preparation

Stock culture of the potential fungal isolate *Trametes elegans H6* was maintained by sub culturing at a regular time interval on 2% Malt Extract (ME) agar plate at 30°C and stored at 4°C. For inoculum preparation, agar disc of 8 mm diameter taken from the stock culture was transferred on 2% Malt Extract (ME) agar plate with a sterilized cup borer and incubated at 30°C for 8 to 10 days. Freshly grown fungal mycelium culture was used to inoculate in SSF flask.

2.3. Enzyme assay

Laccase activity (E.C. 1.10.3.2) was determined by measuring the oxidation of ABTS. Increase in absorbance was measured spectrophotometrically for 3 minutes at 420 nm. Supernatant obtained after centrifugation containing the enzyme extract was mixed with 100µl of 50 mM ABTS and 800 µl of 20 mM Sodium acetate buffer (pH 5.0) and 100 µl of appropriately diluted enzyme extract. One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 µM of substrate per minute at room temperature [14].

2.4. Optimization of laccase production by statistical method

2.4.1. Selection of significant parameters by Plackett -Burman (PB)

A set of 20 experiments was designed using the Plackett-Burman design for 14 variables (Table 1) that were analyzed as possible factors affecting production based on literature search. The parameters evaluated were as follows:.X1-Mannitol, X2-Urea, X3-$CuSO4 \cdot 5H_2O$, X4-Ammonium nitrate, X5-$CoCl_2 \cdot 6H_2O$, X6-$MnSO_4 \cdot 7H_2O$,X7-$ZnSO_4 \cdot 7H_2O$, X8-Ammonium ferric chloride, X9-$MgSO_4 \cdot 7H_2O$, X10-$CaCl_2 \cdot 2H2O$, X11-KH_2PO_4, X12-Tween 80, X13-L-Aspargine, X14-Thiamin hydro-chloride. In each experiment, rice straw was taken as the solid substrate (5g) in 250 ml Erlenmeyer flask. Concentration levels were decided on the basis of literature reports on laccase production, with each variable being represented at two levels high (+) and low (-) and five dummy variables in 20 trials as shown in tables 1 and 2. The effect of each variable was determined by equation:

$$E(xi) = 2(\Sigma Mi+ - Mi-)/N \quad (1)$$

Where, *E*(*xi*) is the concentration effect of the tested variable, *Mi*+ and *Mi*– are the laccase production.

The variable (*xi*) measured was estimated by calculating the variance among the dummy *variables by following formula:*

$$Veff = \Sigma (Ed^2)/n \quad (2)$$

*V*eff is the variance of the concentration effect, *Ed* is the concentration effect for the dummy variables and n is the number of dummy variables. The standard error (S.E.) of the concentration effect was the square root of the variance of an effect and the significance level (*p* value) of each concentration effect was determined using student's t test.

$$t(xi) = Exi/S.E.$$

Where, *Exi* is the effect of variable *x*i.

From the trial the factors showing highest positive effects were selected for optimization using Central Composite Design of Response Surface Methodology.

2.4.2. Optimization of laccase production using response surface methodology (RSM)

Based on the results obtained in the Plackett-Burman

design, the effect of three factors Viz Mannitol, Urea and $CuSO_4$ was studied on laccase production using CCD design. Central composite design (CCD) of RSM was used from software Design expert version 12 to further optimize the levels of significant variables .The effect of each components on laccase production was studied at five different levels viz; -α, -1, 0, +1, +α. (Table 5).

From the data obtained, a regression analysis was performed. The results of the CCD experiments were then fitted with a quadratic equation by multiple regression procedure which resulted in an empirical model which related response to the independent variable of the experiment. The behavior of the system was explained by equation

$$Y = \beta 0 + \Sigma \beta ixi + \Sigma \beta ijxixj + \Sigma \beta iixi^2$$

Where, Y is predicted response, β0 is offset term, βi is the linear coefficient, βii is squared coefficient, βij is interaction effect, xi is the dimensionless coded value of Xi.

Laccase activity was recorded as response. Response data were fed and analyzed by the software to generate 3D plots indicating the optimum conditions and interaction among these factors. The quality of fitting by the polynomial model equation was expressed using coefficient of determination R^2.

3. RESULTS AND DISCUSSION

3.1. Selection of significant parameters by Plackett-Burman (PB)

A total of fourteen parameters were analyzed with respect to their effect on laccase production using Plackett-Burman design. Each independent variable were varied with two levels, a high (+) and a low (-) level and the ranges selected for each parameter are given in table 1. The Plackett-Burman experimental design for each variable with two levels of concentration for 20 trials showing corresponding laccase production in terms of unit per gram of solid substrate is represented in table 2. The variables denoted as X1-X14 represents the medium components while D1-D5 are dummy variables. The effect, standard error, t(xi), p-value and confidence level for each component based on laccase production (U/gm of dry substrate) is described in table 3.

Table 1: Variables showing medium components used in Plackett-Burman design

Variables	Medium component	+ values gm/lit	- values gm/lit
X1	Mannitol	10	1
X2	Urea	0.5	0.05
X3	$CuSO_4 \cdot 5H_2O$	0.007	0.0007
X4	Ammonium nitrate	0.5	0.05
X5	$CoCl_2 \cdot 6H_2O$	0.007	0.0007
X6	$MnSO_4 \cdot 7H_2O$	0.035	0.0035
X7	$ZnSO_4 \cdot 7H_2O$	0.0462	0.00462
X8	Ammonium ferric chloride	0.085	0.0085
X9	$MgSO_4 \cdot 7H_2O$	0.05	0.005
X10	$CaCl_2 \cdot 2H_2O$	0.0132	0.00132
X11	KH_2PO_4	0.2	0.02
X12	Tween 80	0.1	0.01
X13	L-Aspargine	1.0	0.1
X14	Thiamine hydrochloride	0.0025	0.00025

The components were screened at a confidence level of 99%. On the basis of their effects the confidence level for Mannitol, Urea, Copper sulphate showed confidence level of 99.46% 99.44% and 99.35% respectively and were all considered significant. While the confidence level for rest all the component were below 99% and hence considered insignificant. All the component showed confidence level of above 99% or 99% -Mannitol, Urea, Copper sulphate have positive effect on laccase production thus higher concentration is required in further step. The result obtained shows the efficiency of the Plackett-Burman design in identifying the factors that are influencing the laccase production.

3.2. Optimization of laccase production using response surface methodology (RSM)

Response surface Methodology (RSM) is a collection of mathematical and statistical techniques useful for analyzing problems where several independent variables influence a dependent variable or response, and the goal

is to optimize this response. A total of 20 experiments with three variables (components of the medium) and five coded levels (five different concentrations) were performed. Based on the results obtained from the Plackett-Burman Design three variables were selected namely Mannitol, Urea and Copper sulphate. All these three variables showed positive influence on Laccase activity. Thus increasing concentrations of Mannitol, Urea and Copper sulphate resulted in high Laccase activity. The other components of the production medium namely Ammonium nitrate, $CoCl_2 \cdot 6H_2O$, $MnSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, Ammonium ferric chloride, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, KH_2PO_4, Tween 80, L-Aspargine and Thiamine hydrochloride were found to be insignificant, so their concentrations were set at their middle level in Central Composite Design.

The experimental values for the regression coefficient were obtained by quadratic polynomial equation, where only significant coefficients ($P<0.05$) were considered. The smaller *P*-values indicate the higher significance of the corresponding coefficient. The predicted responses; Y for Laccase activity was obtained as follows:

$Y = 5861.30 + 2569.16\ A + 1198.73\ B + 935.66\ C + 19.50\ AB - 147.75\ AC - 27.50\ BC + 23.52\ A^2 - 36.23\ B^2 + 48.45\ C^2$

Where, Y is the Laccase activity (U/gm of dry substrate) and A, B, C are coded values of the independent variables Mannitol, Urea and Copper sulphate respectively.

The statistical significance of the quadratic model for the experimental responses was evaluated by the analysis of variance (ANOVA). According to the ANOVA results (table 4), the model was significant with an *F*-test of a very low probability for Laccase activity indicate that model terms are significant. The P value serves as a tool for checking the significance of each of the coefficients and is indicative of the interaction strength of each independent variable. Low values of $P<0.05$ indicate high significance of the corresponding coefficients. P-values less than 0.0500 indicate model terms which are significant.

R^2 value gives a measure of how much variability in the observed response can be explained by the experimental parameters and their interactions. The Predicted R^2 of 0.9542 is in reasonable agreement with the Adjusted R^2 of 0.9872; *i.e.* the difference is less than 0.2 suggested that the model is suitable and practicable. The "Lack of Fit F-value" of 3.07 for Laccase activity, implies the Lack of Fit is not significant relative to the pure error. The significant lack of fit is bad because we want the model to fit.

Table 2: Plackett-Burman matrix of fourteen variables (X1-X14) and five dummy variables (D1-D5) along with observed response (laccase production)

Run no	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	D1	D2	D3	D4	D5	Laccase activity (U/gm of substrate)
1	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	6893.33
2	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	8501.01
3	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	7908
4	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	4844
5	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	4177.78
6	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	1570
7	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	423.33
8	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	416.61
9	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	1133
10	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	988.89
11	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	5666.67
12	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	2066.66
13	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	4144.44
14	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	6223.336
15	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	9762.23
16	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	8533.34
17	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	5022
18	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	1206.658
19	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	2070
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	373.3184

Table 3: Statistical analysis of the medium components in relation to laccase production as per Plackett-Burman design

Factors	Medium Component	Effect (Exi)	S.E	t(xi)	*p-value*	Confidence level (%)
X1	Mannitol	32432.09	6916.05	4.69	0.01	99.46
X2	Urea	32100.55	6916.05	4.64	0.01	99.44
X3	$CuSO_4$	31059.65	6916.05	4.49	0.01	99.35
X4	Ammonium nitrate	-2189.95	6916.05	0.32	0.76	23.81
X5	$CoCl_2$	-4003.85	6916.05	0.58	0.59	41.29
X6	$MnSO_4$	-474.47	6916.05	0.07	0.95	5.31
X7	$ZnSO_4$	6618.9776	6916.05	0.96	0.38	61.89
X8	Ammonium Ferric Citrate	8839.0736	6916.05	1.28	0.26	74.33
X9	$MgSO_4$	1686.8576	6916.05	0.24	0.82	18.01
X10	$Cacl_2$	-12059.086	6916.05	1.74	0.14	85.77
X11	KH_2PO_4	-4705.6904	6916.05	0.68	0.53	47.33
X12	Tween 80	8291.2896	6916.05	1.20	0.28	71.61
X13	L-Aspargine	864.6376	6916.05	0.13	0.90	9.84
X14	Thiamine hydrochloride	-3983.1504	6916.05	0.58	0.59	41.29

Table 4: Analysis of variance (ANOVA) for the quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	1.220E+08	9	1.355E+07	164.31	< 0.0001
A-Mannitol	9.014E+07	1	9.014E+07	1092.91	< 0.0001
B-Urea	1.962E+07	1	1.962E+07	237.93	< 0.0001
C-Copper Sulphate	1.196E+07	1	1.196E+07	144.96	< 0.0001
AB	3042.00	1	3042.00	0.0369	0.8516
AC	1.746E+05	1	1.746E+05	2.12	0.1763
BC	6050.00	1	6050.00	0.0734	0.7920
A^2	7973.00	1	7973.00	0.0967	0.7623
B^2	18915.73	1	18915.73	0.2293	0.6423
C^2	33824.52	1	33824.52	0.4101	0.5363
Residual	8.248E+05	10	82480.42	-	-
Lack of Fit	6.220E+05	5	1.244E+05	3.07	0.1221
Pure Error	2.029E+05	5	40570.17	-	-
Cor Total	1.228E+08	19	-	-	-

Three-dimensional (3-D) graphs were generated for regression analysis of CCD design, using pair wise combination of variables for Laccase production. These 3-D response surface plots describe the effects of the independent variables and combined effect of each independent variable upon the response (fig. 1a-c). Interaction effects of coefficient term between Mannitol, urea and copper sulphate have also been studied. 3-D response surface plots were constructed by plotting the response (Laccase activity) on the Z-axis against any two independent variables, while maintaining other variables at their optimal levels to determine the optimal levels of each variable for maximum Laccase production, As shown in fig. 1a, The increment of Mannitol and urea concentration from Lower to higher increased the Laccase activity. A similar profile was observed in fig. 1b (effect of copper sulphate and Mannitol) and fig. 1c (Copper sulphate and Urea), where Laccase activity increased with increasing concentration of the variables. The predicted optimal concentrations for different variables were 8.17572 gm/lit (Mannitol), 0.408786 gm/lit (urea) and 0.005723 gm/lit ($CuSO4.5H_2O$) with predicted maximum laccase activity of 10289 U/gm of dry substrate. The relationship between the actual laccase activity and predicted values determined by the model equation for *Trametes elegans* H6is shown in table 5. Most of the points were nearby which indicates that experimentally determined values were similar to those determined by the model.

Table 5: Central Composite Design matrix with coded values and actual values for laccase production

Run No	A-Mannitol		B-Urea		C-Copper Sulphate		Laccase activity U/gm of dry substrate	
	Coded value	Actual value gm/lit	Coded value	Actual value gm/lit	Coded value	Actual value gm/lit	Actual Response	Predicted Response
1	0	5.5	-α	0.05	0	0.0038	3677.44	3742.81
2	-1	2.8242	-1	0.1412	1	0.0057	3013.87	3259.56
3	-1	2.8242	1	0.4087	-1	0.0019	3049.78	3451.19
4	-1	2.8242	1	0.4087	1	0.0057	5612.2	5563.02
5	1	8.1757	-1	0.1412	-1	0.0019	6232.07	6432.55
6	1	8.1757	-1	0.1412	1	0.0057	8314.11	8063.38
7	1	8.1757	1	0.4087	-1	0.0019	9019.23	8924.01
8	0	5.5	0	0.275	0	0.00385	5889.09	5861.3
9	-α	1	0	0.275	0	0.00385	1781.11	1607.02
10	-1	2.8242	-1	0.1412	-1	0.0019	1222.04	1037.73
11	0	5.5	0	0.275	-α	0.0007	4544.01	4424.73
12	0	5.5	0	0.275	0	0.00385	5634.03	5861.3
13	0	5.5	α	0.5	0	0.00385	8055.07	7774.84
14	0	5.5	0	0.275	A	0.007	7667.13	7571.92
15	α	10	0	0.275	0	0.00385	10289.16	10248.63
16	0	5.5	0	0.275	0	0.00385	5801.07	5861.3
17	0	5.5	0	0.275	0	0.00385	6119.04	5861.3
18	0	5.5	0	0.275	0	0.00385	5644.14	5861.3
19	0	5.5	0	0.275	0	0.0038	6044.23	5861.3
20	1	8.1757	1	0.4087	1	0.0057	10109.56	10444.84

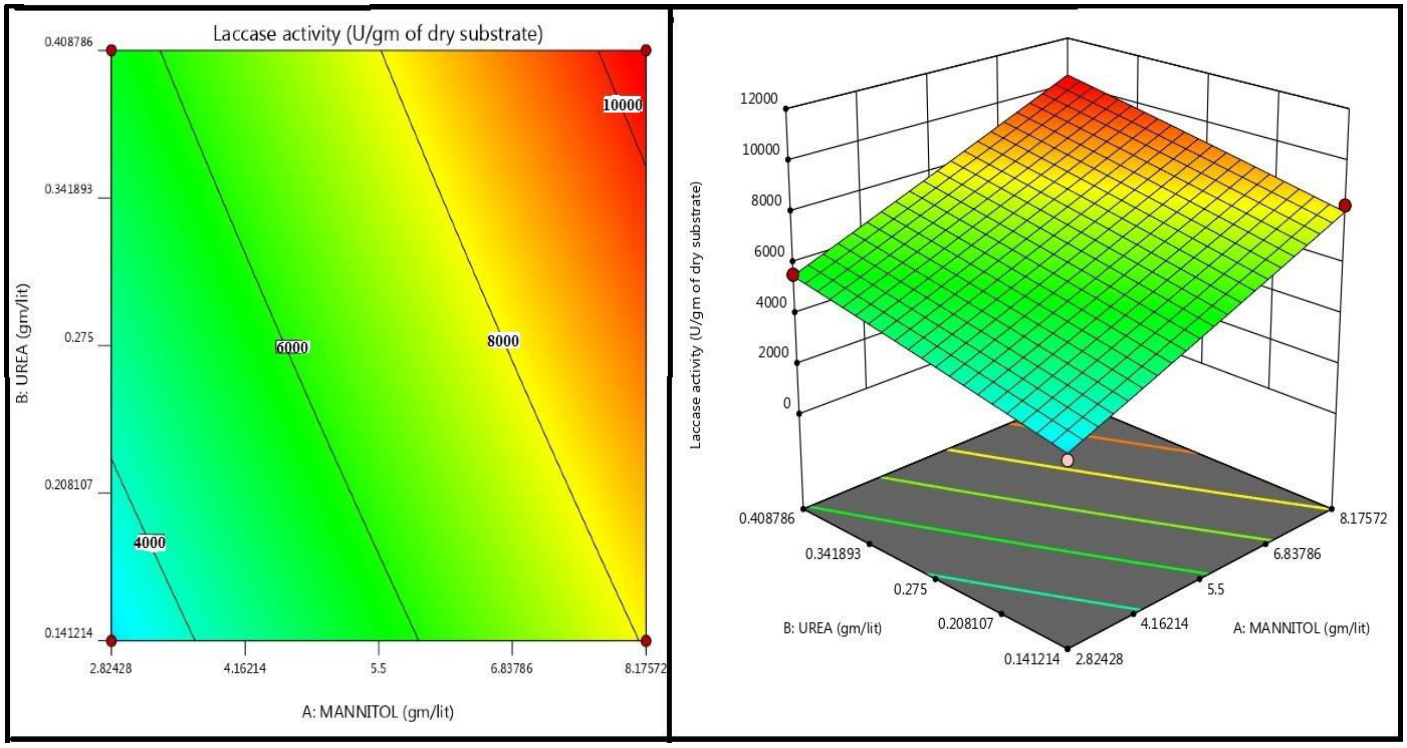

Fig. 1a: 3-D plot showing the interaction between Mannitol and Urea on laccase activity

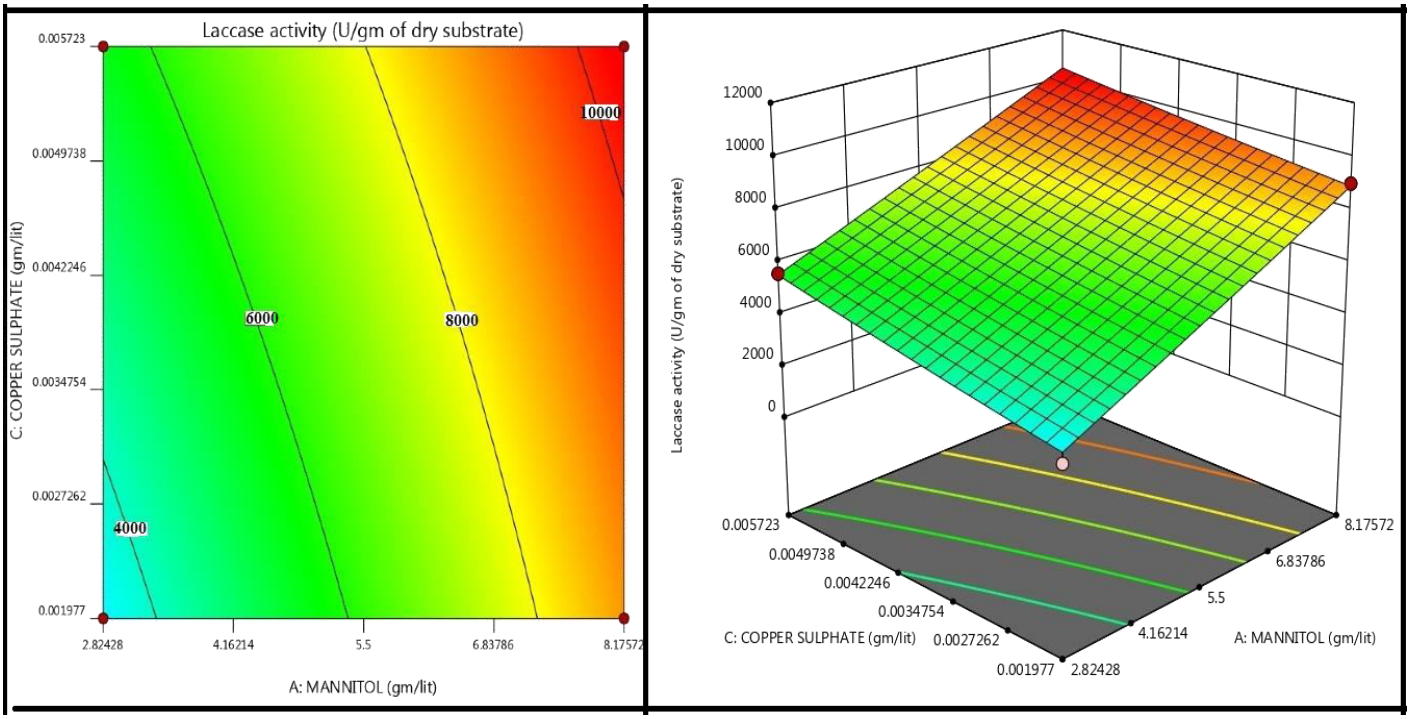

Fig. 1b: 3-D plot showing the effect of interaction between Mannitol and Copper sulphate on laccase activity

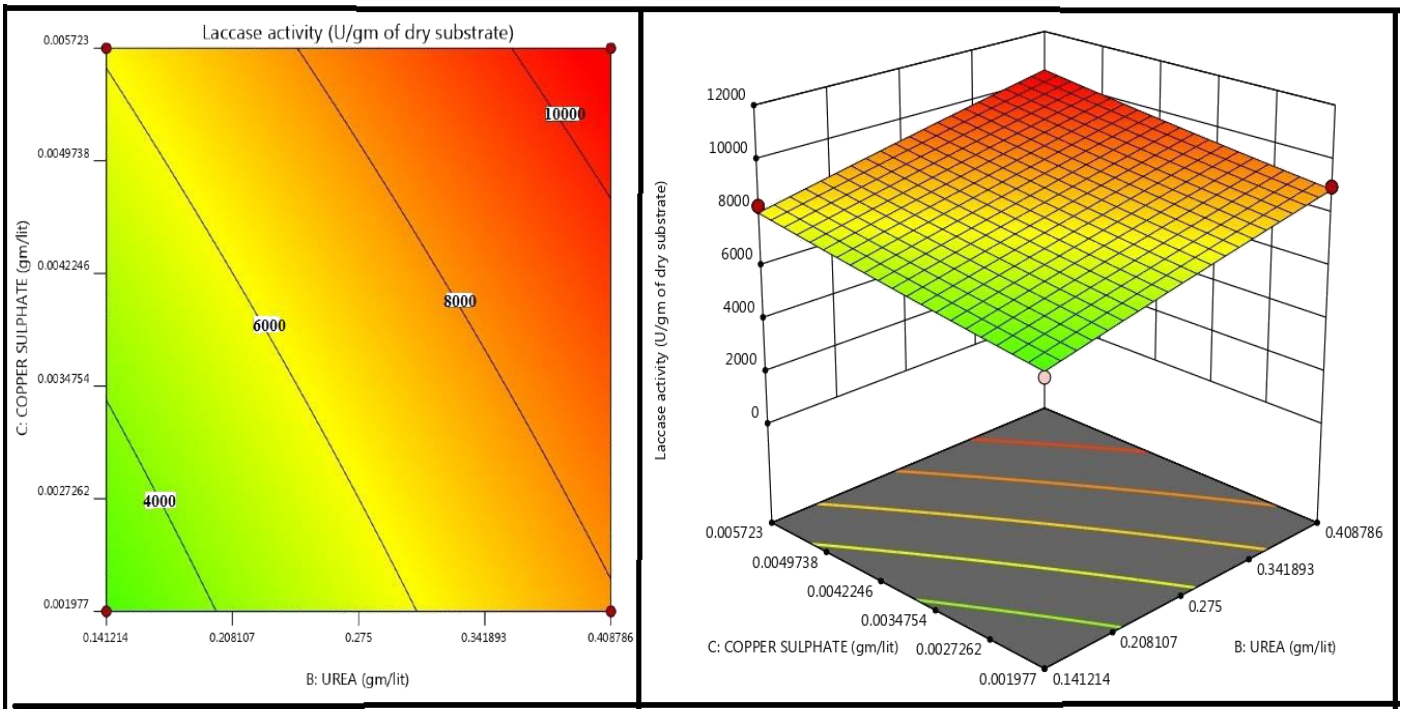

Fig. 1c: 3-D plot showing the effect of interaction between Urea and Copper sulphate on laccase activity

RSM incorporates the interaction effects of variables and aids us in simultaneously optimizing several process parameters within a minimal number of experimental runs. Such statistically assisted experimental designs can lead to significantly enhanced activity .In the present study 1.65 fold increased in the laccase activity is achieved compared to un-optimized conditions. Chhaya & Gupte also reported 16 times increase in laccase production by *Fusarium incarnatum* LD-3 [15]. However, Gao et al. in his study has reported 59.68 times increase

in yield of laccase production by *Trichoderma harzianum* ZF-2 using medium optimized by Response Surface Methodology [16]. In another study, 3-fold higher laccase production by *Streptomyces psammoticus* was achieved by Niladevi et al. using an optimized medium under SSF [17].

4. CONCLUSION

Statistical optimization method *i.e.* placket Burman and RSM has many advantages when compared to classical methods. It needs fewer experiments to study the effects of all the factors and the optimum combination of all the variables can be revealed. The interaction (the behavior of one factor may be dependent on the level of another factor) between factors can be determined. The present investigation had led to the conclusion that The maximum Laccase activity (10289 U/gm of dry substrate) was observed in the presence of 8.17572 gm/lit (Mannitol), 0.408786 gm/lit (urea) and 0.005723 gm/lit ($CuSO_4.5H_2O$), when inoculated with an 10 agar plug of 8mm size and incubated for 14 days after optimization of medium component parameter using RSM. These results revealed that *Trametes elegans H6* is good source of Laccase and it can be use for industrial application.

Conflict of interest

None declared

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