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Research Article

STATISTICAL OPTIMIZATION OF LACCASE PRODUCING *STREPTOMYCES CHARTREUSIS* BY SOLID STATE FERMENTATION

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ABSTRACT
The plan of this work was to optimize the cultural and production parameters during the statistical approach for the production of laccase enzyme by *Streptomyces chartreusis strain NBRC 12753 (Gen Bank Accession Number JQ086575)* through solid state fermentation process (SsF). The process parameters influencing the enzyme production were identified using Plackett-Burman design. Among the variables screened, the yeast extract, dextrose and pyrogallol were found most considerable. The most favorable levels of these significant parameters were determined employing the Response Surface Methodology and the Central Composite Design. The most important variables were determined as Pyrogallol and dextrose. By using the optimal fermentation medium, the laccase production was improved approximate ten times higher over the previous production with un-optimized medium.

Key Words: *Streptomyces chartreusis, Laccase, Response Surface Methodology (RSM), Solid State Fermentation, Plackett-Burman Design*

INTRODUCTION
Laccases are widely distributed in higher plants, bacteria, fungi, and insects. In plants, laccase are found in cabbages, turnip, potatoes, pears, apples, and other vegetables. In addition to plants, fungi and bacteria, laccases or laccase-like activities have been found in some insects, where they have been suggested to be active in cuticle sclerotization (Dittmer *et al.,* 2004). Laccases are multicopper containing enzymes which reduce molecular oxygen to water and simultaneously perform one electron oxidation of various aromatic substrates like diphenols, methoxy substituted monophenols, and aromatic amines (Thurston, 1994). Laccases have acknowledged a lot of scientific awareness over last couple of decade for their potential to oxidize phenolic and nonphenolic lignin related compounds as well as extremely recalcitrant environmental pollutants. At present laccases are used in pulp delignification, textile dye bleaching, effluent detoxification, washing powder components, exclusion of phenolics from cork stoppers, transformation of antibiotics and steroids, as well as in nanobiotechnology for the development of biosensors to detect various phenolic compounds, oxygen or azides (Chhaya and Gupte , 2010). The claim of laccase in biotechnological processes requires the production of high amount of enzyme at low cost and hence the current focus of research is oriented towards the identification and optimization of such an efficient production system. Solid State Fermentation (SsF) is an important mode of fermentation where microorganisms grow on the substrates in the absence or near-absence of free water and excrete aimed product efficiently (Pandey *et al.,* 2000 and Veronique *et al.,* 2003). In view of the lower energy supplies, simplicity of cultivation and media equipment, high product titres and lower waste water output, SsF method ignites the interest of researchers again in the production of enzymes, fine chemicals, and antibiotics etc (Zubeyde *et al.,* 2003; Kumar *et al.,* 2003 and Adinarayana *et al.,* 2003). There are large numbers of reports on the optimization of carbon and nitrogen source by the classical method of medium optimization that changes one independent variable, while fixing other variables at definite levels. Optimizing all the moving parameters by statistical experimental design, Plackett-Burman and Response Surface Methodology can eliminate the limitations of single-factor optimization process collectively (Gohel *et al.,* 2006; Felse and Panda, 1999 and Joshi *et al.,* 2007). The present work attempts to formulate

a appropriate production medium using statistical optimization that can increase the laccase production from *Streptomyces chartreusis* using Plackett-Burman (Plackett and Burman, 1944) and Central Composite Design.

MATERIALS AND METHODS

Chemicals

2, 2-Azino-bis (3ethylbenzthiozoline-6-sulphonic acid) (ABTS) was purchased from Sigma (St. Louis M.O., U.S.A.). Casein enzyme hydrolysate, Yeast extract powder, Sodium chloride and Dextrose were procured from Hi-Media (Mumbai, India). The o-anisidine and p-anisidine were procured from CDH (Mumbai, India). All other chemicals were of analytical grade procured from Qualigens (Mumbai, India).

Screening and Isolation of Bacterial Strain

The samples of soil with decomposed litter were collected from the region near the forest localities in Thol, Gujarat, India. Soil samples were duly diluted and plated on Bennet's agar medium (Chhaya & Modi, 2013a) and incubated at 30°C. After 2 days of incubation bacterial cultures were transferred on Actinomycete isolation agar (Chhaya and Modi, 2013a) incorporated with o- anisidine (0.01%w/v). Bacterial isolates showing positive Bavendamm's reaction were maintained on Bennet's medium at 30°C and stored at 4°C. The cultures were transferred to fresh media once in a month.

Media Preparation and Inoculation for Ssf Study

Five grams of rice bran in 20 ml basal medium were added to a 250 ml Erlenmeyer flask and was moistened with a salt solution containing (g/1000 ml) yeast extract, 1; $(NH_4)_2SO_4$; 0.2, $MgSO_4$; 0.2, $CaCO_3$; 0.04 and $CuSO_4$; 0.002. The requisite volume of media constituents were pipetted out from their stock solution of higher concentration and were mixed together before sterilization. The o-anisidine and p- anisidine were both filter sterilized and added separately to the medium before inoculation. Rice bran was separately sterilized at 15 psi for 30 minutes and mixed aseptically with the medium before inoculation. The pH of the medium was adjusted to 8.0 and sterilized by autoclaving at 15 psi for 15 minutes. Each flask was inoculated with four mycelial agar plugs of 8 mm in diameter (cut from the edge of an actively growing colony on malt extract agar plates), and incubated under static condition at 30°C for 3 days. The media components for laccase production and their composition are given in Table 1 and 2 as per Plackett-Burman Design.

Enzyme Assay

Laccase activity (E.C.1.10.3.2) (Bourbonnais and Paice, 1990) was measured by monitoring the oxidation of 500µM 2, 2- Azino-bis (3ethylbenzthiozoline-6-sulphonic acid) (ABTS). Boost in absorbance for 2 min was measured spectrophotometrically (Make:-Wensor, Model:-WSP-UV800A) at 420 nm(å $=36000cm^{-1}\ M^{-1}$). The reaction mixture contained 100µl of 50mM ABTS and 800µl of 20mM Sodium Phosphate butter (pH-7.5) and 100µl of appropriately diluted enzyme extract. One unit of enzyme was defined as amount of enzyme that oxidized 1µM of substrate per minute (Chhaya and Gupte, 2010).

Statistical Optimization for Laccase Production by SsF

Identification of nutrient components by Placket and Burman Design

The optimization of medium components for laccase production was accomplished in two stages. The Plackett-Burman design was used to find the nutrient components considerably influencing laccase production by *Streptomyces chartreusis* strain NBRC 12753. Total 12 components (variable k=12) were chosen for the study with each variable being represented at two levels, high (+) and low (-) and three dummy variables in 16 trials as shown in Table 1 and 2. The numbers of positive and negative signs per trial are (k+1)/2 and (k-1)/2 respectively. The effect of each variable was determined by the equation as $E(xi) = 2(\sum Mi^{+} - Mi^{-})/N$, where E (xi) is the concentration effect of the tested variable M_i^{+} and M_i^{-} are the laccase production from the trial examination where the variable (xi) calculated was estimated by the variance among the dummy variables as follows: $Veff = \sum (Ed^2)/n$, where Veff 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 measured using student's t test, t(xi) =Exi/S.E where, Exi is the effect of variable xi.

The selected variables for the present study were carbon sources (dextrose, sucrose); nitrogen sources (yeast extract); inducers (copper sulfate, o- anisidine, p- anisidine and pyrogallol) and process parameters like pH, time course, moisture content, particle size and temperature (Table 1). These twelve variables were chosen based on the earlier experiments (Chhaya and Modi, 2013b) and were evaluated in successive experiments. All experiments were performed in duplicate and the average of laccase activity was taken as response. From Table 3, the variables showing highest positive effect on each category were considered to have greater impact on laccase production and hence selected for further optimization using Central Composite Design of Response Surface Methodology (Palvannan and Sathishkumar, 2010).

Optimization of Screened Components Using Central Composite Design

Response Surface Methodology was used to optimize the screened components for enhanced laccase production using Central Composite Design (CCD). The performance of the method was explained by the quadratic equation as $Y=\beta 0+\Sigma\beta ixi+\Sigma\beta ijxixj+\Sigma\beta iixi$, *Where* Y is predicted response, $\beta 0$ is offset term, βi is linear offset, βii is squared offset and βij is interaction effect. *Xi* is dimensionless coded value of *Xi*. The above equation was solved by using the software Design-Expert (Version 7.0.2, Stat ease inc., USA). A factorial design with a total number of 20 trials was employed. The coded and actual values of the variables at various levels are given in Table 4 (Chhaya and Gupte , 2010).

RESULTS

Screening and Isolation of Bacterial Cultures

Extracellular laccase activity was found in twenty isolates. The isolates showed a brown colored zone surrounding the growth on Actinomycete isolation agar plate containing o-anisidine, which is a characteristic of phenol oxidase production on the solid medium (Chhaya and Modi, 2013a). The culture designated as Strain R1 showing 72U/g of laccase (Chhaya and Modi, 2013b) was found to be the best amongst the 20 isolates tested. The identification of Strain R1 was further corroborated by studies on its partial 16S rRNA gene sequencing carried out by Department of Animal Biotechnology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand, Gujarat, India. The isolate was identified as a *Streptomyces chartreusis* strain NBRC 12753 (Genbank Accession no JQ086575).

Screening of Medium Components by SsF as per Plackett and Burman Design

Streptomyces chartreusis produces 72 U/g of laccase (Chhaya and Modi, 2013b) during primary screening at shake flask level under SsF. To enhance the production of laccase, statistical method of medium optimization suggested by Plackett and Burman was tried. Table 1 represents the independent variables and their respective high and low concentration used in the optimization study, while Table 2 the Plackett-Burman experimental design for 16 trials with two levels of concentrations for each variable and the corresponding laccase production in terms of units per gram of rice bran in SsF. The variables K1 to K12 represent medium components while D1 to D3 represents dummy variables. Table 3 represents the effect, standard error, t (xi), p value and confidence level for each component based on the units per gram of dry substrate of laccase. The components were screened at a confidence level of 95% on the basis of their effects. The confidence level for moisture content, time course, pH, temperature, o- anisidine, p-anisidine, sucrose and $CuSO_4.5H_2O$ were below 95% and hence were considered insignificant, while the remaining components dextrose, pyrogallol and yeast extract showed confidence level at or above 95% and were considered to be significant. Here a positive effect means an increase in the laccase production while negative effect means reduction in the laccase production. As a result the variables pyrogallol, dextrose and yeast extract showed confidence level of 98.4%, 95.5% and 95.1% respectively and were all considered significant. These results indicate the effectiveness of the Plackett-Burman design in

identifying the factors with a significant influence on the laccase production. There after the exact optimal values for the individual factors were determined using Central Composite Design experiments.

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

Variables	Medium components	+ Values (g/l)	- Values (g/l)
K1	CuSO4.5H2O	0.2	0.02
K2	Moisture content	65	50
K3	Time Course	72	24
K4	o-anisidine	0.1	0.01
K5	Particle size	350 usp	250 usp
K6	Sucrose	2	0.2
K7	Temperature	40	30
K8	Pyrogallol[a]	0.5	0.05
K9	Yeast extract	2.0	0.02
K10	Dextrose	5.0	0.5
K11	p-anisidine	0.1	0.01
K12	pH	8.0	7.0

([a] ml/l, usp - unseived particles)

Table-2: Plackett-Burman design matrix of twelve variables (K1-K12) and three dummy variables (D1-D3) along with observed response (Laccase production)

Run No.	K1	K2	K3	K4	K5	K6	K7	K8	K9	K10	K11	K12	D1	D2	D3	U/gm
R1	+	-	+	+	-	+	-	+	-	+	-	-	+	-	+	456
R2	+	+	-	+	+	-	+	-	+	-	+	-	-	+	-	34
R3	-	+	+	-	+	+	-	+	-	+	-	+	-	-	+	121.56
R4	+	-	+	+	-	+	+	-	+	-	+	-	+	-	-	33.45
R5	-	+	-	+	+	-	+	+	-	+	-	+	-	+	-	700
R6	-	-	+	-	+	+	-	+	+	-	+	-	+	-	+	290.77
R7	+	-	-	+	-	+	+	-	+	+	-	+	-	+	-	31.23
R8	-	+	-	-	+	-	+	+	-	+	+	-	+	-	+	56.14
R9	+	-	+	-	-	+	-	+	+	-	+	+	-	+	-	46.78
R10	-	+	-	+	-	-	+	-	+	+	-	+	+	-	+	96.30
R11	+	-	+	-	+	-	-	+	-	+	+	-	+	+	-	90.14
R12	-	+	-	+	-	+	-	-	+	-	+	+	-	+	+	28.15
R13	+	-	+	-	+	-	+	-	-	+	-	+	+	-	+	60.56
R14	+	+	-	+	-	+	-	+	-	-	+	-	+	+	-	98.14
R15	-	+	+	-	+	-	+	-	+	-	-	+	-	+	+	61.27
R16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11.10

Table 3: Statistical analysis of medium components in Relation to Laccase production as per Plackett- Burman design

Factors	Medium components	effect	S.E.	t(xi)	p-value	Confidence level (%)
K1	$CuSO_4.5H_2O$	-64.37	37.95	1.69	0.18	81.2
K2	moisture content	21.94	37.95	0.57	0.60	39.6
K3	Time Course	13.18	37.95	0.34	0.75	24.9
K4	o-anisidine	92.36	37.95	2.43	0.09	90.7
K5	Particle size	76.66	37.95	2.01	0.13	86.3
K6	Sucrose	0.42	37.95	0.01	0.99	0.8
K7	Temp	8.71	37.95	0.22	0.83	16.7
K8	Pyrogallol	187.93	37.95	4.95	0.01	98.4
K9	Yeast extract	121.46	37.95	3.2	0.04	95.1
K10	Dextrose	126.03	37.95	3.32	0.04	95.5
K11	p-anisidine	107.55	37.95	2.83	0.06	93.4
K12	pH	9.51	37.95	0.25	0.81	18.2

Optimization of Screened Medium Components for Laccase Production by Ssf Using Central Composite Design

The variables showing positive effect with a confidence level of 98.4% (pyrogallol), 95.5% (dextrose) and 95.1% (yeast extract) in the Plackett-Burman design were selected. Additional optimization was achieved using a central composite design; Contour graphs (Figure: 1, 2, 3) and 3-D surface plots (Figure: 4, 5, 6) were obtained and analyzed based on feeding data on the laccase production in to the Design-Expert® software. The software allows the laccase production to be predicted within the studied range for the all three components of medium. Here each 3- D surface plot represents the effect of three medium.

Components at their studied concentration range. Based on the results of Plackett-Burman Design the component with a significant confidence level (dextrose, pyrogallol and yeast extract) were set at their higher level, while the components with a confidence level below 95% were set at their middle level. Table 4 represents the experimental design for Central Composite Design and the result obtained for laccase production. The variables used for factorial analysis were dextrose, pyrogallol and yeast extract for laccase production. The actual and coded factor levels of laccase production are presented in Table 4. The data were analyzed by a quadratic multiple regression using a Design- Expert® software (version 7.0.2, Stat ease Inc., USA) and the following equation was obtained.

$$Y = 234.30-65.24A-19.21B+33.27C+91.13AB-68.38AC-23.37BC+77.49A^2-35.12B^2-26.99C^2 \ldots (1)$$

Here Y is the predicted response and A, B, C, are the coded variables for dextrose, pyrogallol and yeast extract respectively. To validate the Regression coefficient, an analysis of variance (ANNOVA) of the laccase production was performed (Table-5). The values of the lack of fit, model F and model P>F were found to be 0.9620, 21.99 and <0.0001 respectively indicating that model was significant. The values of the adjusted determination coefficient (Adj R^2=0.9519) was also very high reconfirming the significance of the model. The lack of fit (0.9620) is found to be not significant. This indicates an excellent correlation between the experimental and predicted values of laccase production. At the same time relatively low coefficient variation (CV=16.26%) (Table 5) confirms the precision and reliability of the experiment performed. Figure 7 represents the relationship between the actual laccase production and predicted values determined by the model equation (1) for *Streptomyces chartreusis* .Clearly most of the points were near by the line adjustment which meant that the experimentally determined values were similar to those determined by the model. The model predicted that the maximum production of laccase using above optimum concentration of variables would be 439.12 U/g of Rice bran. To verify this, prediction experiments were carried out using optimized medium and the result showed a higher yield of laccase production i.e. 410 U/g which was 10 times higher than non optimized medium.

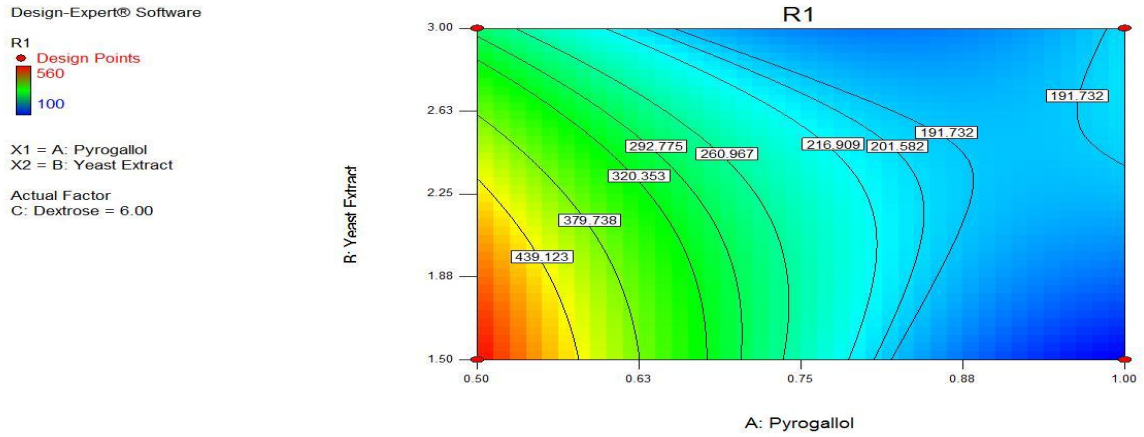

Figure: 1 Three dimensional Contour graph effect of Pyrogallol and Yeast extract at 6.0 (g/l) of Dextrose

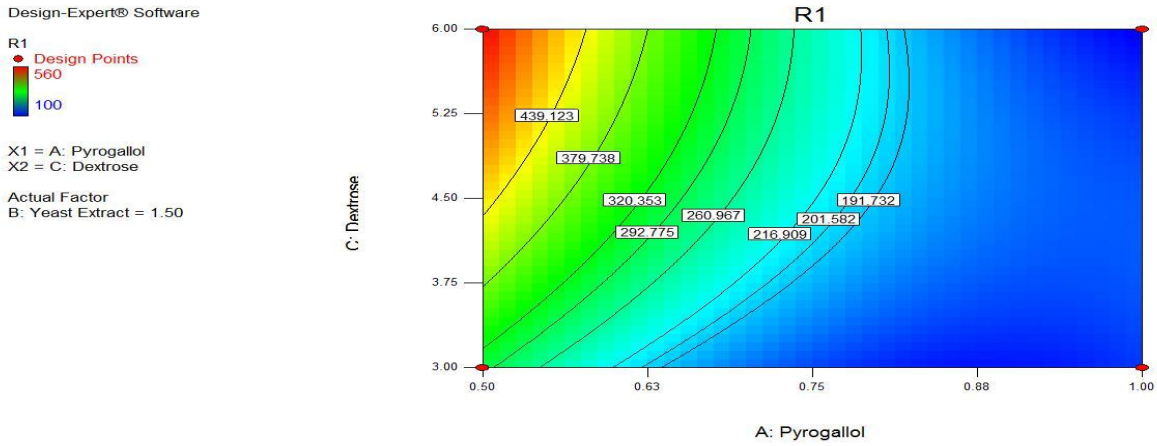

Figure 2: Three dimensional Contour graph effect of Pyrogallol and Dextrose at 1.50 (g/l) of Yeast extract

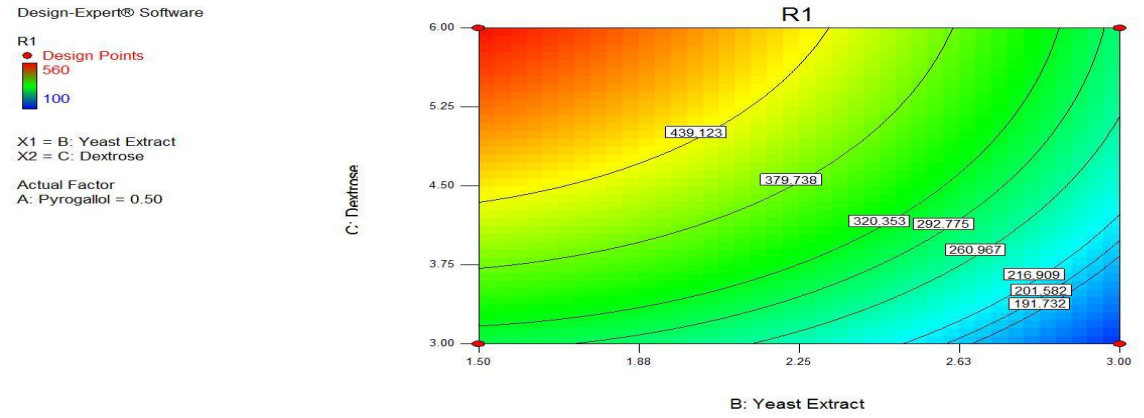

Figure 3: Three dimensional Contour graph effects of Yeast extract and Dextrose at 0.50 (ml/l) of Pyrogallol.

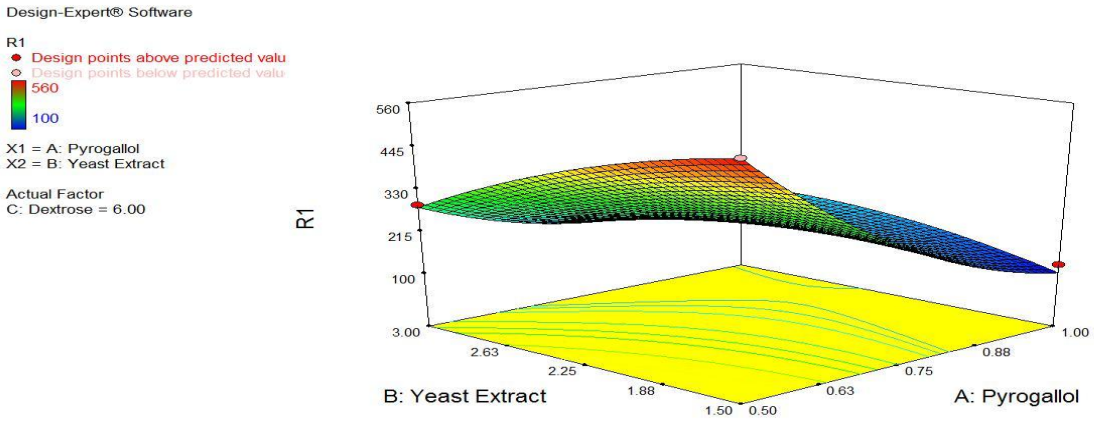

Figure 4: 3D Surface Plot effect of Pyrogallol and Yeast extract at 6.0 (g/l) of Dextrose

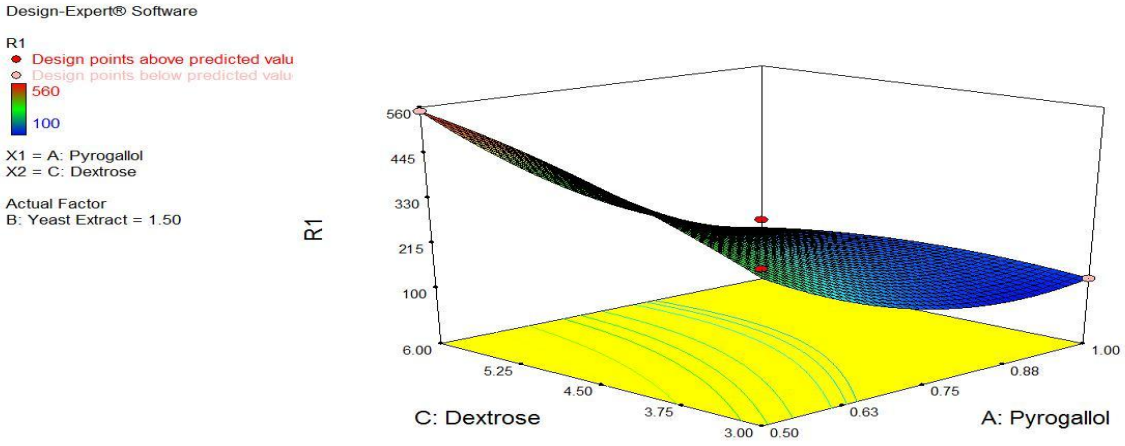

Figure 5: 3D Surface Plot effect of Pyrogallol and Dextrose at 1.50 (g/l) of Yeast extract

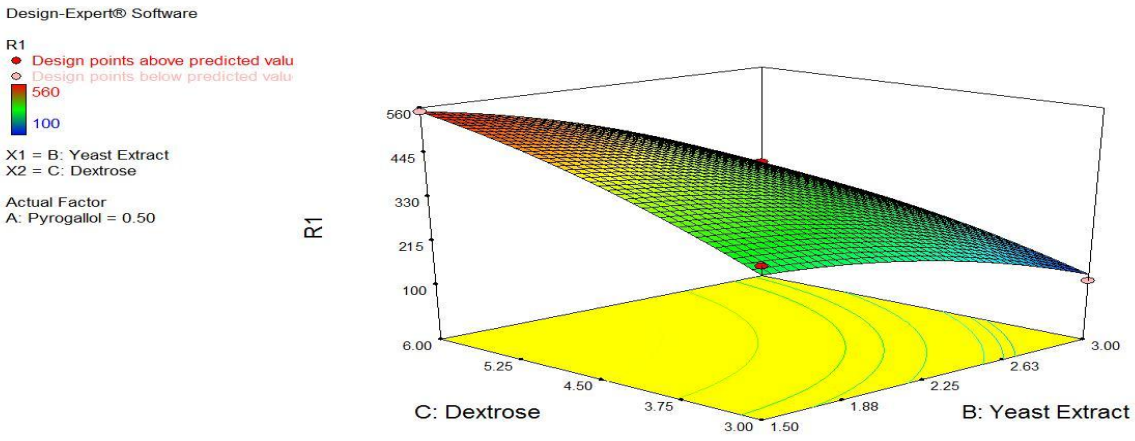

Figure 6: 3D Surface Plot effect of Yeast extract and Dextrose at 0.50 (ml/l) of Pyrogallol

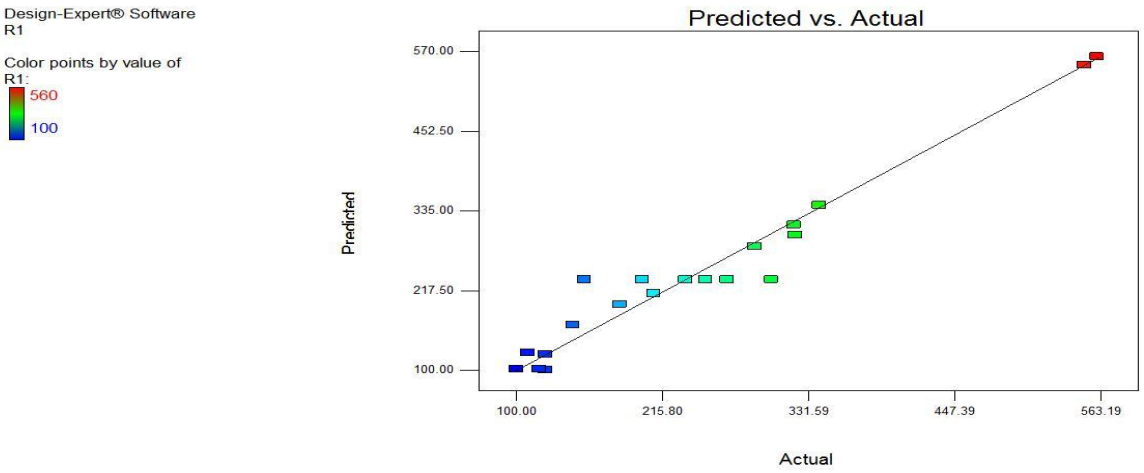

Figure: 7 Predicted v/s Actual Laccase productions from *Streptomyces chartreusis strain NBKC 12753.*

Table-4: Central Composite Design matrix with coded values and actual values for Laccase production.

Run No.	Pyrogallol		Yeast extract		Dextrose		Laccase in U/g	
	Coded value	Actual value	Coded value	Actual value	Coded value	Actual value	Actual value	Predicted value
1	+ 2	1.17	0	2.25	0	4.50	321.00	300.24
2	0	0.75	0	2.25	-2	1.98	123.00	124.26
3	- 2	0.33	0	2.25	0	4.50	109.00	126.31
4	0	0.75	+2	3.51	0	4.50	320.00	314.83
5	- 1	0.50	-1	1.50	+1	6.00	550.00	550.27
6	0	0.75	0	2.25	+2	7.02	123.00	100.79
7	+1	1.00	+1	3.00	-1	3.00	289.00	282.84
8	0	0.75	-2	0.99	0	4.50	182.00	197.86
9	0	0.75	0	2.25	0	4.50	560.00	563.19
10	-1	0.50	+1	3.00	+1	6.00	340.00	343.74
11	+1	1.00	-1	1.50	-1	3.00	145.00	167.28
12	0	0.75	0	2.25	0	4.50	118.00	102.65
13	+1	1.00	-1	1.50	+1	6.00	100.00	102.02
14	0	0.75	0	2.25	0	4.50	209.00	213.91
15	-1	0.50	-1	1.50	-1	3.00	302.00	234.30
16	+1	1.00	+1	3.00	+1	6.00	267.00	234.30
17	0	0.75	0	2.25	0	4.50	250.00	234.30
18	0	0.75	0	2.25	0	4.50	154.00	234.30
19	0	0.75	0	2.25	0	4.50	234.00	234.30
20	-1	0.50	+1	3.00	-1	3.00	200.00	234.30

Table 5: Analysis of variance (ANNOVA) for the quadratic model

Sum of Source	Squares	Mean df	F Square	p-value	Prob > F
Model	3.136E+005	9	34847.71	21.99	< 0.0001
A-Pyrogallol	58129.86	1	58129.86	36.68	0.0001
B-Yeast Extract	5042.02	1	5042.02	3.18	0.1048
C-Dextrose	15113.47	1	15113.47	9.54	0.0115
AB	*66430.13*	1	*66430.13*	*41.91*	*< 0.0001*
AC	*37401.12*	1	*37401.12*	*23.60*	*0.0007*
BC	*4371.12*	1	*4371.12*	*2.76*	*0.1278*
A^2	*86524.95*	1	*86524.95*	*54.59*	*< 0.0001*
B^2	*17776.58*	1	*17776.58*	*11.22*	*0.0074*
C^2	*10497.85*	1	*10497.85*	*6.62*	*0.0277*
Residual	15849.83	10	1584.98		
Lack of Fit	*2326.33*	5	*465.27*	*0.17*	*0.9620*
Pure Error	*13523.50*	5	*2704.70*		
Cor Total	3.295E+005	19			

Where,

Sum of Source	Value	Sum of Source	Value
Std. Dev.	39.81	R-Squared	0.9519
Mean	244.80	Adj R-Squared	0.9086
C.V. %	16.26	Pred R-Squared	0.8826
PRESS	38682.80	Adeq Precision	16.425

Conclusion

Different statistical methods for medium optimization have been employed to improve laccase production by Streptomyces chartreusis. The medium for laccase production was optimized by Response Surface Methodology using Central Composite Design, and a 10 times increase (410 U/g) in the laccase activity was achieved. The methodology of Plackett-Burman was found to be very useful in determining the relevant variables for further optimization making it possible to consider large number of variables and avoid information loss, both of which are essential in the optimization process. As a result the important medium components with a significant effect on laccase production by Streptomyces chartreusis strain NBRC 12753 were identified. Notably p-value of yeast extract was found to be less effective during ANNOVA for Response surface methodology. In conclusion the methodology of Plackett-Burman and Central Composite Design proved to be very effective in improving laccase production.

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