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APPLICATION OF RESPONSE SURFACE METHODOLOGY (RSM) – CCD FOR THE PRODUCTION OF LACCASES USING SUBMERGED FERMENTATION

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ABSTRACT

Laccases are multi-copper oxidases widely distributed in plants, animals, insects and microorganism. These enzymes catalyze the one-electron oxidation of a wide range of organic and inorganic substances, including mono, di and poly phenols, aminophenols, methoxy phenols, aromatic amines and ascobate with the concomitant four electron reduction of oxygen to water. Laccase has the focus of much attention because of its diverse applications, such as delignification of lignocellulosics, cross linking of polysaccharides, bioremediation application etc. The main objective of the paper is to optimize the condition for maximum production of laccase enzyme produced from bacteria – *Pseudomonas aeruginosa* using Response surface methodology. Laccases activity was estimated by Bourbonnais and Paice method and the temperature, pH were optimized separately, the design of experiment for nutritional requirement was designed to 50 run and the activity level were estimated using Response surface methodology. Models were developed by Central composite design (CCD) with the selected parameters. The regression analysis (R2) of RSM showed 97%. The development of rapid and efficient method for media preparation was established using RSM-CCD.

KEYWORDS: Laccase, *Pseudomonas aeruginosa,* RSM-CCD

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INTRODUCTION

Earlier from 19th century Laccases (E.C.1.10.3.2) are multi copper enzyme belongs to the group oxidases were studied due to their wide applications in oxidation of phenolic compounds and degradation of synthetic dyes[1]. Laccases are widely distributed in both prokaryotes and eukaryotes (mainly from fungi) organisms and it is mostly secreted as extra cellular enzyme. The first discovery of laccase was in Japanese lacquer tree *Rhus vernicifera*[2,3]. Even though there are significance production of laccase from fungi bacterial laccase are also contribute preponderantly in the industrial scale. Production of laccase were reported in few bacteria like *Azospirillum lipoferum*[4], *Bacillus subtilis*[5], and in some species of Actinomyctes such as *S.cyaneus*[6], *S.lavendulae*[7] and *S.coelicolor*[8]. *Ps.putida, Ps.desmolyticum* NCIM 2112, *Ps.syringae, Ps.aeruginosa*[23] were also reported to have laccase production. It was also reported that laccase isolated from White rot fungi has anticancer activity[9]. The productivity of the enzyme laccase was enhanced in the precise composition of the media. Hence by using Response surface methodology (RSM) optimization technique media component were standardized for a maximum production of enzyme laccase. RSM is a statistical technique with empirical model building useful for developing, improving and optimizing the response variable. So based on these recent modelling techniques in response surface methodology, an experimental design - Central composite design CCD used for building second order quadratic mode was used to analyse the composition of media. Other functions of laccase include the cleaning the industrial effluents, mostly from industries like paper industry, pulp, textile & petrochemical industries. Laccase are also used in the medical diagnostics and for cleaning herbicides, pesticides and some explosives in soil. Laccase has many applications in agricultural, medicinal and industrial areas[11]. One of application of Laccases is to clean the water in many purification systems. It has also applications in medical side to prepare certain drugs like anticancer drugs and it is added in cosmetics to minimize their toxic effects. Laccase has the enormous ability to remove xenobiotic substances and produce polymeric products and that is why they are being used for many bioremediation purposes[12].

MATERIALS AND METHODS

(1) Soil samples

Soil samples were collected from 5 different locations in the Harur forest (latitudes N 11 47' and 12 33' and longitudes E 77 02' and 78 40'), in the district of Dharmapuri District, Tamilnadu. Soil samples were taken from approximately 1 dm depth.

(2) Isolation and identification of microorganisms

1 g of each soil sample was suspended in 10 mL physiological saline. 1 mL 10^{-2} or 10^{-3} dilutions of soil suspension of the 5 different soil samples were placed on different lignin agar plates. The plates were incubated for 2 to 3 days at room temperature until colonies appeared. Strains were purified by reinoculation for 3 times onto new plates. The strains were considered pure on the basis of the colony morphology.

(3) Characterization

Identification of microorganism was done by performing scheduled biochemical tests and 16S rRNA sequencing[13]. 16S rRNA sequencing was done by isolation of DNA from the organism and the large fragment of the 16S rRNA gene was amplified by PCR using the universal primers BAC-F-(5'-AGA GTT TGA TC(AC) TGG CTC AG-3') BAC-R (5'AAG GAG GTG (AT)TC CA(AG) CC-3'). The PCR products were purified using a Wizard PCR Preps DNA Purification System according to the manufacturer's instructions. The PCR product after purification is sequenced using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and Automatic sequencer. The neighbouring known relatives of the freshly isolates were determined by performing a sequence database search. The sequences of closely associated strains were reclaimed from GENBANK and the Ribosomal Database Project (RDP) libraries.

(4) Nutritional requirement

After incubation, 0.5 ml of the supernatant was inoculated into minimal media containing KH_2PO_4 (3 g/L), Na_2HPO_4 (6 g/L), NH_4Cl (2 g/L), NaCl (5 g/L) and Mg SO_4 (1g/L). After 2 days of cultivation at 37°C, 0.5 ml of the culture broth was diluted with 50 ml of minimal media (100- fold dilution). These procedures were repeated for three times in order to stimulate an enrichment culture. After the enrichment steps, the culture broth was spread out on minimal media solidified with 2% agar. This strain was stored in minimal media with 25% glycerol at -70°C. The isolated culture was subjected for further study.

(5) Enzyme extraction

The crude laccase was extracted by mixing 10g of fermented materials with distilled water, stirred for 30 minutes in the shaker, filtered and then centrifuged for 30 minutes. The supernatant was used as the crude enzyme and then studied for enzymatic measurements by appropriate method[14].

(6) Laccase assay

Laccase activity was determined by the oxidation of ABTS method[15]. The nonphenolic dye ABTS (2, 20-azino-bis- [3 – ethyl benzothiazoline – 6 –sulphonic acid]) (Sigma) is oxidized by laccase to the more stable and preferred state of the cation radical. The concentration of the cation radical responsible for the intense blue-green color can be correlated to enzyme activity and is read at 420 nm[16]. The assay mixture contained 0.5 mM ABTS, 0.1 M sodium acetate (pH 4.5), and a suitable amount of enzyme. Oxidation of ABTS was monitored by determining the increase in A420 (ε420, 3.6×10^4 $M^{-1}cm^{-1}$). The reaction mixture contained 0.5 mM substrate (ABTS), 2.8 mL of 0.1 M sodium acetate buffer of pH 4.5, and 100 µL of culture supernatant and incubated for 5 min. Absorbance was read at 420 nm in a spectrophotometer against a suitable blank. One unit was defined as the amount of the laccase that oxidized 1 µmol of ABTS substrate per min. The absorbance was read after 10 min interval using UV/VIS spectrophotometer (Varian Cary® 100 UV-Vis).Protein concentration was determined by the Lowy method using BSA as standard[17].

(7) Calculations and statistics

A statistically significant difference between means was determined according to Student's t-test at a probability level of 0.05. The statistical analyses were performed by SPSS Inc., 2014.

(8) Optimization of laccase Production

(i) Effect of incubation time and carbon source on laccase[12].

Equal volume of the bacterial isolate was inoculated in minimal media containing either any one of the following carbohydrate i.e. Glucose, lactose. This was incubated at different time of incubation viz., 12, 24, 36, 48, 60, 72, 84 and 96. The laccase production was estimated after incubation.

(ii) Effect of initial pH on laccase production[12]

Equal volume of the bacterial isolate was inoculated in minimal media with various initial pH viz., 3, 4, 5, 6, 7, 8, 9 and 10. The flasks were incubated at 30°C for 60 h. The laccase production was estimated after incubation. The initial pH at which maximum production of laccase was observed was chosen and maintained in the following studies.

(iii) Effect of temperature on laccase production[12]

Bacterial isolate was inoculated into minimal media and incubated at different temperature viz., 26, 28, 30, 32, 34, 36, 38 and 40°C for 72 h. The laccase was estimated after incubation.

(9) RSM – CCD

Statistical experimental designs are powerful tools for searching the key factors rapidly from a multivariable system. Plackett – Burman design[18] 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. Optimization of media components for the production of laccase by response surface methodology has been reported in the case of different fungal strains [20, 21, 22]. Response Surface Methodology is a statistical

experimental technique applied under appropriate experimental design to resolve multi-variable equations[18]. Statistical analysis RSM was used to investigate the main effects of dependable variables on the production of laccase by the organism. Glucose (A), K_2HPO_4 (B), KH_2PO_4(C), $MgSO_4$(D), and NaCl (E) were selected as independent variables. Central composite design (CCD) was used for the experimental data and data were fitted to a second order polynomial model and regression coefficients obtained. The data were subjected to Analysis of Variance (ANOVA) and 3D response surface graphs were constructed using Design expert, version 7 (Stat-ease) programs to study the responses. Regression analysis from many model specify that has more than 95% of variation in the data can be considered as best fit quadratic model which is considered significant. The ANOVA and F ratio test have been performed to justify the goodness of fit of the developed mathematical models. The calculated values of F ratios for lack-of-fit have been compared to standard values of F ratios corresponding to their degrees of freedom to find the adequacy of the developed mathematical models.

RESULTS AND DISCUSSION

The soil samples collected from different parts of Dharmapuri, Tamilnadu were subjected for serial dilution and grown on Plate Count Agar (PCA), after incubation these organisms were purified and screened for the ability of producing laccase. The isolated organism *Pseudomonas aeruginosa* was proved to have maximum ability of producing laccase was confirmed by conventional method and further by 16SrDNA sequencing and the sequence was submitted in GENBANK.

Effect of the pH, Temperature and incubation time in the production of laccase enzyme

The organism was found to produce more laccase at 30ºC at pH 6 with incubation time of 60 hours and it shows more activity on Glucose containing medium.

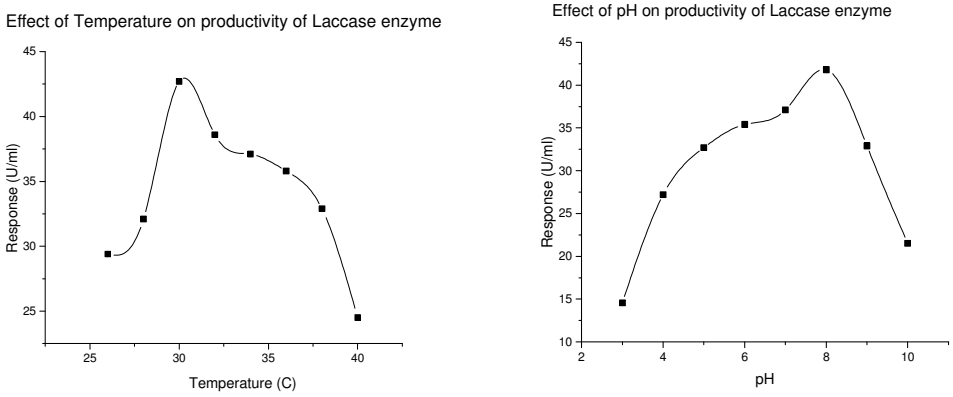

Figure 1
Influence of temperature and pH on production of Laccase.

From the above data, temperature and pH for the Response surface methodology was confirmed and the media components pooled according to the DOE.

Table 1
Experimental variables in coded and actual units

Independent variable	Coded unit		
	-1	0	1
K_2HPO_4	0.2	0.9	1.6
$NaNO_3$	0.5	1	1.5
$MgSO_4$	0.2	0.4	0.5
NH_4Cl	0.5	1.25	2
Glucose	1	2	3

Table 2
Design of experiment (DOE) for optimization of Laccase production using Pseudomonas aeruginosa by RSM – CCD

Std	Run	Factor 1 A:K_2HPO_4	Factor 2 B:$NaNO_3$	Factor 3 C:$MgSO_4$	Factor 4 D:NH_4Cl	Factor 5 E:Glucose	Response (R) IU/ml
16	1	(1) 1.6	(1) 1.5	(1) 0.4	(1) 2	(-1) 1	31.8794
42	2	(0) 0.9	(0) 1	(0) 0.3	(0) 1.25	4.38	32.0058
20	3	(1) 1.6	(1) 1.5	(-1) 0.2	(-1) 0.5	(1) 3	31.7831
3	4	(-1) 0.2	(1) 1.5	(-1) 0.2	(-1) 0.5	(-1) 1	28.9986
12	5	(1) 1.6	(1) 1.5	(-1) 0.2	(1) 2	(-1) 1	30.229
7	6	(-1) 0.2	(1) 1.5	(1) 0.4	(-1) 0.5	(-1) 1	35.649
48	7	(0) 0.9	(0) 1	(0) 0.3	(0) 1.25	(0) 2	46.7403
29	8	(-1) 0.2	(-1) 0.5	(1) 0.4	(1) 2	(1) 3	23.3791
6	9	(1) 1.6	(-1) 0.5	(1) 0.4	(-1) 0.5	(-1) 1	31.4406
30	10	(1) 1.6	(-1) 0.5	(1) 0.4	(1) 2	(1) 3	24.8212
5	11	(-1) 0.2	(-1) 0.5	(1) 0.4	(-1) 0.5	(-1) 1	30.4985
24	12	(1) 1.6	(1) 1.5	(1) 0.4	(-1) 0.5	(1) 3	33.9335
36	13	(0) 0.9	2.19	(0) 0.3	(0) 1.25	(0) 2	29.1967
44	14	(0) 0.9	(0) 1	(0) 0.3	(0) 1.25	(0) 2	46.7403
2	15	(1) 1.6	(-1) 0.5	(-1) 0.2	(-1) 0.5	(-1) 1	37.0403
1	16	(-1) 0.2	(-1) 0.5	(-1) 0.2	(-1) 0.5	(-1) 1	28.5981
26	17	(1) 1.6	(-1) 0.5	(-1) 0.2	(1) 2	(1) 3	32.9209
31	18	(-1) 0.2	(1) 1.5	(1) 0.4	(1) 2	(1) 3	37.0296
47	19	(0) 0.9	(0) 1	(0) 0.3	(0) 1.25	(0) 2	46.7403
41	20	(0) 0.9	(0) 1	(0) 0.3	(0) 1.25	-0.38	31.7862
13	21	(-1) 0.2	(-1) 0.5	(1) 0.4	(1) 2	(-1) 1	28.0367
18	22	(1) 1.6	(-1) 0.5	(-1) 0.2	(-1) 0.5	(1) 3	35.8826
33	23	-0.76	(0) 1	(0) 0.3	(0) 1.25	(0) 2	24.6675
11	24	(-1) 0.2	(1) 1.5	(-1) 0.2	(1) 2	(-1) 1	32.5369
15	25	(-1) 0.2	(1) 1.5	(1) 0.4	(1) 2	(-1) 1	37.6873
40	26	(0) 0.9	(0) 1	(0) 0.3	3.03	(0) 2	30.3469
4	27	(1) 1.6	(1) 1.5	(-1) 0.2	(-1) 0.5	(-1) 1	16.9408
32	28	(1) 1.6	(1) 1.5	(1) 0.4	(1) 2	(1) 3	32.9718
39	29	(0) 0.9	(0) 1	(0) 0.3	-0.53	(0) 2	31.4451
38	30	(0) 0.9	(0) 1	0.54	(0) 1.25	(0) 2	33.1288
27	31	(-1) 0.2	(1) 1.5	(-1) 0.2	(1) 2	(1) 3	33.8792
9	32	(-1) 0.2	(-1) 0.5	(-1) 0.2	(1) 2	(-1) 1	30.6364
50	33	(0) 0.9	(0) 1	(0) 0.3	(0) 1.25	(0) 2	46.7403
10	34	(1) 1.6	(-1) 0.5	(-1) 0.2	(1) 2	(-1) 1	33.8285
8	35	(1) 1.6	(1) 1.5	(1) 0.4	(-1) 0.5	(-1) 1	31.0912
28	36	(1) 1.6	(1) 1.5	(-1) 0.2	(1) 2	(1) 3	33.3214
22	37	(1) 1.6	(-1) 0.5	(1) 0.4	(-1) 0.5	(1) 3	30.283
49	38	(0) 0.9	(0) 1	(0) 0.3	(0) 1.25	(0) 2	46.7403
23	39	(-1) 0.2	(1) 1.5	(1) 0.4	(-1) 0.5	(1) 3	36.7414
14	40	(1) 1.6	0.5	(1) 0.4	(1) 2	(-1) 1	27.7289
19	41	(-1) 0.2	(1) 1.5	(-1) 0.2	(-1) 0.5	(1) 3	31.091
45	42	(0) 0.9	(0) 1	(0) 0.3	(0) 1.25	(0) 2	46.7403
34	43	(-1) 2.56	(0) 1	(0) 0.3	(0) 1.25	(0) 2	25.1245
46	44	(0) 0.9	(0) 1	(0) 0.3	(0) 1.25	(0) 2	46.7403
25	45	(-1) 0.2	(-1) 0.5	0.2	(1) 2	(1) 3	27.9787

35	46	(0) 0.9	-0.19	(0) 0.3	(0) 1.25	(0) 2	22.5953
17	47	(-1) 0.2	(-1) 0.5	(-1) 0.2	(-1) 0.5	(1) 3	29.6905
43	48	(0) 0.9	(0) 1	(1) 0.3	(0) 1.25	(0) 2	46.7403
21	49	(-1) 0.2	(-1) 0.5	(1) 0.4	(-1) 0.5	(1) 3	27.5908
37	50	(0) 0.9	(0) 1	0.06	(0) 1.25	(0) 2	33.6631

The combination of K_2HPO_4 – 0.9 gm/l, $NaNO_3$-1 gm/l, $MgSO_4$-0.3 gm/l, NH_4Cl- 1.25 gm/l and Glucose -2 gm/l shows maximum influence on the production of laccase enzyme. According to Niladevi *et al.* (2009) work 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[24]. Incubation temperature, incubation period, agitation rate, concentrations of yeast extract, $MgSO_4.7H_2O$, and trace elements were found to influence laccase production significantly.

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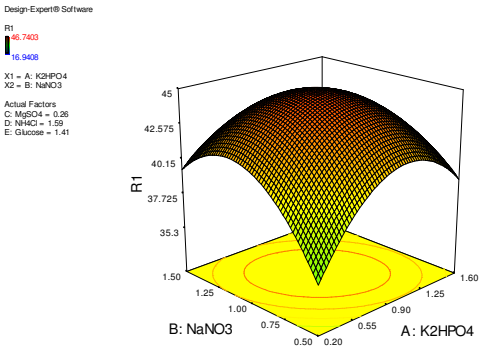
Table 3
Test of significance for regression coefficient

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	2394.02	15	159.6	71.93	< 0.0001	Significant
B-$NaNO_3$	60.31	1	60.31	27.18	< 0.0001	
AB	108.78	1	108.78	49.03	< 0.0001	
AC	13.78	1	13.78	6.21	0.0177	
AE	13.78	1	13.78	6.21	0.0177	
BC	157.53	1	157.53	71	< 0.0001	
BD	63.28	1	63.28	28.52	< 0.0001	
BE	52.53	1	52.53	23.68	< 0.0001	
CD	26.28	1	26.28	11.85	0.0015	
CE	19.53	1	19.53	8.8	0.0055	
DE	16.53	1	16.53	7.45	0.01	
A^2	842.88	1	842.88	379.9	< 0.0001	
B^2	768.1	1	768.1	346.19	< 0.0001	
C^2	317.96	1	317.96	143.31	< 0.0001	
D^2	446.3	1	446.3	201.15	< 0.0001	
E^2	392.36	1	392.36	176.84	< 0.0001	
Residual	75.44	34	2.22			
Lack of Fit	75.44	27	2.79			Not significant
Pure Error	12.58	7	1.29			
Cor Total	2469.45	49				

Table 4
Analysis of variance (ANOVA) for regression

Std. Dev.	1.49	R-Squared	0.9695
Mean	33.28	Adj R-Squared	0.956
C.V. %	4.48	Pred R-Squared	0.9314
PRESS	169.29	Adeq Precision	30.657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16.9408
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E: Glucose = 1.41
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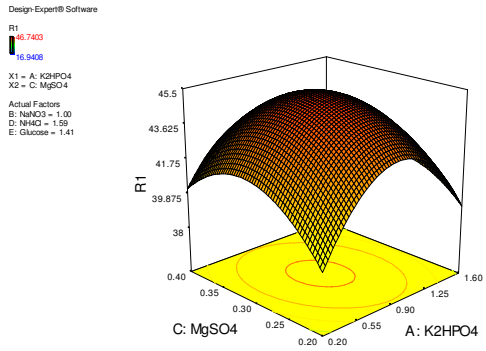
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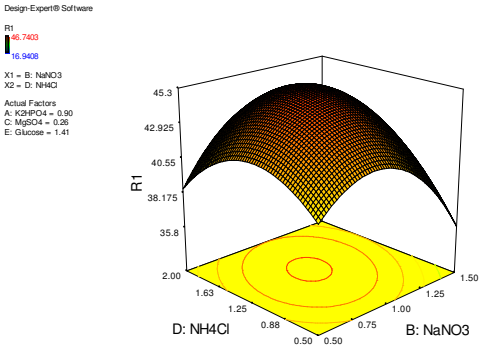
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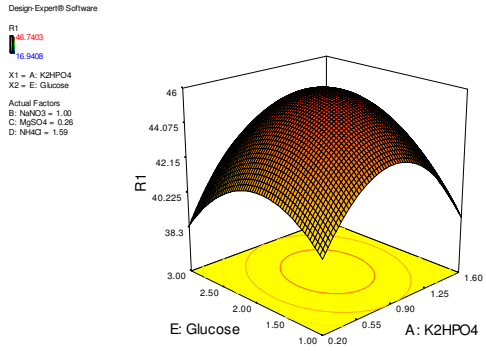
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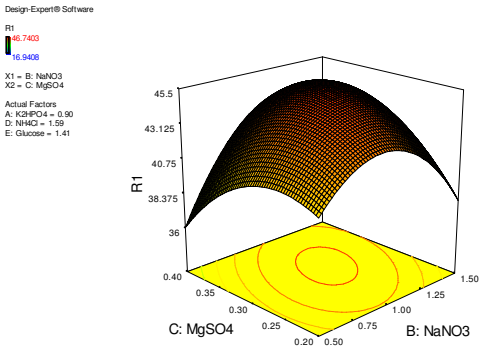
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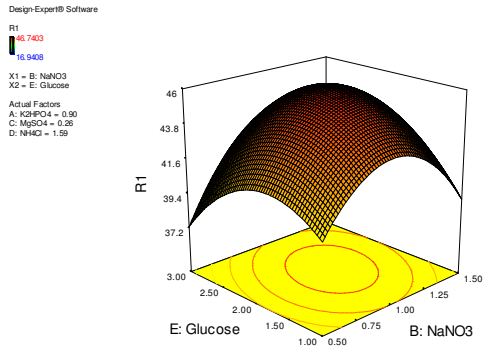
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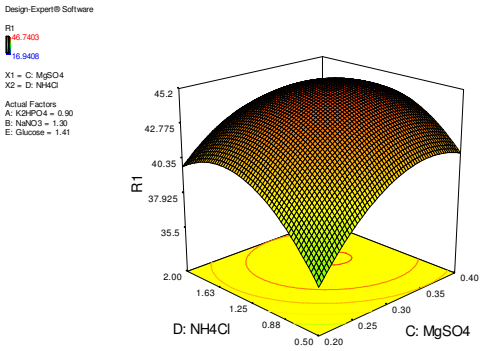
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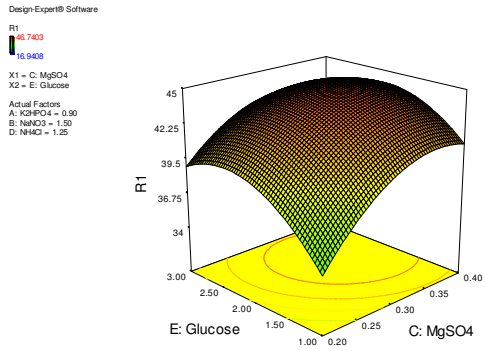
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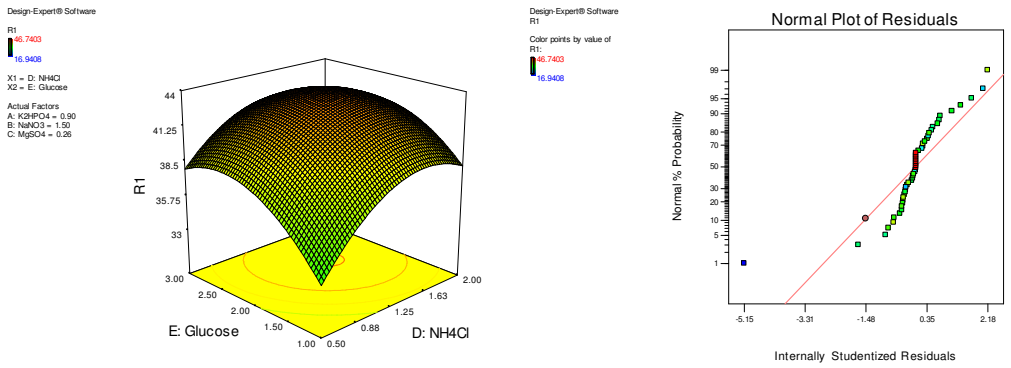

Figure 2

Surface and contour plot for the response of laccase production with respect to the interaction of A:K_2HPO_4, B:$NaNO_3$, C:$MgSO_4$, D:NH4Cl, and E:Glucose.

The data obtained from RSM on laccase production were subjected to the analysis of variance (ANOVA). The results of RSM were used to fit a second-order polynomial equation that represents the behavior of the system R1 =46.7 + 1.18B - 1.84A B - 0.66AC + 0.66AE +2.22BC + 1.41BD +1.28B E - 0.91CD - 0.78CE- 0.72DE - 3.89A^2 -3.72B^2 -2.39 C^2 - 2.83D^2 -2.66E^2 ANOVA results of these quadratic models are represented in Tables 3 and 4, indicating that these models can be used to navigate the design space. Fig. 2 showed that the experimental value for enzyme activity in response. Thus it was inferred that the experimental values were nearly significant with those predicted values. The t and P-values were employed to evaluate the significance of each coefficient. Larger t-test value and smaller the P-value suggests higher significance of the corresponding coefficient. The coefficient of determination (R square) was 0.969 indicating the response model could explain 0.9314 of total variation. Value of R (96.95) indicated high agreement between the experimental and predicated value. High value of adjusted determination coefficient (R^2_{ad} = 0.956) indicated the significance of model. The result of ANOVA as shown in Table4 indicates the predictability of the model was at 99% confidence interval. A pa value less that 0.0001 indicates the model was statistically significant. P value lower that 0.05 indicates that the mode was statistically significant. This shows a significant increase of the laccase production. The enzyme yield was enhanced further by response surface methodology and the model validation performed with RSM suggested that the model was valid with good reproducibility of the results. On the whole, there was increase in laccase yield was attained after statistical optimization. The study has confirmed the aptness of using statistical methods for enhancing laccase production by this strain.

CONCLUSION

Response surface methodology (RSM) was applied successfully for the optimization of operational conditions for laccase production. All five nutritional parameters, KH_2PO_4, Na_2HPO_4, NH_4Cl, NaCl and Mg SO_4 had a significant effect on laccase production. From the present study, it was observed that use of statistical optimization approach (RSM), had helped to identify the most significant operating factors and optimum levels for laccase production with minimum effort by *Pseudomonas aeruginosa* ADN04.

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