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Optimization of laccase production media by *Bacilllus cereus* TSS1 using Box-Behnken design

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ABSTRACT

A *Bacilllus cereus* TSS1isolated from textile industry effluent was used for the laccase production. The seven selected media components were optimized at three different levels using Box-Behnken design. The media components that were optimized for the laccase production were magnesium sulphate (1.50 mM), yeast extract (0.3g/100 ml), ammonium sulphate (1 mM), agitation(144 rpm), incubation hour (48 hr), inoculum size (1 %) and sodium chloride (0.9g /100 ml). The optimized media resulted in four fold increase in laccase production compared to the unoptimized media.

Keywords: Laccase, Response surface methodology, Box-Behnken, *Bacillus cereus*.

1. INTRODUCTION

Laccase enzyme typically contains 15–30% carbohydrate. It has an acidic isoelectric point and has a molecule mass of 60–90 kDa. Laccases are the model enzymes for multi-copper oxidases and participate in cross-linking of monomers, degradation of polymers, and ring cleavage of aromatic compounds. For catalyzing the oxidation of non-phenolic substrates, laccase requires the presence of a mediator in the medium. A mediator is a small molecule that behaves like an 'electron shuttle' between laccase and substrate and these small molecular-mass compounds are converted into stable radicals by means of enzymatic oxidation. [1,2]

Laccase has broadsubstrate specificity towards aromatic compounds containing hydroxyl and amine groups. These enzymes were known to catalyze the oxidation of a wide range of phenolic compounds and aromatic amines. Laccases have attracted increasing scientific attention in the recent years due to their application in diverse industrial sectors such as food, cosmetics, textile, paper, decolorize dyes, beverage production industries and also they have a role in bioremediation of contaminated soil. [3]

The first report on bacterial laccase came on the year 2000[4], however the studies on bacterial enzyme is very negligible compared to fungal laccase. Most of the bacterial laccases studied were spore coat protein [5] and only few are extracellular laccase. [6,7] For the application of laccase in industrial process large amount of enzyme is required. The synthesis of laccase is influenced by the culture conditions, type and concentration of nutrients. [8]

Niladevi *et al*. (2009) [9] reported that laccases expression in *Streptomycetes* is influenced by culture conditions, media composition, pH, temperature and presence of inducers. 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. [10] Optimizing all parameters by statistical experimental design, Response Surface Methodology can eliminate the limitations of single-factor optimization process collectively. [11] Hence the present study aimed at optimizing the culture conditions for laccase production by the isolated *Bacillus cereus* TSS1 using Box-Behnken design.

2. MATERIALS AND METHOD

2.1. Culture maintenance and production of inoculum

The bacterial strain was isolated from textile industry effluent and it was identified by biochemical test as *Bacillus cereus* and designated as *Bacillus cereus* TSS1. The stock culture was maintained on nutrient agar slants at 4°C with

periodic transfer. For production of inoculum, a loopful of bacterial culture was inoculated into 5 ml of Luria-Bertani (LB) broth and incubated at 37^0C, 100 rpm for overnight. 5 ml of overnight grown culture was added to 25 ml of LB broth incubated at 37^0C, for 12- 16 hr. From this 1 ml was inoculated into Minimal Salt Medium. The Composition of Minimal Salt Medium (g/L) is Yeast extract – 5g; Glucose – 1g; Magnesium sulphate – 1.5 mM; K_2HPO_4 – 0.8g; KH_2PO_4 – 0.4g, Sodium chloride – 3g, 1mM Copper sulphate, Ammonium sulphate – 0.01 g.

2.2. Optimization by response surface methodology

The response surface methodology using Box-Behnken design was used to determine the optimum concentration of the selected variables. The variables selected were magnesium sulphate, yeas extract, ammonium sulphate, sodium chloride, inoculum size, incubation period and agitation. A total of 62 experiments were formulated using the statistical software package 'minitab 15.0'. Each selected variable was analyzed at three levels namely low, medium and high coded as -1, 0 and +1 in total of 62 runs (Table 1)

Table - 1: Box-Behnken design for the selected experimental variables with the coded and uncoded values.

Variables	levels		
	+1	0	-1
Magnesium Sulphate (A)(mM)	1.5	1.25	1.0
Yeast extract (B) (g/100ml)	0.7g	0.5g	0.3g
Ammonium sulphate (C) (mM)	2.0	1.5	1.0
Incubation period (D)	48 hr	36 hr	30 hr
Agitation (E)	165 rpm	145 rpm	125 rpm
Inoculum size (F)	3 %	2 %	1 %
Sodium chloride (G) (g/100ml)	0.9g	0.6g	0.3g

2.3. Preparation of crude enzyme extract and laccase assay

The culture medium was centrifuged at 10000 x g for 15 min at 4^0 C after fermentation at each experimental set up and the supernatant was used as the crude enzyme extract. Bacterial laccase activity was determined using 2mM guaiacol as substrate, at room temperature in 0.1M phosphate buffer (pH 7.0). The changes in absorbance due to oxidation of guaiacol in the reaction mixture was monitored at 470 nm(€=27,000 M^{-1} cm^{-1}) for 10 min incubation. [12] Laccase activity is expressed as the amount of enzyme needed to oxidize 1 µmol of the substrate per minute. The growth of bacterial culture was estimated in terms of biomass by measuring optical density at 600 nm for each of the experiment.

2.4. Statistical analysis

Box-Behnken design was generated and analyzed using Minitab 15.0 (trial version).

3. RESULTS AND DISCUSSION

3.1. Optimization of laccase production by Box-Behnken design

The experimental and predicted values of the laccase production in the 62 experimental setup designed by Box-Behnken method are presented in table 2. The results of the second order response surface model fitting in the form of analysis of variance (ANOVA) are given in table 3.

The determination coefficient R^2, R^2 (predicted), R^2 (adjusted) and model significance (F-value) were used to judge the adequacy of the model. According to Table – 3, the high F-value (10.11), low probability value ($P<F<0.05$) and lack of fit (0.000) for the developed model indicated that the predicted value was in good agreement with the experimental value.

The regression equation obtained from the ANOVA showed that the R^2 (multiple correlation coefficient) was 0.8008 (a value >0.75 indicates fitness of the model). This is an estimate of the fraction of overall variation in the data accounted by the model, and thus the model cannot explain only 19.92% variations of the total model. The predicted determination coefficient (R^2 predicted) of 0.6053 can be taken into considerable agreement with the adjusted determinant coefficient of 0.7213. For a good statistical model, the value of R^2 (predicted) and R^2 (adjusted) should be in the range of 0 – 1.0. Hence in the present study, the proposed model was found to be statistical significant.

The P value ($P\leq0.05$) is used to determine whether the effect of each factor in the model is statistically significant. The significance of each coefficient in the experimental model was determined by T value and the probability of $P<T$ was calculated using Minitab 15.0. The P value and T values of all linear, all squared and all interaction effects are shown in table 4.

The linear effect of magnesium sulphate, yeast extract, agitation, inoculum size and Sodium chloride was found to be significant ($P<0.05$). The quadratic effect of yeast extract was found to be

significant. The interactive effect of magnesium sulphate and ammonium sulphate, magnesium sulphate and incubation period, magnesium sulphate and agitation, magnesium sulphate and inoculum size, magnesium sulphate and sodium chloride, yeast extract and ammonium sulphate, yeast extract and incubation period, yeast extract and agitation, yeast extract and inoculum size, yeast extract and sodium chloride, ammonium sulphate and incubation period, ammonium sulphate and agitation, ammonium sulphate and inoculum size, ammonium sulphate and sodium chloride, incubation period and agitation, incubation period and inoculum size, was found to be significant.

Sondhi *et al.* (2014) [6] reported that most significant factor for laccase production by *Bacillus tequilenius* SN4 is $MnSO_4$, $FeSO_4$ and alcohol. Kaushik and Thakur (2014) [13] reported the major factor contribution for laccase production by Bacillus sp. is copper sulphate and distillery spent compared to carbon and nitrogen source.

The significant medium components were further analyzed using 3D response surface plots, which are the graphical representations of the regression model. The optimum values of the variables were obtained by simulating the experimental results using the empirical model. From the 3D response surface plots, the interactions between any two factors is convenient to understand and their optimum levels could also been obtained.

The laccase production was observed as response variable to the interaction of A (Magnesium sulphate) and C (Ammonium sulphate), the rest of the variables is set as zero. The higher concentration of magnesium sulphate (1.25 to 1.75g/100ml) and lower concentration of ammonium sulphate from 1.0 to 1.5 g/100ml was found to significantly increase the laccase production. The increase in the concentration of magnesium sulphate was found to increase the laccase production (Figure 1). The rest of the media components interactions had the same path.

Table – 2: Box-Behnken design matrix for observed and predicted values of laccase production by *Bacillus cereus* TSS1

Run	A	B	C	D	E	F	G	Biomass	Observed value (U/ml)	Predicted value (U/ml)
1.	0	1	0	0	-1	0	1	1.71	0.80	0.70
2.	0	1	0	0	-1	0	-1	1.79	3.06	3.62
3.	1	-1	0	-1	0	0	0	1.265	2.60	3.51
4.	0	0	0	-1	-1	-1	0	1.705	2.28	1.87
5.	0	1	1	0	0	-1	0	1.24	3.05	2.43
6.	0	0	0	-1	-1	1	0	0.95	3.46	2.96
7.	-1	0	-1	0	1	0	0	1.09	2.16	2.55
8.	0	0	0	0	0	0	0	1.64	1.54	1.66
9.	0	0	0	1	1	1	0	1.6	1.33	1.15
10.	0	0	0	-1	1	1	0	1.62	1.88	1.85
11.	-1	0	0	0	0	1	-1	1.335	2.55	2.43
12.	0	-1	1	0	0	1	0	1.105	5.01	4.23
13.	1	0	1	0	1	0	0	1.545	1.08	2.43
14.	-1	-1	0	-1	0	0	0	1.255	6.54	5.35
15.	1	0	0	0	0	1	1	1.465	5	5.51
16.	1	0	0	0	0	-1	1	1.31	4.94	4.50
17.	0	0	0	0	0	0	0	1.715	2.65	3.36
18.	1	0	-1	0	1	0	0	1.37	1.58	2.37
19.	0	-1	-1	0	0	-1	0	1.365	7.15	5.95
20.	1	1	0	1	0	0	0	1.475	1.84	1.54
21.	-1	0	1	0	-1	0	0	1.65	2.71	3.90
22.	0	1	0	0	1	0	-1	1.02	3.36	2.80
23.	0	-1	1	0	0	-1	0	1.38	6	4.94
24.	0	0	1	1	0	0	-1	1.575	1.23	0.95
25.	-1	1	0	-1	0	0	0	1.65	1.92	2.23
26.	0	-1	0	0	-1	0	1	1.67	3.35	2.43

27.	0	-1	0	0	1	0	1	1.715	3.34	3.10
28.	0	0	1	1	0	0	1	1.71	1.25	1.77
29.	0	0	0	1	-1	-1	0	1.65	2.87	2.16
30.	0	0	0	1	1	-1	0	1.64	2.76	2.21
31.	0	-1	0	0	-1	0	-1	1.845	1.15	1.70
32.	-1	0	0	0	0	-1	-1	1.585	0.36	0.38
33.	1	0	-1	0	-1	0	0	1.8	3.11	4.03
34.	0	0	-1	1	0	0	-1	1.68	1.94	2.43
35.	0	0	1	-1	0	0	1	1.245	2.64	2.92
36.	0	0	0	-1	1	-1	0	1.345	1.46	1.64
37.	0	0	0	0	0	0	0	1.65	2.20	2.42
38.	0	0	1	-1	0	0	-1	1.785	0.88	0.90
39.	1	0	1	0	-1	0	0	1.605	1.65	2.16
40.	-1	0	-1	0	-1	0	0	1.63	2.11	3.32
41.	0	0	-1	-1	0	0	-1	1.235	2.51	3.69
42.	0	1	-1	0	0	1	0	1.64	3.68	2.43
43.	-1	-1	0	1	0	0	0	1.57	5.15	6.21
44.	1	1	0	-1	0	0	0	1.73	2.28	2.41
45.	-1	0	1	0	1	0	0	1.7	3.58	4.19
46.	0	0	0	1	-1	1	0	1.605	3.26	2.18
47.	-1	0	0	0	0	1	1	1.785	0.44	1.52
48.	0	1	-1	0	0	-1	0	1.445	3.39	3.49
49.	1	0	0	0	0	-1	-1	1.475	2.68	2.17
50.	0	-1	-1	0	0	1	0	1.675	5.11	3.93
51.	0	0	-1	1	0	0	1	1.765	2.73	2.21
52.	0	0	0	0	0	0	0	1.78	3.701	3.09
53.	0	0	-1	-1	0	0	1	1.77	0.50	0.74
54.	-1	1	0	1	0	0	0	1.555	2.28	2.43
55.	0	-1	0	0	1	0	-1	1.64	2.51	2.91
56.	0	1	1	0	0	1	0	1.775	4.87	3.95
57.	-1	0	0	0	0	-1	1	1.66	0.56	0.17
58.	0	0	0	0	0	0	0	1.8	1.19	1.17
59.	1	0	0	0	0	1	-1	1.8	0.88	0.76
60.	0	1	0	0	1	0	1	1.03	0.93	0.81
61.	0	0	0	0	0	0	0	1.73	3.06	2.84
62.	1	-1	0	1	0	0	0	1.76	4.32	4.76

Table - 3: Analysis of variance of response surface methodology for the laccase production by isolated *Bacillus cereus* TSS1

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Model	35	228.796	228.796	6.5370	10.11	0.000
Linear	7	56.573	56.573	8.018	12.50	0.000
Square	7	7.256	7.256	1.0365	1.60	0.145
Interaction	21	164.968	164.968	7.856	12.15	0.000
Residual Error	88	56.910	56.910	0.6467	-	-
Lack-of-Fit	21	47.946	47.946	2.2831	17.06	0.000
Pure Error	67	8.964	8.964	0.1338	-	-
Total	123	285.71	-	-	-	-

S = 804.182 PRESS = 112773313 R-Sq = 80.08% R-Sq (pred) = 60.53% R-Sq (adj) = 72.16%

Table - 4: Student's T test of the selected variables

Source	Coef	SECoef	T	P
Linear Effects				
Constant	2.43218	0.2312	10.477	0.000
Magnesium sulphate	-0.3884	0.1161	-3.346	0.001[a]
Yeast extract	0.7626	0.1161	6.570	0.000[a]
Ammonium sulphate	-0.1082	0.1161	-0.939	0.350
Incubation period	-0.1634	0.1161	-1.408	0.163
Agitation	0.2389	0.1161	2.058	0.043[a]
Inoculum size	-0.5246	0.1161	-4.520	0.000[a]
Sodium chloride	0.2745	0.1161	2.365	0.020[a]
Squared				
Magnesium sulphate*Magnesium sulphate	0.1269	0.1548	0.820	0.415
Yeast extract*Yeast extract	0.3458	0.1548	2.234	0.028[a]
Ammonium sulphate*Ammonium sulphate	-0.2699	0.1548	-1.744	0.085
Incubation period*Incubation period	0.1375	0.1548	0.889	0.377
Agitation*Agitation	0.7470	0.1548	0.483	0.631
Inoculum size * Inoculum size	0.1241	0.1548	0.802	0.425
sodium chloride*sodium chloride	0.117	0.1548	0.722	0.472
Interaction Effect				
Magnesium sulphate*Yeast extract	-0.2419	0.201	-1.203	0.232
Magnesium sulphate*Ammonium sulphate	-0.8284	0.201	-4.120	0.000[a]
Magnesium sulphate*Incubation period	0.5893	0.201	2.931	0.004[a]
Magnesium sulphate*Agitation	0.6088	0.201	3.028	0.003[a]
Magnesium sulphate* Inoculum size	0.6729	0.201	3.347	0.001[a]
Magnesium sulphate*Sodium chloride	1.59	0.201	7.948	0.000[a]
Yeast extract*Ammonium sulphate	0.6429	0.201	3.198	0.002[a]
Yeast extract*Incubation period	-0.7589	0.201	-3.775	0.000[a]
Yeast extract*Agitation	1.46	0.201	7.281	0.000[a]
Yeast extract* Inoculum size	0.6252	0.201	3.110	0.003[a]
yeast extract*sodium chloride	-0.0453	0.201	-0.225	0.822
Ammonium sulphate*Incubation period	0.4914	0.201	2.444	0.017[a]
Ammonium sulphate*Agitation	-0.1903	0.201	-0.946	0.347
Ammonium sulphate* Inoculum size	0.7320	0.201	3.641	0.000[a]
Ammonium sulphate*Sodium chloride	-0.4035	0.201	-2.007	0.048[a]
Incubation period*Agitation	-0.7821	0.201	-3.890	0.000[a]
Incubation period* Inoculum size	-0.5175	0.201	-2.574	0.012[a]
Incubation period*Sodium chloride	-0.3334	0.201	-1.659	0.101
Agitation* Inoculum size	-0.2595	0.201	-1.291	0.200
Agitation*Sodium chloride	-0.1515	0.201	-0.753	0.453
Inoculum size *Sodium chloride	0.5093	0.201	2.533	0.013[a]

[a] - P< 0.05 are significant factors

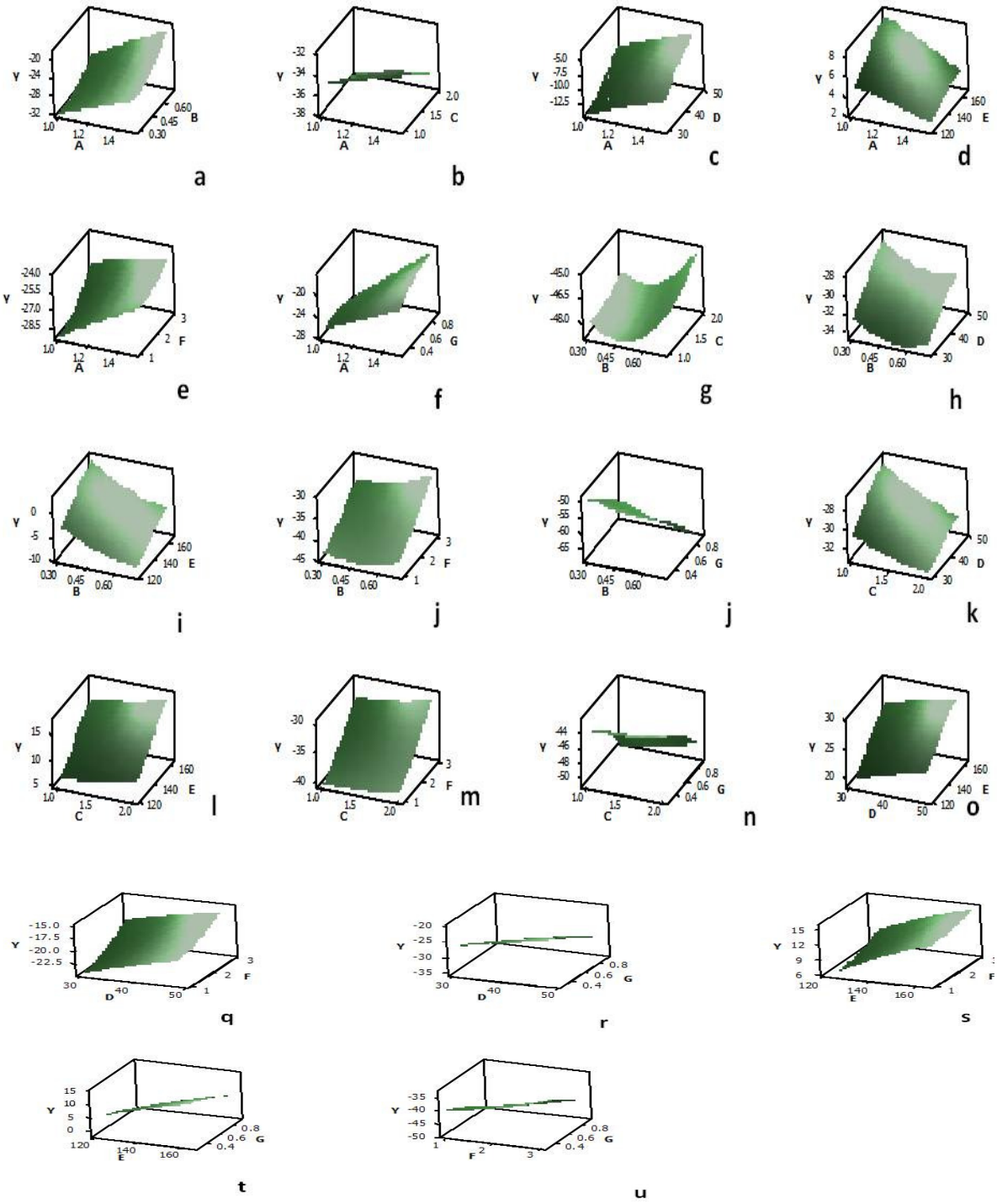

Figure - 1: Response surface plot for the combinatory effect (**a)** A and B **b)** A and C **c)** A and D **d)** A and E **e)** A and F **f)** A and G **g)** B and C **h)** B and D **i)** B and E **j)** B and F **k)** B and G **l)** C and D **m)** C and E **n)** C and F **o)** C and G **p)** D and E **q)** D and F **r)** D and G **s)** E and F **t)** E and G **u)** F and G).

3.2. Validation of the optimized media components

The model predicted the maximum laccase production of 9.403 U/ml when the concentration of magnesium sulphate was 1.5 mM, yeast extract was 0.30 g/100ml, ammonium sulphate was 1mM, agitation was 144 rpm, incubation hour of 48 hrs, Inoculum size was 1 % and sodium chloride was 0.9g /100 ml. To verify the predicted results, validation experiment was performed in triplicates. The observed experimental production of laccase was 9.03 U/ml. It suggested that experimental and

predicted values were in good agreement. The enzyme production in unoptimized media was 2.05 U/ml whereas in optimized media was 9.03 U/ml.

4. CONCLUSION

The laccase production media for the isolated *Bacillus cereus* TSS1 was optimized using response surface methodology. The optimized media (9.03 U/ml) was found to have four fold increase in laccase production compared to unoptimized media (2.05 U/ml). In the present study the significant factors responsible for laccase production were found to be magnesium sulphate, yeast extract, agitation, inoculum size and sodium chloride for the isolated strain *Bacillus cereus* TSS1. The conditions optimized for laccase production from the isolated bacterial strain could be selected to obtain large amount of enzyme and could be exploited for various biotechnological applications.

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