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Optimization of cellulase production by *flavobacterium bolustinum* under submerged fermentation using response surface methodology

Suman Malik*, Rishi Pal Mandhan, Ajay Kansal
Department of Biotechnology, Kurukshetra University, Kurukshetra, India
E-Mail: sumanmalik16@gmail.com
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ABSTRACT

A 2 step *Response Surface Methodology* (RSM) employing central composite design (CCD) was applied for the optimization of cellulase production by a newly isolated strain *Flavobacterium bolustinum.* Initially, Plackett Burman revealed the 4 significant factors (Temperature, Pineapple peel, Rice straw and NH_4Cl) out of 9 on the basis of their F-value. Secondly, a 2^3 full factorial central composite design and RSM were applied to determine the optimal concentration of each significant factor. The result showed that linear effect of temperature and pineapple peel and their interactions (as $P<0.05$) were more significant than the linear and interactive effect of the other variables. It showed the optimum condition for cellulase production as Pineapple peel 2%, Rice straw 0.6%, NH_4Cl 0.15%, $MgSO_4$ 0.4%, Glycerol 1% pH 9 Incubating at 40°C for 24 hrs at 200 rpm resulted in 2.26 fold increase in activity(601.08U/ml) as compared to initial level (265U/ml). Analysis of variance (ANOVA) showed a high coefficient of determination (R^2) value 91.87 ensuring a satisfactory adjustment of quadratic model with the experimental data. Using Agro waste, Pineapple and Rice straw as carbon source reduces the cost of enzyme production thus making the process economically sound, a major challenge in cellulase production.
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KEYWORDS

Cellulase;
Flavobacterium bolustinum;
Pineapple peel;
Response Surface Methodology.

INTRODUCTION

Cellulase is a synergistic enzyme that is used to break up cellulose into glucose or to other oligosaccharides[1]. Cellulase can be produced by fungi, bacteria and actinomyctes but the common producer is fungi. High cost of cellulase due to expansive substrate used in production such as cellulose and carboxymethylcellulose by fungi and its slow growth lead to search for other producers those overcome the limitation faced by using fungus. Answer lie in bacteria having high growth rate and has good potential to be used for cellulase production. Moreover, cellulase produced by bacteria are more effective catalyst and less inhibited by the feedback inhibition. One of the major parameter influencing the cost of cellulase production is the type of substrate used in production of the enzyme[2]. The enzymatic hydrolysis of cellulose require the use of cellulase (1, 4-(1, 3)-β-D-glucan glucanohydrolase, EC(3.2.1.4), a multiple enzyme system consisting of endo 1,4,-β-D-glucanase[1,4-β-D-glucanases(CMCase, EC3.2.14)] and exo 1,4-β-D-glucanase(1,4-β-D-glucan cellobiohydrolase, FPA, EC 3.2.1.91) along with cellobiase (β-D-glucoside glucanohydrolase, EC 3.2.1.21)[3,4].

The cost of the enzyme production is one of the main factors determining the economics of the process. Reducing the cost of the enzyme production by optimizing the medium and the process is the goal of the basic research for industrial application. Recently, statistical designs (RSM) have been successfully employed for the optimization of various industrially important microbiological and biotechnological products[5]. It involves the collection of the statistical techniques for designing of experiment, models, evaluating the effect of factors and searching for optimum conditions for desirable responses ensuring the maximum enzyme activity[6,7]. These methods of optimization have proved to be powerful, useful and less time consuming. Cellulase is one of the most useful enzyme in various industries such as animal feed, textile, fuel, chemical, pulp and paper, waste management[8,9,10,11].

In this present study, the various medium components and physical variables affecting the cellulase production for isolate *Flavobacterium bolustinum* under submerged fermentation were optimized using Response Surface Methodology. The parameters with significant effects on cellulase production were identified using a fractional factorial design and their levels were optimized using a central composite response surface method to improve cellulase yield. Thus by using RSM, we can reduce the cost of enzyme production and increase enzyme activity which is the basic requirement for the application of enzyme in industries.

MATERIAL AND METHODOLOGY

Microorganism

Soil sample was collected from sugarcane mill and colonies exhibiting cellulase activity were isolated from medium containing Carboxymethylcellulose (CMC) as substrate. On the basis of morphological, physiological and biochemical characteristics strain was identified as *Flavobacterium bolustinum* by IMTECH, Chandigarh having MTCC no.10203[12] (Malik et.al, 2010). The organism was sub cultured over the interval of 1 month and stored at 4°C.

Medium and cultivation

The seed medium consisting of CMC (0.1%), Peptone (0.5%), Beef extract (0.15%) , Yeast extract (0.15%) , NaCl (0.5%) , KH_2PO_4 (0.1%), wheat bran (1%) pH 9 was sterilized by autoclaving at 121°C for 30 minutes. After cooling the medium was inoculated with 1% of inoculum of age 20 hrs and was incubated at 37°C for 24 hrs at 200rpm in orbital shaking-cum BOD incubator. By using conventional method of optimization, medium screened for production included pineapple peel (1.5%), fructose (0.25%), NH_4Cl (0.4%), $MgSO_4$ (0.2%), Glycerol (1%).

Enzyme assay

The culture was harvested by centrifugation at 10,000 rpm for 20 min at 4°C using Refrigerated centrifuge (REMI). The supernatant was used as the crude extra cellular enzyme source. Cellulase activity was determined at 65°C by using carboxymethylcellulose (Sodium salts, Sigma, India) as substrate. A reactive mixture contained 450μl of 1 %(w/v) substrate in 0.1M Glycine-NaOH (pH 9) and 50μl of culture supernatant. The mixture was incubated at 65°C for 10 min. The reducing sugar released was measured using 3,5-dintrosalicyclic acid (DNSA)[13]. One unit of enzyme activity was expressed as the amount of enzyme required to release 1 μmol reducing sugars per ml under the above assay condition by using glucose as a standard.

Optimization of parameters for cellulase production by RSM

Optimization of parameters for cellulase production was performed in two stages. Initially, 9 variables were screened using a fractional factorial design to identify the parameters, which significantly influenced enzyme production and in the second stage the levels of these parameters were optimized using a Central Composite design (CCD) [14,15].

Plackett Burman

This screening design allows the testing of multiple independent variables within a single experiment[16]. The effect of each variable was identified by the difference between the average of the + and – response. The significant level of the effect of each variable was determined by *F- test*. The variables evaluated are listed in TABLE 1.

Here 9 variables out of which 1 dummy variable

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(Inoculum volume) were screened in 12 trails as shown in TABLE 2 .1.The effect of an independent variable was the difference between average response for 6 experiments at high level and average value for 6 experiments at low level as in eq 1.

TABLE 1: Variables evaluated for PB design

Variable Code	Variable	(+) Level	(-) Level
A	Temperature	45°C	35°C
B	Time	30 hrs	24 hrs
C	pH	9	6
D	Glycerol	1%	0.5%
E	Pineapple Peel	1.5%	0.5%
F	Rice Straw	1%	0.5%
G	Fructose	0.25%	0.1%
H	NH_4 Cl	0.4%	0.2%
I	Inoculum Volume (Dummy Variable)	2%	1%

A = ΣA (H)/6 – ΣA (L)/6 **(1)**

Then estimated the mean square value of the variable called variance effect by eq 2

A = (Σ A (H) – Σ A (L))^2/12 **(2)**

Experimental error was calculated by averaging the mean square of dummy variable. *F-effect* was calculated by eq 3

F effect = Factor mean square / Error mean square **(3)**

Central composite design (CCD)

It is a response surface design[17] for improving cellulase production and the variable having the greatest influence (Temperature, Pineapple peel, Rice straw and NH_4Cl) were chosen for this study. The statistical software package *Minitab 15* was used to design and analyze the experimental results. Each variable was studied for five different levels (-2, -1, 0, 1, 2) as shown in TABLE 3.

A set of 31 experiments were performed and enzyme activities were calculated. Regression analysis was performed on the data obtained as in TABLE 4. The results of CCD were used to fit a second order polynomial equation as follow:

$$Y = \beta o + \Sigma_{i=o}^{n} \beta i\, xi + \Sigma_{i=o}^{n} \beta n\, xi^2 + \Sigma_{i>j}^{n} \beta ii\, xi\, xj$$

Where Y = Predicted response, βo = Intercept, βi = Linear coefficient , βn = Squared coefficient, βii = Interaction coefficient

ANOVA was used to analyze experiments and to generate response surface graphs[18]. The ideal levels and combinations of parameters were identified by optimization functions in the software and experiments were run at these levels for validation of the model.

RESULTS AND DISCUSSION

Plackett Burman

TABLE 2.1: Experimental design and results of PB design

Runs	Variables / Levels									Activity (U/ml)
	A	B	C	D	E	F	G	H	I	
1	+	-	+	-	-	-	+	+	+	221.06
2	-	+	+	-	+	-	-	-	+	387.68
3	+	+	+	-	+	+	-	+	-	438.38
4	-	-	-	-	-	-	-	-	-	208.38
5	-	-	-	+	+	+	-	+	+	296.24
6	+	-	-	-	+	+	+	-	+	505.12
7	-	+	+	+	-	+	+	-	+	268.94
8	+	+	-	+	-	-	-	+	+	198.17
9	-	-	+	+	+	-	+	+	-	295.34
10	-	+	-	-	-	+	+	+	-	303.91
11	+	+	-	+	+	-	+	-	-	355.67
12	+	-	+	+	-	+	-	-	-	275.67

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TABLE 2.2: Analysis of results of TABLE 2.1

Variables	Mean of H-level	Mean of L-level	Difference	Square mean	Variance effect	F-values
A	332.34	293.41	38.93	1515.54	126.29	1578625
B	325.45	294.57	30.88	953.57	79.46	993250
C	314.51	311.24	3.27	10.69	0.891	111375
D	344.13	311.67	32.46	1053.65	87.80	1097500
E	379.73	246.02	133.71	133.71	1489.95	18623750
F	348.04	277.71	70.33	70.33	412.21	5152625
G	329.55	300.75	28.80	28.80	69.13	864125
H	333.57	292.27	-41.3	1705	142.14	1776750
I	312.86	312.89	-.031	.00096	.00008	1

TABLE 3: Range of variables at different levels for the fractional factorial design

Independent variables	Levels				
	+2	+1	0	-1	-2
Temperature	44	42	40	38	36
Pineapple peel (%)	2	1.5	1	0.5	0
Rice straw (%)	1	0.8	0.6	0.4	0.1
NH_4Cl (%)	0.25	0.2	0.15	0.1	0.05

Total 9 variables were analyzed and resulting effects of these variables are presented in TABLE 2.2. One the basis of their F-test, variables (Temperature, Pineapple peel, Rice straw and NH_4Cl) were found to be significant. Pineapple[19] and rice straw[20] was used as novel substrate for cellulase production. Greater the F-value, greater is the effect of the variable as shown in TABLE 2.2

Central Composite design

4 variables which were found to be significant by PB design were subjected to CCD. TABLE 4 gives the design and results of the experiment carried out by CCD design. The final response equation that represented a suitable model for cellulase production is given below by using coefficients values as shown in TABLE 5.

$Y = -9281.85 + 467.44A + 551.60B + 265.61C + 1772.63D + (-5.76) A^2 + (-70.86) B^2 + (-96.21) C^2 + (-1471.17) D^2 + (-6.34) AB + (-33.79) BC + (- 188.17) CD + (-25) AD + (-2.97) AC + (-121.3) BD$

The regression equation indicates that coefficient of determination (R^2) was 0.9187 and thus the model could explain more than 91.87% of variability in the response. Coefficient of determination (R^2) is defined as the ratio of explained variation to the total variation and is a measurement of the degree of fitness[21]. The R^2 value always remained between 0 and 1. The closer the R^2 is to 1, the stronger the model and better it predicts[22]. A small value of R^2 indicates a poor relevance of the dependent variables in the model. The low probability p-value (<0.05) indicates that model terms were significant. A, B, A^2, B^2 were found as significant model terms as shown in TABLE 5. This implies that linear coefficient ($p<0.002$) and square coefficient ($p<0.001$) were found to be significant as compared to the interaction once as shown in TABLE 6 (Analysis of model). Rashid *et al*[23] had done statistical optimization for cellulase production using palm oil mill effluent from filamentous fungus, *Trichoderme reesei* RUT-C30. Maximum CMCase activity obtained was 18.53 U/ml with 99.5% regression coefficient (R^2) of the Design.

The three dimensional response surfaces had been plotted to study the interaction among the various factors selected. The fitted response for the above regression model had been plotted as shown in Figure (1.3). 3D graphs had been generated for various combinations of the two factors while keeping the other two at their optimum levels. Graphs were given here to highlight the role played by various factors. Figure 1 showed that with the increase in pineapple peel concentration (B), the cellulase activity increases with the increase in temperature (A) but only up to its middle value (40°C). Figure 2 showed the effect of Rice straw (C) and pineapple peel (B) on cellulase activity while temperature and NH_4Cl were fixed at their middle level. Graph showed that with increase in peel concentration enzyme activity increases but the requirement for rice straw decreases and reached to optimum level of 0.6%.This may be due to formation of thick suspension and im-

TABLE 4: Results of CCD using four independent variables and eight central points showing observed and predicted response

Runs	Temperature (°C)A	Pineapple peel (%w/v)B	Rice straw (%w/v)C	NH_4Cl (%w/v)D	Observed values (U/ml)	Predicted values (U/ml)
1	40	1	0.6	0.05	501.95	478.65
2	38	0.5	0.8	0.1	390.21	387.03
3	40	1	0.6	0.15	504.95	504.95
4	42	0.5	0.4	0.2	401.6	400.05
5	40	2	0.6	0.15	601.08	555.12
6	40	1	0.6	0.15	504.95	504.95
7	42	1.5	0.8	0.1	487.98	485.63
8	38	1.5	0.8	0.2	503.49	521.56
9	42	1.5	0.4	0.1	465.38	506.03
10	42	0.5	0.4	0.1	409.19	381.56
11	36	1	0.6	0.15	457.91	432.58
12	38	1.5	0.8	0.1	491.49	516.80
13	40	1	0.6	0.15	504.95	504.95
14	40	1	0.2	0.15	498.85	482.62
15	38	1.5	0.4	0.1	525.62	529.97
16	42	0.5	0.8	0.1	381.42	381.99
17	40	1	0.6	0.25	494.12	501.89
18	40	1	0.6	0.15	504.95	504.95
19	42	1.5	0.8	0.2	477.57	480.50
20	40	1	0.6	0.15	504.95	504.95
21	40	1	1	0.15	455.87	457.84
22	40	0	0.6	0.15	281.42	313.11
23	42	1.5	0.4	0.2	518.66	518.66
24	38	0.5	0.8	0.2	420.89	404.02
25	38	0.5	0.4	0.1	359.16	380.02
26	40	1	0.6	0.15	504.95	504.95
27	38	1.5	0.4	0.2	522.59	546.12
28	40	1	0.6	0.15	504.95	504.95
29	42	0.5	0.8	0.2	402.33	388.43
30	38	0.5	0.2	0.2	415.61	408.40
31	44	1	0.6	0.15	381.99	393.06

proper mixing of substrate in shake flask[24,25]. This high level of cellulase production with increased concentration of pineapple peel could be due to fact the peel act as good reservoir for cellulolytic organism as peel is rich in cellulose, hemicellulose and nitrogen source. The plots clearly showed that in any condition cellulase activity increases with increasing pineapple peel concentration. Thus p value (<0.04 and 0.001) and the plots had shown that the most important factor for cellulase production was the pineapple peel concentration in production medium.

Incubation temperature was a critical factor effecting cellulase production by *Flavobacterium bolustinum* as its linear and square coefficient p value was < 0.000 and < 0.000 respectively. Singh *et al*[26] had also identified that temperature was critical factor as its (P<0.0001) less than 0.05 for cellulase production by fungal strain *A. heteromorphus* using rice straw. Good cellulase yield could be obtained by using ammonium chloride as the nitrogen source. Though the

TABL5: Results of the regression analysis of the CCD

Factors	Coefficient	P-values
A	467.44	.000
B	551.60	.044
C	256.61	.550
D	1772.63	.496
A*A	-5.76	.000
B*B	-70.86	.001
C*C	-96.21	.078
D*D	-1471.17	.436
A*B	-6.34	.318
B*C	-33.79	.422
C*D	-188.17	.652
D*A	-25	.690
A*C	-2.97	.775
B*D	-121.3	.629
Constant	-9281.85	.000

TABLE 6: Analysis of variance (ANOVA) for response surface model for cellulase production

Sources	Sum of square	Degree of freedom	Mean square	F- value	P-value**	Significance*
Model	116038	14	8288.42	13.7	0.001	Significant
Linear	91969	4	4330.24	7.6	0.002	Significant
Square	22588	4	5647.02	9.33	0.001	Significant
Interaction	1480	6	246.75	0.41	0.863	
Residual error	9679	16	604.95			
Lack of fit	9679	10	967.9	8.56	0.0015	Significant
Pure error	0	6				

*** Statistically significant at 95% of probability level.**
****Value of "P>F" less than 0.0500 indicate model terms are significant.**
R-Sq = 91.87%, R-Sq (pred) = 53.19%, R-Sq (adj) = 84.76%

addition of organic nitrogen sources such as beef extract and peptone resulted in increased growth and enzyme production as was reported before (Sun *et al*, 2008)[27]. Figure 3 depicts that at the middle values of all the factors (pineapple peel, 1%; temperature, 40°C; rice straw, 0.6% and NH_4Cl, 0.15%) showed maximum activity which also correspond to experimental data except for pineapple peel concentration showing optima at 2% concentration (w/v). The 3D graphs results correspond to Regression analysis results and to our experimental results, thus showing that the model was accurate.

Over the last few decades, even though several papers regarding optimization of cellulase production have been reported, but little information is available about the optimization of cellulase production using response surface methodology (RSM). Earliar fungal species *Trichoderma longibrachiatum, Aspergillus niger and Saccharomyces cerevisiae* were reported using pineapple peel as carbon source for cellulase production. No bacterial strain has been reported till date using pineapple peel as carbon source and producing good enzyme activity. This is first ever report of producing cellulases by the bacteria with high titer of enzyme activity. The response surface showed the two factors temperature and pineapple peel were most effective for cellulase production in submerged fermentation (SmF), and maximum activity produced was 601.08 U/ml.

CONCLUSION

As very less reports were available on optimization of cellulase production using Response Surface Methodology. Thus, the Statistical optimization method can overcome the limitations of classic empirical methods and has proved to be a powerful tool for the optimization of cellulase production with minimum number of experimental trails. Under optimal conditions (pineapple, 2%; rice straw, 0.6%; NH_4Cl, 0.15% and temperature, 40°C) cellulase activity increased up to 2.26 fold. Moreover, nutrient which were found to be effective for cellulase production are inexpensive agro wastes showing important economic advantages.

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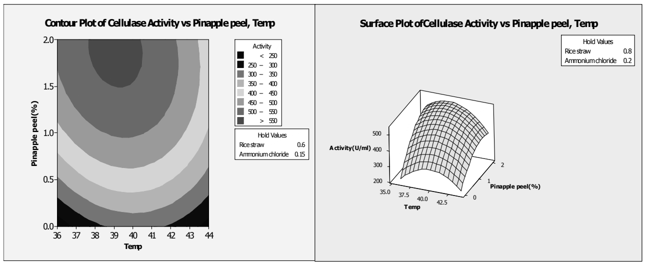

Figure 1.1

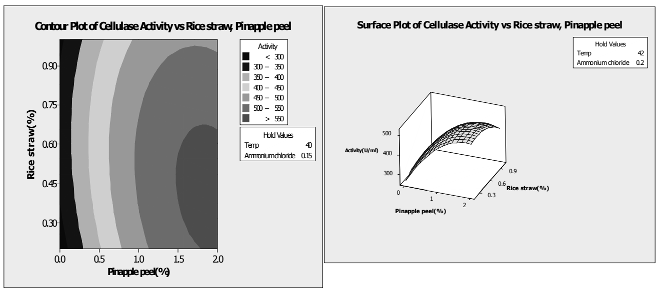

Figure 1.2

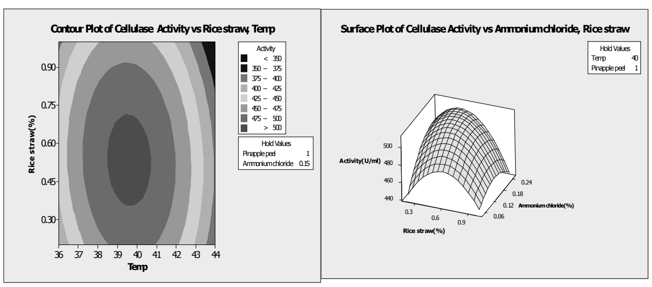

Figure 1.3

Figure 1: Shows the contour and surface plots of various combinations of factors

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