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OPTIMIZATION OF CELLULASE PRODUCTION BY SOIL BACTERIA USING STATISTICAL DESIGN

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ABSTRACT

Cellulase is an enzyme complex which breaks down cellulose to glucose. It has been used widely in commercial food processing, textile industry and laundry detergents. Majority of studies on cellulase production have focused on fungi, with relatively lesser emphasis on bacterial sources. The current study has focused on isolating bacteria from paddy soil, capable of producing free cellulase and optimization of culture media for increasing cellulase production. Cellulase producing bacteria was isolated from paddy soil and was identified using 16S rDNA sequencing and BLAST search. Screening of nutrients and their influence on the cellulase production was studied using a Plackett-Burman design. Five variables $(NH_4)_2SO_4$, Cellulose, K_2HPO_4, $CaCl_2.2H_20$, pH were screened to evaluate their effect on cellulase production, of only two variable K_2HPO_4 andcellulose were found to have significant effect on cellulase production. Two components with significant effect on cellulase production were further optimized with Response Surface Methodology (RSM) using Central Composite Design (CCD).The optimal levels of these medium components determined were K_2HPO_4 (2g/ml) andCellulose (2g/ml). The enzyme activity with nutritional medium was 0.148 U/ mL, but a 2 fold increase in the cellulase activity in optimized medium was observed.

Keywords: Cellulase, Central Composite Design, Optimization, Plackett-Burman design, Response Surface Methodology, 16S rDNA sequencing.

INTRODUCTION

Cellulose is the most abundant natural polysaccharide, consisting of hundreds to over ten thousand β-1, 4 linked glucose units. Cellulolysis is the process by which cellulose is degraded by an enzymatic reaction into smaller polysaccharides called cellodextrins or completely into glucose units. Breaking down of cellulose is rather a difficult process when compared to other polysaccharides and known to require synergistic actions of three enzymes including endo-β-1,4-glucanase (EC 3.2.1.4, EG, randomly cleaving internal linkages), cellobiohydrolase (EC 3.2.1.91, CBH, specifically hydrolyzing cellobiosyl units from non-reducing ends) and β-D-glucosidase (EC 3.2.1.21, hydrolyzing glucosyl units from cello-oligosaccharides)[1,2].

Cellulases are class of enzymes produced primarily by fungi, bacteria, and protozoans. Majority of studies on cellulase production have focused on fungi, with relatively lesser emphasis on bacterial sources. Most commonly studied cellulolytic organisms include: fungal species- *Trichoderma, Humicola, Penicillium, Aspergillus;* bacterial species- *Bacillus*, *Clostridium*, *Cellulomonas*, *Thermomonospora*, *Ruminococcus*, *Bacteroides*, *Erwinia*, *Acetovibrioand* actinomycetes in particular *Streptomyces* species have been reported[3]. The isolation and characterization of novel cellulolytic bacteria is still a dynamic area of research, because bacteria have a higher growth rate than fungi and capability to produce enzyme stable under extreme conditions[4]. Such an enzyme can increase rate of enzymatic hydrolysis, fermentation and product recovery which may be applied in biofuel and bioproduct industries[5]. Since very few isolates available for cellulose hydrolysis and the bacterial cellulase yield is low, active research for newer isolates is on.Therefore, there has been much research aimed at obtaining new bacterial isolates giving higher yield of enzyme and with higher specific activities.

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Table 1: Experimental codes, range and levels used in Plackett-Burman design.

Sr.No.	Variables	Units	Symbol Code	Experimental value	
				Lower	Higher
1.	$(NH_4)_2SO_4$	g/100ml	X_1	0.1	0.5
2.	Cellulose	g/100ml	X_2	0.5	2.0
3.	K_2HPO_4	g/100ml	X_3	0.5	2.0
4.	$CaCl_2.2H_20$	g/100ml	X_4	0.05	0.5
5.	pH	g/100ml	X_5	6.0	8.0

Table 2: Biochemical characteristics of the cellulase producing bacterial isolates.

Fermentation of Sugars	C2	BA	T1	RC
Glucose	+	+	+	+
Galactose	+	-	-	-
Mannitol	+	-	-	+
Maltose	-	+	+	+
Sucrose	-	+	-	+
Xylose	-	-	-	-
IMViC				
Indole	-	-	-	-
MR	-	-	+	+
VP	-	+	-	-
Citrate	+	+	+	+
Nitrate red.	+	-	-	+
TSI Slant	ALK	A	ALK	ALK
Butt	ALK	A	ALK	A
Gas	-	-	-	-
H2S	-	-	-	-
Oxidase	-	+	-	-
Catalase	+	-	+	+
Gram Character	Gram negative rods	Gram positive rods	Gram positive cocci	Gram positive cocci
Motility	+	+	-	-

In the present study an attempt has been made to isolate, identify and optimize the culture conditions for cellulase producing bacteria isolated from paddy soil. The statistical experimental design was used (Plackett-Burman)[6] to evaluate which nutritional variables affects cellulase production significantly. Sequentially, the optimum levels of the most significant identified variables were determined through application of response surface methodology.

MATERIALS AND METHODS

Enrichment and Isolation

Rhizosphere soil sample was collected from the paddy field in Umbardegaon, Kalyan (West). For enrichment of sample 100ml of sterile enrichment broth containing filter paper was used. 1 gram of soil sample was transferred aseptically to sterile enrichment broth and the flask was placed on rotary shaker at 28±2^{0}C for 7 days. Enrichment was repeated once more using 1ml of the enriched broth. Enriched culture was isolated on sterile 1% Cellulose agar plates. Plates were incubated at 28±2^{0}C for 7 days. The replica plates were prepared separately for staining. Replica plates were flooded with 0.1% aqueous solution of Congo red for 15mins, poured off and plates were then flooded with 1M NaCl for 15mins. The cellulase producing bacteria showing maximum clear zone around the colonies were selected from master plate[7]. The selected cultures were maintained on sterile cellulose agar slants. The culture slants were stored at 4°C and subculture every 10–15 days.

Morphological and Biochemical Characterizations of the Bacterial Isolates

Morphological and biochemical properties of the isolate were identified, evaluated, and compared, as described in Bergey's Manual of Determinative Bacteriology.

Table 3: Zone of clearance around different isolates.

Sr.No.	Isolates	Zone of Clearance around growth (mm)
1.	C2	13
2.	BA	3
3.	T1	9
4.	RC	5

Table 4:Cellulase Activity of selected isolates

Names of Isolate	Cellulase activity (FPU/ml)
C2	0.148
T1	0.074
RC	0.083
BA	0.059

Table 5: Twelve trial Plackett-Burman design for five variables along with FPU/ml

Run Order	$(NH_4)_2SO_4$	Cellulose	K_2HPO_4	$CaCl_2.2H_20$	pH	FPU/ml
1	1	-1	1	1	-1	0.138
2	-1	1	1	1	-1	0.233
3	-1	-1	-1	1	1	0.064
4	1	1	-1	1	1	0.160
5	-1	-1	-1	-1	-1	0.033
6	-1	-1	1	1	1	0.097
7	1	1	1	-1	1	0.245
8	1	1	1	1	-1	0.222
9	1	-1	-1	-1	1	0.064
10	-1	1	-1	-1	1	0.222
11	1	-1	1	-1	-1	0.113
12	-1	1	-1	-1	-1	0.125

Table 6: Statistical analysis of Plackett Burman design showing Coefficient values, T-and P-value for each variable.

Predictor	Coef	T	P
Constant	0.143000	21.52	0.000
$(NH_4)_2SO_4$	0.014000	2.11	0.080
Cellulose	0.058167	8.75	0.000
K_2HPO_4	0.031667	4.77	0.003
$CaCl_2.2H_20$	0.009333	1.40	0.210
pH	-0.001000	-0.15	0.885

S = 0.0230181 R-Sq = 94.6% R-Sq (adj) = 90.2%

Qualitative estimation

All the isolates of cellulase producing bacteria isolated from paddy soil were inoculated loop fully on sterile cellulose agar plate. Plate was incubated at 37°C for 7 days. For selecting cellulase producing bacteria plates were flooded with 0.1% aqueous solution of Congo red for 15minutes. After 15minutes Congo red solution was poured off and plate was then flooded with 1M NaCl for 15minutes. Isolate showing maximum clear zone around the colony was consider as highest cellulase producing bacterial isolate as compared to other isolates on the basis of qualitative estimation[7].

Quantitative estimation

All the isolates, were grown in 100 mL sterile enzyme production medium (at pH 7.0) containing the following components (g/100mL): $(NH_4)_2SO_4$ (0.2), Cellulose (1.0), K_2HPO_4 (1.0), $CaCl_2.2H20$ (0.1), $FeSO_4.6H_2O$(0.0167), $ZnSO_4.7H_2O$ (0.00018), $CuSO_4.5H_2O$ (0.00016), Agar (3.0), Distilled water (100ml),pH (7.0). 100mL sterile medium (containing 1% inoculum) was taken in 250 mL Erlenmeyer flask and incubated at 28±2^{0}C on rotary shaker for 7 days. After 7 days, the cultures were centrifuged at 3000rpm for 20 min. The cell-free culture broth containing the crude enzyme was used for estimation of Filter paper assay[8]. Based on the higher cellulase activity, an isolate was selected for further study.

16S ribosomal DNA (rDNA) analysis

Highest cellulase producing isolate obtained after qualitative and quantitative estimation was sent to 16S Ribosomal DNA (rDNA) analysis at

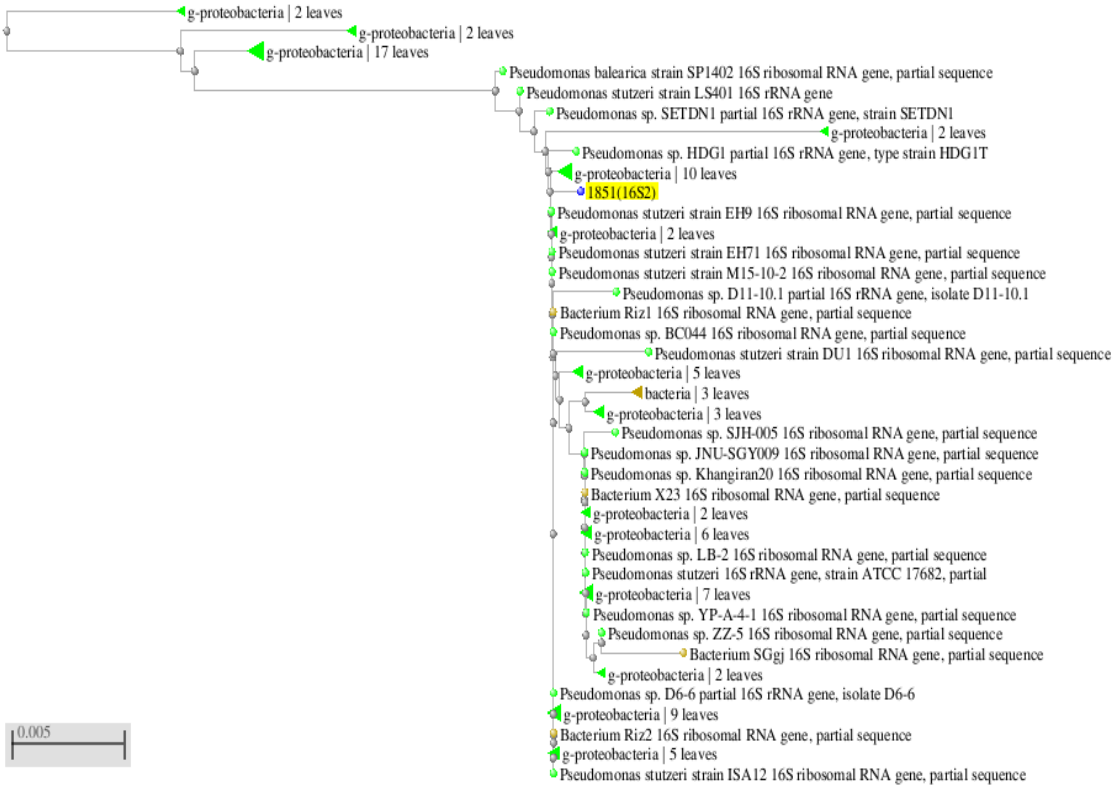

Figure 1: Neighbor-joining tree based on 16S rDNA sequences of isolate C2.

geneOmbio lab (Pune). Bacterial genomic DNA was isolated using geneO-spin Microbial DNA isolation kit (geneOmbio technologies, Pune; India). Bacterial 16S region gene was amplified using standard PCR reaction. The primer pair 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') was used in a PCR reaction with an annealing temperature of 57˚C. After amplification, products were purified by using a geneO-spin PCR product Purification kit (geneOmbio technologies, Pune; India) and were directly sequenced using an ABI PRISM BigDye Terminator V3.1 kit (Applied Biosystems, USA). The sequences were analyzed using Sequencing Analysis 5.2 software. BLAST analysis was performed at BlastN site at NCBI server.

Table 7: Design and result of Central Composite Design (CCD)

RunOrder	K_2HPO_4	Cellulose	FPU/ml
1	-1	-1	0.054
2	0	-1	0.074
3	-1	1	0.129
4	0	0	0.148
5	0	0	0.148
6	0	1	0.198
7	1	-1	0.123
8	0	0	0.148
9	0	0	0.148
10	0	0	0.148
11	1	1	0.296
12	-1	0	0.123
13	1	0	0.168

Screening for the most significant medium components by Plackett-Burman design

Plackett-Burman design was employed to screen most significant component of basal medium affecting cellulase production[9]. Five factors were considered under study to evaluate

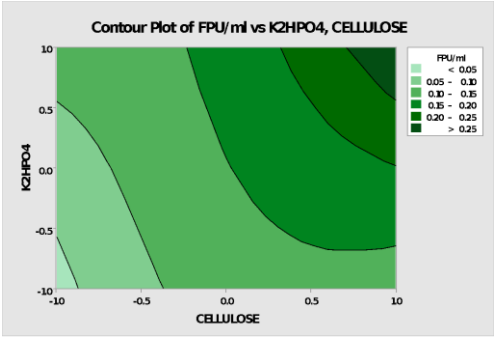

Figure 2: Contour Plot of enzyme production of isolate C2 showing effect of K_2HPO4 and Cellulose

Table 8: Analysis of variance (ANOVA) for the quadratic model

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	0.038724	0. 038724	0. 007745	27.44	0.000
Linear	2	0. 036224	0. 036224	0. 018112	64.17	0.000
Square	2	0. 000098	0. 000098	0. 000049	0.17	0.844
Interaction	1	0. 002401	0. 002401	0. 002401	8.51	0.022
Residual Error	7	0. 001976	0. 001976	0. 000282		
Lack-of-Fit	3	0. 001976	0. 001976	0. 000659		
Pure Error	4	0. 000000	0. 000000	0. 000000		
Total	12	0. 040699				

R-Sq = 95.15% R-Sq(pred) = 52.22% R-Sq(adj) = 91.68%

whether they significantly affect the cellulase production. Each factor was examined at two levels: +1 as the high and -1 as the low level (Table 1). Plackett-Burman experimental design is based on the first-order polynomial model:

$$Y = \beta 0 + \beta iXi$$

Where, Y is the response (enzyme activity), β0 is the model intercept, βi is the linear coefficient, and Xi is the level of the independent variable. This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response. From the regression analysis the variables, which were significant at or above 95% level (p-value <0.05), were considered to have greater impact on cellulase activity and were further optimized by central composite design.

Optimization of significant variable using response surface methodology

Response Surface Methodology using Central Composite Design was used to further optimize the variables found to have significant impact on cellulase production from Plackett-Burman design[10]. A central composite design consists of a "cube" portion made up of the design points from a 2 factorial or 2 fractional factorial design; 2K axial or "star" points, and center points (where K is the number of factors). The regression analysis is performed to estimate the response function as second-order polynomial:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i} \sum_{j} \beta_{ij} X_i X_j,$$

Where, β_0 is the interception coefficient, βi is the coefficient for the linear effect, βii is the coefficient for the quadratic effect, βijij is the coefficient of the interaction effect, and XiXj are input variables that influences the response variable Y. In order to visualize relationship between experimental variables and responses contour plot is generated.

RESULT AND DISCUSSION

Four cellulase secreting bacterial isolates were obtained from the paddy soil. On the basis of morphological and biochemical characters (Table 2), the bacterial isolates C2, BA, T1 and RC were identified as *Pseudomonas*, *Bacillus, Streptococcus* and *Micrococcus,* respectively. Similar bacterial cellulase producing bacteria have been isolated by many researchers[11-14].

By observing zone of clearance around each colony after staining with congo red solution it was found that isolate C2 (qualitative estimation) was giving higher cellulase activity as compared to BA, T1, RC (Table 3). This was further confirmed by filter paper assay (quantitative estimation) (Table 4).

16S ribosomal DNA (rDNA) sequencing identified isolate C2 as *Pseudomonas balearica*DSM 6083with 99% sequence homology. *Pseudomonas balearica*is closely related to *Pseudomonas stutzeri*sharing many basic phenotypic traitswith itand on the basis of 16S rRNA analysis *Pseudomonas balearica*is placed in *Pseudomonas stutzeri*group[15].

Five medium components (variables) i.e., $(NH_4)_2SO_4$, Cellulose, K_2HPO_4, $CaCl_2.2H_20$, pH were screened according to Plackett-Burman design to determine significant effect of each variable on the production of enzyme cellulase. The data in Table 5 indicated that there was a wide variation of cellulase activity from 0.06 FPU/mL to 0.245 FPU/mL in twelve trials. This variation reflected the significance of variables on the enzyme activity. From the derived regression equation for the optimization of medium components indicated that the cellulase activity (Y) is a function of the concentration of Cellulose (X_2), K_2HPO_4 (X_3). The

first-order polynomial equation derived is as follow:

$$Y = 0.143 + 0.0140\ X_1 + 0.0582\ X_2 + 0.0317\ X_3 + 0.00933\ X_4 - 0.00100\ X_5$$

Where, Y= FPU/ml, X_1 = $(NH_4)_2SO_4$, X_2 = Cellulose, X_3 = K_2HPO_4, X_4 = $CaCl_2.2H_20$ and X_5 = pH

The result of first-order polynomial model Plackett-Burman design in tabulated in Table6. Generally, a large t-value associated with a low p-value (<0.05) of a variable indicates a high significance of the corresponding coefficient. In our case, Cellulose and K_2HPO_4 is observed to have positive effect on cellulase production with higher t-value and low p-value (<0.05) when compared to other components and thus was considered to have significant effect on cellulase production.

Furthermore, cellulose and K_2HPO_4 were chosen for further optimization, since these factors had the most significant effect on the cellulase activity. All other variables used in all the trials were kept to the median level. The different combination of cellulose and K_2HPO_4 were designed and their effect on cellulase activity was determined by central composite design of response surface methodology. Total of 13 experiments were at different combinations of the two factors and cellulase activity associate with these combinations is shown in Table 7. The result of response surface methodology in the form of ANOVA is given in Table 8. The derived second order regression equation indicated that cellulase activity is function of interaction of cellulose and K_2HPO_4 at high level which is presented in the following equation as follows:

$$Y = 0.145655 + 0.046833X_1 + 0.062000X_2 + 0.005707X_1^2 - 0.003793X_2^2 + 0.024500X_1 .X_2$$

Where, Y = FPU/ml, X_1 = K_2HPO_4, X_2 = Cellulose.

To test the fit of the model equation, the regression-based determination coefficient R^2 was evaluated. The nearer the value of R^2 to 1, the stronger the model is and would explain better for variability of experimental values to the predicted values. The model under study presented a value of R^2 = 0.9515 explaining 95.15% of the variability in the response was attributed to the given independent variables. The ANOVA for quadratic regression model exhibited significant model [(Pmodel>F) = 0.0001]. The computed F-value was observed to be much higher than tabulated F-value [(F_{model} = 27.44) > ($F_{0.01}$ (5,7) = 7.460)] thus, indicating significant model terms. Contour graph was plotted which showed elliptical nature which indicates significant interaction between the corresponding variables (Figure 2).

The above result suggests the importance of potassium, phosphorus and carbon source in production of enzyme cellulase from bacterial species. While increasing cellulose concentration in media is known to increase cellulase production using both fungal and bacterial culture, importance of K_2HPO_4 remains to be established. Studies using cellulase producing fungal isolates have shown importance of K_2HPO_4 in cellulase production. Elimination of K_2HPO_4 from culture condition has been observed to reduced cellulase production using *Trichoderma reesei*[16], while incorporating K_2HPO_4in culture medium along with other essential components has been shown to increase cellulase production by several times using *Clostridium thermocopriae*[17] and *Trichoderma reesei*[18]. With bacterial isolates K_2HPO_4being component of media composition, studies by Deka *et al*[18], Gururaj[19]and Shankar *et al*[20]show no significant impact of K_2HPO_4on cellulase production. The present study using *Pseudomonasbalearica* as a highest cellulase producing bacterial isolate indicates the importance of combine effect of $K_2HPO_4$and cellulose on cellulase production, where increasing concentration of $K_2HPO_4$and cellulose significantly increase the enzyme production two fold.

The present study has shown that *Pseudomonas balearica* is a potential cellulase producing bacterial isolate.The yield of cellulase can be enhanced by increasing the concentration of cellulose and K_2HPO_4, indicating their importance in media and reliability of model for enhancing cellulase production using *Pseudomonas balearica*.

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