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Statistical *In vitro* Model for Upscaling Biofilm of *Chroococcidiopsis cubana* by Media Optimization and its Protocol for DNA Extraction

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ABSTRACT

Manual process for the optimization of different salts for growth of bacteria is labour intensive work and has low precision. In this study, we had used design expert software for biofilm yield optimization of a strain of *Chroococcidiopsis cubana*. *C. cubana* was exposed on outer surface of the monuments due to adverse environmental conditions and formed the blackish biofilm on it. This biofilm was grown in culture media and their DNA was extracted for strain confirmation. Strain was confirmed by 16s rRNA gene sequence using sanger sequencing. The response surface method was used to optimize the concentration of two main components $NaNO_3$ and K_2HPO_4 among various salts of BG 11 media. RSM was studied by ANOVA coefficient estimation using F – test with very low probability value. The obtained goodness of fit was significant (R^2 = 0.99). Estimation of coefficient was used for calculation of t and p – values and decided their significancy. The model having p value less than 0.05 was considered for optimization. Desirability of optimization was resolved from contour plot having concentration of $NaNO_3$ = 13 and K_2HPO_4 = 6.50 for optimum biofilm yield. Using these parameters, the *in vitro* model of *Chroococcidiopsis cubana* resulted in a yield of 20 g/l biofilm in 10 days.

KEY WORDS: ANOVA, ENDOLITHIC BIOFILM, RESPONSE SURFACE METHOD, 16S RRNA GENE SEQUENCING.

INTRODUCTION

Biofilm is defined as sessile microorganisms growing on solid surface and embedded in matrix of extracellular polymeric substances (Garrett et al., 2008). These microorganisms include several bacteria like *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* etc as well as some blue green algae like *Nostoc* sp., *Phormidium* sp., *Microspora* sp., *Chroococcidiopsis* sp. etc (Hancock 2013; Ljaljevic-Grbic et al., 2010; Miller et al., 2009). *Chroococcidiopsis* sp., a blue-green alga, has desiccation tolerance due to the thick polysaccharide sheath on the outer surface of the cells (Knowles and Castenholz, 2008). In some species such sheaths have the ability to self-recognise the surfaces. Due to this, cells auto-aggregate mostly at the bottom of the broth culture flask. Hence, exopolysaccharides are also known as auto-agglutinins. This is the first stage in the whole process of biofilm formation (Trunk et al., 2018).

The study of biofilm formation has importance in a broad scale of industrial application such as health and cosmetics products, food, water, paper mills, medical health and pharmaceutical (Molobela and Ilunga,

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2012). *In vitro* production of biofilm is a laborious and cumbersome task. Hence, it is necessary to optimise some components for enhanced biofilm yield through a reliable and successfully working statistical tool like response surface methodology (RSM) (Bratchell, 1989; Lundstedt et al, 1998; Mirhosseini and Tan, 2009; Khuri and Mukhopadhyay, 2010). Growth rates and yield of the species are dependent on the concentration level of $NaNO_3$ and K_2HPO_4 (Rippka et al., 1988).

In the present work, *Chroococcidiopsis* sp. was isolated from biofilm and obtained pure culture by streak plate method. Central composite design (CCD) of RSM has been applied to obtain the optimized concentration of two main salts components $NaNO_3$ and K_2HPO_4 of BG 11 media for in vitro biofilm yield. DNA was extracted from biofilm and was amplified using 16s rRNA primer for strain confirmation.

MATERIAL AND METHODS

Isolation of Microorganism: Surface biofilm of the endolithic *Chroococcidiopsis* sp. was collected during post monsoon period from the dome in the main building of Faculty of Arts, The M.S. University of Baroda, Vadodara, India. The biofilm was immersed in petri plate having sterile distilled water and kept for 24h. Clump of biofilm was segregated with the help of needle and isolated by streak plate method in agar (Rippka et al., 1988). Culture was maintained on agar plate and sub cultured every two weeks.

Growth medium: BG 11 medium as described by Rippka et al., (1988) having pH 7.2 was prepared as control. A 250 ml Erlenmeyer flask containing 100 ml of media was inoculated and incubated at 27 – 30 ^{0}C temperature in 2500 Lux white light for 14h and 2h rotatory shaker at 100 RPM for 10 days. The biofilm was separated by centrifuging at 4000 RPM for 10 mins. Supernatant was discarded and yield of biofilm was measured. The same procedure was repeated for all the experimental flasks.

Experimental design for optimization: CCD was selected to optimize the concentration of $NaNO_3$ and K_2HPO_4 for enhancing the yield of *Chroococcidiopsis* sp. The experiment was designed by the Design Expert 7.0.2 (stat-ease, USA). The range of the variables of $NaNO_3$ and K_2HPO_4 were selected from preliminary study. The lowest and highest values of the variables have mentioned in table 1. Central composite design with 22 factorial gives 4 star points and axial points having 5 replicate at the centre as central point leading to 13 runs were engaged for the optimization of the concentration (Bradley, 2007). For statistical calculation, the variables were coded by equation 1:

$$x_i = {}^{x_1 - x_0}/_{\Delta x} \quad (i = 1, 2, .. q) \qquad (1)$$

x_i is the dimensionless value of variable 1, x_1 is real value of variable 1, x0 is centre point value of x_1 and x is step change.

CCD is second order level design. In this design, the dependent variable gives information about interaction between factors in their relation to the dependent variable. 2nd order polynomial equation 2:

$$\hat{Y}_i = \beta_0 + \sum_{i=1}^{2} \beta_i x_i + \sum_{i=1}^{2} \beta_{ii} x_i^2 + \sum_{i,j=1}^{2} \beta_{ij} x_i x_j \qquad (2)$$

$\hat{Y}_i$ denoted predicted response, xi and xj input variables, β_0 intercept term, β_i linear effect, β_{ii} squared effect and β_{ij} interaction term. Design expert software was performed for solving the regression equation and analysis of the response surface contour plot (Zheng et al., 2008).

Table 1. Range and Levels of variables

Variables		Range and Levels				
		-2	-1	0	1	2
X1	K_2HPO_4 (g/l)	5	7	9	11	13
X2	$NaNO_3$ (g/l)	10	13	16	19	22

Identification of strain by 16s rRNA gene sequences: DNA was isolated from biofilm cells using the protocol followed by Tillett and Neilan, (2000) with some minor modifications. 50 µl of TER buffer (10 Mm Tris HCl, pH 7.4; 1 mM EDTA pH 8; 100 µg/ml RNase A) was added into tube containing cell pellets. 750 µl freshly prepared XS buffer (1% Potassium ethyl Xanthogenate, 100 mM Tris HCl pH 7.4, 20 mM EDTA pH 8; 1% SDS; 800 mM ammonium acetate) and 10 µl RNase A were added and mixed by pipetting. After proper mixing, the solution tube was incubated at 70 ^{0}C for 60 min. After incubation, tube was kept on ice for 30 min. Cell debris was removed by centrifugation at 14,000 rpm for 10 min. DNA was precipitated in supernatant by adding chilled ethanol. The DNA pellet was obtained by centrifuging at 12,000 rpm for 10 min. DNA pellet was washed with 70% ethanol and air dried. Then, TE buffer was added (Tris HCl 10 mM and EDTA 1 mM) to dissolve the pellet.

Amplification process was carried out by primers DNM1 F 5'GAAAGCCTGACGGAGCAATA 3' and DNM1 R 5' CGGGACTTAACCCAACATCT 3'. PCR reaction mixture (20 µl) was prepared by mixing 10 µl Dream Taq master mix, 0.5 µl forward and reverse primers from 1:10 stock, 1 µl template DNA and 8 µl MilliQ water. 35 PCR cycles were performed, initial denaturation at 94 ^{o}C for 4mins, denaturation at 94^o C for 30 secs, elongation at 55 ^{o}C for 45 secs, extension at 72 ^{o}C for 2 min and final extension 7mins at 72 ^{o}C. DNA purification was carried out by GeneJET Gel Extraction (Thermo scientific). The purified DNA amplicon was subjected to Sanger sequencing. The identity of the strain was confirmed by BLAST analysis of obtained sequences with NCBI database (https://blast.ncbi.nlm.nih.gov).

RESULT AND DISCUSSION

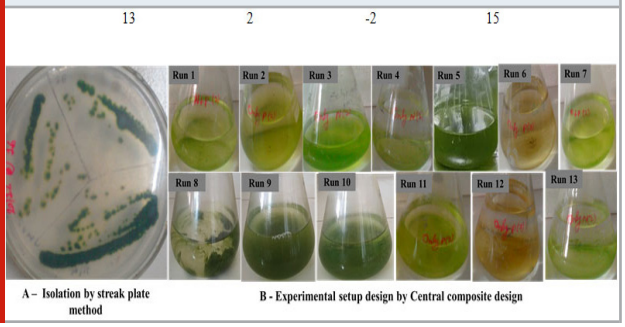
Isolation, Biofilm yield and Strain confirmation of

***Chroococcidiopsis* sp. by 16s rRNA gene sequences:** *Chroococcidiopsis* sp. isolation was done through streak plate method and individual colonies were observed on agar plates (Fig 1). The BLAST analysis of sequence data revealed the identity of the isolated organism as *C. cubana*. The sequence was deposited in NCBI GenBank data base with accession number MN950976. The biofilm yield of single colony grown in control and experimental flasks for 10 days were recorded (Table 2). Except run 4, the biofilm yield of all experimental flasks were significantly higher than of control.

Table 2. Experimental design with experimental yield (at 10thday)

Runs	X_1 (B)	X_2 (A)	Biofilm yield(g/l)
1	-2	0	10
2	2	0	18
3	0	0	20
4	-1	1	05
5	0	0	20
6	0	2	10
7	-1	-1	16
8	0	0	20
9	0	0	20
10	0	0	20
11	1	1	19
12	1	-1	12
13	2	-2	15

Figure 1: Isolation and experimental set up of CCD in lab



ANOVA for Response surface model: The effect of two independent variables were studied on one dependent response. The results of the second order response surface model for increase yield based on analysis of variance (ANOVA) is given in table 3. Regression equation of yield having empirical function of test variables in coded unit is shown in equation 3:

$$\hat{Y}_i = 20 + 2.66X_1 - 1.38X_2 + 4.50\,X_1X_2 - 3.06\,X_1^2 - 3.81\,X_2^2$$

Where $\hat{Y}_i$ is the predicted yield, X1 is K_2HPO_4 and X_2 is $NaNO_3$.

ANOVA uses F – statistic to test the equality of means. F – test had very low probability value [(Prob>F) < 0.0001]. Hence, this model was highly significant for this experiment (Zheng et al., 2008). Goodness of fit of model was determined by determination coefficient (R^2). Determination coefficient, (R^2) value was 0.99 owing to which 99 % sample variation was qualified the variables, only 1% of the total variance could not be explained by this model. Adjusted determination coefficient also had similar value. Hence, the significance of the model was confirmed at required confidence level. Standard deviation (SD) was 0.47. Smaller the value of standard deviation, more precise the data. Because larger value of SD increases the acceptable range within the deviation. Coefficient of variation or pure error was 2.95%. Pure error lower than 5% considered to be an acceptable range of error (Box et al., 1978). Moreover, adequate precision had a value of 48.72. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable (Ahmadkhaniha et al., 2015). Because the model has a high signal it could be a so it's more reliable for optimization.

Table 3. ANOVA for response surface quadratic model

Source	Sum of Square	Degree of freedom	Mean Square	F – value	Probe>F (p value)
Model	300.79	5	60.16	277.10	<0.0001
Residual	1.52	7	0.22		
Lack of fit	1.52	3	0.51		
Pure error	0.00	4	0.00		
Total	302.31	12			

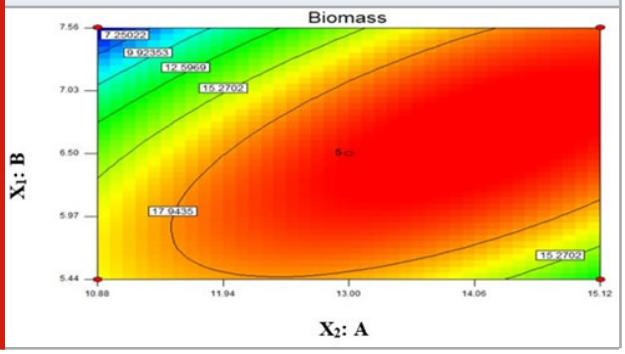
Standard deviation. = 0.47; Coefficient of variation = 2.95%; Mean = 15.77; R^2 = 0.99; Adj. R^2 = 0.99; Pred. R^2 = 0.96

Coefficient estimation for model: The significance of coefficient was determined by t value and p value. Both these values were computed and mentioned in Table 4. Higher the value of t – test and smaller the p value, the results are more significant (Lee and Wang, 1997). Our model intercept had large t value suggesting this model is highly significant. In model, variable X_1 had small p value as compare to variable X_2. Hence variable A was more significant than variable B in this model. The 2D contour plot represents graphical view of the regression equation (Wang et al., 2007). It had shown the effect of both the variables on yield of biofilm. Based on centre point of the contour plot, the obtained value of variable A ($NaNO_3$) and variable B (K_2HPO_4) were 13 and 6.5 respectively (Fig 2). Hence, both these values were found to be optimal for biofilm yield in 10 days. For optimization, the Design Expert 7.0.2 software suggested 0.95 and 1 as the two desirability standards. In current study, we proceeded for desirability 1 obtained value for A ($NaNO_3$) = 13 from lower limit 10 and upper limit 15 and value for B (K_2HPO_4) = 6.5 from lower limit 5 and upper limit 8. It was given 20 g/l yield in 10 days.

Table 4. Coefficient estimation of model

Factor	Coefficient	Std. error	Computed t – value	Computed p - value
Intercept	20	0.21	95.23	0.003
A	2.66	0.16	16.625	0.019
B	-1.38	0.16	-8.625	0.036
AB	4.50	0.23	19.57	0.016
A2	-3.06	0.18	-17.0	0.018
B2	-3.81	0.18	-21.0	0.015

Figure 2: 2D contour plot for *in vitro* biofilm yield of *Chroococcidiopsis cubana*



CONCLUSION

From the above results, it was concluded that variables A ($NaNO_3$ = 13 g/l) and B (K_2HPO_4 = 6.5 g/l) were giving similar results in experimental design data and for desirability of optimization. Hence, these values of the variables were used for upscaling the biofilm yield. The optimized model was successfully employed for *in vitro* cyanobacterial biofilm yield of *Chroococcidiopsis cubana*.

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Conflict of interest: There is no conflict of interest.

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