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RESEARCH ARTICLE

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Statistical optimization of critical medium components for biosurfactant production by *Bacillus subtilis*

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ABSTRACT

In the present study optimization of the critical medium components for biosurfactant production by *Bacillus subtilis* using statistical experimental design was studied. Response surface methodology (RSM) was employed to determine the optimal level of the four medium variables (sucrose, yeast extract, $FeSO_47H_2O$ and KH_2PO_4). Central composite design (CCD) of RSM was applied to study the four variables at five levels and biosurfactant concentration was measured as response. Regression coefficients were calculated by regression analysis and the model equation was determined. R^2 value for biosurfactant (g/L) was calculated as 0.835 and it indicates that the model was well fitted with the experimental results. Surface plots were made and the maximum biosurfactant production (10 g/L) was predicted at the optimized values of sucrose 70 g/L, yeast extract 5 g/L, $FeSO_47H_2O$ 0.055 g/L and KH_2PO_4 0.15 g/L. The obtained mathematical model was verified by performing the experiment with the predicted optimized values and the yield of bio-surfactant was found to be 9.78 g/L. Validation of the predicted model was fitted 97.8% with the experimental results conducted at the optimum conditions. Results of this statistical analysis showed that sucrose and yeast extract had found significant medium components for biosurfactant production.

Key words: *Bacillus subtilis*, biosurfactant, central composite design, response surface methodology

Introduction

Surfactants are usually organic compounds that are amphiphilic in nature containing both hydrophobic and hydrophilic groups and it is used to lower the interfacial tension between two liquids (Desai & Banat, 1997; Mulligan, 2005). Surfactants may act as detergents, wetting, emulsifiers, foaming agents, and dispersants (Singh et al., 2007). Biosurfactants derived from microorganisms are found to be better alternate for the synthetic surfactants. They are complex molecules that can be classified based on different structures that include lipopeptides, glycolipids, polysaccharide-protein complexes, fatty acids and phospholipids (Neto et al., 2008).The major advantages of using biosurfactants are biodegradability, low toxicity and can be produced from renewable and cheaper substrates (Mohan et al., 2006). Biosurfactants are mainly used for bioremediation to treat hydrocarbon polluted sites and also for oil recovery. They are also used as one of the ingredient in formulation of pesticides, healthcare and cosmetics, pulp and paper and food industries (Hickey et al., 2007; Ghojavand et al., 2008). Microorganisms such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilis*, and *Pseudomonas putida* are capable of producing biosurfactant (Priya et al., 2005; Sanket et al., 2008; Guerra de Oliveira & Garcia-Cruz, 2013; Abbasi et al., 2013). Lipopeptides derived from *Bacillus subtilis* are particularly interesting because of their high surface activity and therapeutic potential (Besson & Michel 1992; Sandrin et al., 1990).

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Optimization of medium and fermentation conditions is a critical step in bioprocess development and it involves several factors (Chakravarthi & Sahai, 2002). One factor at a time optimization is accepted method, but it has many disadvantages like more experimental runs and time (Haaland, 1989). Response surface methods (RSM) is a collection of statistical tools to design and analyses the experiments focused on optimization (Myers & Montgomery, 1995). RSM is successfully employed to determine the optimal conditions of the selected variables involved in the process (Tamilarasan et al., 2012; Khayati et al., 2013; Abbasi et al., 2013). The main advantage of using RSM is to evaluate the interaction effect of the variables under study with the help of response surface plots generated by the software.

The objective of this study is to determine the optimal levels of the medium components for biosurfactant production from *Bacillus subtilis* by response surface methodology.

Materials and Methods

Microorganism

The microorganism *Bacillus subtilis* MTCC 1427 used in this study was obtained from Institute of Microbial Type Culture Collection Chandigarh, India. The culture was maintained in LB agar plates incubated at 37°C and subcultured at regular intervals. Inoculum was prepared by transferring a loopful of culture to 100 mL of sterilized Luria Bertani (LB) broth and kept in rotary shaker incubator at 180 rpm at 37°C for 24 h. All the chemicals used in the study are of analytical grade and procured from Hi-Media, India.

Fermentation conditions

Two percent of the seed culture was inoculated in the production media containing (g/L): glucose - 40, yeast extract - 0.5, KH_2PO_4 - 10.5, $FeSO_4$ $7H_2O$ - 0.05, $NaHCO_4$ - 1.5 and $MgSO_4.7H_2O$ - 0.05. The initial pH of the medium was adjusted to 8.0 (Kim et al., 1997). All fermentations were carried out at 37°C in shaker flask held on rotary platform shaker at 180 rpm. For statistical optimization experiments, 100 mL of medium was prepared in 250 mL conical flask according to the central composite design given in Table.1.

Biosurfactant precipitation

1.5 mL of fermented broth was collected in 2 mL Eppendorf tube and centrifuged at 10000 rpm for 10 minutes. After centrifugation, supernatant was used for the extraction of biosurfactant. 6N HCl was added in the Eppendorf containing supernatant and kept it for overnight incubation. Then, the sample was centrifuged at 6000 rpm for 10 min and the precipitated biosurfactant was collected in a form of pellet. The precipitated biosurfactant was dried in hot air oven at 80°C for overnight and weight of the crude biosurfactant was determined.

Experimental design

Four medium variables (sucrose, yeast extract, $FeSO_4$ $7H_2O$ and KH_2PO_4) were selected for RSM optimization studies based on preliminary screening studies. The range of level of four variables was given in Table 1. Thirty experiments were carried out according to central composite design (CCD) shown in Table 2.The relation between the variables and the response is generally represent by the second order polynomial equation (Eqn. 1).

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{44}X_4^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{14}X_1X_4+\beta_{23}X_2X_3+\beta_{24}X_2X_4+\beta_{34}X_3X_4 \quad (1)$$

Factors (g/L)	Symbol	+2	+1	0	-1	-2
Sucrose	A	70	60	50	40	30
Yeast extract	B	7	6	5	4	3
$FeSO_47H_2O$	C	0.065	0.06	0.055	0.05	0.045
KH_2PO_4	D	0.25	0.2	0.15	0.1	0.05

Table 1. *Range of variable levels for RSM experiment*

Results and Discussion

Response surface optimization

Statistical optimization for biosurfactant production was carried out according to central composite design of RSM using Design expert software (Trial version). The response, biosurfactant concentration was estimated for thirty experiments and represented in Table.2.The response data were subjected to regression analysis to estimate regression coefficient. The estimated coefficients were presented in Table 3 and a second order polynomial equation (Eqn. 2) for biosurfactant production was constructed by using the coefficients.

$$Y_{\text{Surfactant (g/L)}} = 7.556+0.639\ A\ -1.194\ B\ +0.306\ C-0.028\ D+0.257\ A^2-0.493\ B^2-0.160\ C^2+0.257\ D^2+0.042\ A*B+0.125\ A*C+0.042\ A*D-0.208\ B*C-0.125\ B*D+0.292\ C*D \quad ...(2)$$

The adequacy of the model was checked using analysis of variance (ANOVA) and the results were shown in Table 3. The Model F-value of 5.42 implies the model is significant. High value of F-test for regression indicating that the model is fit well and can adequately explain the variation observed in biosurfactant concentration with the designed levels of variables. Probability value ($p<0.05$) is usually used to check the statistical significance of the parameters. Results represented in Table 3 explained that the individual effect of glucose (A), yeast extract (B) and square effect of yeast extract (B^2) were found significant in the production of biosurfactant. R^2 value was observed as 0.835 and this value shows that the model was fitted for 83.5% of biosurfactant production. These results showed that the model chosen can satisfactorily explain the linear effects and square effects of the variables selected for the biosurfactant production.

Table 2. *Central composite design matrix with the experimental and predicted values of biosurfactant produced by B. subtilis*

Run order	Medium components				Surfactant (g/L)	
	A	B	C	D	Experimental	Predicted
1	40	4	0.05	0.1	7.33	7.86
2	60	4	0.05	0.1	9.33	8.72
3	40	6	0.05	0.1	6.00	6.06
4	60	6	0.05	0.1	7.33	7.08
5	40	4	0.06	0.1	8.00	8.06
6	60	4	0.06	0.1	9.33	9.42
7	40	6	0.06	0.1	5.33	5.42
8	60	6	0.06	0.1	6.00	6.95
9	40	4	0.05	0.2	7.33	7.39
10	60	4	0.05	0.2	8.67	8.42
11	40	6	0.05	0.2	5.33	5.08
12	60	6	0.05	0.2	5.33	6.28
13	40	4	0.06	0.2	8.67	8.75
14	60	4	0.06	0.2	9.33	10.28
15	40	6	0.06	0.2	4.00	5.61
16	60	6	0.06	0.2	8.00	7.31
17	30	5	0.055	0.15	8.00	7.31
18	70	5	0.055	0.15	10.00	9.86
19	50	3	0.055	0.15	8.00	7.97
20	50	7	0.055	0.15	4.00	3.20
21	50	5	0.045	0.15	6.00	6.30
22	50	5	0.065	0.15	8.67	7.53
23	50	5	0.055	0.05	8.67	8.64
24	50	5	0.055	0.25	9.33	8.53
25	50	5	0.055	0.15	8.00	7.56
26	50	5	0.055	0.15	7.33	7.56
27	50	5	0.055	0.15	6.67	7.56
28	50	5	0.055	0.15	7.33	7.56
29	50	5	0.055	0.15	7.33	7.56
30	50	5	0.055	0.15	8.67	7.56

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Table 3. *ANOVA statistics for biosurfactant production by Bacillus subtilis*

Factors	**Sum of squares**	**Degrees of freedom**	**Mean square**	**F-value**	**p-value**	**Significance**
Model	61.68	14	4.41	5.41	0.0012	Significant
A-Sucrose	9.79	1	9.79	12.03	0.0034	Significant
B-Yeast extract	34.25	1	34.25	42.09	< 0.0001	Significant
C-$FeSO_4$ $7H_2O$	2.25	1	2.25	2.77	0.1170	
D-KH_2PO_4	0.019	1	0.019	0.023	0.882	
AB	0.028	1	0.028	0.034	0.8558	
AC	0.25	1	0.25	0.31	0.5894	
AD	0.028	1	0.028	0.034	0.8552	
BC	0.69	1	0.69	0.85	0.3702	
BD	0.25	1	0.25	0.31	0.5857	
CD	1.36	1	1.36	1.68	0.2151	
A^2	1.81	1	1.81	2.23	0.1567	
B^2	6.67	1	6.67	8.21	0.0118	Significant
C^2	0.70	1	0.7	0.86	0.3691	
D^2	1.81	1	1.81	2.23	0.1567	
Residual	12.20	15	0.81			
Lack of Fit	9.83	10	0.98	2.07	0.2187	Not significant
Pure Error	2.38	5	0.48			
Total	73.88	29				

Surface plots

Surface plots are used to study the interaction between the variables graphically (Bas & Boyaci, 2007).

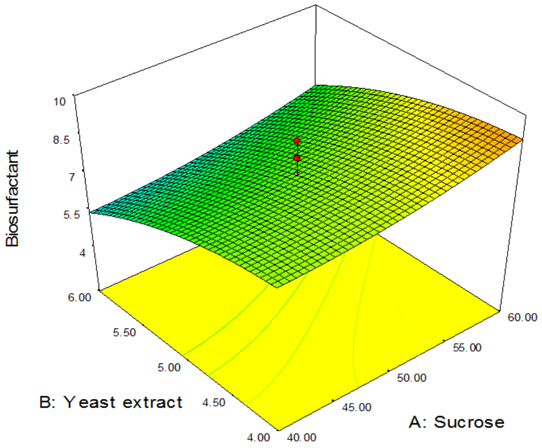

Figure 1. *3D surface plots showing the mutual effect between pair of variables sucrose (A) and yeast extract (B) on biosurfactant production.*

Figure 1 represent the combined effect of sucrose and yeast extract and maximum biosurfactant production (9 g/L) was observed at low level of yeast extract (4 g/L). There was a significant increase in the product concentration when sucrose concentration increased from 40 g/L to 60 g/L. Makkar & Cameotra (2002) reported that sucrose was most suitable carbon source for biosurfactant production by *B. subtilis* among the other carbohydrates studied. Several researchers concluded that presence of yeast extract in low concentration increases the biosurfactant synthesis (Gandhimathi et al., 2009). Supplementation of yeast extract (4 g/L) in the production medium was sufficient for enhancing biosurfactant production as the amino acids are required for the formation of the lipopeptide biosurfactant by *Bacillus* species. Casas & Garcia-Ochoa (1999) also reported that low level of yeast extract enhances the biosurfactant production. Figure 2 demonstrated that increase in both sucrose and $FeSO_47H_2O$ improves the biosurfactant production. It was observed that the $FeSO_47H_2O$ in the medium plays a significant role in productivity. When sucrose concentration increases from low to high level, the productivity was also increased whereas increase in concentration of KH_2PO_4 does not shown any impact in the biosurfactant production (Figure 3).

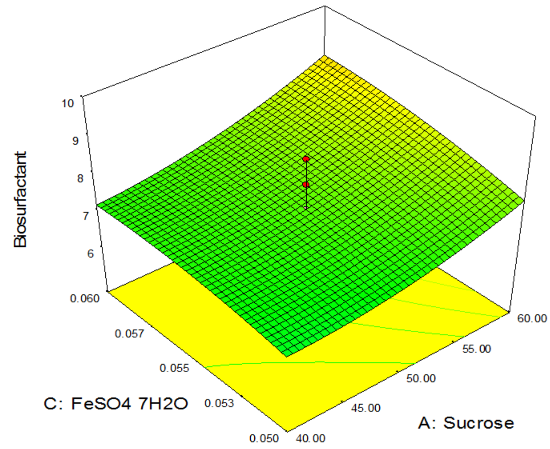

Figure 2. *3D surface plots showing the mutual effect between pair of variables sucrose (A) and* $FeSO_47H_2O$ *(C) on biosurfactant production.*

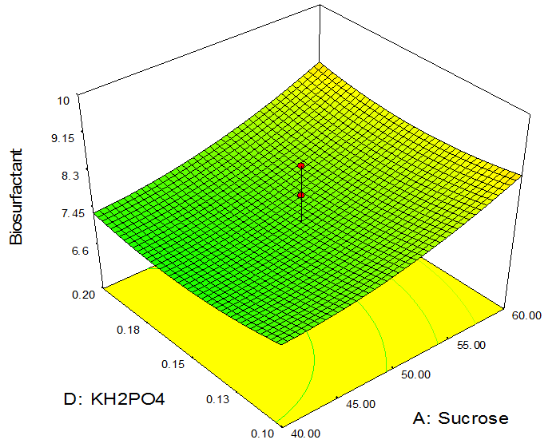

Figure 3. *3D surface plots showing the mutual effect between pair of variables sucrose (A) and* KH_2PO_4 *(D) on biosurfactant production.*

From Figure 4, it was observed that the production of biosurfactant decreased when the yeast extract increased from low to high level stating that 4 g/L is sufficient for optimum productivity, whereas the productivity increased when the concentration of $FeSO_47H_2O$ increased from low to high level.

In Figure 5, biosurfactant production was decreased when yeast extract concentration increased from low to high whereas static condition is prevailed in KH_2PO_4 indicating the contribution for biosurfactant production by KH_2PO_4 is minimum. It is observed that the productivity of biosurfactant increased when the concentration of ferrous sulphate increased from low to high (Figure 6).

Point prediction tool of Design Expert software was used to determine the optimal level of each variable in the process. The maximum biosurfactant concentration (10 g/L) was predicted by the software at optimal level of Sucrose - 70 g/L, yeast extract - 5 g/L, $FeSO_47H_2O$ - 0.055 g/L and KH_2PO_4 - 0.15 g/L.

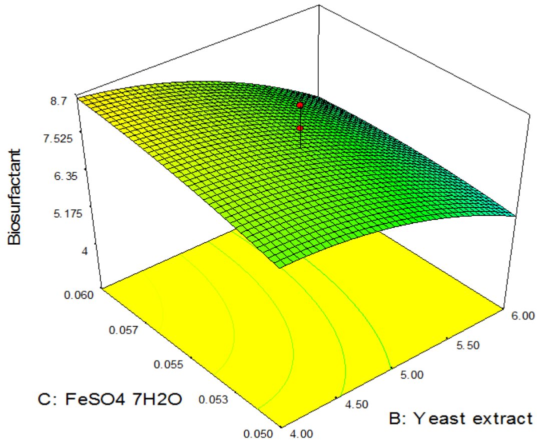

Figure 4. *3D surface plots showing the mutual effect between pair of variables yeast extract (B) and* $FeSO_47H_2O$ *(C) on biosurfactant production.*

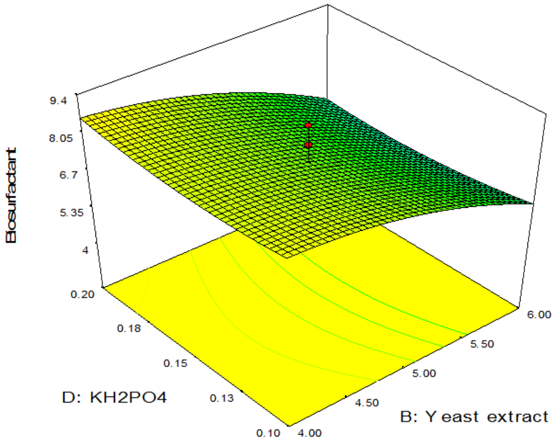

Figure 5. *3D surface plots showing the mutual effect between pair of variables yeast extract (B) and* KH_2PO_4 *(D) on biosurfactant production.*

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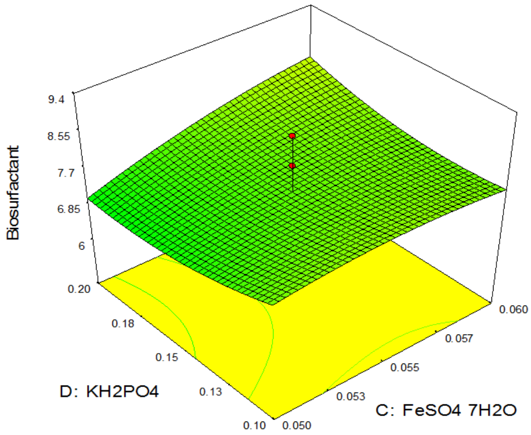

Figure 6. *3D surface plots showing the mutual effect between pair of variables* $FeSO_4\ 7H_2O$ *(C) and* KH_2PO_4 *(D) on biosurfactant production.*

Model validation

To check the accuracy of the predicted model, experiments were carried out at the predicted optimal concentration of sucrose - 70 g/L, yeast extract - 5 g/L, $FeSO_4\ 7H_2O$ - 0.055 g/L and KH_2PO_4 - 0.15 g/L.

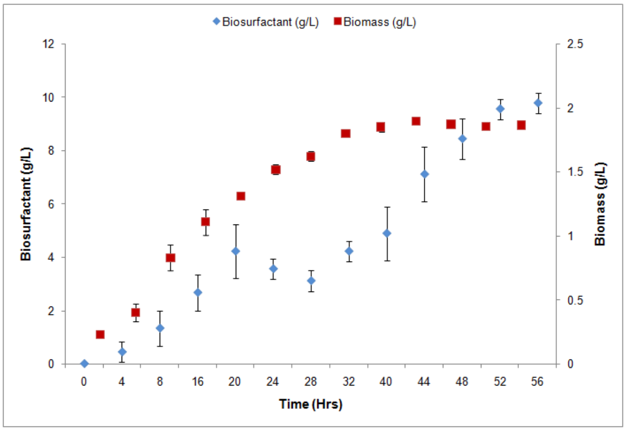

Figure 7. *Time course profile of biosurfactant and biomass production by B. subtilis using predicted optimal level of the selected medium components in validation experiment.*

In validation experiment, maximum biosurfactant concentration of 9.78 g/L was obtained. The time course profile of biosurfactant and biomass production by *B. subtilis* at predicted optimal level of the medium components is shown in Figure 7. The validation result indicates that predicted model was fitted 97.8% with the experimental results.

Conclusion

Response surface methodology was successfully applied to optimize the four media components to enhance the biosurfactant production. Four variables (sucrose, yeast extract, $FeSO_47H_2O$ and KH_2PO_4) were optimized according to central composite design of RSM. Surface plots were made and the optimized values obtained for the maximum production of biosurfactant were sucrose - 70 g/L, yeast extract - 5 g/L, ferrous sulphate - 0.055 g/L and KH_2PO_4 - 0.15g/L. Validation of the experiment was performed and it indicates that the model was well fitted with the experimental results. Application of RSM illuminates the optimal levels for enhanced production of biosurfactant with less experimental runs and interaction effects of the variables.

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