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Statistical Optimization of Medium Components for the Production of Biosurfactant by *Bacillus licheniformis* K51

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Abstract The nutritional medium requirement for biosurfactant production by *Bacillus licheniformis* K51 was optimized. The important medium components, identified by the initial screening method of Plackett-Burman, were H_3PO_4, $CaCl_2$, H_3BO_3, and Na-EDTA. Box-Behnken response surface methodology was applied to further optimize biosurfactant production. The optimal concentrations for higher production of biosurfactants were (g/l): glucose, 1.1; $NaNO_3$, 4.4; $MgSO_4 \cdot 7H_2O$, 0.8; KCl, 0.4; $CaCl_2$, 0.27; H_3PO_4, 1.0 ml/l; and trace elements (mg/l): H_3BO_3, 0.25; $CuSO_4$, 0.6; $MnSO_4$, 2.2; Na_2MoO_4, 0.5; $ZnSO_4$, 6.0; $FeSO_4$, 8.0; $CoCl_2$, 1.0; and Na-EDTA, 30.0. Using this statistical optimization method, the relative biosurfactant yield as critical micelle dilution (CMD) was increased from 10× to 105×, which is ten times higher than the non-optimized rich medium.

Key words: *Bacillus licheniformis*, biosurfactant, Plackett-burman, Box-Behnken, critical micelle dilution (CMD)

The unique properties of biosurfactants allow their use and possible replacement of chemically synthesized surfactants in a great number of industrial operations [6, 8]. Virtually all surfactants are chemically synthesized. Nevertheless, in recent years, much attention has been directed towards biosurfactants owing to their different advantages such as, lower toxicity, higher biodegradability, better environmental compatibility, higher foaming, high selectivity and specific activity at extreme temperatures, pH and salinity, and the ability to be synthesized from renewable feed stocks [6]. However, applications of these biomolecules are limited by their cost of production, which can be reduced by optimizing the concentrations of medium components for biosurfactant production.

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There are a large number of reports on the optimization of C and N sources on the classical method of medium optimization by changing one independent variable while fixing all the others at a fixed level. This is extremely time consuming and expensive for a large number of variables and requires a large number of experiments to determine optimum levels, which are unreliable. Optimizing all the affecting parameters by statistical experimental designs can eliminate these limitations of a single factor optimization process collectively by statistical experimental design using Plackett-Burman design and response surface methodology (RSM) [9]. Initial screening of the ingredients is done to understand the significance of their effect on the product formation and then a few better ingredients are selected for further optimization. Different types of statistical methods are available for such optimization experiments [15] including the nonstatistical self-optimization technique [7]. The Plackett-Burman design is a well-established and widely used statistical design technique for the screening of the medium components in shake flask [16]. Multifactorial designs will be difficult, as large numbers of variables are to be screened in terms of number of experiments (2k; where k is the total number of variables). Hence, two-level fractional factorial designs like Plackett-Burman will be of choice that screens the k variable in just k+1 experiment. Moreover, the design is orthogonal in nature and thus gives a pure effect of each variable not confounded with interactions among variables. The variables screened by Plackett-Burman design were further optimized in a 2^3 factorial Box-Behnken design methodology [3].

There are very few reports on the statistical optimization for the production of biosurfactants. This report attempts to formulate a suitable production medium using statistical optimization that can substantially increase the biosurfactant production by *Bacillus licheniformis* K51, using Plackett-Burman and Box-Behnken methods.

Table 1. Variables showing medium components used in Plackett-Burman design.

Variables	Medium components	+ Values (g/l)	- Values (g/l)
X_1	Glucose	20	2
X_2	$NaNO_3$	8	0.8
X_3	$MgSO_4 \cdot 7H_2O$	0.8	0.08
X_4	KCl	0.4	0.04
X_5	$CaCl_2$	0.3	0.03
X_6	H_3PO_4	1.0[a]	0.1[a]
X_7	H_3BO_3	2.5[b]	0.25[b]
X_8	$CuSO_4$	0.6[b]	0.06[b]
X_9	$MnSO_4$	4.0[b]	0.4[b]
X_{10}	Na_2MoO_4	1.0[b]	0.1[b]
X_{11}	$ZnSO_4$	6.0[b]	0.6[b]
X_{12}	$FeSO_4$	8.0[b]	0.8[b]
X_{13}	$CoCl_2$	1.0[b]	0.1[b]
X_{14}	Na-EDTA	300[b]	30[b]

[a]ml/l; [b]mg/l.

MATERIALS AND METHODS

Organisms and Culture Conditions

The biosurfactant-producing isolate was isolated in our laboratory from an oil well, in Gujarat, India. It was identified as *Bacillus licheniformis* K51 by morphological and biochemical tests and 16S rRNA gene sequencing. It was grown in Luria Bertani (LB) broth and maintained in 40% glycerol and stored below 0°C.

Inoculum and Media Preparation

LB was used for preparation of the inoculum. The culture was grown for 8–10 h at 45°C ($OD_{600\,nm}$ 0.8–0.9). The seed culture at 2% (v/v) was used as an inoculum into 50 ml of the glucose-based minimal production medium in a 250-ml Erlenmeyer flask. The requisite volume of the media constituents were pipetted out from their stock solutions of higher concentrations and were mixed together before sterilization. Glucose was sterilized separately and mixed with the rest of the medium before inoculation in all the experiments. The pH of the media were adjusted to 7.0 and sterilized by autoclaving at 15 psi for 15 min. Flasks were incubated on a temperature controlled shaker incubator at 160 rpm and 45°C for 72 h. The media components for biosurfactant production and their composition are given in Tables 1 and 2.

Analysis of Biosurfactant

Surface tension measurements were made with a surface tensiometer (Khushboo sci. co., India) employing the principles of Du-Nuoy's ring method. Biosurfactant yield was assayed by an indirect method, which involves measurement of surface tension of diluted broth samples from the Box-Behnken experiment. Relative surfactant concentrations were determined by serially diluted broth until the critical micelle concentration (CMC) was reached. The dilution at which the surface tension starts rising abruptly was denoted by CMD and was considered proportional

Table 2. Plackett-Burman design matrix of fourteen variables (X1-X14) and five dummy variables (D1-D5) along with the observed response (surface tension).

Run	Variables																			Surface tension (mN/m)
	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	D1	D2	D3	D4	D5	
1	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	32
2	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	30
3	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	54
4	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	30
5	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	30
6	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	30
7	-	-	-	+	+	-	+	+	-	-	+	+	+	+		+	-	+	-	36
8	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	30
9	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	60
10	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	64
11	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	37
12	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	43
13	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	42
14	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	39
15	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	47
16	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	30
17	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	58
18	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	31
19	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	33
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	63

to the amount of surfactant present in the original sample [5, 17].

Optimization Procedure

The optimization of medium constituents for biosurfactant production by *Bacillus licheniformis* K51 was carried out in two stages.

Identification of Important Nutrient Components. To find out the important medium components, a Plackett-Burman design was followed [16] as shown in Table 2. Total of 14 components [variables, k=14] were selected for the study, with each variable being represented at two levels, high (+) and low (-), and five dummy variables in 20 trials, as shown in Tables 1 and 2. The numbers of positive and negative signs per trial are (k+1)/2 and (k-1)/2, respectively. Each row represents a trial and each column represents an independent (assigned) or dummy (unassigned) variable. The effect of each variable was determined by the following equation:

$$E(x_i)=2(\Sigma M_i^+ - M_i^-)/N \quad (1)$$

where E (x_i) is the concentration effect of the tested variable. M_i^+ and M_i^- are the biosurfactant production from the trials where the variable (x_i) measured was present at high and low concentrations, respectively, and N is the number of trials (20). Experimental error was estimated by calculating the variance among the dummy variables as follows:

$$V_{eff}=\Sigma(E_d)^2/n \quad (2)$$

where V_{eff} is the variance of the concentration effect, E_d the concentration effect for the dummy variable, and n is the number of dummy variables. The standard error (SE) of the concentration effect was the square root of the variance of an effect and the significance level (p value) of each concentration effect was determined using the Student's t test:

$$t(x_i)=E(x_i)/SE \quad (3)$$

where E (x_i) is the effect of variable x_i.

Optimization of Screened Components. Response surface methodology was used to optimize the screened components for enhanced biosurfactant production using a Box-Behnken design [3]. The behavior of the system was explained by the following quadratic equation:

$$Y=\beta_0+\Sigma\beta_i x_i+\Sigma\beta_{ij}x_i x_j+\Sigma\beta_{ii}x_i^2 \quad (4)$$

where Y is the predicted response, β_0 is the offset term, β_i is the linear offset, β_{ii} is the squared offset, β_{ij} is the interaction effect, and x_i is the dimensionless coded value of X_i.

A trial version of statistical software package Design-Expert (Version 7.0.2, State-Ease, Minneapolis, MN, U.S.A.) was used to design and analyze the experiment. A 2^3 factorial design, with five replicates at the center point, with a total number of 29 trials was employed. The coded and uncoded values of the variables at various levels are given in Table 4.

Nucleotide Sequence Number

The nucleotide sequence of *Bacillus licheniformis* K51 has been assigned as GenBank Accession No. DQ922951.

RESULTS AND DISCUSSION

Screening of Important Media Components for Biosurfactant Production

Since the temperature in the oil well was in the range of 50°C, initial experiments were done in LB medium for the production of biosurfactant at different temperatures (30°C, 40°C, 45°C, 50°C, and 55°C; data not shown). Maximum biosurfactant production was observed at 45°C (in terms of surface tension reduction). The surface tension value in LB medium was 35 mN/m, after 72 h. Hence,

Table 3. Statistical analysis of medium components on biosurfactant production as per Plackett-Burman design.

Factors	Medium components	Effect	SE	t(xi)	*P*-value	Confidence level (%)
X_1	Glucose	1.10	1.787	0.61	0.585	41.50
X_2	$NaNO_3$	1.30	1.787	0.73	0.518	48.20
X_3	$MgSO_4 \cdot 7H_2O$	4.10	1.787	2.3	0.105	89.50
X_4	KCl	5.10	1.787	2.86	0.065	93.50
X_5	$CaCl_2$	8.10	1.787	4.55	0.0199	98.01
X_6	H_3PO_4	12.9	1.787	7.25	0.0054	99.46
X_7	H_3BO_3	-6.90	1.787	3.87	0.0305	96.95
X_8	$CuSO_4$	7.10	1.787	3.98	0.0284	97.16
X_9	$MnSO_4$	0.90	1.787	0.51	0.6452	35.48
X_{10}	Na_2MoO_4	-1.30	1.787	0.73	0.5182	48.18
X_{11}	$ZnSO_4$	7.70	1.787	4.33	0.0227	97.73
X_{12}	$FeSO_4$	6.30	1.787	3.54	0.0384	96.16
X_{13}	$CoCl_2$	6.30	1.787	3.54	0.0384	96.16
X_{14}	Na-EDTA	-6.70	1.787	3.76	0.0329	96.71

Table 4. Box-Behnken experimental design matrix with experimental and predicted values of biosurfactant production.

Trial number	Variables/levels								Biosurfactant production (CMD)	
	H_3PO_4		$CaCl_2$		H_3BO_3		Na-EDTA			
	Coded value	Actual value (ml/l)	Coded value	Actual value (mg/l)	Coded value	Actual value (mg/l)	Coded value	Actual value (mg/l)	Experimental	Predicted
1	−1	0.1	−1	0.03	0	1.38	0	165	35	42
2	+1	1	−1	0.03	0	1.38	0	165	45	46
3	−1	0.1	+1	0.3	0	1.38	0	165	10	18
4	+1	1	+1	0.3	0	1.38	0	165	70	72
5	0	0.55	0	0.16	−1	0.25	−1	30	80	82
6	0	0.55	0	0.16	+1	2.5	−1	30	36	40
7	0	0.55	0	0.16	−1	0.25	+1	300	25	30
8	0	0.55	0	0.16	+1	2.5	+1	300	30	37
9	−1	0.1	0	0.16	0	1.38	−1	30	50	46
10	+1	1	0	0.16	0	1.38	−1	30	87	81
11	−1	0.1	0	0.16	0	1.38	+1	300	25	24
12	+1	1	0	0.16	0	1.38	+1	300	50	47
13	0	0.55	−1	0.03	−1	0.25	0	165	60	56
14	0	0.55	+1	0.3	−1	0.25	0	165	50	45
15	0	0.55	−1	0.03	+1	2.5	0	165	30	28
16	0	0.55	+1	0.3	+1	2.5	0	165	42	39
17	−1	0.1	0	0.16	−1	0.25	0	165	45	43
18	+1	1	0	0.16	−1	0.25	0	165	70	75
19	−1	0.1	0	0.16	+1	2.5	0	165	35	28
20	+1	1	0	0.16	+1	2.5	0	165	55	55
21	0	0.55	−1	0.03	0	1.38	−1	30	50	53
22	0	0.55	+1	0.3	0	1.38	−1	30	55	57
23	0	0.55	−1	0.03	0	1.38	+1	300	32	28
24	0	0.55	+1	0.3	0	1.38	+1	300	30	26
25	0	0.55	0	0.16	0	1.38	0	165	50	48
26	0	0.55	0	0.16	0	1.38	0	165	45	48
27	0	0.55	0	0.16	0	1.38	0	165	48	48
28	0	0.55	0	0.16	0	1.38	0	165	48	48
29	0	0.55	0	0.16	0	1.38	0	165	48	48

further optimization study was done at 45°C. with LB being a complex and costly medium to be used for biosurfactant production, a glucose-based minimal medium was formulated for economical biosurfactant production. Table 1 represents the independent variables and their respective high and low concentrations used in the optimization study, and Table 2 represents the Plackett-Burman experimental design for 20 trials with two levels of concentrations for each variable and corresponding biosurfactant production in terms of ST values. The variables X1-X14 represent the medium constituents and D1-D5 represent the dummy variables/unassigned variables. Table 3 represents the effect, standard error, t (xi), P, and confidence level of each component from the result of ST values in Table 2.

The components were screened at the confidence level of 95% on the basis of their effects. The confidence level of components glucose, $NaNO_3$, $MgSO_4 \cdot 7H_2O$, KCl, $MnSO_4$, and Na_2MoO_4 were below 95% in biosurfactant production and hence, were considered insignificant. The rest of the components, $CaCl_2$, H_3PO_4, H_3BO_3, $CuSO_4$, $ZnSO_4$, $FeSO_4$, $CoCl_2$, and Na-EDTA, showed confidence level at or above 95% and were considered to be significant. Here, positive effect means reduction in surface tension and negative effect means increase in surface tension. Hence, the effect for each component was considered as opposite from calculated values, *i.e.*, lower surface tension means positive and higher value means negative effect, unlike the usual way of calculating the effect for each component. The variables $CaCl_2$, H_3PO_4, H_3BO_3, and Na-EDTA showed confidence level at 98.01%, 99.46%, 96.95%, and 96.71%, respectively, and were considered significant.

The above results indicated that the Plackett-Burman design is a powerful tool for identifying factors, which had significant influence on biosurfactant production. The exact optimal values of the individual factors are still unknown but can be determined by the subsequent Box-Behnken experiment.

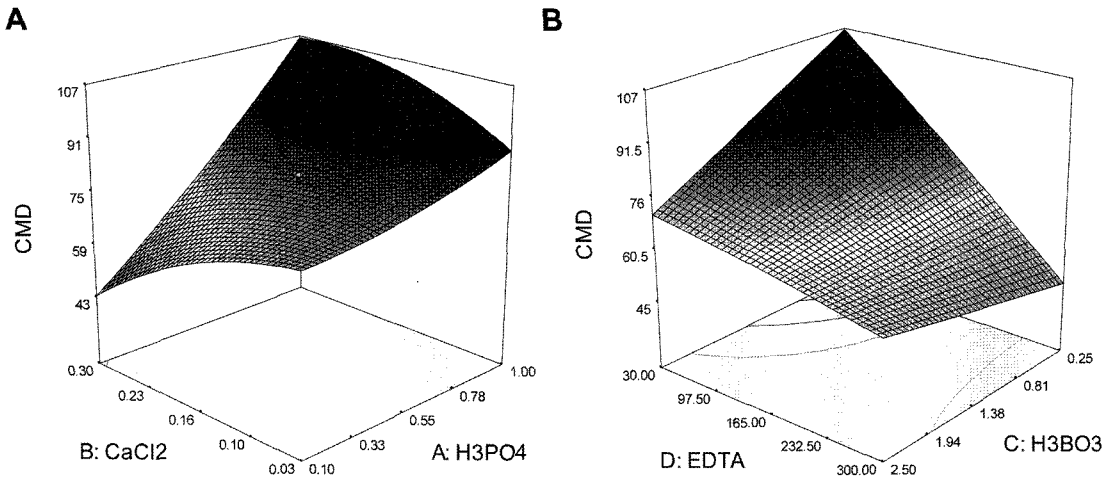

Fig. 1. Three-dimensional response plot showing the effect of (**A**) $CaCl_2$, H_3PO_4 at 0.25 mg/l H_3BO_3 and 30 mg/l Na-EDTA and (**B**) H_3BO_3, Na-EDTA at 0.27 g/l $CaCl_2$ and 1.0 ml/l H_3PO_4 on biosurfactant yield (CMD).

Optimization of Screened Medium Components for Biosurfactant Production

The variables showing positive effect with confidence level above 98% ($CaCl_2$ and H_3PO_4) and variables with negative effect above 96% (H_3BO_3 and Na-EDTA) in the Plackett-Burman design were selected and further optimization was done using Box-Behnken design. Three-dimensional surface plots were obtained when the data of biosurfactant production as CMD (X times dilution) were fed into the design expert software, and analyzed by it. The software has the function by which we can predict the production of biosurfactant within a studied range of all four medium components. Here, each 3D surface plot represents the effect of two medium components at their studied concentration range and at fixed concentration of the rest of the two components. The value of the rest of the two components were varied for that situation with the software and the optimum values were determined. Based on the results of Plackett-Burman design, components having a confidence level above 95% ($CuSO_4$, $ZnSO_4$, $FeSO_4$, and $CoCl_2$) were set at their higher level and components having a confidence level below 95% were set at their middle level in the Box-Behnken design.

Table 4 represents the experimental design and the results obtained for biosurfactant production. The variables used for the factorial analysis were $CaCl_2$, H_3PO_4, H_3BO_3, and Na-EDTA for biosurfactant production. The center point in the design was repeated five times for estimation of error. The actual and coded factor levels are represented in Table 4 for biosurfactant production. Data were analyzed by quadratic multiple regression using the trial version, Design-Expert (Version 7.0.2; Stat-Ease, Inc.), and the following equation was obtained.

$$\begin{aligned} Y = &+47.80+14.75\times A+0.42\times B-8.50\times C \\ &-13.83\times D+12.50\times A\times B-1.25\times A\times C \\ &-3.00\times A\times D+5.50\times B\times C-1.75\times B\times D \\ &+12.25\times C\times D+2.52\times A2-5.98\times B2+0.14\times C2 \\ &-0.86\times D2 \end{aligned} \quad (5)$$

where Y is the predicted response and A, B, C, and D are the coded values of H_3PO_4, $CaCl_2$, H_3BO_3, and Na-EDTA, respectively.

Isoresponse 3D surface plots showing the effect of H_3PO_4, $CaCl_2$, and H_3BO_3, Na-EDTA at different concentrations of the other two variables are shown in Fig. 1. The statistical optimal values of variables are obtained when moving along the major and minor axis of the contour and the response at the center point yields maximum biosurfactant production. From the study of the 3D plots, the optimal concentration of A, B, C, and D (H_3PO_4, $CaCl_2$, H_3BO_3, and Na-EDTA) were found to be 1.0 ml/l, 0.27 g/l, 0.25 mg/l, and 30 mg/l, respectively.

To validate the regression coefficient, analysis of variance (ANOVA) for biosurfactant production was performed

Table 5. Analysis of variance (ANOVA) for the Quadratic model[a].

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*p*>F
Model	7,503.15	14	535.94		
Error	12.80	4	3.20	17.10	<0.0001
Corrected total	7,941.86	28			

[a]R^2=0.6886; R=0.9448; Adj. R^2=0.8895; CV=12.15%.

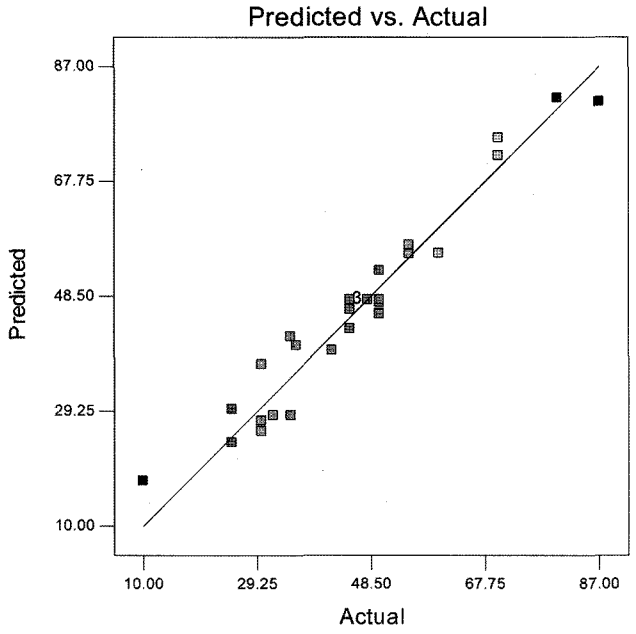

Fig. 2. Plot of predicted *vs* actual biosurfactant concentration (CMD values) for *B. licheniformis* K51.
The biosurfactant concentration is the response variable of interest. The predicted biosurfactant values are determined by the model equations determined for the Box-Behnken design.

(Table 5). The values of correlation coefficient, Model *F* and Model *P>F*, were found to be 0.9448, 17.10, and <0.0001, respectively, which implies that the model is significant. The value of the adjusted determination coefficient (Adj. R^2=0.8895) was also very high to advocate for a high significance of the model. A higher value of correlation coefficient, R (0.9448), justifies an excellent correlation between experimental and predicted values of biosurfactant production. At the same time, a relatively lower value of the coefficient of variation (CV=12.15%) indicates a better precision and reliability of the experiments carried out [2].

Fig. 2 represents the relationship between the actual biosurfactant concentration values and the predicted values determined by the model equation (5) for *B. licheniformis* K51. It can be observed that most points are near the line adjustment, which means that the values determined experimentally are similar to those determined by the model.

The model predicted that the maximum production of biosurfactant that can be obtained using the above optimum concentrations of the variables was 107×CMD. The vitrification of the results using the optimized medium was acomplished by carrying out shake flask experiments, which showed a higher yield of biosurfactant by *B. licheniformis* K51 with reduction in ST value as 29 mN/m and concentration as 105×CMD (×, times dilution factor), which is ten times higher than non-optimized rich LB medium.

With the use of a statistical optimization method, the chitnase production by *Alcaligenes xylosoxydans* was found to increase from 12 to 29 U/ml [18]; there was a 50.33% increase in lactic acid production by *Lactobacillus* sp. [4], and compactin production was increased from 250 μg/ml to 400 μg/ml in a *Penicillium* sp. strain [13]. There are very few reports on optimization of media components for biosurfactants by different organisms. Abalos *et al.* [1] reported the utilization of response surface methodology to optimize the culture media for the production of rhamnolipids by *Pseudomonas aeruginosa* AT10. A 2^4 full factorial, central composite rotational design and response modeling method (RSM) was used to enhance rhamnolipid production in mineral medium with waste fatty acids as carbon source. The maximum concentration of rhamnolipid, 18.7 g/l, was attained in optimized medium. R. K. Sen carried out the optimization of fermentation media for maximization of surfactin production by *Bacillus subtilis* DSM 3256 [17]. He used a 2^4 full factorial central composite experimental design followed by multistage Monte-Carlo optimization in the design of experiments and in the analysis of results. He expressed the relative surfactin concentration as the reciprocal of the critical micelle concentration (CMC^{-1}) by diluting the broth until the CMC was reached, beyond which the surface tension starts rising abruptly, and the maximal predicted yield in terms of CMC^{-1} was 45.5×. Jacques *et al.* [10] reported the optimization of biosurfactant lipopeptide production from *B. subtilis* S499 by Plackett-Burman design. The amount of biosurfactant lipopeptide in the supernatant of a culture carried out in this optimized medium was about five times higher than that obtained in non-optimized rich medium [10]. However, no reports are available for optimization of biosurfactant production by *B. licheniformis*. The biosurfactant produced by *B. licheniformis*, lichenysin, is a cyclic lipopeptide, which is the most effective biosurfactant discovered so far [11, 12, 14, 19]. This report is an attempt to formulate an optimized medium for the biosurfactant production by *B. licheniformis* K51. The FTIR spectrum of the partially purified biosurfactant from *B. licheniformis* K51 showed similarity in structure to lichenysin (data not shown).

The methodology of Plackett-Burman was found to be very useful for the determination of relevant variables for further optimization. This made it possible to consider a large number of variables and avoid the loss of information, which might be essential in the optimization of the process. The use of these techniques has helped in finding out the important medium components that have significant effect on biosurfactant production by *B. licheniformis* K51. In conclusion, the methodologies of Plackett-Burman and Box-Behnken designs have proved to be very effective in improving biosurfactant production.

REFERENCES

1. Abalos, A., F. Maximo, M. A. Manresa, and J. Bastida. 2002. Utilization of response surface methodology to optimize the culture media for the production of rhamnolipids by *Pseudomonas aeruginosa* AT10. *J. Chem. Technol. Biotechnol.* **77:** 777–784.
2. Box, G. E. P. and K. B. Wilson. 1951. On the experimental attainment of optimum conditions. *J. Roy. Stat. Soc.* **B13:** 1–45.
3. Box, G. E. P. 1952. Multi-factor designs of first order. *Biometrika* **39:** 49–57.
4. Chauhan, K., U. Trivedi, and K. C. Patel. 2006. Application of response surface methodology for optimization of lactic acid production using date juice. *J. Microbiol. Biotechnol.* **16:** 1410–1415.
5. Cooper, D. G., C. R. Macdonald, S. J. B. Duff, and N. Kosaric. 1981. Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. *Appl. Environ. Microbiol.* **42:** 408–412.
6. Desai, J. D. and I. M. Banat. 1997. Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.* **61:** 47–64.
7. Felse, P. A. and T. Panda. 1999. Self-directing optimization of parameters for extracellular chitinase production by *Trichoderma harzianum* in batch mode. *Proc. Biochem.* **34:** 563–566.
8. Fiechter, A. 1992. Integrated systems for biosurfactant synthesis. *Pure. Applied Chem.* **64:** 1739–1743.
9. Gohel, V., T. Chaudhary, P. Vyas, and H. S. Chhatpar. 2006. Statistical screening of medium components for the production of chitinase by the marine isolate *Pantoea dispersa*. *Biochem. Eng. J.* **28:** 50–56.
10. Jacques, P., C. Hbid, J. Destain, H. Razafindralambo, M. Paquot, E. Pauw, and P. Thonart. 1999. Optimization of biosurfactant lipopeptide production from *Bacillus subtilis* S499 by Plackett-Burman design. *Appl. Biochem. Biotech.* **77–79:** 223–233.
11. Javaheri, M., G. E. Jenneman, M. J. McInerney, and R. M. Knapp. 1985. Anaerobic production of biosurfactant by *Bacillus licheniformis* JF-2. *Appl. Environ. Microbiol.* **50:** 698–700.
12. Jenny, K., O. Kappeli, and A. Fiechter. 1991. Biosurfactants from *Bacillus licheniformis*: Structural analysis and characterization. *Appl. Microbiol. Biotechnol.* **36:** 5–13.
13. Konya, A., A. Jekkel, J. Suto, and J. Salat. 1998. Optimization of compactin fermentation. *J. Ind. Microbiol. Biotechnol.* **20:** 150–152.
14. Lin, S. C., M. A. Minton, M. M. Sharma, and G. Georgiou. 1994. Structural and immunological characterization of a biosurfactant produced by *Bacillus licheniformis* JF-2. *Appl. Environ. Microbiol.* **60:** 31–38.
15. Montogomery, D. C. 2000. *Design and Analysis of Experiments*, 5th Ed. John Wiley & Sons, Singapore.
16. Plackett, R. L. and J. P. Burman. 1944. The design of optimum multifactorial experiments. *Biometrika* **33:** 305–325.
17. Sen, R. K. 1997. Response surface optimization of the critical media components for the production of surfactin. *J. Chem. Tech. Biotech.* **68:** 263–270.
18. Vaidya, R., P. Vyas, and H. S. Chhatpar. 2003. Statistical optimization of medium components for the production of chitinase by *Alcaligenes xylosoxydans*. *Enzyme Microb. Technol.* **33:** 92–96.
19. Yakimov, M. M., K. N. Timmis, V. Wrey, and H. L. Fredrickson. 1995. Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. *Appl. Environ. Microbiol.* **61:** 1706–1713.