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Statistical Optimization of Significant Variables for Exopolysaccharide Production by *Lactobacillus plantarum*

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Abstract: The optimal level of the key variables (Dextrose, peptone, $MgSO_4$, glutamine, inoculum concentration) used to determine the effect of their interactions on EPS production by *Lactobacillus plantarum* using the statistical tool (CCD of RSM). The second-order quadratic model with the optimum conditions (Dextrose-1.0%, peptone-0.7%, $MgSO_4$-0.02%, glutamine-0.2%, inoculum concentration- 2.0%). The nearness of the coefficient of determination (R^2=0.8716) to 1 ensures the satisfactory adjustment of the quadratic model to the experimental data. The bacterial EPS recovered from optimized medium was 258 ± 0.4 mg/100ml of dry weight.

Key words: Exopolysaccharide · CCD · RSM · *Lactobacillus plantarum*

INTRODUCTION

The physiological role of EPS depends on the ecological niches and the natural environment in which microorganisms have been isolated. Indeed, the EPS production is a process that requires a noticeable energy cost of up to 70% of total energy reserve, representing a significant carbon investment for microorganisms. However, the benefits related to EPSs production are significantly higher than costs considering the increasing growth and survival of microorganisms in their presence [1]. In their natural environment, most bacteria occur in microbial aggregates whose structural and functional integrity is based on the presence of a matrix of extracellular polymeric substances and the EPS production seems to be essential for their survival [2]. A vast number of microbial EPSs were reported over the last decades and their composition, structure, biosynthesis and functional properties have been extensively studied. In recent years the increased demand for natural polymers for pharmaceutical, food and other industrial applications has led to a remarkable interest in polysaccharides produced by microorganisms [3]. Further, this method is time consuming and requires a large number of experiments to determine the optimum levels in the production medium. These limitations of the single factor optimization method can be overcome by developing a non-linear multivariate process model. Response surface methodology (RSM) is a well-known method applied in the optimization of medium constituents and other critical variables responsible for the production of biomolecules [4]. Response surface methodology (RSM) is an efficient statistical strategy for designing experiments, building models, searching optimum conditions of factors for desirable responses and evaluating the relative significance of several affecting factors even in the presence of complex interactions [5, 6]. The importance of this method is that it states the amount

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of similarity between the organisms using statistical analysis. Based on this similarity percentage, measured in terms of Pearson correlation, we can predict the strain type, differentiate up to the sub species level, which was not possible with biochemical characterization. Statistical experimental designs can be employed at various phases of optimization process, such as for screening experiments and for finding optimum conditions for a desired response. This method has been successfully applied in the optimization of media composition [7, 8] and fermentation processes [9, 10]. The optimal level of the key variables is used to determine the effect of their interactions on EPS production using the statistical tool (CCD). The bacterial EPS was recovered from optimized medium [11]. This present work was carried out to determine the effect of different nutrient resources on microbial growth and EPS yield that was thoroughly studied from *Lactobacillus plantarum* by statistical optimizaton using Central Composite Design (CCD).

MATERIALS AND METHODS

Optimization of significant variables for EPS production using central composite design (CCD): To find the optimal cultivation conditions for EPS production, CCD with five coded levels was used for locating the true optimum. For the four factors, this trial was essentially a full 24 factorial design with six axial points (α= 2) and six replication of the center points, resulting in a total number of 30 experiments. The levels of the variables and the experimental design are shown in Table 1 & 2. The results of CCD was expressed as the following second-order polynomial Eq. 2 using a multiple regression technique.

$$Y = \beta_0 + \Sigma\, \beta i\chi i + \Sigma\, \beta ii\chi i2 + \Sigma\, \beta oij\chi i\chi j$$

where Y is the predicted response, β_0 the intercept term, βi the linear coefficients, βii the quadratic coefficients, βij the interactive coefficients and χi and χj the coded independent variables [12].

EPS production by optimized parameters: Mass production of EPS was carried out in the fermentor by using optimized parameters. After 72 hours incubation at pH 7.0, 40°C on optimized medium (Dextrose-1.0%, (Carbon source), peptone-0.7% (Nitrogen source), $MgSO_4$-0.02% (Metal ions), glutamine-0.2% (Aminoacid), inoculum concentration- 2.0% were centrifuged at 5000 rpm for 20 min. The EPS was precipitated from the supernatant by addition of equal amount of ethanol. The mixture was agitated with addition of methanol to prevent local high concentration of the precipitate and left overnight at 4°C and centrifuged at 7000 rpm for 20 mins. After centrifugation the precipitate was collected in a petri plate and dried at 60°C [13].

Statistical Analysis Software: Experimental designs and the polynomial coefficients were calculated and analyzed using a trial version of Design-Expert software (version 7.1.6., Stat-Ease Inc., Minneapolis, USA). Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

RESULTS

Central Composite Design (CCD) and Response Surface Methodology (RSM): The optimal level of the key variables (Dextrose, Peptone, Magnesium sulphate and Tween 80) and the effect of their interactions on EPS production were further explored using the CCD of RSM. The experiments were conducted by five different levels employed simultaneously covering the spectrum of variables for the production of EPS in the Central Composite Design. Table 1 indicates the range and levels of the independent variables selected for the production of EPS. To understand the effects of the parameters such as carbon source, nitrogen source, metal ions, surfactants and their interactions on the production of EPS process, statistically designed experiments were used.

Central Composite Design: Response surface methodology was used to optimize the levels of the significant variables identified by the 2-level fractional factorial design. A CCD matrix was developed depending on the number of factors considered for optimization. Based on the identification of variables by the 2-level fractional factorial, a central composite design was developed for variables significantly affecting citric acid production. All the non-significant factors were maintained at central points ('0' coded level) of the levels used in the 2-level fractional factorial design.

Table 4 shows the five levels of variables chosen for trials in CCD. Response surface methodology (RSM) was used to optimize cultivation conditions for EPS production, 30 experimental runs with different combinations of four factors and five levels were carried out (Table 3). The variables used for the factorial analysis were carbon source, nitrogen source, metal ions and surfactants, named X1, X2, X3 and X4, respectively.

Table 1: Independent variables and their coded level for the central composite design used for EPS production by *Lactobacillus plantarum*

Variables	$-\alpha$	Low value	Coded variable	High value	$+\alpha$
Carbon source	0.5	0.75	1.0	1.25	1.5
Nitrogen source	0.3	0.4	0.5	0.6	0.7
Metal ions	0	0.01	0.02	0.03	0.04
Surfactants	0	0.1	0.2	0.3	0.4

Table 2: Central composite design for EPS production by *Lactobacillus plantarum*

Std	Run	Factor 1 Dextrose	Factor 2 Peptone	Factor 3 $MgSO_4$	Factor 4 Tween 80
2	1	1.25	0.40	0.01	0.10
24	2	1.00	0.50	0.02	0.40
30	3	1.00	0.50	0.02	0.20
18	4	1.50	0.50	0.02	0.20
8	5	1.25	0.60	0.03	0.10
26	6	1.00	0.50	0.02	0.20
25	7	1.00	0.50	0.02	0.20
11	8	0.75	0.60	0.01	0.30
1	9	0.75	0.40	0.01	0.10
10	10	125	0.40	0.01	0.30
13	11	0.75	0.40	0.03	0.30
5	12	0.75	0.40	0.03	0.10
6	13	1.25	0.40	0.03	0.10
4	14	125	0.60	0.01	0.10
21	15	1.00	0.50	0.00	0.20
15	16	0.75	0.60	0.03	0.30
3	17	0.75	0.60	0.01	0.10
20	18	1.00	0.70	0.02	0.20
28	19	1.00	0.50	0.02	0.20
23	20	1.00	0.50	0.02	0.00
9	21	0.75	0.40	0.01	0.30
22	22	1.00	0.50	0.04	0.20
16	23	1.25	0.60	0.03	0.30
14	24	1.25	0.40	0.03	0.30
12	25	1.25	0.60	0.01	0.30
27	26	1.00	0.50	0.02	0.20
17	27	0.50	0.50	0.02	0.20
7	28	0.75	0.60	0.03	0.10
19	29	1.00	0.30	0.02	0.20
29	30	1.00	0.50	0.02	0.20

The effects of the four independent variables on EPS production and the experimental response along with the predicted response obtained from the regression equation for each run are shown in Table 5. It can be seen from Table 5, that there was a considerable variation in the EPS production depending on the four chosen variables. The maximum EPS production (2.030 OD) was achieved. This adequately indicated that choosing appropriate cultivation conditions could evidently enhance the yield of EPS. By applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to explain the EPS production by only considering the significant terms and was shown as below:

Final Equation in Terms of Coded Factors:

R1 = + 1.55+0.089 * A + 0.075 * B + 0.018 * C + 0.083 * D + 0.015 * A * B + 7.812E – 003 * A * C - 0.067 * A * D- 0.093 * B * C - 4.375E – 004 * B * D - 0.16 * C * D -0.076 * A^2 + 0.085 * B^2 - 0.18 * C^2 - 0.051 * D^2

Final Equation in Terms of Actual Factors:

R1 = - 1.14824 + 2.96208 * Carbon source - 6.52896 * Nirogen source + 150.45625 * Metalions + 8.81979 * Surfactants + 0.60750 * Carbon source * Nirogen source -3.12500 * Carbon source * Metal ions - 2.69750 * Carbon source * Surfactants -93.43750 * Nirogen source * Metal ions 0.043750 * Nirogen source * Surfactants - 162.06250 * Metal ions * Surfactants -1.21650 * Carbon source^2 + 8.54687 * Nirogen source^2 - 1815.31250 * Metal ions^2 - 5.06563 * Surfactants^2.

Table 3: The matrix of the CCD experiment and the corresponding experimental data.

Std	Run	Factor 1A: Carbon source	Factor 2 B: Nitrogen source	Factor 3 C: Metal ions	Factor 4 D: Sufactants	Response 1 R1 OD
2	1	1.25	0.40	0.01	0.10	1.039
24	2	1.00	0.50	0.02	0.40	1.501
30	3	1.00	0.50	0.02	0.20	1.556
18	4	1.50	0.50	0.02	0.20	1.418
8	5	1.25	0.60	0.03	0.10	1.599
26	6	1.00	0.50	0.02	0.20	1.556
25	7	1.00	0.50	0.02	0.20	1.556
11	8	0.75	0.60	0.01	0.30	1.721
1	9	0.75	0.40	0.01	0.10	0.774
10	10	1.25	0.40	0.01	0.30	1.404
13	11	0.75	0.40	0.03	0.30	1.292
5	12	0.75	040	0.03	0.10	1.294
6	13	1.25	0.40	0.03	0.10	1.589
4	14	1.25	0.60	0.01	0.10	1.401
21	15	1.00	0.50	0.00	0.20	1.772
15	16	0.75	0.60	0.03	0.30	1.201
3	17	0.75	0.60	0.01	0.10	1.076
20	18	1.00	0.70	0.02	0.20	2.030
28	19	1.00	0.50	0.02	0.20	1.556
23	20	1.00	0.50	0.02	0.00	1.182
9	21	0.75	0.40	0.01	0.30	1.383
22	22	1.00	0.50	0.04	0.20	0.864
16	23	1.25	0.60	0.03	0.30	1.305
14	24	1.25	0.40	0.03	0.30	1.301
12	25	1.25	0.60	0.01	0.30	1.759
27	26	1.00	0.50	0.02	0.20	1.556
17	27	0.50	0.50	0.02	0.20	1.062
7	28	0.75	0.60	0.03	0.10	1.233
19	29	1.00	0.30	0.02	0.20	1.742
29	30	1.00	0.50	0.02	0.20	1.556

Table 4: Analysis of variance (ANOVA) for quadratic model for EPS production

Source	Sum of squares	df	Mean square	f-value	p-value prob>f
Model	2.53	14	0.18	610.23	<0.0001^{C}
A-Carbon source	0.19	1	0.19	640.64	<0.0001^{C}
B-Nitrogen source	0.13	1	0.13	452.85	<0.0001^{C}
C-Metal ions	8.103E-003	1	8.103-003	27.33	0.0001^{C}
D-Surfactants	0.17	1	0.17	561.63	<0.0001^{C}
AB	3.691E-003	1	3.691E-003	12.45	0.0030^{C}
AC	9.766E-004	1	9.766E-004	3.29	0.0896
AD	0.073	1	0.073	245.45	<0.0001^{C}
BC	0.14	1	0.14	471.19	<0.0001^{C}
BD	3.062E-006	1	3.062E-006	0.010	0.9204
CD	0.42	1	0.42	1417.48	<0.0001^{C}
A^2	0.16	1	0.16	534.83	<0.0001^{C}
B^2	0.20	1	0.20	675.85	<0.0001^{C}
C^2	0.90	1	0.90	3048.87	<0.0001^{C}
Residual	4.447E-003	15	2.965E-004		
Lack of fit	2.674E-003	10	2.674E-004	0.75	0.6718
Pure error	1.773E-003	5	3.547E-004		
Cor Total	2.54	29			

R^2=0.9982; AdjR^2=0.9966; C.V%=1.25; ‘model terms are significant

The independent variables were fitted to the second order model equation and examined for the goodness of fit. Several indicators were used to evaluate the adequacy of the fitted model and the results are shown in Table 4. The determination coefficient R^2 value, correlation coefficient R value, coefficients of variation (CV) and model significance (F-value) were used to judge the adequacy of the model. R^2, or coefficient of determination, is the proportion of variation in the response attributed to the model rather than to random error, Suggested for a good fit of a model, R^2 should be at least 80%. The determination coefficient (R^2) implies that the sample variation of 95.94% for EPS production using substrate is attributed to the independent variables and only about 4.06% of the total variation cannot be explained by the model. The closer value of *R* (Correlation coefficient) to 1, the better is the correlation between the experimental and predicted values. Here the value of R^2 (0.9982) for Eq. 2 being close to 1 indicated a close agreement between the experimental results and the theoretical values predicted by the model equation. The coefficient of variation (CV) is the ratio of the standard error of estimate to the mean value of the observed response, expressed as a percentage. A model can be considered reasonably reproducible if the CV is not greater than 10%. Usually, the higher value of CV, the lower is the reliability of experiment. Here, a lower value of CV (1.25) indicated a greater reliability of the experiments performed. The model significance (*F*-value) indicates the level of confidence that the selected model cannot be due to experimental error. Linear and quadratic terms were significant at 1% level. Therefore, the quadratic model was selected in this optimization study. The Student *'t'* distribution and the corresponding *P* value, along with the parameter estimate, are given in Table 4. The *P*-values are used as a tool to check the significance of each of the coefficients which, in turn, are necessary to understand the pattern of mutual interactions between the best variables. The estimates of and parameter the corresponding *P*-values showed that among the independent variables, X1 (Carbon source), X2 (Nitrogen source), X3 (Metal ions) and X4 (Surfactants) had a significant effect on EPS production. So, compared with the traditional 'one- variable at- a-time' approach which is unable to detect the frequent interactions occurring between two or more factors although they often do occur, RSM has immeasurable effects and tremendous advantages.

The significant factors identified by manual optimization design were considered for the next stage in the medium optimization using response surface optimization technique for future study. The analysis of variance (ANOVA) was employed (Shown in Table 4) for the determination of significant parameters. ANOVA consists of classifying and cross classifying statistical results and testing whether the means of a specified classification differ significantly. The F-value is the ratio of the mean square due to regression to the mean square due to error and indicates the influence (Significance) of each controlled factor on the tested model.

Where, Y1 was EPS production, X1 carbon source, X2 nitrogen source, X3 metal ions and X4 surfactants, the Model F-value of 610.23 implies the model is significant. There is only a 0.01% chance that in a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate model terms are significant. In this case A, B, C, D, AB, AD, BC, CD, A^2, B^2, C^2 and D^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Pred R-Squared" of 0.9929 is in reasonable agreement with the "Adj R-Squared" of 0.9966."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 105.133 indicates an adequate signal. This model can be used to navigate the designspace.

The above model can be used to predict the EPS production within the limits of the experimental factors. Fig. 1 shows that the actual response values agree well with the predicted response values.

The shapes of the contour plots, circular or elliptical, indicate whether the mutual interactions between the variables are significant or not. A circular contour plot of response surfaces indicates that the interaction between the corresponding variables can be ignored, while an elliptical or saddle nature of the contour plot suggests that the interaction between the corresponding variables is significant.

The conventional method (i.e., change-one-factor-at-a-time) traditionally used for optimization of multifactor experimental design had limitations because (i) it generates large quantities of data which are often difficult to interpret (ii) it is time consuming and expensive (iii) ignores the effect of interactions among factors which have a great bearing on the response. To overcome these problems, a central composite design (CCD) and RSM were applied to determine the optimal levels of process variables on citric acid production. Only 30 experiments were necessary and the obtained model was adequate ($P < 0.001$). By solving the regression equation, the optimum process conditions were determined; substrate concentration, 1% carbon source, 0.7% nitrogen source, 0.02% metal ions and 0.2% surfactants. A maximum EPS yield of 2.03 OD was obtained at the optimized process conditions. The research results indicated that RSM not only helps us locate the optimum conditions of the process variables in order to enhance the maximum EPS production, but also proves to be well suited to evaluating the main and interaction effects of the process variables on EPS production from residues.

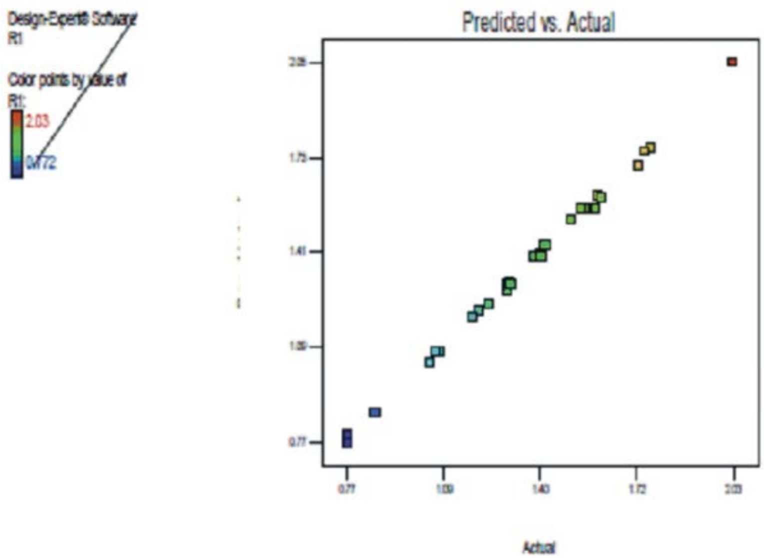

Fig. 1: Predicted response versus actual value

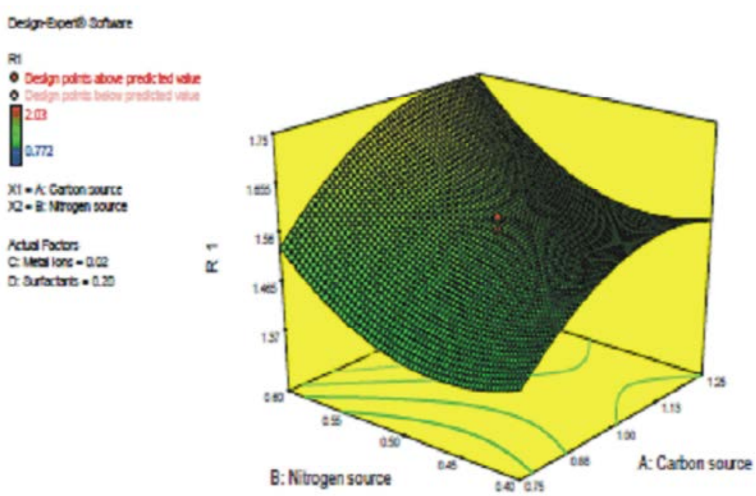

Fig. 2: 3D plot showing the effect of carbon source and nitrogen source on EPS production

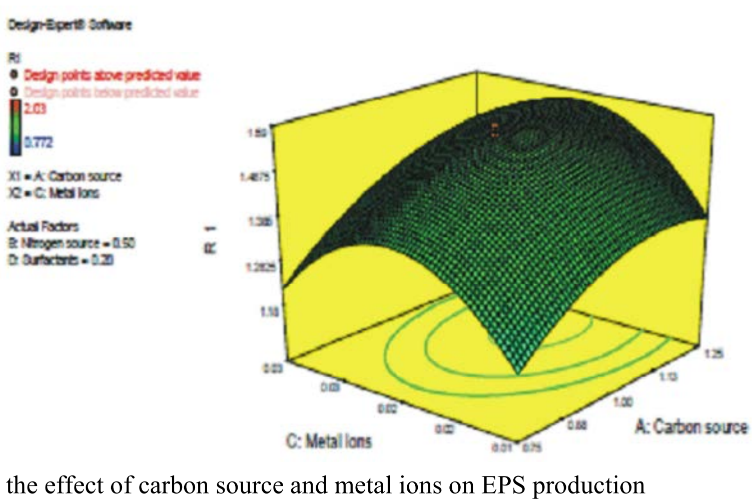

Fig. 3: 3D plot showing the effect of carbon source and metal ions on EPS production

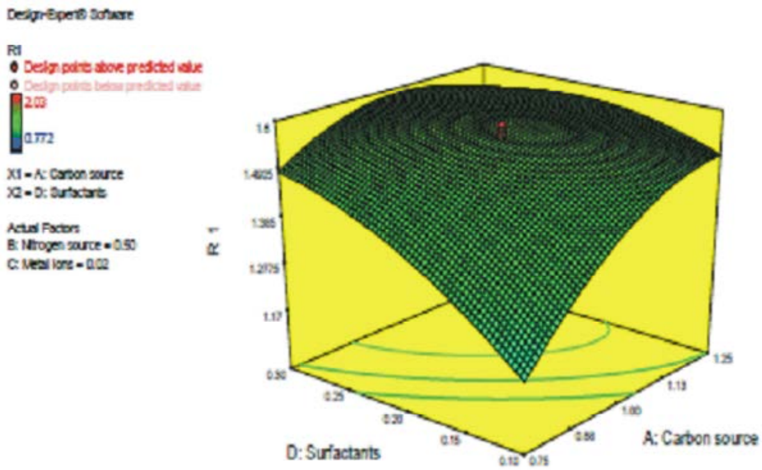

Fig. 4: 3D plot showing the effect of carbon source and surfactants on EPS production

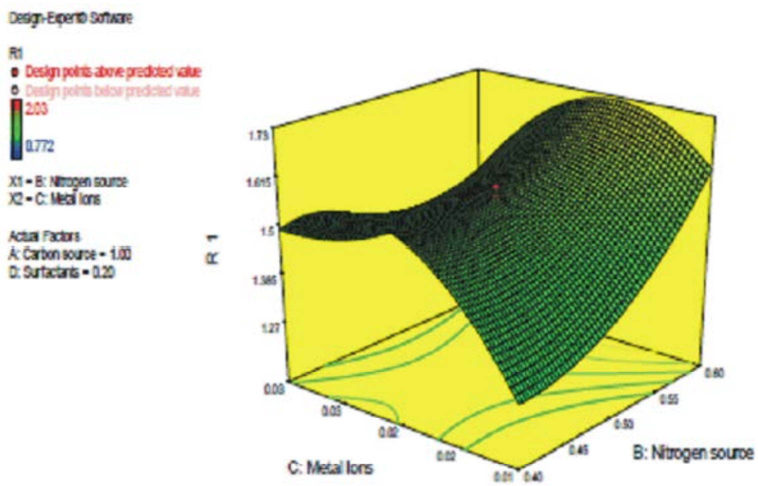

Fig. 5: 3D plot showing the effect of metal ions and nitrogen source on EPS production

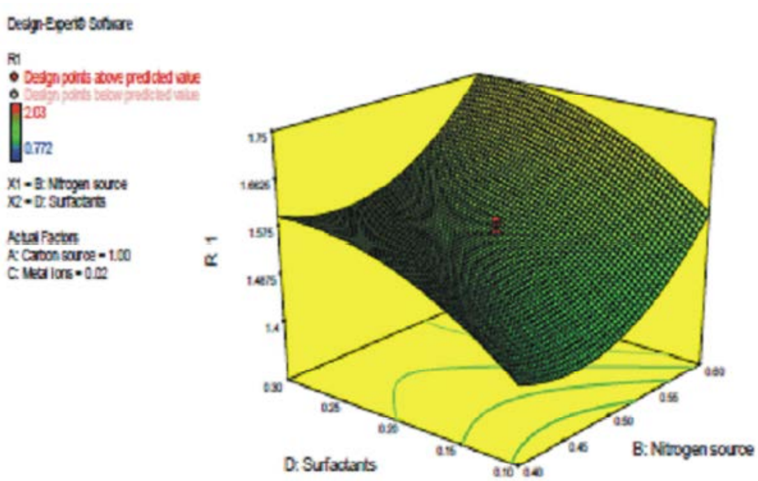

Fig. 6: 3D plot showing the effect of nitrogen source and surfactants on EPS production

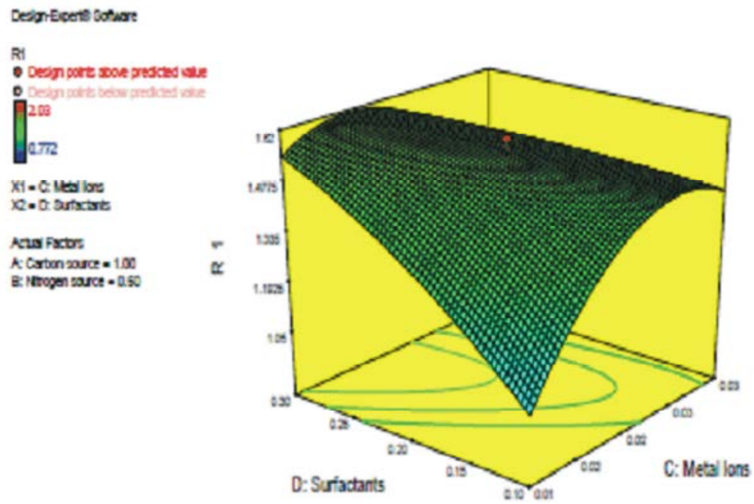

Fig. 7: 3D plot showing the effect of surfactants and metal ions on EPS production

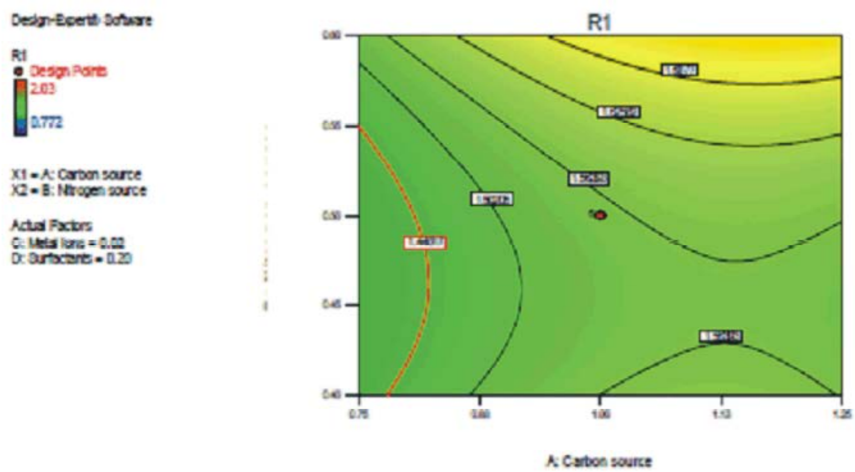

Fig. 8: Contour plot showing the effect of carbon source and nitrogen source on EPS production

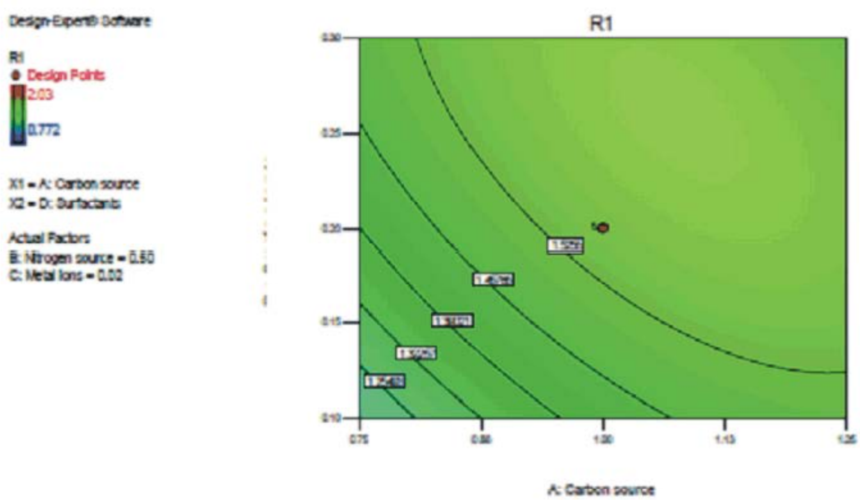

Fig. 9: Contour plot showing the effect of carbon source and surfactants on EPS production

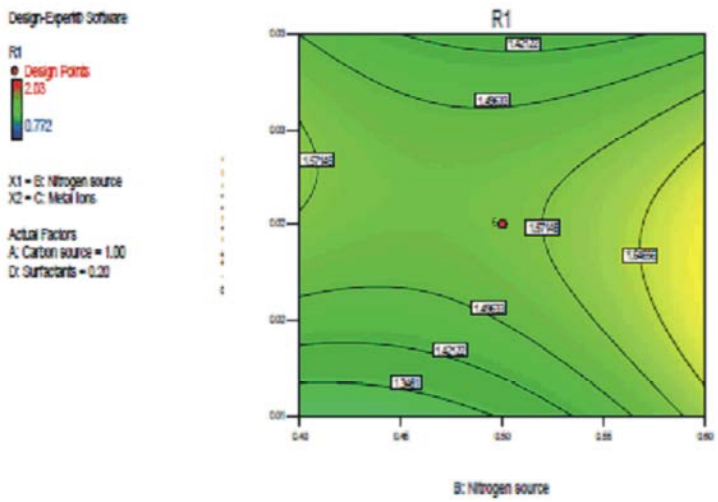

Fig. 10: Contour plot showing the effect of nitrogen source and metal ions on EPS production

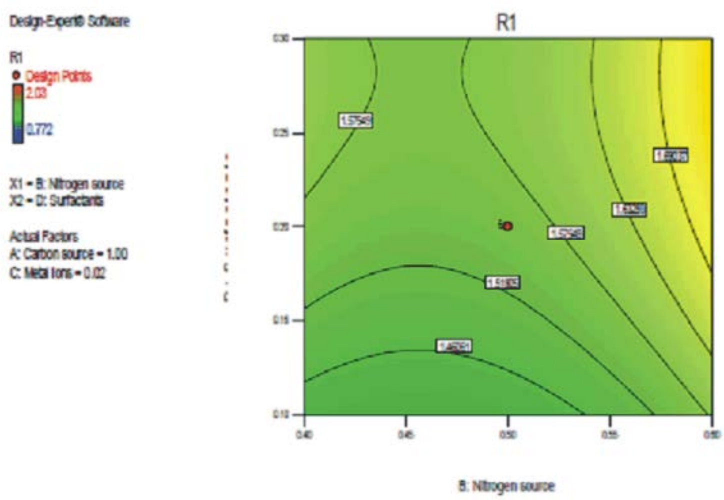

Fig. 11: Contour plot showing the effect of nitrogen source and surfactants on EPS production

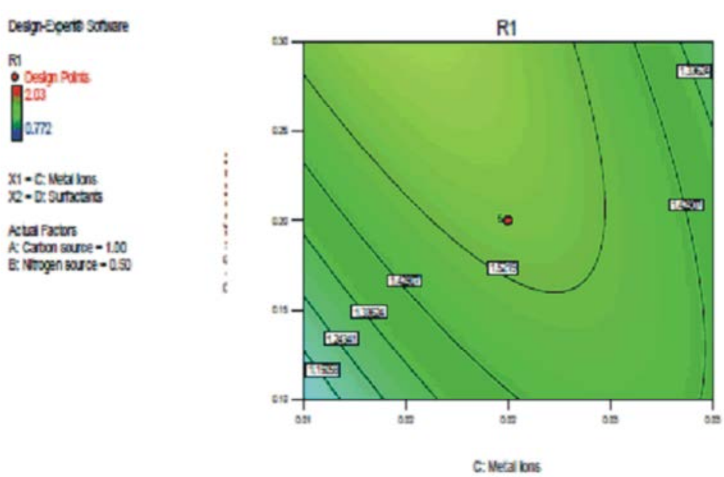

Fig. 12: Contour plot showing the effect of metal ions and surfactants on EPS production

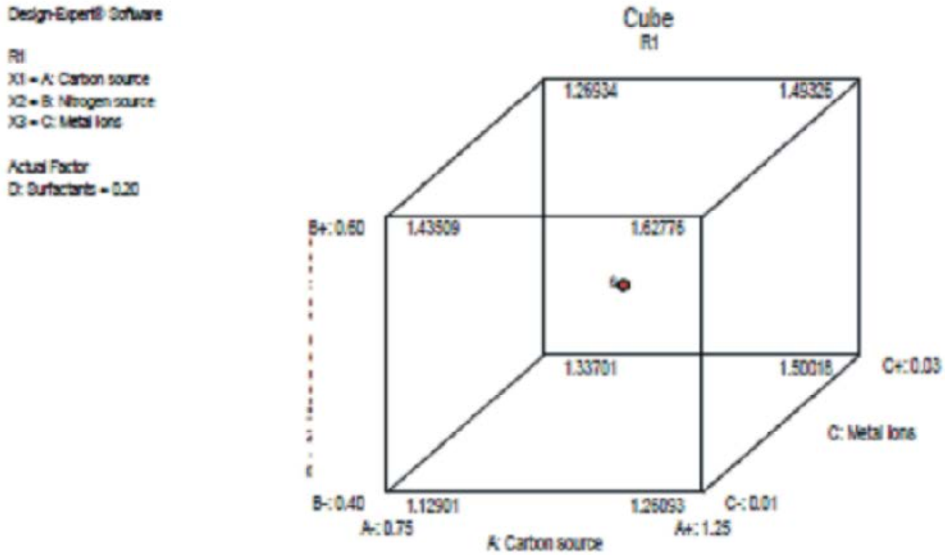

Fig. 13: Cube model showing the effect of carbon source, nitrogen source and metal ions on EPS production

DISCUSSION

In the present study, model of RSM was employed in the optimization of major EPS producing conditions such as dextrose, peptone, glutamine, $MgSO_4$. The second-order quadratic model with the optimum conditions (Dextrose 1%, $MgSO_4$ 0.02%, peptone 0.7% and glutamine 0.2%). The nearness of the coefficient of determination (R^2=0.9982) to 1 ensures the satisfactory adjustment of the quadratic model to the experimental data. The bacterial EPS recovered from statistical optimized medium was 258 ± 0.4mg/100ml of dry weight. Model of response surface methodology (RSM) was employed in the optimization of major encapsulation conditions such as concentration of sodium alginate, calcium chloride and curing time for the EPS producing microorganism *Lactobacillus plantarum*. The second-order quadratic model with the optimum conditions (Sodium alginate 2% (By mass per volume), calcium chloride 0.5 M and curing time 3 h) resulted in a maximum titer of (0.9 ± 0.1) g/L of exopolysaccharides (EPS) at 72 hours. The nearness of the coefficient of determination (R2=0.97) to 1 ensures the satisfactory adjustment of the quadratic model to the experimental data [14].

Likewise model of RSM was employed in the optimization of major EPS producing conditions such as jaggery, glutamine, ferric chloride. The second-order quadratic model with the optimum conditions (Jaggery-1%, ferric chloride-0.02% and glutamine-0.2%). The nearness of the coefficient of determination (R^2=0.8716) to 1 ensures the satisfactory adjustment of the quadratic model to the experimental data [11].

This study correlated to the optimum parameters for keratinase production by *Bacillus thuringiensis* using response surface methodology (RSM) based on central composite design (CCD) model. Optimum conditions for keratinase production by *Bacillus thuringiensis* were: pH 10, temperature 50°C and mannitol (1%). By optimizing with coded factor the maximum keratinase production experiential by the model was 63.01 U/ml [15].

Shankar and Isaiarasu reported that the optimum conditions for cellulase production by *Bacillus pumilus* EWBCM1 were: galactose of 1.0 g/L, malt extract of 0.5 g/L and incubation time of 72hrs. Fisher's statistical testing was performed for the analysis of variance (ANOVA) for quadratic regression equations. By optimizing with coded factor the maximum cellulase production observed by the model was 0.5751 IU/ml [16].

Shankar *et al.* also reported that the optimal level of the key variables (orange peel, yeast extract and methionine) used to determine the effect of their interactions on invertase production using the statistical tool (CCD of RSM) by *Saccharomyces cerevisiae* MK. The second-order quadratic model with the optimum conditions was orange peel - 4%; yeast extract - 0.5% and methionine - 0.5%. The nearness of the coefficient of determination (R =0.9994) to 1 ensures 2 the satisfactory adjustment of the quadratic model to the experimental data. The maximum invertase production was calculated as 0.50 IU/ml [17]. Optimum conditions for keratinase production by *Bacillus cereus* were: pH 9, temperature 50°C and starch- 1%. By optimizing with coded factor the maximum keratinase production observed by the model was 60.67 U/ml [18].

CONCLUSIONS

In the present study the Statistical optimization (RSM-CCD) was carried out using Stat Ease – Design Expert software, Minneapolis, USA. The bacterial EPS recovered from statistical optimized medium was 258 ± 0.4 mg/100ml of dry weight. The physicochemical characterization was done for the EPS crude extract obtained from *Lactobacillus plantarum* and it was purified by column chromatography.

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