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Optimization of Culture Medium for Exopolysaccharide Production by *Enterobacter cloacae* Z0206 Using Response Surface Methodology

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The quantitative effects of maltose, tryptone and beef extract concentration in the medium on yield of exopolysaccharide produced by *Enterobacter cloacae* Z0206 were investigated using response surface methodology. The experimental data obtained were fitted to a second-order polynomial equation using multiple regression analysis and also analyzed by appropriate statistical methods. By solving the regression equation and analyzing the response surface contour plots, the optimal medium was determined: maltose 37.150 g/L, tryptone 5.101 g/L and beef extract 5.627 g/L. Under the optimal conditions, the corresponding response value predicted for exopolysaccharide production was 12.950 g/L of fermentation liquor, which was also verified by validation experiments.

Key Words: Optimization, Exopolysaccharide, *Enterobacter cloacae*, Response surface methodology, Box-Behnken design.

INTRODUCTION

In recent years, a large number of biopolymers produced by microorganisms, most of which are polysaccharides, have been isolated because of their specific functions. Those macromolecules are widely used in the food, pharmaceutical and chemical industries and functions as bioflocculants, bioabsorbents, heavy metal removal agents, drug delivery agents, *etc.*[1,2].

Enterobacter cloacae Z0206, a bacterial strain, can produce large amounts of exopolysaccharide. In our previous studies[3], we found that exopolysaccharide-1, the major component of exopolysaccharide, could provide protection against cyclophosphamide-induced immunosuppression and oxidative damage in mice model. It has also been reported that glycoproteins from *E. cloacae* showed antitumor effects on mice with S180 tumors[4]. Besides, Prasertsan *et al.*[5], reported *E. cloacae* WD7 could produce exopolysaccharide possessing high flocculating activity with the yield of 2.27 g/L.

It is well known that optimization of cultivation medium plays an important role in enhancement of the desired products[6]. There were many reports on improving fermentation medium performance for producing microbial metabolites by statistical optimization techniques[7]. However, optimization of fermentation medium for exopolysaccharide production by *E. cloacae* is limited to conventional techniques such as one-factor-at-a-time method[8], which is extremely laborious, time-consuming and unable to detect the frequent interactions between two or more factors[9]. Response surface methodology (RSM) is a comprehensive methodology employing factorial designs to optimize chemical production processes such as medium composition[10] and cultivation conditions[11]. It is a powerful technique for testing multiple variables because fewer experimental trials are needed and interaction between variables can be identified and quantified[12].

In our preliminary experiments, the suitability of various carbon and nitrogen sources for the effective production of exopolysaccharide by *E. cloacae* Z0206 was evaluated[13]. The results indicated that the concentration of carbon source (maltose) and nitrogen source (a combination of tryptone and beef extract) were the major constituents affecting the yield of exopolysaccharide produced by *E. cloacae* Z0206. The objective of this investigation is to optimize the medium composition of above three factors to increase the yield of exopolysaccharide using response surface methodology.

EXPERIMENTAL

Microorganism: *E. cloacae* Z0206 was kept in our laboratory and it has been collected by China General Microbiological Culture Collection Center (CGMCC). It was maintained on potato-dextrose-agar (PDA) slant subcultured every 4 weeks.

Shake flask cultivation: *E. cloacae* Z0206 was initially grown on potato-dextrose-agar medium at 32 ºC for 1 day and

then transferred to 250 mL flasks containing 80 mL of seed culture medium (fresh potato, 20 % (w/v); dextrose, 2 %; peptone, 0.2 %; yeast extract, 0.3 %) and incubated on a rotary shaker at 250 rpm for 18 h at 32 ºC.

0.05 % KNO_3, 0.3 % K_2HPO_4 and 0.15 % $MgSO_4$ were treated as composition of the basal cultivation medium, concentrations of maltose, tryptone and beef extract in the medium was varied according to the experimental design. The optimization study experiments were carried out in 250 mL flask with 50 mL of cultivation medium at 220 rpm for 48 h at 32 ºC. The initial pH was 7.5 and the inoculation volume was 4.0 % (v/v).

Estimation of exopolysaccharide: After cultivation, the broth was centrifuged at 4500 × g for 20 min to remove the mycelia. The supernatant was precipitated upon addition of 4 volumes of cold 95 % ethanol and kept at -20 °C for 2 h. The resulting precipitate was collected by centrifugation at 7600 × g for 15 min at 4 ºC, dried for 12 h at 80 ºC and weighted to constant weight after cooling in a desiccator. Exopolysaccharide content was determined by the phenol-sulfuric acid method[14].

Response surface methodology experimental design and statistical analysis: A three level (-1, 0, 1) three factor Box-Behnken factorial design[15] was applied to optimize the exopolysaccharide production. The three variables, consisted of the concentrations of maltose, tryptone and beef extract, were designed as X_1, X_2 and X_3. The range and level of these variables are given in Table-1. A total of 15 experimental sets with appropriate combinations of three factors were conducted (Table-2).

TABLE-1
VARIABLES AND EXPERIMENTAL DESIGN LEVELS FOR RESPONSE SURFACE

Variables (g/L)	Coded symbols	Levels		
		-1	0	1
Maltose	X_1	20	30	40
Tryptone	X_2	2	4	6
Beef Extract	X_3	2	4	6

TABLE-2
BOX-BEHNKEN DESIGN MATRIX AND THE RESPONSES OF THE DEPENDENT VARIABLES EPS

Runs	Maltose (X_1)	Tryptone (X_2)	Beef Extract (X_3)	EPS (g/L)	
				Experimental	Predicted
1	-1	-1	0	7.760	7.733
2	1	-1	0	8.870	8.836
3	-1	1	0	9.760	9.794
4	1	1	0	12.533	12.560
5	-1	0	-1	9.693	9.370
6	1	0	-1	10.960	10.644
7	-1	0	1	9.580	9.896
8	1	0	1	12.170	12.493
9	0	-1	-1	7.520	7.870
10	0	1	-1	11.487	11.776
11	0	-1	1	10.360	10.071
12	0	1	1	12.300	11.950
13	0	0	0	12.153	11.964
14	0	0	0	11.967	11.964
15	0	0	0	11.773	11.964

Average from replicated values of exopolysaccharide production was taken as dependent variable or response Y_{EPS}. To predict the optimal point and the parameter of the mathematical model, a second-order polynomial equation was fitted to correlate the relationship between independent variables and response as follows:

$$Y_{EPS} = \beta_0 + \Sigma\beta_i X_i + \Sigma B_{ii} X_{ii}^2 + \Sigma\beta_{ij} X_{ij} \quad (1)$$

where Y_{EPS}, β_0, β_i, β_{ii}, β_{ij}, represent the predicted response in the form of exopolysaccharide production, the constant process effect in total, the linear, quadratic effect of X_i and the interaction effect between X_i and X_j on exopolysaccharide production.

The software Minitab 15.0 was used for experimental design, data analysis and the quadratic model building. The three-dimensional response surface plots were generated using the software Design Expert 7.0. The optimal medium composition for exopolysaccharide was obtained by solving the regression equation and analyzing the response surface plots using the same software.

RESULTS AND DISCUSSION

Response surface methodology model fitting: In order to investigate the culture medium composition for the optimization of exopolysaccharide production, maltose, tryptone and beef extract were studied to evaluate their effects on the production of exopolysaccharide using response surface methodology based on a three level Box-Behnken factorial design in this study. The yield of exopolysaccharide was selected as the response and the levels of the variables were selected according to the results of the previous experiments[13]. The center point of the corresponding composition was selected to be maltose 30 g/L, tryptone 4 g/L and beef extract 4 g/L.

A total of 15 designed experiments were carried out in the current Box-Behnken design. Each run was performed in duplicate and thus the values of exopolysaccharide given in Table-2 were averages of two sets of experiments, while the predicted values of responses were obtained from quadratic model fitting techniques using the software Minitab 15. Maximum yield of exopolysaccharide (12.533 g/L of fermentation liquor) was recorded under the experimental conditions of maltose 40 g/L, tryptone 6 g/L and beef extract 4 g/L.

Data obtained from the experiments were analyzed by linear multiple regression. The analysis of variance (ANOVA) of the quadratic regression model (Table-3) showed that the *p*-value of the model was smaller than 0.01, which indicated that the model was suitable for use in this experiment. The coefficient of determination (R^2) value was 97.69 %, indicating that only 2.31 % of the total variance could not be explained by the model. The adjusted R^2 value was 93.54 %, which was also satisfactory to confirm the significance of the model. The *p*-value of "lack of fit" was 0.118 ($p > 0.01$), which indicated that "lack of fit" was insignificant relative to the pure error. All of this suggested that the accuracy and general availability of the polynomial model were adequate.

The coefficient estimate for the parameter optimization (Table-4) suggested that all the independent variable studied (X_1, X_2, X_3) and two quadratic terms (X_1^2, X_2^2) significantly affected the exopolysaccharide yield. The model reveals that

TABLE-3
ANALYSIS OF VARIANCE (ANOVA) FOR RESPONSE SURFACE QUADRATIC MODEL

Source	DF[a]	Seq SS[b]	Adj MS[c]	F	P
Regression	9	37.9381	4.2153	23.51	0.001
Linear	3	27.0419	9.0140	50.28	< 0.001
Square	3	8.7401	2.9134	16.25	0.005
Interactions	3	2.1562	0.7187	4.01	0.085
Residual errors	5	0.8964	0.1793	–	–
Lack of fit	3	0.8242	0.2747	7.61	0.118
Pure error	2	0.0722	0.0361	–	–
Total	14	38.8345	–	–	–

[a]DF: degree of freedom, [b]SS: sum of square, [c]MS: mean square.

TABLE-4
MODEL COEFFICIENT ESTIMATED BY MULTIPLIES LINEAR REGRESSION[a]

Factor	Coefficient	Standard error	*t*-Value	*p*-Value
Intercept	11.9643	0.2445	48.943	< 0.001
X_1	0.9675	0.1497	6.463	0.001
X_2	1.4463	0.1497	9.661	< 0.001
X_3	0.5938	0.1497	3.966	0.011
X_1X_1	-1.0248	0.2203	-4.651	0.006
X_2X_2	-1.2088	0.2203	-5.486	0.003
X_3X_3	-0.3388	0.2203	-1.538	0.185
X_1X_2	0.4158	0.2117	1.964	0.107
X_1X_3	0.3307	0.2117	1.562	0.179
X_2X_3	-0.5067	0.2117	-2.394	0.062

[a]R^2 = 97.69 %; R^2 (adjusted) = 93.54 %.

tryptone concentration (X_2) had the largest coefficient and it was the most significant parameter which influenced exopolysaccharide production followed by maltose (X_1) and beef extract (X_3). Positive coefficient of X_1, X_2 and X_3 indicated a linear effect to increase exopolysaccharide yield. However, quadratic terms (X_1^2 and X_2^2) had negative effects. The final predictive corresponding second-order response model for the regression was obtained as following while neglecting the insignificant terms (in term of coded factors):

$$Y_{EPS} = 11.9643 + 0.9675X_1 + 1.4463X_2 + 0.5938X_3 - 1.0248X_1^2 - 1.2088X_2^2 \quad (2)$$

The three-dimensional response surface plots are generally the graphical representation of the regression equation and very useful to see interaction effects of the factors on the responses. The effect of maltose, tryptone concentrations and their mutual effect on the production of exopolysaccharide is shown in Fig. 1a. At the designed range of maltose concentration from 20-40 g/L, the yield of exopolysaccharide increased with tryptone concentration increasing. At a high tryptone concentration, the yield of exopolysaccharide increased significantly from 9.79-12.77 g/L with tryptone concentration increasing. But the exopolysaccharide yield just increased from 7.73-9.71 g/L at lower level of tryptone. Increase of maltose and tryptone concentration at appropriate range resulted in the increase of exopolysaccharide yield.

The effect of maltose and beef extract concentrations on the synthesis of exopolysaccharide is provided in Fig. 1b. At the designed range of maltose concentration from 20-40 g/L, the yield of exopolysaccharide increased with beef extract concentration increasing. And the exopolysaccharide yield was increased too with maltose increase at the design range of beef extract from 2-6 g/L. Exopolysaccharide was affected significantly by maltose concentration.

Fig. 1c shows the effect of interaction of tryptone and beef extract concentration on the yield of exopolysaccharide. It can be seen that with the increase of beef extract concentration, the exopolysaccharide increased from around 7.87-10.07 g/L at low tryptone concentration, but only increased from 11.78-11.95 g/L at high level of tryptone concentration. Increasing the concentration of tryptone and beef extract at appropriate range is beneficial to the yield of exopolysaccharide. This observation can be attributed to the inhibition of mycelium growth and exopolysaccharide production by high level of nitrogen sources.

The results above show that tryptone and beef extract had a significant positive correlation with exopolysaccharide production. Due to their complex components, it is different to find what dominated exopolysaccharide production. Amino nitrogen, the principal component of tryptone and beef extract, might be suggested to explain this result[16]. Organic nitrogen or other unidentified substances in tryptone and beef extract may influence exopolysaccharide production. Further studies will be carried out to clarify the stimulatory effect of tryptone and beef extract on exopolysaccharide production.

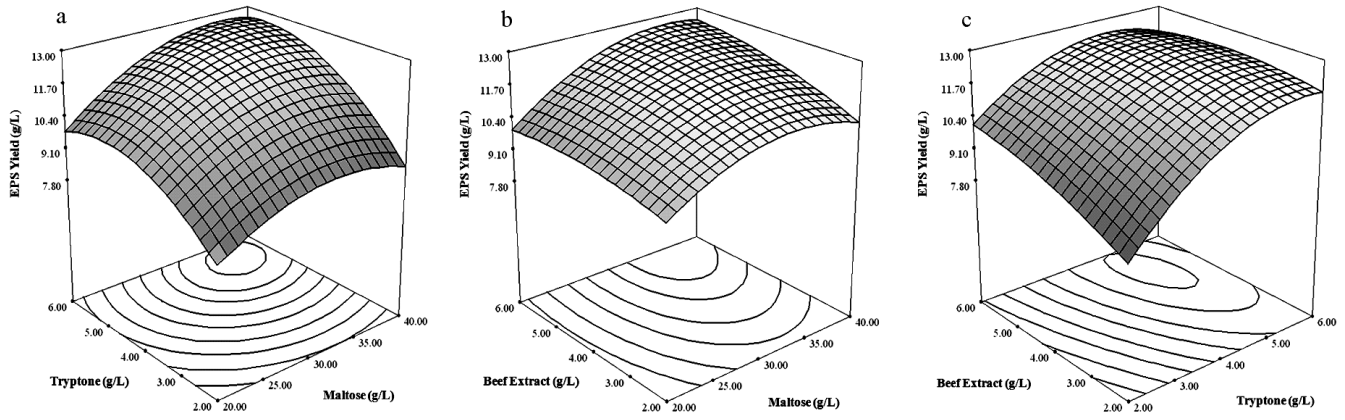

Fig. 1. Response surface plots of the yield of exopolysaccharide as affected by concentrations of maltose, tryptone and beef extract in the medium. Where (a) is maltose and tryptone concentrations (beef extract, 4 g/L); (b) is maltose and beef extract concentrations (tryptone, 4 g/L); (c) is tryptone and beef extract concentrations (maltose, 30 g/L)

Validation of the models: By solving the regression equation (eqn. 2), the optimum values of the selected test variables for exopolysaccharide production were obtained as follows: maltose 37.150 g/L, tryptone 5.101 g/L and beef extract 5.627 g/L. Under the above conditions, the maximum predicted yield of exopolysaccharide was 12.950 g/L of fermentation liquor. In order to verify the prediction of the model, experimental rechecking was performed using this deduced optimal condition of three independent replicates for exopolysaccharide synthesis. The average exopolysaccharide yield of 12.885 ± 0.135 g/L, demonstrated the validation of the response surface methodology model. The excellent correlation between these predicted and measured results confirmed that the response model was adequate for reflecting the expected optimization. As a result, the models developed were considered to be accurate and reliable for prediction the production of exopolysaccharide.

Prasertsan *et al.*[8], reported their results on optimization of exopolysaccharide production using one-factor-at-a-time method and continuous culture studies. With their optimized medium composition and culture conditions, the highest level of exopolysaccharide was 7.28 g/L. The yield of exopolysaccharide obtained in our study (12.885 g/L) was almost 1.8 times the highest yield reported above. To our knowledge, this is the first analysis of exopolysaccharide production by *E. cloacae* using response surface methodology.

Conclusion

In summary, the response surface methodology based on a three-factor Box-Behnken factorial design was used in the present investigation for the yield of exopolysaccharide produced by *E. cloacae* Z0206. By statistical analysis, the optimal concentrations of maltose, tryptone and beef extract in the medium were determined as 37.150, 5.101 and 5.627 g/L, respectively. The maximum exopolysaccharide yield of 12.950 g/L of fermentation liquor can be achieved under the above conditions. The results indicated that response surface methodology is a useful and effective method for optimization of cultural medium for exopolysaccharide production using *E. cloacae* Z0206 and statistical analysis is proved to be satisfactory and powerful in this process.

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