Optimization of succinic acid fermentation with *Actinobacillus succinogenes* by response surface methodology (RSM)[*]

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Abstract: Succinic acid is considered as an important platform chemical. Succinic acid fermentation with *Actinobacillus succinogenes* strain BE-1 was optimized by central composite design (CCD) using a response surface methodology (RSM). The optimized production of succinic acid was predicted and the interactive effects between glucose, yeast extract, and magnesium carbonate were investigated. As a result, a model for predicting the concentration of succinic acid production was developed. The accuracy of the model was confirmed by the analysis of variance (ANOVA), and the validity was further proved by verification experiments showing that percentage errors between actual and predicted values varied from 3.02% to 6.38%. In addition, it was observed that the interactive effect between yeast extract and magnesium carbonate was statistically significant. In conclusion, RSM is an effective and useful method for optimizing the medium components and investigating the interactive effects, and can provide valuable information for succinic acid scale-up fermentation using *A. succinogenes* strain BE-1.

Key words: Succinic acid, Response surface methodology (RSM), Optimization, *Actinobacillus succinogenes*
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1 Introduction

Succinic acid is an intermediate product of the tricarboxylic acid cycle (TCA), as well as one of fermentation products of anaerobic metabolism. It is identified as one of the most important platform chemicals based on the ease of its biotechnological production (Bechthold *et al*., 2008). It has wide industrial applications, such as food, pharmaceuticals, resins, and agriculture. Traditionally, succinic acid has been acquired from petrochemicals, which is costly and may cause pollution. Compared with tra-

ditional synthetic methods by fossil fuels, the production of succinic acid from a naturally-derived biomass would alleviate the dependence on oil supply in the future. Recently, microbial conversion of biomass into succinic acid has been attracting increasing interest as an eco-friendly and energy-saving process (Li *et al*., 2010).

Microorganisms intensively investigated for succinic acid fermentation include *Actinobacillus succinogenes* (Zheng *et al*., 2009), recombinant *Escherichia coli* (Wu *et al*., 2009), *Anaerobiospirillum succiniciproducens* (Lee *et al*., 2003), and *Mannheimia succiniciproducens* (Lee *et al*., 2002). Among them, *A. succinogenes* is able to accumulate higher succinic acid in the bioreactor, and is more resistant to succinic acid than any other previously reported succinic acid producers (Bechthold *et al*., 2008). Therefore, *A. succinogenes* is considered to be the most promising strain.

The theoretical yield is 2 mol succinic acid per mol glucose. However, the succinic acid yield (0.49 g/g glucose) in fermentation is not high enough for industrial production. Given the immense potential of *A. succinogenes*, it is worthwhile to optimize the production of succinic acid for maximum yields. The traditional method for succinic acid fermentation medium component optimization is carried out by changing one parameter at a time and keeping the others at a constant level (viz. 'one-at-a-time' method). This technique not only is time-consuming, but also lacks factor interaction analysis. Therefore, an alternate strategy involving a statistical approach, e.g., response surface methodology (RSM), was adopted to investigate the combined effects between the main factors (Sharma *et al.*, 2009). RSM is a collection of statistical and mathematical techniques for an optimizing process, which can be used to evaluate the interactions between a set of independent experimental factors and observed responses. This methodology can also reduce the replicated experimental units required to determine optimal conditions (Ghosh and Hallenbeck, 2010). Furthermore, it has been used for optimizing the fermentation medium and culture conditions (Mohana *et al.*, 2008; Yuan *et al.*, 2008; Açıkel *et al.*, 2010).

Recently, several studies have described the use of RSM for the fermentation process parameters in succinic acid production. Isar *et al.* (2006) reported on the concentration of succinic acid in fermentation with *E. coli* and interactive effects of five factors (sucrose, tryptone, magnesium carbonate, inoculum size, and incubation period). The concentration of succinic acid was increased from 7.00 to 14.30 g/L. In another project, the production of succinic acid obtained from the fermentation of *Bacteroides fragilis* was improved from 5.40 to 12.50 g/L under the process optimization with RSM (Isar *et al.*, 2007). However, no literature reported the optimization of the medium components in the production of succinic acid with *A. succinogenes* strain BE-1 by RSM. In this work, central composite design (CCD), the standard RSM, is used for the study of optimization of succinic acid fermentation media with *A. succinogenes* strain BE-1 as well as the interactive effects between the main components.

2 Materials and methods

2.1 Microorganism

A. succinogenes strain BE-1, a strain that produces a high concentration of succinic acid, was isolated from bovine rumen and collected in China General Microbiological Culture Collection Center (No. CGMCC2650).

2.2 Culture media and succinic acid fermentation

Inoculum was prepared in an aerobic flask containing 50 ml of 3% tryptic soy broth (TSB) medium (0.03 g/ml; pancreatic digest of casein 17.00 g/L, soy peptone 3.00 g/L, glucose 3.00 g/L, NaCl 5.00 g/L, and K_2HPO_4 2.50 g/L). The seed cultures were grown in a rotary shaker at 37 °C and 180 r/min for 5 h. The fermentation was conducted in 100 ml flask with 50 ml of the medium containing: glucose 30.00 g/L, yeast extract 10.00 g/L, K_2HPO_4 1.37 g/L, KH_2PO_4 1.53 g/L, NaCl 1.50 g/L, $MnCl_2$ 0.07 g/L, $CaCl_2$ 0.38 g/L, and $MgCO_3$ 30.00 g/L. All chemicals used in this study were of analytical level and were purchased from either OXOID (England) or Sinopharm Chemical Reagent Beijing Co., Ltd. (China) unless otherwise described. Glucose was separately sterilized at 115 °C for 20 min and added to the medium to maintain the initial concentration of 30.00 g/L. The initial pH of the sterilized medium was adjusted to 7.00 by H_3PO_4. The fermentation media were inoculated with 5% of the seeds prepared and grown in the rotary shaker at 37 °C and 40 r/min for 12 h.

2.3 Analysis methods

The culture broth was centrifuged at 10000×*g* for 10 min, filtered, and 10 μl of each test sample was run on high-performance liquid chromatography (HPLC; Agilent 1200, USA) equipped with an Agilent model DAD UV-Vis detector and ZORBAX SB-Aq column (25 cm×4.6 mm, 5 μm; Agilent). The column was operated at 25 °C. The mobile phase was 20 mmol/L KH_2PO_4/H_3PO_4 buffer of the pH 2.70 at a flow rate of 1.0 ml/min. And the glucose used in the fermentation was analyzed by SBA-40D Biosensor Analyzer (Biology Institute of Shandong Academy of Sciences, China).

2.4 Key medium components screened for succinic acid production

Experiments were carried out by the conventional 'one-at-a-time' method to select the suitable factors for maximum succinic acid production. The medium components (carbon, nitrogen, and metal ions) were selected while the temperature, pH, medium volume, inoculum size, and fermentation time were examined as the conditions of the fermentation. The optimal concentrations of the selected key factors were further determined to obtain a higher production of succinic acid.

2.5 RSM: CCD as the experimental design

In order to evaluate the effect of factors on the response surface in the region of investigation, a three-factor-five-level CCD was performed (Aktaş et al., 2006). Based on the best results of the one-at-a-time method, the ranges and levels of three variables viz. glucose (A), yeast extract (B), and magnesium carbonate (C) are listed in Table 1. The test variables were coded according to the following equation (Kılıç et al., 2002):

$$x_i = (X_i - X_i^*)/\Delta X_i, \tag{1}$$

where x_i is the coded value of the ith independent variable, X_i is the uncoded value of the ith independent variable, X_i^* is the uncoded ith independent variable at the centre point, and ΔX_i is the step change value.

Table 1 Experimental ranges and levels of the three independent variables used in RSM in terms of actual and coded factors

Coded value	Actual value (g/L)		
	Glucose	Yeast extract	MgCO₃
−1.68	9.89	3.30	9.89
−1	15.00	5.00	15.00
0	22.50	7.50	22.50
+1	30.00	10.00	30.00
+1.68	35.11	11.70	35.11

The statistical software package 'Design-Expert 7.0 (trial version)' was used to analyze the experimental design. The total number of experiments with three factors was 20 (2^k+2k+6, when $k=3$, where k is the number of factors). The design matrix with three

variables (glucose, yeast extract, and magnesium carbonate)×five levels (−1.68, −1, 0, +1, +1.68) is presented in Table 2. All the variables were taken at the coded values. In order to control the error bar, 20 runs were performed in a random order in which there were six replications at the center points to evaluate the pure error. Process performance was evaluated by analyzing the concentration of succinic acid produced after 12 h in the fermentation medium. In optimization, the response can be related to chosen factors by linear or quadratic models. A quadratic model, which also includes a linear model, is given as follows:

$$Y=\beta_0+\beta_1 A+\beta_2 B+\beta_3 C+\beta_{12} AB+\beta_{13} AC+\beta_{23} BC+ \\ \beta_{11} A^2+\beta_{22} B^2+\beta_{33} C^2, \tag{2}$$

where Y is the predicted response; β_0, intercept; β_1, β_2, β_3, linear coefficients; β_{12}, β_{13}, β_{23}, interaction coefficients; β_{11}, β_{22}, β_{33}, squared coefficients. Data were processed for Eq. (2) using the Design-Expert 7.0 program including analysis of variance (ANOVA) to obtain the interactive effects between the process variables and the response. The quality of fit of the polynomial model was expressed by the coefficient of determination R^2, and its statistical significance was checked by the F-test in the same program.

2.6 Validation of the model

The second-order polynomial regression equation obtained from the experimental data can be used to predict the response at any levels of the variables within the range of the experimental design. In order to determine the accuracy of the model, the concentrations of three factors (glucose, yeast extract, and magnesium carbonate), which had a major influence on succinic acid production, were randomly selected within the design space. The remaining components of the medium in this experiment were at fixed levels. Four sets of experiments were carried out.

3 Results and discussion

3.1 Medium components screened for succinic acid production with one-at-a-time method

In order to select the key parameters for succinic acid production with A. succinogenes strain BE-1, the

one-at-a-time method was conducted as described above. It showed that the key parameters were glucose (22.50 g/L), yeast extract (7.50 g/L), magnesium carbonate (22.50 g/L), inoculum size (5%), and fermentation time (12 h). The initial fermentation pH was at 7.00. This resulted in the production of 14.80 g/L of succinic acid in 12 h at 37 °C (data not shown). The by-products were also determined by HPLC, and the yields of formic acid, acetic acid, and lactic acid were 2.8, 3.2, and 2.3 g/L, respectively. Thus, the medium obtained by the one-at-a-time method was: glucose 22.50 g/L, yeast extract 7.50 g/L, K_2HPO_4 1.37 g/L, KH_2PO_4 1.53 g/L, NaCl 1.50 g/L, $MnCl_2$ 0.07 g/L, $CaCl_2$ 0.38 g/L, and $MgCO_3$ 22.50 g/L (pH 7.00).

3.2 CCD

A three-variable-five-level matrix of CCD was employed to determine the optimized conditions and the interactive effects. Glucose, yeast extract, and magnesium carbonate were selected as the factors for CCD. The succinic acid concentrations for each individual run along with the predicted responses are summarized in Table 2. The highest succinic acid concentration of 18.86 g/L was attained at 12 h when the concentrations of glucose, yeast extract, and

magnesium carbonate were 30.00, 10.00, and 30.00 g/L, respectively, with 5% inoculum volume (Run 8). Also, the lowest succinic acid concentration was 0.104 g/L, which was gained when glucose, yeast extract, and magnesium carbonate concentrations were 22.50, 3.30, and 22.50 g/L, respectively (Table 2). Based on the analysis of the software, the optimized concentrations of glucose, yeast extract, and magnesium carbonate were 27.43, 9.56, and 23.32 g/L and the predicted concentration of succinic acid was 19.08 g/L. This was a 28.9% improvement over that attained with the one-at-a-time method.

The response data were analyzed in the Design-Expert software. The application of RSM yielded the following regression equation, which is an empirical relationship between succinic acid and the test variables in coded units:

$$Y=16.41+2.47A+4.07B+0.39C+0.53AB+0.41AC+1.68BC-1.50A^2-3.18B^2-0.89C^2, \qquad (3)$$

where Y is the succinic acid produced as a function of glucose (A), yeast extract (B), and magnesium carbonate (C). The statistical significance of the above equation was checked by the F test, and the ANOVA for the response surface quadratic model is shown in Table 3. The model F value of 14.95 and values of probability (P)>F (0.0001) indicated that the model terms were significant. The regression equation showed that the R^2 was 0.9308 (Table 3), which indicated aptness of the model (Isar *et al*., 2006). This was similar to R^2 of 0.936 reported by Singh and Chhatpar (2010). This result indicates that approximately 93% of the variability in the dependent variable (response) can be explained by this model. The R^2 value is always between 0 and 1. The closer the R^2 is to 1.0, the stronger the model and the better it predicts the response (Aghaie *et al*., 2009). The adjusted R^2, which corrects the R^2 value for the sample size and for the number of terms, was 0.8686. The lack of fit F value was 1.81 (the lack of fit value was not significant in relation to the pure error). Non-significant lack of fit indicated that the model was a good fit (Song *et al*., 2007). The adequate precision value, which measured the signal to noise ratio, was 14.432. The ratio greater than 4 is desirable. Thus, this model could be used to navigate the design space (Ghosh and Hallenbeck, 2010).

Table 2 Design matrix of centered central composite design (CCD) for succinic acid production

Run	Glucose	Yeast extract	MgCO$_3$	Succinic acid (g/L)	
				Observed	Predicted
1	−1	−1	−1	7.884	6.543
2	+1	−1	−1	10.403	9.609
3	−1	+1	−1	11.692	10.267
4	+1	+1	−1	15.127	15.441
5	−1	−1	+1	3.260	3.138
6	+1	−1	+1	6.223	7.840
7	−1	+1	+1	12.601	13.589
8	+1	+1	+1	18.863	20.399
9	−1.68	0	0	6.782	8.021
10	+1.68	0	0	17.804	16.326
11	0	−1.68	0	0.104	0.586
12	0	+1.68	0	14.998	14.278
13	0	0	−1.68	11.226	13.257
14	0	0	+1.68	16.831	14.562
15	0	0	0	14.447	16.421
16	0	0	0	15.435	16.421
17	0	0	0	15.070	16.421
18	0	0	0	17.425	16.421
19	0	0	0	18.541	16.421
20	0	0	0	17.518	16.421

Table 3 Analysis of variance (ANOVA) for the quadratic model[a]

Source	Sum of squares	Degree of freedom	Mean square	F value	Prob>F	Significance
Model	506.70	9	56.30	14.95	0.0001	Significant
Residual (error)	37.66	10	3.77			
Lack of fit	24.26	5	4.85	1.81	0.2652	Not significant
Pure error	13.40	5	2.68			
Total	544.35	19				

[a] Coefficient of determination (R^2)=0.9308; Adjusted R^2=0.8686; Coefficient of variation (CV)=15.39%; Adeq precision=14.432

The F test and the corresponding P values along with the parameters were estimated (Table 4). The smaller the P value, the larger the significance of the corresponding coefficient (Qi *et al.*, 2009). The parameters estimated and the corresponding P values suggest that, among the independent variables, glucose (A) and yeast extract (B) have significant effects on succinic acid production. The quadratic terms of glucose (A), yeast extract (B), and interaction between yeast extract (B) and magnesium carbonate (C) also have significant effects on succinic acid yield.

Table 4 Significance of the coefficients of regression

Model term	Parameter estimated	Standard error	F value	P value
β_0	16.41	0.79		
β_1	2.47	0.53	22.11	0.0008[**]
β_2	4.07	0.53	60.03	<0.0001[**]
β_3	0.39	0.53	0.54	0.4796
β_{12}	0.53	0.69	0.59	0.4602
β_{13}	0.41	0.69	0.36	0.5645
β_{23}	1.68	0.69	6.00	0.0342[*]
β_{11}	−1.50	0.51	8.63	0.0149[*]
β_{22}	−3.18	0.51	38.65	<0.0001[**]
β_{33}	−0.89	0.51	3.02	0.1131

[**] $P<0.01$ indicates that model terms are highly significant at the 1% level; [*] $P<0.05$ indicates that model terms are significant at the 5% level

The response surface curves were then plotted to study the interaction among different factors, and to determine the optimal concentration of each factor for maximum succinic acid production. As shown in Fig. 1a, the effects of glucose and yeast extract on the production of succinic acid were determined when the other factor was at its center point. When yeast extract was at a low level, the yield of succinic acid was low. Significant improvement in succinic acid production can be obtained by increasing the amount of yeast extract to some extent.

The effects of glucose and magnesium carbonate are shown in Fig. 1b. Magnesium carbonate is considered as one of the main factors to control the pH of the fermentation broth, and provides magnesium ions for phosphoenolpyruvate carboxykinase, which is a key enzyme in succinic acid production (Lee *et al.*, 1999). When the glucose was at a low level, an increase in magnesium carbonate did not improve the succinic acid concentration. However, if the glucose and magnesium carbonate were at high levels, more succinic acid could be attained.

The interaction of yeast extract and magnesium carbonate on the concentration of succinic acid, when glucose concentration was at its center point, was statistically significant, as shown in Fig. 1c. When the concentration of magnesium carbonate was 15.00 g/L, the succinic acid concentration increased at some extent with the amount of yeast extract added less than 7.50 g/L; while with yeast extract at a higher concentration (>10.00 g/L), adding enough magnesium carbonate can lead to a higher concentration of succinic acid.

3.3 Validation of model

The model was validated for the three variables within the design space. The conditions and results of four experiments are listed in Table 5. The result shows that under the following conditions: glucose 25.00 g/L, yeast extract 10.00 g/L, and magnesium carbonate 25.00 g/L, the concentration of succinic acid (18.23 g/L) nearly reached the optimized concentration, viz. 19.08 g/L. The predicted values and actual experimental values were compared, and then the residual and percentage error were calculated in Table 5. It was observed that the percentage errors between the actual and predicted values for succinic acid production varied from 3.02% to 6.38%. This range of the percentage error accepted was similar in the confirmation experiments of optimization of
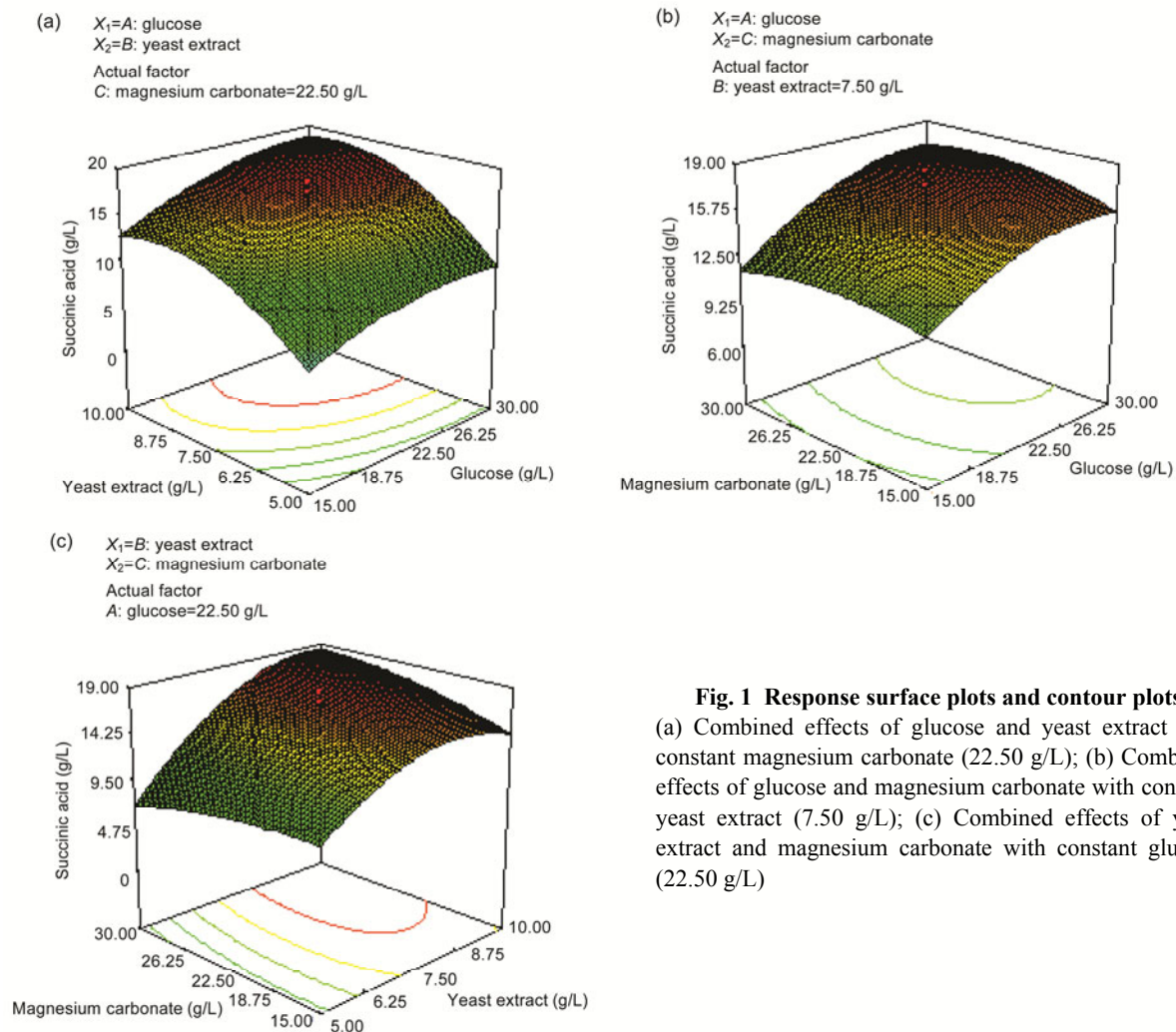
**Fig. 1 Response surface plots and contour plots**
(a) Combined effects of glucose and yeast extract with constant magnesium carbonate (22.50 g/L); (b) Combined effects of glucose and magnesium carbonate with constant yeast extract (7.50 g/L); (c) Combined effects of yeast extract and magnesium carbonate with constant glucose (22.50 g/L)

Table 5 Confirmation experiments

No.	Glucose (g/L)	Yeast extract (g/L)	MgCO₃ (g/L)	Succinic acid concentration			
				Actual (g/L)	Predicted (g/L)	Residual (g/L)	Error (%)
1	20.00	5.00	30.00	6.32	6.040	0.280	4.43
2	20.00	10.00	35.00	15.89	16.905	−1.015	6.38
3	20.00	5.00	20.00	9.22	8.736	0.484	5.25
4	25.00	10.00	25.00	18.23	18.780	−0.550	3.02

enzymatic hydrolysis conditions (Qi *et al*., 2009). Therefore, the empirical models were reasonably accurate, and the RSM analysis is indeed a useful technique to predict and optimize the fermentation media. Usually, it is necessary to check the adequacy of the model to ensure that it provides maximum approximation on relationship between response and

factors. The residuals from the least squares are an important tool for judging the model adequacy. Fig. 2 shows the plot of residuals vs. the predicted response. The residual plots of the model are randomly distributed without any trends. This result indicates good predictions of maximum response along with constant variance and adequacy of the quadratic models.
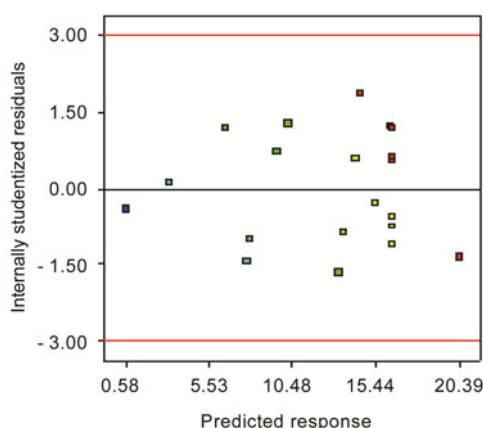
Fig. 2 Plot of internally studentized residuals vs. predicted response

4 Conclusions

RSM was successfully applied to the optimization of succinic acid fermentation medium components. The predicted model of succinic acid production was developed in terms of fermentation factors by RSM, and an ANOVA test was performed. The glucose, yeast extract, and interactive effect of yeast extract and magnesium carbonate were the most significant factors in succinic acid production. The optimum values for glucose, yeast extract, and magnesium carbonate concentrations were found to be 27.43, 9.56, and 23.32 g/L, respectively. This resulted in a predicted value 19.08 g/L, which was increased by 28.9% compared with 14.80 g/L obtained from the one-at-a-time method. The validation of the model was also conducted, and the percentage errors between the actual and predicted values for succinic acid production varied from 3.02% to 6.38%. Therefore, the RSM approach can be quite efficient and useful for the optimization of succinic acid fermentation conditions, and the optimal conditions provide important parameters for scaled-up industry succinic acid fermentation with *A. succinogenes* strain BE-1.

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