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Statistical optimization for the production of recombinant cold-adapted superoxide dismutase in *E. coli* using response surface methodology

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ABSTRACT

Cold-adapted superoxide dismutase (SOD) with higher catalytic activity at lower temperature has great amount of applications in many aspects as an industrial enzyme. The application of recombinant enzyme in gene engineering and microbial fermentation technology is an effective way to obtain high-yield product. In this study, to obtain the recombinant SOD in *E. coli* (*rPs*SOD) with the highest activity, the Box-Behnken design was first applied to optimize the important parameters (lactose, tryptone and Tween-80) affecting the activity of r*Ps*SOD. The results showed that the optimal fermentation conditions were Tween-80 (0.047%), tryptone (6.16 g/L), lactose (11.38 g/L). The activity of *rPs*SOD was 71.86 U/mg (1.54 times) as compared with non-optimized conditions. Such an improved production will facilitate the application of the cold-adapted *rPs*SOD.

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Introduction

Superoxide dismutase (SOD, $_{EC}$ 1.15.1.1) is a class of metalloproteins as natural prosthetic groups, which is considered to be the first line in antioxidant system as an antioxidant enzyme.[1,2] SOD can give protection against oxidative stress and scavenge reactive oxygen species (ROS) which is highly toxic to cells.[3,4] Besides, previous studies have demonstrated that SOD plays a critical role in immune defense to kill invading microorganisms and can be applied in medical treatments, cosmetic and other chemical industrials.[5-9]

The application of recombinant enzymes in gene engineering and microbial fermentation technology is an effective way to obtain high-yield product.[7] The coding gene of the target protein can be transformed into industrial microorganism competent cells for larger production using recombination, which can solve the problems of the complexity of the extraction and the time limitation of specimen collection. *E. coli* is widely used for the recombinant expression of proteins in medicine, foodstuff and other related industries due to its ease of genetic manipulation, its fast growth, its high expression levels, and its ability to grow in inexpensive media, as well as the wealth of available genetic information.[10,11]

The conventional method of medium optimization, one factor at a time, is time-consuming, is expensive, and often leads to the misinterpretation of results when interactions between different components are present.[12] Response surface methodology (RSM) is commonly used for optimizing industrial processes and has been applied in various fields, such as biomedicine, food and agriculture. It is an effective mathematical and statistical technique that can evaluate and determine the interactions between different physiologic parameters.[13-15] The Box-Behnken design (BBD) of the RSM requires fewer experiments and a shorter cycle time for multi-variable optimization. It is an efficient and productive tool to optimize and evaluate the effects of process optimizing parameters and has been used to enhance the production of enzymes such as lipase from *Geotrichum candidum*, alkaline protease from *Bacillus* sp. and phytase from *Rhodotorula mucilaginosa*.[16-18]

In our previous studies, the statistical experimental method has proven to be a very effective tool to

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optimize the production of cold-active enzymes such as protease, lysozyme and lipase.[19-21] The estimated molecular weight of the 193-amino acid cold-adapted recombinant SOD enzyme purified in this study was 21.4 kDa, and it exhibited maximum activity at 30°C and pH 8.0 and high-thermo lability at temperatures greater than 50°C.[22] In addition, compared with normal SOD, low temperature SOD exhibits higher catalytic activity at lower temperatures (0–10°C).[23,24] Furthermore, cold-adapted r*Ps*SOD can be widely used in various fields and has great potential as a natural food preservative to replace chemical preservatives.[25] The aim of the present work was to develop the enzymatic bioprocess for industrial purposes by using a statistical experimental method to optimize the significant factors to improve cold-adapted r*Ps*SOD production.

Materials and methods

Bacterial strain and expression plasmid

A pET-SOD recombinant *E. coli* (BL21) strain was used in this study.[22] All chemicals were of analytical grade and were purchased from Qingdao, China, including lactose, sucrose, glucose, glycerol, soluble starch, citric acid, maize flour, maize steep liquor, urea, casein, tryptone, yeast extract, $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, Tween-80, Triton X-100, sodium dodecyl sulfate (SDS), NaCl and $Na_2HPO_4{\cdot}12H_2O$. The basic medium (Luria-Bertani, LB) for the pET-SOD *E. coli* consisted of 5 g/L yeast extract, 10 g/L NaCl and filtered seawater. This medium was stored at room temperature after high-temperature sterilization for further experiments. Cultures of pET-SOD *E. coli* were incubated until an OD_{600} between 0.5 and 0.7 was reached. IPTG was then added for induction, and the cultures were further incubated for 8 h at 28°C.

Assay of SOD activity

The SOD activity was determined using the method of Giannopolitis and Reis.[26] One unit of SOD activity was defined as the amount of enzyme that caused a 50% inhibition of the nitro blue tetrazolium (NBT) reduction rate at 25°C. SOD activity was expressed as units per milligram protein (U/mg). Protein concentrations were determined based on the Lowry method.[27]

Screening of important influential factors

To identify the important influential factors that affect the expression of SOD, single factor experiments were conducted with carbon sources, nitrogen sources and metal ions. Glucose, sucrose, glycerol, lactose, starch and citric acid at a final concentration of 1% were added to the basic medium, as they are the most common carbon sources for *E. coli*. In terms of nitrogen sources, tryptone, casein, urea, NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$ and NH_4NO_3 at a final concentration of 0.5% were also to the basic medium as the main nitrogen sources for *E. coli*. Finally, other additives were added to the medium: Na_2HPO_4, 0.5% maize flour, 0.5% maize steep liquor, 5 mM $FeCl_3$, 0.05% Tween-80, 0.2% Triton X-100, 1 mM polyethylene glycol 2000 (PEG 2000), 1 mM β-mercaptoethanol and 1% SDS. A climbing experimental combination was designed based on the results of the single factor experiments to identify the best center value for each factor (Table 1).

Experimental design and optimization for the production of cold-adapted SOD

After optimizing the nutritional factor levels using a one-variable-at-a-time approach, the 3 most important factors were analyzed (Tween-80, tryptone and lactose), which mainly controlled the protease production of *E. coli*. Based on these results, a reasonable climbing experimental combination was designed. For the purpose of improving the production of SOD, a BBD design of 3 factors at 3 levels was used, which indicated that 15 experiments were required. Three variables (Tween-80, tryptone and lactose) are represented by A, B, and C, and −1, 0, and 1 represent the 3 levels of each variable. The *Y* value was regarded as a prediction of r*Ps*SOD's activity. Based on the results of the BBD, a second order polynomial model was built between the significant factors and the response

Table 1. The design of the climbing experimental combination.

Run order	A-Tween-80 (%)	B-Tryptone (g/L)	C-Lactose (g/L)	r*Ps*SOD activity (U/mg)
1	0.00	2	6	—
2	0.02	4	8	—
3	0.04	6	10	—
4	0.06	8	12	—
5	0.08	10	14	—
6	0.10	12	16	—

values. In accordance with each calculated component value in each agar medium under the same conditions, 3 parallel experiments were performed, and the activity of r*Ps*SOD was determined.

Statistical analysis

An analysis of variance (ANOVA) and a multiple regression analysis were conducted to fit the model using BBD. The data were measured with various statistical analysis parameters, such as the *F*-value, the degrees of freedom (DF), the sum of squares (SS), the coefficient variation (CV) and the determination coefficient (R^2), to generate the statistical significance of the developed quadratic mathematical model. After fitting the data to the models, the data produced were used to plot the response surfaces and the contour plots. All the data are presented as the mean ± standard error of 3 determinations.

Results and discussion

Optimization of medium composition

Under several different culture medium component conditions, the r*Ps*SOD production was linked to the growth. Therefore, it was very important to regulate the cell growth to increase r*Ps*SOD production (data not shown). After 8 h of induction, the activity of r*Ps*SOD was 44.2 U/mg when 1% lactose was added, which was the highest activity observed among other carbon sources. After an 8 h induction, the highest activity of r*Ps*SOD was 48.7 U/mg when tryptone (0.5%) was added as a nitrogen source. Finally, Tween-80 had the largest positive impact on the growth of pET-SOD, and the activity of r*Ps*SOD could be increased to 46.7 U/mg in the medium supplemented with Tween-80 (data not shown). There have been reports that Tween-80 can be used as the carbon source for the growth of *Monascus* sp and that it promotes the dissolution of oxygen to increase the growth of bacteria.[28,29] Based on our results, lactose, tryptone and Tween-80 were selected as the most important factors for the subsequent experiments.

Optimization by response surface methodology

Based on the results from the previous section, lactose, tryptone and Tween-80 were selected as the significant factors that influenced the growth of pET-SOD and the activity of r*Ps*SOD for the related climbing experimental combinations. The results are presented in Table 2, and Tween-80 (0.04%), tryptone (6 g/L) and lactose (10 g/L) were chosen as the factor centers in the BBD experiments. A complete 3^3 factorial experimental design was generated for the 3 selected important variables. Each factor was marked as A, B, or C, and each level was marked as −1, 0, or 1. The results of the 3^3 factorial experimental designs are shown in Table 3. Each experiment was performed 3 times to remove as much experimental error as possible.

Table 2. The results of the climbing experimental combination.

Run order	A-Tween-80 (%)	B-Tryptone (g/L)	C-Lactose (g/L)	r*Ps*SOD activity (U/mg)
1	0.00	2	6	53.7
2	0.02	4	8	55.8
3	0.04	6	10	65.8
4	0.06	8	12	62.3
5	0.08	10	14	55.4
6	0.10	12	16	50.1

Using the data in Table 3, a regression analysis was performed by Design-expert. A quadric multiple regression equation was used:

$$Y = 69.8 + 0.47A + 1.72B - 1.45C + 4.35AC + 1.20BC - 5.17A^2 - 13.03B^2 - 12.38C^2$$

where Y represents the activity of r*Ps*SOD, and A, B, and C represent the coded values of the 3 factors. The factors and the interacted factor coefficients are shown in Table 4.

An ANOVA is a statistical technique that subdivides the total variation in a set of data into component parts associated with specific sources of variation for the purpose of testing hypotheses on the

Table 3. The results of the BBD.

Run no.	A	B	C	r*Ps*SOD activity (U/mg)
1	0	0	0	69.6
2	1	−1	0	50.3
3	1	1	0	53.6
4	0	1	−1	46.4
5	0	0	0	70.4
6	−1	0	−1	57.5
7	1	0	1	55.7
8	0	0	0	69.4
9	−1	1	0	52.9
10	−1	0	1	45.8
11	0	−1	−1	45.2
12	1	0	−1	50.0
13	0	1	1	46.0
14	0	−1	1	40.0
15	−1	−1	0	49.6

Table 4. The estimate of the regression coefficients for r*Ps*SOD.

Parameter	Parameter estimate	DF	Standard error
constant	69.80	1	0.22
A	0.47	1	0.14
B	1.72	1	0.14
C	−1.45	1	0.14
AB	0.00	1	0.19
AC	4.35	1	0.19
BC	1.20	1	0.19
A^2	−5.17	1	0.20
B^2	−13.03	1	0.20
C^2	−12.38	1	0.20

parameters of the mode.[30] The ANOVA of the fitting model generated by the Design-Expert software version 10 is presented in Table 5. The probability p of the whole model was less than 0.05, the lack of fit (0.8725 > 0.05) was not significant, and 87.25% of the lack of fit was caused by error, which indicated that the equation showed good fit for the experiment and that the experimental error was small. In general, the exploration and optimization of a fitted response surface may produce poor or misleading results, unless the model exhibits a good fit, which makes checking of the adequacy essential.[31] In addition, the value of the determination coefficient ($R^2 = 0.9994$) indicated the significant regression of the fitting equation and adequate model discrimination, and only 0.06% of the total variations could not be explained by the fitting equation.[32] The coefficient of variation (CV) was 0.72, which demonstrated the precision of the whole experiment, and the smaller the value was, the higher the fitting degree could be. Considering the value of each factor, it could be proved that the entire model was significant.

Table 5. The analysis of variance (CV) of the BBD regression model.

Source	Sum of Squares	DF	Mean square	*F*-value	Prob > *F*
Model	1272.88	9	141.43	942.87	<0.0001
A-Tween-80 (%)	1.81	1	1.81	12.03	0.018
B-yeast extract (g/L)	23.80	1	23.80	158.70	<0.0001
C-Lactose (g/L)	16.82	1	16.82	112.13	0.0001
AB	0.00	1	0.00	0.000	0.087
AC	75.69	1	75.69	504.60	<0.0001
BC	5.76	1	5.76	38.40	0.0016
A^2	98.88	1	98.88	659.22	<0.0001
B^2	626.40	1	626.40	4176.02	<0.0001
C^2	565.44	1	565.44	3769.62	<0.0001
Residual	0.75	5	0.15	—	—
Lack of fit	0.19	3	0.063	0.23	0.8725
Pure error	0.56	2	0.28		
Total	1273.63	14			

The remark of the model was significant.

In this model, the interactions of 3 single factors A, B and C were significant, Prob > *F* of AC, BC, A^2, B^2, and C^2 were all less than 0.05, which indicated that their interactions were significant while the AB interaction was not. The data were analyzed using Design-Expert software version 10, and the figures of the response surface and contour plots of the 3 interacted factors could be obtained. Based on the contour plots and the 3-D response surface plots, the stable points were all in the experimental area (Figs. 1–3). In the analysis of response surface values, the oval and saddle contour showed that the interaction of the factors was significant, whereas the circle was considered to be not significant. The interaction between Tween-80 and lactose was the most significant interaction (Fig. 3).

The first partial derivative of the regression equation was calculated for the optimal values of each factor, which meant that A was 0.33, B was 0.08, and C was 0.69. On the basis of the values, Tween-80 (0.047%), tryptone (6.16 g/L) and lactose (11.38 g/L) were added to

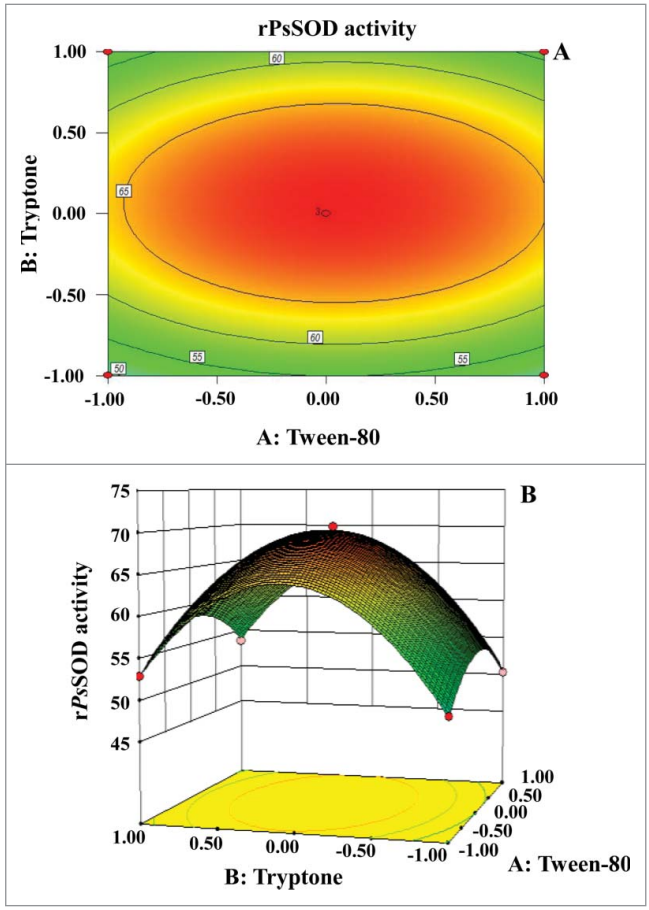

Figure 1. The response surface plot (A) and the contour plot (B) of the activity of r*Ps*SOD with Tween-80 and tryptone.

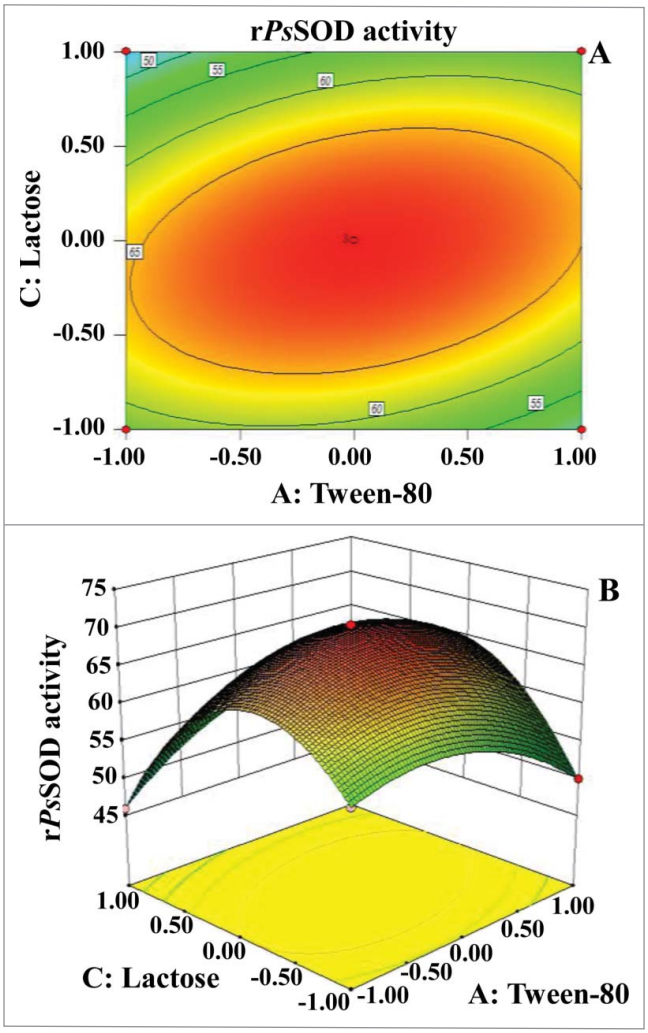

Figure 2. The response surface plot (A) and the contour plot (B) of the activity of r*Ps*SOD with Tween-80 and lactose.

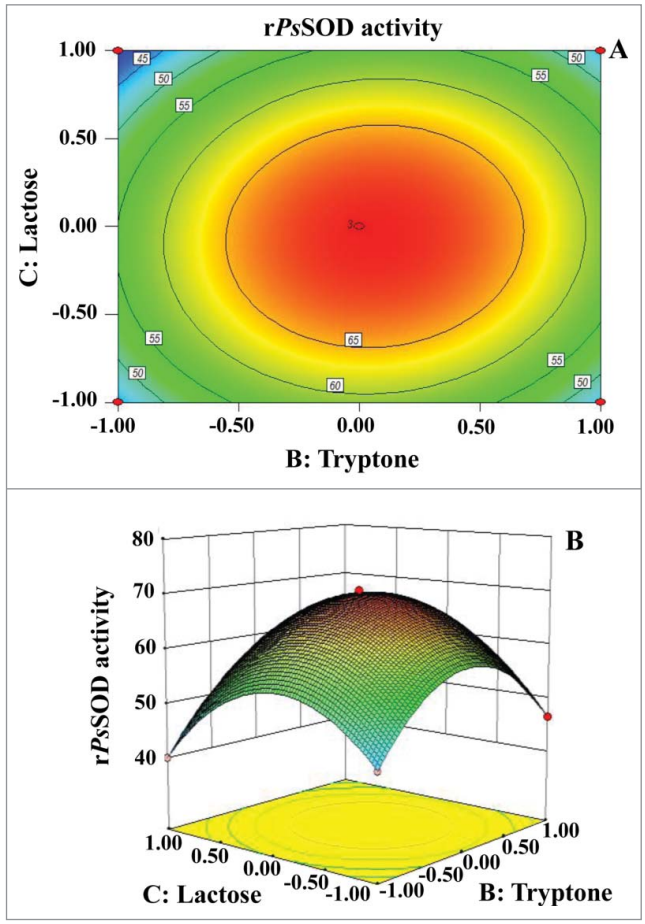

Figure 3. The response surface plot (A) and the contour plot (B) of the activity of rPsSOD with lactose and tryptone.

the medium (yeast extract powder 5 g/L, NaCl 10 g/L), and the predicted maximum response *Y* was 72.57 U/mg.

Verification experiment

A verification experiment was repeated 3 times based on the calculation of the optimal conditions. The bacteria density OD_{600} of the induced culture was 2.77, and the r*Ps*SOD activity was 71.86 U/mg, which is 1.54 times higher than that observed before optimization and close to predicted values.

The application of different methods and definitions of SOD units by various researchers to estimate the enzyme activity, along with the use of various substrates and optimum temperatures, makes comparisons difficult. In previous studies, the activity of the novel cold-active SOD from the Antarctic strain *Aspergillus glaucus* 363 was 51.1 U/mg, which is slightly lower than that of statistically optimized r*Ps*SO.[33] The value is comparable to cold-adapted SOD isolated from cucumber seedlings (30.0 U/mg) and low temperature *Ph*SOD from *Pseudoalteromonas haloplanktis* (14.8 U/mg).[34,35] In addition, the activity of SOD purified from *Camellia sinensis* (L.) was 56.66 U/mg, which is close to the activity of optimized r*Ps*SOD.[25] Therefore, response surface methodology was an efficient technique and a productive tool for enhancing cold-adapted SOD production. At present, SOD is a research hotspot around the world; however, statistical experimental methods for obtaining recombinant cold-adapted SOD production, which would offer some important parameters for large-scale fermentation, are applied in very few cases.

Conclusion

As an industrial enzyme, SOD has been used extensively in many fields. The components of the cold-adapted r*Ps*SOD culture medium were studied using BBD experiments. The results showed that lactose, tryptone and

Tween-80 were the major factors that influenced microorganism growth and r*Ps*SOD fermentation. A second-degree polynomial regression model of the production of r*Ps*SOD was built, and optimal fermentation conditions for retaining the activity of r*Ps*SOD were identified and verified. In addition, the optimal components of the fermentation culture medium were identified as Tween-80 (0.047%), tryptone (6.16 g/L), lactose (11.38 g/L), yeast extract powder (5.0 g/L) and NaCl (10.0 g/L). The bacteria density OD_{600} of the induced culture was 2.77, and the r*Ps*SOD activity was 71.86 U/mg, which is 1.54 times higher than that observed before optimization and close to predicted values according to the verification experiment. To the best of our knowledge, this is the first report in which RSM was used for the optimization of recombinant cold-adapted SOD production.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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