

## Medium Optimization for Nitrilase Production by Newly Isolated *Rhodococcus erythropolis* ZJB-0910 Using Statistical Designs

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The objective of this work is to investigate the effects of the medium components including carbon sources, nitrogen sources and metal ions on nitrilase production from *R. erythropolis* ZJB-0910, which was applied in stereoselective hydrolysis of racemic ethyl 4-cyano-3-hydroxybutyrate to (*R*)-ethyl-3-hydroxyglutarate [(*R*)-EHG]. In this study, glucose, yeast extract and MgSO<sub>4</sub> were selected as the suitable components, and the concentrations of these three factors for the zero coded levels of variables in the subsequent optimization were confirmed through the empirical 'one-factor-at-a-time' experiments. The combination of response surface methodology (RSM) and Box-Behnken factorial design was carried out to establish a quadratic model to fit the nitrilase activity and the variables. The maximum nitrilase activity of 77.482 U g<sup>-1</sup> was predicted when glucose concentration was 20.986 g L<sup>-1</sup>, yeast extract concentration was 8.468 g L<sup>-1</sup>, and MgSO<sub>4</sub> concentration was 0.22 g L<sup>-1</sup>. Validation experiments were carried out under the optimized conditions for verification of the model, and the nitrilase activity was found to be improved by 1.77 fold, compared to the value before optimization. The strain growth curve and nitrilase activity alteration in the course of culture were also examined, and the cells were suitably harvested after being cultured for 45–48 h.

### Key words:

Nitrilase, stereoselective hydrolysis, (*R*)-ethyl-3-hydroxyglutarate, optimization, response surface methodology, Box-Behnken

## Introduction

(*R*)-ethyl-3-hydroxyglutarate [(*R*)-EHG] is a key precursor for synthesis of chiral side chain of rosvastatin, which so far has been the most potent statin drug for lowering the level of LDL (low-density lipoprotein) cholesterol to cure hyperlipidemia.<sup>1–3</sup> Few biosynthetic routes for (*R*)-EHG have been reported and most focus on hydrolysis of the corresponding diester with commercial ester hydrolases including lipase, esterase and protease.<sup>1,4,5</sup> However, these methods are not suitable for application in the industrial manufacture of (*R*)-EHG because of the high cost of enzymes and low optical purities. In contrast, nitrilase has gained great interest as it can catalyze the hydrolysis of nitriles to the carboxylic acids with high yield and selectivity.<sup>3</sup> A novel nitrilase-producing strain ZJB-0910, identified as *Rhodococcus erythropolis*, was screened using Co<sup>2+</sup> colorimetric screening method and was successfully employed to stereoselectively hydrolyze racemic ethyl 4-cyano-3-hydroxybutyrate to (*R*)-EHG without breaking the ester bond; the product was further confirmed to be (*R*)-EHG (*ee* > 99 %) by determination of its structure and configuration.<sup>3</sup>

This biotransformation is largely superior to other synthetic methods for (*R*)-EHG not only due to cheaper raw material sources, but due to high optical purity of (*R*)-EHG. To produce (*R*)-EHG on an industrial scale, it is necessary to improve nitrilase production by *R. erythropolis* ZJB-0910 for maximum synthesis of (*R*)-EHG through optimization of culture medium.

Owing to many variables in culture conditions affecting the production of enzymes, such as carbon source, nitrogen source and so on, and there also exist some interactions between these variables, the traditional 'one-factor-at-a-time' approach is not suitable for optimization of the culture conditions.<sup>6–8</sup> In practical research and applications, statistical experimental designs have been proven very effective for medium optimization, because the optimized values of statistical experimental designs are close to the actual values by simultaneous study of several variables and the interactions between these variables, and the statistical methods can also reduce the development time and overall costs.<sup>9,10</sup> As a widely used statistical method, response surface methodology (RSM) based on Box-Behnken experimental design can optimize the identified controllable variables collectively to eliminate the limitations of a single factor optimization process,

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and build the models to study the interaction between the variables in a shorter time and at lower cost.<sup>6,11</sup> Box-Behnken design is always adopted to fit a second order polynomial by a least squares technique, which produces an equation to demonstrate how the test variables affect the response.

In this study, the ‘one-factor-at-a-time’ approach and RSM based on Box-Behnken design were combined to optimize the culture conditions for the production of nitrilase from *R. erythropolis* ZJB-0910. Three medium components including glucose, yeast extract and MgSO<sub>4</sub> were selected as independent variables, while the specific nitrilase activity was chosen as the response value. In addition, the time courses of strain growth and nitrilase activity alteration were also intensively investigated.

## Materials and methods

### Chemicals

Ethyl 4-cyano-3-hydroxybutyrate was presented from Donggang Pharmaceuticals (Zhejiang, China). (*R*)-EHG (*ee* > 99 %) was prepared in our laboratory. All other chemicals were of analytical grade and commercially available.

### Microorganism and culture conditions

*R. erythropolis* ZJB-0910 was isolated in our lab, and deposited in the China Center for Type Culture Collection (CCTCC M 209244, Wuhan, China).<sup>3</sup> The microorganism was incubated aerobically at 30 °C for 48 h in the initial medium with the composition (in g L<sup>-1</sup>): 10, glucose; 5, yeast extract; 0.2, MgSO<sub>4</sub> · 7H<sub>2</sub>O; 0.5, K<sub>2</sub>HPO<sub>4</sub>; 0.5, KH<sub>2</sub>PO<sub>4</sub>. The cells were harvested by centrifugation under 4 °C at 9000 · g for 10 min, and washed with saline (0.85 %), the remaining wet cells were stored at 4 °C for further use.

### Optimization of culture conditions by one-factor-at-a-time approach

The selection of medium components including carbon sources, nitrogen sources and metal ions were investigated through the traditional ‘one-factor-at-a-time’ approach. Various carbon sources, nitrogen sources and metal ions were tested in this study. The ingredients of carbon sources, nitrogen sources and metal ions were replaced by varied factors, while other ingredients were kept unchanged. The biomass of the cells was calculated by the dry cell mass (DCM) in 1 L fermentation broth.

## Experimental design and optimization by RSM

Based on the previous experiment, glucose, yeast extract and MgSO<sub>4</sub> were confirmed as three suitable ingredients of the medium. A Box-Behnken factorial design with three factors and three levels including three replicates at the centre point was used for fitting a second-order response surface. The factors and their values in three levels were shown in Table 1. The uncoded value of each variable ( $X_i$ ) was deduced from the real value of variable ( $x_i$ ) by equation  $X_i = (x_i - x_{i0})/\Delta x_i$ , of which  $i = 1, 2, 3$ ;  $x_{i0}$  was the value of variables at zero level; and  $\Delta x_i$  was the step length of the variables. The relationships and interrelationships of the variables were determined by fitting the second-order polynomial equation as below:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 \quad (1)$$

Where  $Y$  is the specific nitrilase activity of the cells (U g<sup>-1</sup> DCM);  $X_1$  is the uncoded value of glucose concentration;  $X_2$  is the uncoded value of yeast extract concentration;  $X_3$  is the uncoded value of MgSO<sub>4</sub> concentration;  $a_0$  is a constant;  $a_1$ ,  $a_2$  and  $a_3$  are linear coefficients;  $a_{11}$ ,  $a_{22}$  and  $a_{33}$  are squared coefficients; and  $a_{12}$ ,  $a_{13}$  and  $a_{23}$  are interaction coefficients. The coefficients were calculated and analyzed using Design Expert software (Version 8.0.1, Stat-Ease Inc., USA). The response surface graphs were plotted to determine their optimum levels for maximal nitrilase activity.

Table 1 – Factors and levels for response surface by Box-Behnken design

Independent variables	Coded symbols	Levels		
		–1	0	1
Glucose concentration (g L <sup>-1</sup> )	$X_1$	15	20	25
Yeast extract concentration (g L <sup>-1</sup> )	$X_2$	6	8	10
MgSO <sub>4</sub> concentration (g L <sup>-1</sup> )	$X_3$	0.1	0.2	0.3

### *R. erythropolis* ZJB-0910 growth and cellular activity alteration

The microorganism was aerobically incubated at 30 °C in the optimized medium components. The cells were harvested at intervals during the culture time. The biomass of the strain and specific activity of the nitrilase were determined at each time point.

### Enzymatic activity assay

The bioconversion was performed at 30 °C in 50-mL Erlenmeyer flasks on a rotary shaker at

180 rpm. The reaction mixture (10 mL) consisted of 0.016 g DCM resting cells in 20 mmol L<sup>-1</sup> sodium phosphate buffer (pH 7.5), and 0.032 g substrate was added to initialize the reaction. After 30 min, samples (1 mL each) of the reaction media were withdrawn and analyzed by high performance liquid chromatography (HPLC).<sup>3</sup> A LC-10AS instrument (Shimadzu, Japan) equipped with a hypersil ODS C18 column (250 mm · 4.6 mm, 5 μm) and a UV-detector (Spd-10A Vp plus; Shimadzu) was used. The mobile phase solution was composed of acetonitrile and 0.5 % (v/v) aqueous triethylamine solution (1 : 4, v/v), the flow rate and the detection wavelength were set as 1 mL min<sup>-1</sup> and 260 nm, respectively. One unit of nitrilase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol (*R*)-EHG per minute under the experimental conditions described above. The relative activity is calculated by comparison with the maximal activity (100 %) in each experiment.

## Results and discussion

### Medium optimization by one-factor-at-a-time approach

#### Suitable carbon source selection

Carbon source is the most important medium component in the period of growing and metabolic processes of the microorganism. Therefore, the effects of various organic and inorganic carbon sources on the nitrilase production by *R. erythropolis* ZJB-0910 were investigated first. As shown in Fig. 1, the biomass and nitrilase production from *R. erythropolis* ZJB-0910 alterations were associ-

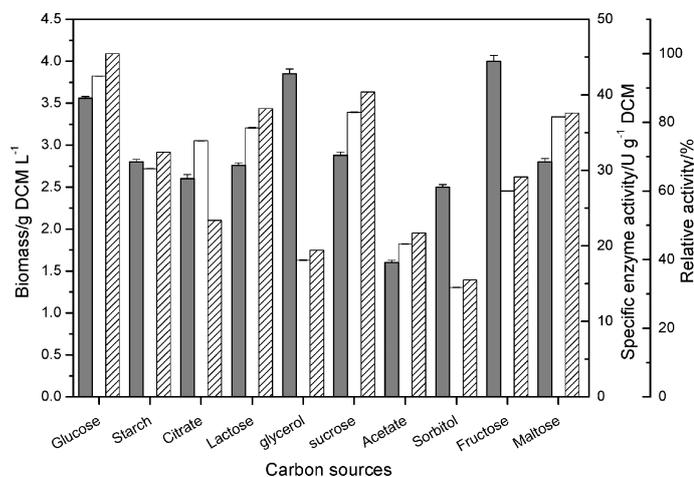


Fig. 1 – Effects of carbon sources on growth and nitrilase activity of *R. erythropolis* ZJB-0910. Biomass (■), specific activity (□), relative activity (▨). Other culture conditions: 5 g L<sup>-1</sup> yeast extract, 0.5 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.5 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 30 °C, initial pH, 100 mL nutrient solution in 500 mL flask.

ated with changes in different carbon sources (10 g L<sup>-1</sup>). Among these carbon sources, acetate and citrate produce lower biomass and nitrilase activity; by contrast, although fructose and glycerol supported more strain growth, they could not produce more nitrilase activity than other carbon sources; the highest nitrilase activity (42.5 U g<sup>-1</sup> DCM) was produced by *R. erythropolis* ZJB-0910 in the presence of glucose, which also produced rather high biomass (3.56 g DCM L<sup>-1</sup>). Therefore, in this study, glucose was selected as the most suitable carbon source. And 20 g L<sup>-1</sup> of glucose was further confirmed as the suitable concentration by investigating the effect of glucose concentration on biomass and nitrilase activity (Fig. 2), and such glucose concentration was selected as the zero coded level of glucose in the RSM experiments.

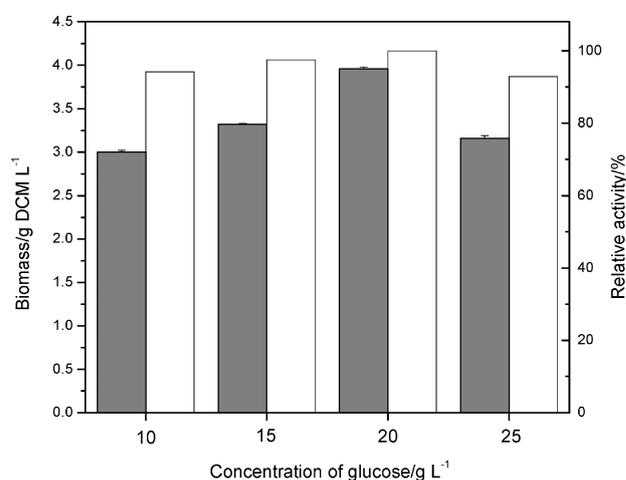


Fig. 2 – Effects of glucose concentration on growth and nitrilase activity of *R. erythropolis* ZJB-0910. Biomass (■), relative activity (□). Other culture conditions: 5 g L<sup>-1</sup> yeast extract, 0.5 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.5 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 30 °C, initial pH, 100 mL nutrient solution in 500 mL flask.

#### Suitable nitrogen source selection

As one of the major ingredients in the culture mediums, the effects of various nitrogen sources (5 g L<sup>-1</sup>) including organic, inorganic nitrogen sources and both combinations on biomass and nitrilase production were investigated in this study. As shown in Fig. 3, the lowest biomass and nitrilase activity were observed when (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was used as the nitrogen source; in contrast, the organic nitrogen sources such as yeast extract, beef extract and peptone favored more cell mass and nitrilase activity; however, when each organic nitrogen source was mixed with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> by an equal percentage to be added in the culture medium, both the biomass and nitrilase activity dropped at certain degrees compared to the corresponding sole organic nitrogen source, indicating that the inorganic nitro-

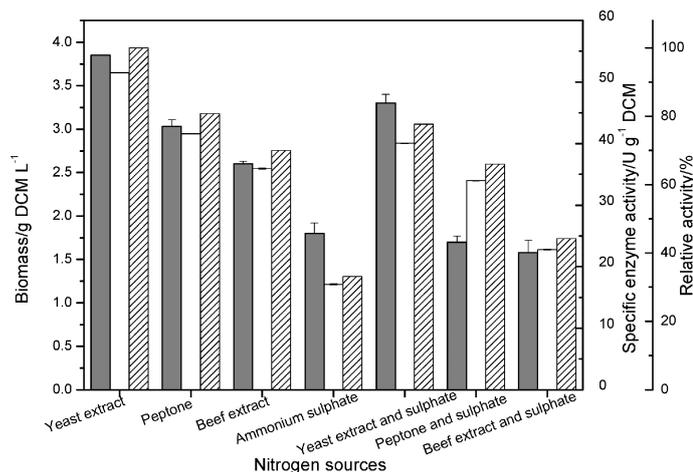


Fig. 3 – Effects of nitrogen source on growth and nitrilase activity of *R. erythropolis* ZJB-0910. Biomass (■), specific activity (□), relative activity (▨). Other culture conditions: 20 g L<sup>-1</sup> glucose, 0.5 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.5 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 30 °C, initial pH, 100 mL nutrient solution in 500 mL flask.

gen source did not support the cell growth and nitrilase production. The results showed that the highest biomass (3.85 g DCM L<sup>-1</sup>) and nitrilase activity (51.5 U g<sup>-1</sup> DCM) were produced when yeast extract was used as nitrogen source, so it was chosen as the suitable nitrogen source for further study. Thereafter, as shown in Fig. 4, the concentration of yeast extract was examined and 8 g L<sup>-1</sup> of yeast extract was selected as the zero coded level in the RSM experiments.

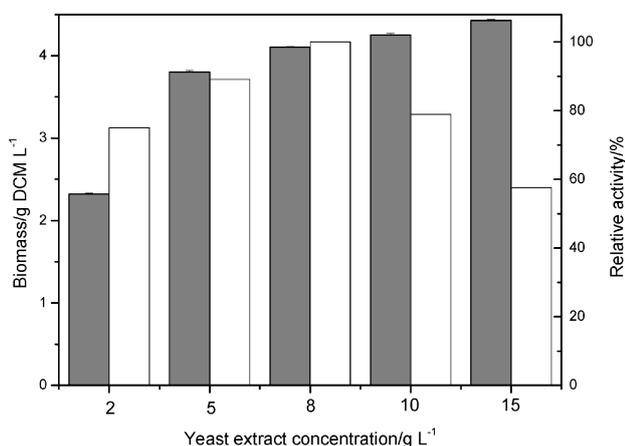


Fig. 4 – Effects of yeast extract concentration on growth and nitrilase activity of *R. erythropolis* ZJB-0910. Biomass (■), relative activity (□). Other culture conditions: 20 g L<sup>-1</sup> glucose, 0.5 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.5 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 30 °C, initial pH, 100 mL nutrient solution in 500 mL flask.

### Effects of metal ions on nitrilase production and biomass

Traces of metal ions are always regarded as the activators supporting cell growth and enzymatic production. In this study, the effects of various

metal ions (0.2 g L<sup>-1</sup>) on biomass and nitrilase activity were also investigated. The medium without addition of metal ion was taken as the control, and the other ingredients were unchanged. As shown in Fig. 5, except the addition of Mg<sup>2+</sup> supporting the biomass and nitrilase production, the other metal ions were confirmed to inhibit the cell growth and nitrilase activity at various degrees, especially as Ni<sup>2+</sup> was added, the growth of *R. erythropolis* ZJB-0910 was almost inhibited during the culture process. The results indicated that the addition of Mg<sup>2+</sup> could slightly increase biomass and nitrilase production, so 0.2 g L<sup>-1</sup> of Mg<sup>2+</sup> was selected as the zero coded level in the RSM experiments.

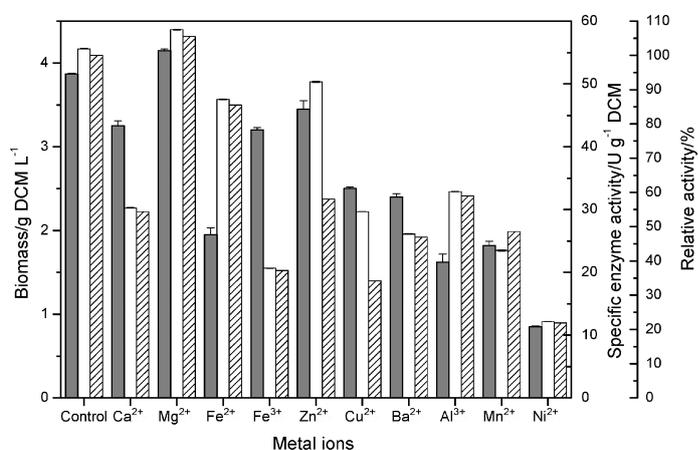


Fig. 5 – Effects of metal ions on growth and nitrilase activity of *R. erythropolis* ZJB-0910. Biomass (■), specific activity (□), relative activity (▨). Other culture conditions: 20 g L<sup>-1</sup> glucose, 8 g L<sup>-1</sup> yeast extract, 0.5 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.5 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 30 °C, initial pH, 100 mL nutrient solution in 500 mL flask.

### Optimization of the nitrilase production by RSM

As shown in Table 2, 15 experimental runs with different combinations of three factors were carried out to optimize the nitrilase production. The results showed that the minimum specific nitrilase activity of 48.3 U g<sup>-1</sup> DCM was observed in run 1 under the conditions of 15 g L<sup>-1</sup> of glucose, 6 g L<sup>-1</sup> of yeast extract and 0.2 g L<sup>-1</sup> of Mg<sup>2+</sup>; on the other hand, the maximum specific activity of 77.4 U g<sup>-1</sup> DCM was achieved in run 14 (20 g L<sup>-1</sup> of glucose, 8 g L<sup>-1</sup> of yeast extract and 0.2 g L<sup>-1</sup> of Mg<sup>2+</sup>). By applying multiple regression analysis on the experimental data (uncoded values) in Table 2, the following second-order polynomial equation was found to best explain the nitrilase production from *R. erythropolis* ZJB-0910, and the second-order polynomial coefficients were calculated and analyzed using the Design Expert 8.0.1 software:

Table 2 – Box-Behnken design and the results of the experiment

Std	Run	$X_1$	$X_2$	$X_3$	Specific activity (U g <sup>-1</sup> DCM)
		Glucose concentration (g L <sup>-1</sup> )	Yeast extract concentration (g L <sup>-1</sup> )	MgSO <sub>4</sub> concentration (g L <sup>-1</sup> )	
1	10	25	8	0.1	63.5 ± 0.85
2	5	20	6	0.1	56.5 ± 0.92
3	3	25	6	0.2	56.7 ± 1.06
4	13	20	8	0.2	75.6 ± 0.98
5	2	15	10	0.2	54.6 ± 0.76
6	6	20	6	0.3	65.9 ± 1.15
7	12	25	8	0.3	66.2 ± 1.37
8	8	20	10	0.3	69.5 ± 0.85
9	15	20	8	0.2	76.2 ± 1.28
10	1	15	6	0.2	48.3 ± 1.12
11	11	15	8	0.3	58.4 ± 1.32
12	7	20	10	0.1	66.2 ± 1.25
13	4	25	10	0.2	65.7 ± 1.36
14	14	20	8	0.2	77.4 ± 1.53
15	9	15	8	0.1	55.4 ± 1.26

$$Y = -294.075 + 18.718X_1 + 35.788X_2 + 215.125X_3 - 0.458X_1^2 - 2.156X_2^2 - 415X_3^2 + 0.068X_1X_2 - 0.3X_1X_3 - 3.125X_2X_3 \quad (2)$$

Analysis of variance (ANOVA) was performed to determine the accuracy and significance of the model, and the results were presented in Table 3. The  $P$ -values less than 0.05 suggest the significant model terms, and the corresponding coefficient will be more significant as it is smaller.<sup>12,13</sup> In this case, the significant model was established attributing to the model  $F$ -value of 103.71, which was significant due to the  $P$ -value of  $< 1.0 \cdot 10^{-4}$ . As to the first order, it was found that glucose, yeast extract and MgSO<sub>4</sub> had significant effect on nitrilase production; all the second orders of the three variables were significant to the model; however, the interactions between these three variables were found to be insignificant to the model. It was showed that the lack of fit test was insignificant due to the value of 1.561, so the model was supposed to be adequate for prediction within selected variables range. The  $R^2$  value of 0.995 indicated that only about 0.5 % of the variation was unable to be explained, and the model was confirmed to be significant. A relatively low coefficient of variation (CV) of 2.21 suggested better precision and reliability of the experiments.

Table 3 – Analysis of variables (ANOVA) for the regressive equation

Source	df	Sum of squares	Mean of squares	F value	$P$ -value Prob > F
Model	9	1047.732	116.415	103.710	$1.0 \cdot 10^{-4}$
$X_1$	1	154.001	154.001	137.195	$1.0 \cdot 10^{-4}$
$X_2$	1	129.605	129.605	115.461	$1.0 \cdot 10^{-4}$
$X_3$	1	26.281	26.281	26.281	$4.7 \cdot 10^{-3}$
$X_1X_2$	1	1.823	1.823	1.624	0.259
$X_1X_3$	1	0.09	0.09	0.08	0.788
$X_2X_3$	1	1.563	1.563	1.392	0.291
$X_1^2$	1	484.071	484.071	431.243	$1.0 \cdot 10^{-4}$
$X_2^2$	1	274.673	274.673	244.698	$1.0 \cdot 10^{-4}$
$X_3^2$	1	63.591	63.591	56.651	$7.0 \cdot 10^{-4}$
Residual	5	5.613	1.123		
Lack of fit	3	3.933	1.310	1.561	0.414
Pure error	2	1.68	0.84		
Total	14	1053.344			

Coefficient of variation (CV) = 2.21;  
determination coefficient ( $R^2$ ) = 0.995.

Three-dimensional response surface plots were constructed through plotting the specific nitrilase activity on the Z-axis against two independent variables, and another variable was fixed at a middle level. Fig. 6 showed the response and contour curves for glucose, yeast extract and  $\text{MgSO}_4$ . The effects of glucose and yeast extract on the nitrilase production were shown in Fig. 6(a), while  $\text{MgSO}_4$  concentration was fixed at  $0.2 \text{ g L}^{-1}$ . The results showed that the nitrilase activity improved as the glucose concentration increased from  $15 \text{ g L}^{-1}$  to about  $20 \text{ g L}^{-1}$ , then it dropped with the carbon source continued increasing, indicating that the excessive glucose inhibited nitrilase production;<sup>7</sup> and it maintained the same variation trend with yeast extract, the nitrilase activity raised as the concentration of yeast extract increased from  $6.0 \text{ g L}^{-1}$  to about  $8.0 \text{ g L}^{-1}$ , while it decreased as the concentration of yeast extract continued to increase. From the analysis by Design-Expert software, it was suggested that the optimal glucose concentration was  $20\text{--}21 \text{ g L}^{-1}$ , and the optimal yeast extract concentration was  $8.0\text{--}8.5$  for the nitrilase production. In addition, Fig. 6(b) and Fig. 6(c) also presented the effects of yeast extract and  $\text{MgSO}_4$ ,  $\text{MgSO}_4$  and glucose on the nitrilase production. Among these three factors, glucose had the most powerful effect on the nitrilase production from *R. erythropolis* ZJB-0910, yeast extract as the secondary, and the ion of  $\text{Mg}^{2+}$  as the least. This order of effects agrees with the general orders of effects of medium components on nitrilase production. Based on the model, the optimum medium composition was obtained with  $20.986 \text{ g L}^{-1}$  of glucose,  $8.468 \text{ g L}^{-1}$  of yeast extract and  $0.220 \text{ g L}^{-1}$  of  $\text{MgSO}_4$ . In comparison with the obtained culture conditions for nitrilase from other strains by RSM method, appropriately increasing yeast extract concentration was helpful to the nitrilase production from *R. erythropolis* ZJB-0910.<sup>7</sup> The addition of  $\text{Mg}^{2+}$  ion supported the nitrilase production in the study, this was inconsistent with the results in other studies that metal ions showed negative or few effects on nitrilase activity.<sup>7,14,26</sup> Under the optimized conditions, the nitrilase activity was predicted to be  $77.482 \text{ U g}^{-1} \text{ DCM}$ . The validation experiments were carried out in 5 replicates under the optimum conditions, and the average nitrilase activity of  $75.235 \text{ U g}^{-1} \text{ DCM}$  was close to the predicted value, which affirmed the rationality of the model. The results showed that the medium optimization produced an evident increase by 1.77-fold in the nitrilase activity compared to that before optimization.

*R. erythropolis* ZJB-0910, a nitrilase-producing strain, is firstly reported to stereoselectively hydrolyze racemic  $\beta$ -hydroxy aliphatic nitrile to chiral  $\beta$ -hydroxy aliphatic acid without breaking the ester

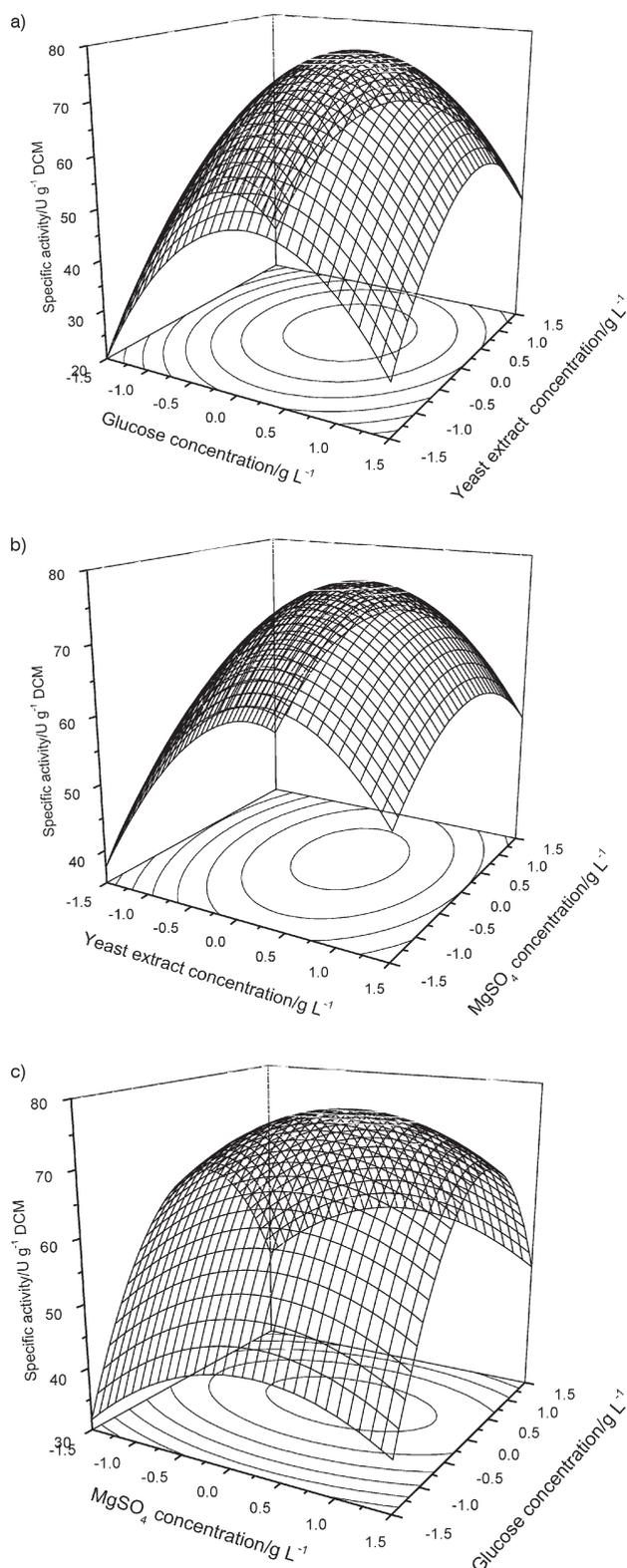


Fig. 6 – (a) Three-dimensional contour plots showing the effects of glucose and yeast extract on nitrilase activity at  $\text{Mg}^{2+}$  coded level of zero; (b) Three-dimensional contour plots showing the effects of yeast extract and  $\text{Mg}^{2+}$  on nitrilase activity at glucose coded level of zero; (c) Three-dimensional contour plots showing the effects of  $\text{Mg}^{2+}$  and glucose on nitrilase activity at yeast extract coded level of zero.

bond of the substrate, which is accepted as difficult to realize because few enzymes show chiral recognition toward  $\beta$ -hydroxy nitriles bearing a remote chiral center from the reacting cyano moiety.<sup>15,16</sup> In addition, the synthesized product of (*R*)-EHG is a novel and promising precursor for preparation of rosuvastatin, which is a potent LDL-lowering drug with good sales prospects.<sup>1,17</sup>

In order to improve the yield of (*R*)-EHG and realize its industrial manufacture, the nitrilase activity enhancement through optimization of culture medium components is the first priority.<sup>7</sup> Using conventional techniques such as the ‘one-factor-at-a-time’ method to perform the optimization is extremely laborious and time consuming.<sup>18,19</sup> Moreover, such methods can not guarantee the determination of optimum conditions, and detect synergistic interactions between two or more factors; however, one advantage of this method is that it quickly selects and determines the aim factor from several similar factors with relatively lighter workload.<sup>20</sup> In the present study, non-statistical and statistical experimental methods were combined to investigate the different variables’ effects on the nitrilase production as well as the interaction of these factors. Firstly, through the ‘one-factor-at-a-time’ experiments, 20 g L<sup>-1</sup> of glucose, 8 g L<sup>-1</sup> of yeast extract and 0.2 g L<sup>-1</sup> of MgSO<sub>4</sub> were selected as suitable carbon source, nitrogen source and metal ion concentration for zero level of the variables in the Box-Behnken design, respectively. RSM was employed to optimize the culture conditions for improving nitrilase production. RSM, which has been extensively applied in the optimization of fermentation processes, is a collection of statistical techniques for experiment designing, model development, factor evaluating and optimal conditions searching.<sup>21–23</sup> RSM could overcome the shortcoming of the classical or empirical methods such as the ‘one-factor-at-a-time’ technique, which is incapable of searching for the global optimal condition, especially when interactions between independent factors exist.<sup>24</sup> However, most researchers have focused on the isolation, characterization and purification of the novel nitrilase from various new strains, or committed themselves to improving the nitrilase activity by mutation breeding and genetic modification methods.<sup>25–27</sup> In contrast, the optimization method for improving the nitrilase production by using RSM with more certainty was seldom reported. Furthermore, this is the first time to optimize the culture conditions for high yields of nitrilase used in stereoselective hydrolysis of  $\beta$ -hydroxyaliphatic nitriles to the corresponding chiral aliphatic acids using statistical experimental designs. The experimental values close to the predicted nitrilase activity revealed the accuracy and

applicability of RSM to optimize the process for nitrilase production from *R. erythropolis* ZJB-0910.

### *R. erythropolis* ZJB-0910 growth and cellular activity alteration

The time courses of strain growth and its nitrilase activity at 30 °C were shown in Fig. 7. The results showed that the growth of *R. erythropolis* ZJB-0910 went through three periods. Above all, the cells grew slowly and the nitrilase activity kept very low during the lag phase from 0 to about 10 h; after this period, the biomass increased exponentially in the next 35 h, and a swift growth in the nitrilase activity was also observed during this stage; the change on biomass kept stable after 45 h, indicating that it entered the stationary growth period. Meanwhile, the nitrilase activity peaked at the culture time near 45–48 h with the value of 75.6 U g<sup>-1</sup> DCM, and it decreased with the elongated culture time subsequently.

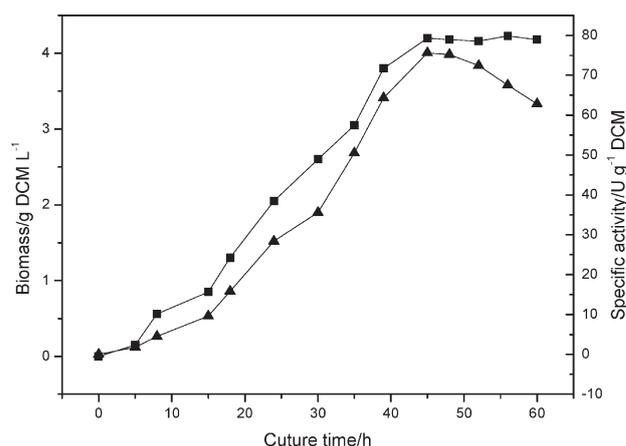


Fig. 7 – Time course of the strain growth and nitrilase activity alteration during the culture time in fermentation broth. Biomass (■), specific activity (▲). Culture conditions: 20.986 g L<sup>-1</sup> glucose, 8.468 g L<sup>-1</sup> yeast extract, 0.22 g L<sup>-1</sup> MgSO<sub>4</sub>, 0.5 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.5 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 30 °C, initial pH, 100 mL nutrient solution in 500 mL flask.

## Conclusions

The RSM technology was employed to optimize the medium components for nitrilase production from *R. erythropolis* ZJB-0910 based on the results of the ‘one-factor-at-a-time’ experiments in this study. The nitrilase activity was improved by 1.77-fold compared to the initial activity under the optimum culture conditions of glucose 20.986 g L<sup>-1</sup>, yeast extract 8.468 g L<sup>-1</sup> and MgSO<sub>4</sub> 0.22 g L<sup>-1</sup>. The results showed that RSM was helpful in optimizing the culture conditions and improving nitrilase production with less workload, and it was also in favor of improving the yields of (*R*)-EHG and realizing its industrial manufacture on a large scale.

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