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Medium Optimization for Enzymatic Production of L-Cysteine by *Pseudomonas* sp. Zjwp-14 Using Response Surface Methodology

Guo-Ying Lv[1,2], *Pu Wang*[1]*, *Jun-Yao He*[1] *and Xiao-Nian Li*[3]

[1]Institute of Biopharmaceutical Engineering, Zhejiang University of Technology, CN-310014 Hangzhou, PR China

[2]Zhejiang Academy of Agricultural Sciences, CN-310021 Hangzhou, PR China

[3]College of Chemical Engineering and Materials Science, Zhejiang University of Technology, CN-310014 Hangzhou, PR China

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Summary

Response surface methodology was applied to optimize medium constituents for enzymatic production of L-cysteine from DL-2-amino-Δ^2-thiazoline-4-carboxylic acid (DL-ATC) by a novel *Pseudomonas* sp. Zjwp-14. With the Plackett-Burman design experiment, glycerol, DL-ATC, yeast extract, and pH were found to be the most powerful factors among the eight tested variables that influence intracellular enzyme activity for biotransformation of DL-ATC to L-cysteine. In order to investigate the quantitative effects for four variables selected from Plackett-Burman design on enzyme activity, a central composite design was subsequently employed for further optimization. The determination coefficient (R^2) was 0.9817, and the results show that the regression models adequately explain the data variation and represent the actual relationships between the parameters and responses. The optimal medium for *Pseudomonas* sp. Zjwp-14 was composed of (in g/L): glycerol 16.94, DL-ATC 4.59, yeast extract 6.99, NaCl 5.0, peptone 5.0, beef extract 5.0, $MgSO_4{\cdot}7H_2O$ 0.4, and pH=7.94. Under the optimal conditions, the maximum intracellular enzyme activity of 918.7 U/mL in theory and 903.6 U/mL in the experiment were obtained, with an increase of 15.6 % compared to the original medium components. In a 5-litre fermentor, cultivation time for *Pseudomonas* sp. Zjwp-14 was cut down for 6 h and the maximum enzyme activity reached 929.6 U/mL.

Key words: DL-2-amino-Δ^2-thiazoline-4-carboxylic acid (DL-ATC), L-cysteine, medium optimization, *Pseudomonas* sp. Zjwp-14, response surface methodology

Introduction

L-cysteine, an important and well-known S-containing amino acid, has been widely used as a medical intermediate, and food or cosmetic additive (*1*). Traditionally, industrial L-cysteine production mainly depends on acid or alkaline hydrolysis of human or animal hairs. However, due to high energy cost, unpleasant odour and intractable waste products in this process, an alternative microbial method for L-cysteine production has been developed, which is more economical, efficient and environmentally friendly. Some bacteria can asymmetrically hydrolyze a chemically synthesized substrate, DL-2-amino-Δ^2-thiazoline-4-carboxylic acid (DL-ATC), to L-cysteine, with the genus *Pseudomonas* being the most extensively studied, especially *P. ovalis*, *P. cohaerens*, *P. thiazolinophi-*

*Corresponding author; Phone: ++86 571 88 320 389; E-mail: wangpu@zjut.edu.cn

lum, *P. putida* and *P. desmolytica* (*1–3*). Because of the limited intracellular enzyme activities, 2–6 g/L of DL-ATC were used as substrate in the bioconversion process (*3*,*4*). Some studies have attempted to improve enzyme activities using mutant strains or recombinant *Escherichia coli* (*5–7*). It was reported that when fermented with recombinant *Escherichia coli*, the yield of L-cysteine was 10 % (*8*).

In our previous study, a novel strain Zjwp-14, showing asymmetric hydrolytic activity from DL-ATC to L-cysteine, was isolated from a soil sample using DL-ATC as the sole nitrogen source and inducer for enzyme formation in the screening medium (*9*). The bacterial strain Zjwp-14 exhibited morphological and physiological characteristics of the *Pseudomonas* genus. Therefore, biochemical tests of this strain were done, and it was positive to catalase test, oxidase test, Voges-Proskauer (VP) test, indole test and hydrogen sulphide test, but negative to methyl red (MR) test. The results from the ATB system analysis suggest that Zjwp-14 strain can utilize L-proline, acetate, DL-lactate, L-alanine, 3-hydroxybutyrate, propionate, decanoate, D-glucose, pentanoate, citrate, histidine and L-serine as carbon sources, but not salicin, D-ribose, mannitol, D-sorbitol, 5-ketoglyconate, malonate, suberate, rhamnose, sucrose, maltose, N-acetylglucosamine, glycogen, 3-hydroxybenzoate, 2-ketoglyconate, inositol, L-fucose, itaconate, D-melibiose, L-arabinose or 4-hydroxybenzoate. The partial sequencing of the 16S ribosomal DNA gene of the Zjwp-14 strain was amplified by PCR. The DNA fragments of 1456 bp were detected and the sequence homology was compared to the GenBank database (*10*) by the use of BLAST. The results demonstrated that the sequence of 16S rDNA of Zjwp-14 strain, which was deposited in the GenBank database (accession number EF032875), has 99.79 % homology to *Pseudomonas* sp. The multiple sequence alignments of Zjwp-14 strain were compared with those of the 10 most closely related species of *Pseudomonas* genus present in the GenBank database by the use of ClustalX. The system evolutionary tree was constructed with the aid of Mega 211 software. Phylogenetic tree demonstrated that Zjwp-14 strain was closely related to *P. putida*. This newly isolated strain was deposited in China Center for Type Culture Collection (CCTCC) as *Pseudomonas* sp. Zjwp-14 CCTCC M 206104.

In order to develop a process for the maximum biocatalytic activity for this newly isolated strain, it is crucial to optimize the medium constituents.

Single variable optimization methods are tedious and can also lead to misinterpretation of results because of overlooking the interaction between different factors involved (*11*). Response surface methodology (RSM) is an alternative and more efficient approach applied in the optimization of medium constituents and other critical variables responsible for the production of biomolecules (*12*,*13*). In this study, statistical optimization of medium constituents was employed to enhance intracellular enzyme activities for L-cysteine production. To the best of our knowledge, there is not enough information concerning optimum nutritional requirements for bioconversion of DL-ATC to L-cysteine using statistical experimental designs.

Materials and Methods

Materials

Pseudomonas sp. Zjwp-14 strain, used throughout the investigation, was isolated from the soil collected near a chemical factory (Hangzhou, China). Chemically synthesized DL-ATC used for L-cysteine production was provided by Tianjin Chemical Co. Ltd., China. All other reagents were of analytical grade.

Medium and culture conditions

Firstly, *Pseudomonas* sp. Zjwp-14 was cultured in seed medium containing (in g/L): glycerol 10, yeast extract 5, beef extract 5, peptone 5, and DL-ATC 2 (used as inducer for enzyme production). The initial pH of the medium was adjusted to 7.0 with 3 M NaOH. Cultures were incubated at 30 °C for about 10 h on a rotary shaker (200 rpm), with A_{600} (10-fold dilution) reaching 0.40–0.50. Subsequently, a 1.0-mL inoculum was transferred into 250-mL Erlenmeyer flasks containing 50 mL of fermentation medium and further incubated at 30 °C, on a rotary shaker at 200 rpm for 12 h, with A_{600} (10-fold dilution) in the range of 0.45–0.60. The original fermentation medium was composed of (in g/L): glycerol 20, yeast extract 5, peptone 5, beef extract 5, DL-ATC 4, NaCl 5, $MgSO_4{\cdot}7H_2O$ 0.5. The initial pH of the medium was adjusted to 7.5 with 3 M NaOH.

Biotransformation conditions of bioconversion from DL-ATC to L-cysteine

The incubated cells were harvested from the fermentation broth by centrifugation at 4000×*g* for 15 min, and washed twice with sterile saline solution (0.8 %). Cells harvested from 50-mL fermentation broth were resuspended into 250-mL Erlenmeyer flasks containing 25 mL of reaction buffer (0.1 M, pH=8.0, sodium phosphate buffer with 0.25 g of DL-ATC as substrate). Enzymatic production of L-cysteine from DL-ATC was undertaken at 42 °C for 2 h. Erlenmeyer flasks (250 mL) were shaken at 100 rpm for 3 min, with an interval of 30 min for dispersing cells. The reaction was quenched by the addition of 5 mL of 1 M HCl solution. The reaction mixture was centrifuged at 10 000×*g* for 10 min, and the obtained supernatant was assayed for L-cysteine.

Determination of bacterial growth

After dilution of 0.5 mL of cultured broth with 4.5 mL of distilled water, absorbance was measured at 600 nm with 722S VIS spectrophotometer.

Enzymatic activity assay

The formed L-cysteine was measured by the method of Gaitonde (*14*). A volume of 0.5 mL of sample (containing L-cysteine) was thoroughly mixed with 0.5 mL of acetic acid and 0.5 mL of ninhydrin solution (60 mL of acetic acid, 40 mL of 37 % hydrochloric acid and 2.5 g of ninhydrin) and heated in a boiling water bath for 10 min. The solution was then rapidly cooled in tap water, and diluted to 5 mL with 95 % ethanol. A spectral measurement of the reacted solution at 560 nm was made, and the amount of L-cysteine was calculated from a standard curve.

One unit of enzyme activity was defined as the amount of enzyme that produces 1 μg of L-cysteine per h under assay conditions (*15*).

Plackett-Burman design

Plackett-Burman design is a powerful and efficient mathematical approach to determine the effect of medium constituents on enzyme activity (*16*). In this study, glycerol, DL-ATC, yeast extract, NaCl, peptone, beef extract, $MgSO_4 \cdot 7H_2O$ and pH were selected as the independent variables. These variables were investigated and 12 experiments were carried out. Each variable was set at two levels, high level and low level. The experimental design is given in Table 1. SAS software (version 8.0) was used for regression analysis of the obtained experimental data. The significance of regression coefficients was tested by *t*-test.

Central composite design

A central composite design (CCD) was applied to determine the optimum concentration of 4 significant variables screened from Plackett-Burman design. The effect of these variables (glycerol, DL-ATC, yeast extract and pH) on enzyme activity was studied at 5 experimental levels: $-\alpha$, -1, 0, +1, $+\alpha$, where $\alpha=2^{n/4}$, and n is the number of variables and 0 corresponds to the central point. The experimental levels for these variables were selected from our preliminary work, which indicated that an optimum could be found within the level of parameters studied.

A 2^4 full factorial central composite experimental design with 7 replicates at the central points and resulting in a total of 31 experiments was used to investigate the 4 chosen variables. The experiment was designed by using the SAS software (version 8.0). The levels of variables used for CCD are presented in Table 2. Enzyme activity was analyzed using a second-order polynomial equation and the data were fitted into the equation by multiple regression procedure. The model equation for analysis is given below:

$$Y=\beta_0+\Sigma\beta_i X_i+\Sigma\beta_{ii} X_i^2+\Sigma\beta_{ij} X_i X_j \qquad /1/$$

where Y represents the response variable, β_0 is the interception coefficient, β_i is the coefficient of the linear effect, β_{ii} is the coefficient of quadratic effect and β_{ij} the coefficient of interaction effect, while X_i and X_j denote the coded levels of variables. The variable x_i is coded as X_i according to the Eq. 2:

$$X_i=x_i-x_0/\Delta x_i \qquad /2/$$

where X_i is coded value of the variable x_i, while x_0 is the real value of x_i at the center point (zero) level, and the Δx_i is the step change value.

The multiple coefficients of correlation R and the determination coefficient of correlation R^2 were calculated to evaluate the performance of the regression equation, and the statistical significance of the regression equation was determined by the *F*-test.

Results and Discussion

Plackett-Burman design

A wide variation of enzyme activity was observed in Plackett-Burman experiment (Table 1). The p-value of the variable was at 5 % level ($p<0.05$), when it was considered to have a greater impact on the enzyme activity. The data of regression analysis for Plackett-Burman design are shown in Table 3. Out of 8 variables studied, 4 variables (glycerol, DL-ATC, yeast extract and pH) had significant influence on enzyme activity as evidenced by their p-value ($p<0.05$, significant at 5 % level), obtained from the regression analysis. However, the optimal concentrations of medium components that significantly affect enzyme activity could not be obtained. Further work needs to be done to find out this information.

Central composite design

CCD provides important information regarding the optimum level of each variable along with their interactions with other variables and their effects on enzyme activity. Based on Plackett-Burman design, glycerol (X_1), DL-ATC (X_2), yeast extract (X_3) and pH (X_4) were selected

Table 1. Plackett-Burman design and the experimental data

Run no.	Variables								Enzyme activity
	γ(DL-ATC) g/L	γ(glycerol) g/L	γ(yeast extract) g/L	pH	γ(NaCl) g/L	$\gamma(MgSO_4 \cdot 7H_2O)$ g/L	γ(peptone) g/L	γ(beef extract) g/L	U/mL
1	5	10	7	6.5	5	0.4	7	7	772.4
2	5	30	5	7.5	5	0.4	5	7	661.7
3	2	30	7	6.5	7	0.4	5	5	668.2
4	5	10	7	7.5	5	0.6	5	5	533.6
5	5	30	5	7.5	7	0.4	7	5	803.5
6	5	30	7	6.5	7	0.6	5	7	479.8
7	2	30	7	7.5	5	0.6	7	5	587.9
8	2	10	5	7.5	7	0.4	7	7	802.3
9	2	10	5	7.5	7	0.6	5	7	810.6
10	5	10	5	6.5	7	0.6	7	5	796.8
11	2	30	5	6.5	5	0.6	7	7	831.0
12	2	10	5	6.5	5	0.4	5	5	540.6

Table 2. Central composite design and the results

Run no.	Variable level				Enzyme activity U/mL	
	γ(glycerol) g/L	γ(DL-ATC) g/L	γ(yeast extract) g/L	pH	Experimental	Predicted
1	15	2.0	4.0	7.0	663.4	670.1
2	15	2.0	4.0	8.0	691.2	700.3
3	15	2.0	6.0	7.0	474.2	480.5
4	15	2.0	6.0	8.0	678.7	709.6
5	15	4.0	4.0	7.0	592.6	603.3
6	15	4.0	4.0	8.0	512.0	536.9
7	15	4.0	6.0	7.0	661.1	684.3
8	15	4.0	6.0	8.0	821.4	816.9
9	25	2.0	4.0	7.0	663.4	672.6
10	25	2.0	4.0	8.0	776.0	787.9
11	25	2.0	6.0	7.0	324.2	334.3
12	25	2.0	6.0	8.0	654.4	648.5
13	25	4.0	4.0	7.0	584.0	588.1
14	25	4.0	4.0	8.0	608.3	606.8
15	25	4.0	6.0	7.0	524.8	520.4
16	25	4.0	6.0	8.0	709.8	738.1
17	10	3.0	5.0	7.5	625.0	591.2
18	30	3.0	5.0	7.5	521.0	514.9
19	20	1.0	5.0	7.5	673.9	654.6
20	20	5.0	5.0	7.5	697.9	677.4
21	20	3.0	3.0	7.5	781.8	764.0
22	20	3.0	7.0	7.5	727.7	705.6
23	20	3.0	5.0	6.5	459.8	446.8
24	20	3.0	5.0	8.5	721.3	694.6
25	20	3.0	5.0	7.5	807.7	818.8
26	20	3.0	5.0	7.5	799.4	818.8
27	20	3.0	5.0	7.5	846.1	818.8
28	20	3.0	5.0	7.5	798.1	818.8
29	20	3.0	5.0	7.5	833.3	818.8
30	20	3.0	5.0	7.5	823.7	818.8
31	20	3.0	5.0	7.5	819.1	818.8

Table 3. Results of regression analysis for the Plackett-Burman design

Variables	Levels –1	Levels 1	*t*-value	p>|*t*|	Significance
γ(DL-ATC)/(g/L)	2.0	5.0	9.2268	0.00269	3
γ(glycerol)/(g/L)	10.0	30.0	10.5424	0.00182	1
γ(yeast extract)/(g/L)	5.0	7.0	19.4760	0.00249	2
pH	6.5	7.5	7.2579	0.00540	4
γ(NaCl)/(g/L)	5.0	7.0	0.4058	0.7121	8
γ($MgSO_4 \cdot 7H_2O$)/(g/L)	0.4	0.6	–2.1162	0.1246	7
γ(peptone)/(g/L)	5.0	7.0	–2.5244	0.0858	6
γ(beef extract)/(g/L)	5.0	7.0	–2.8252	0.06645	5

for CCD optimization. The concentrations of these major tested variables are presented in Table 2. Concentrations for other variables were set at their low level used in Plackett-Burman design. The experimental and predicted data are shown in Table 2. The experimental results were fitted with a second-order polynomial equation. The values of regression coefficients were calculated and the fitted equations for predicting enzyme activity are as follows:

$$Y=818.8-19.08X_1+5.69X_2-14.60X_3+61.96X_4-66.44X_1^2-38.20X_2^2-21.00X_3^2-62.04X_4^2-4.42X_1X_2-37.18X_1X_3+21.26X_1X_4+67.66X_2X_3-24.14X_2X_4+49.74X_3X_4 \qquad /3/$$

where Y is the response value (enzyme activity), and X_1, X_2, X_3 and X_4 are the coded levels of glycerol, DL-ATC, yeast extract and pH, respectively.

The goodness of fit can be checked by different criteria. The coefficient of determination R^2 was 0.9817, indicating that the response model could explain 98.17 % of the total variations. The value of R (0.9608) indicates high agreement between the experimental and predicted values. The value of adjusted determination coefficient (R^2_{Adj}=0.9657) was also high enough to indicate the significance of the model.

The corresponding analysis of variance (ANOVA) is shown in Table 4. ANOVA gives the value of the model and can explain whether this model adequately fits the variation observed in the enzyme activity with the designed nutrient level. If the *F*-test for the model is significant at the 5 % level ($p<0.05$), then the model is fitted and can adequately explain the variation observed. Generally, the calculated *F*-value should be several times greater than the tabulated *F*-value if the model was a good prediction of the experimental results. The calculated F of 61.24249 in our study was much greater than the tabulated *F*-value ($F_{14,16}$=3.45) at 0.01 level, and $p>F$=0.0001 was very low. This indicated that the second-order polynomial was highly significant.

Table 4. Analysis of variance (ANOVA) for the four factorial designs

Source	Degrees of freedom	Sum of squares	Mean square	*F*-value	p>*F*
Model	14	492904.30	35207.45	61.24249	0.0001
Error	16	9198.18	574.89		
Total	30	502102.50			

The three-dimensional response surface plots were employed to demonstrate the interaction among the medium components and to determine the optimum levels of components supplemented into the basal medium, which have significant effects on enzyme activity (*17*). The response surface plots are shown in Figs. 1–6, which illustrate the relationships between response and the experimental data. As can be seen from Figs. 1 and 2, enzyme activity was predominantly affected by glycerol concentration, which was used as a carbon source. Enzyme activity increased with the increase of glycerol concentration, reached a maximum when glycerol concentration was 16.94 g/L, and then decreased. This result conformed well to the information obtained from our Plackett-Burman design. The reason might be that

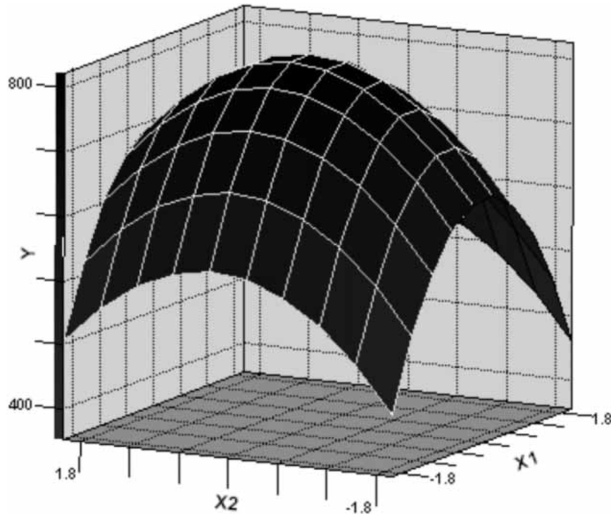

Fig. 1. Effects of glycerol (X_1), DL-ATC (X_2) and their interaction on enzyme activity (Y), with other variables set at central level

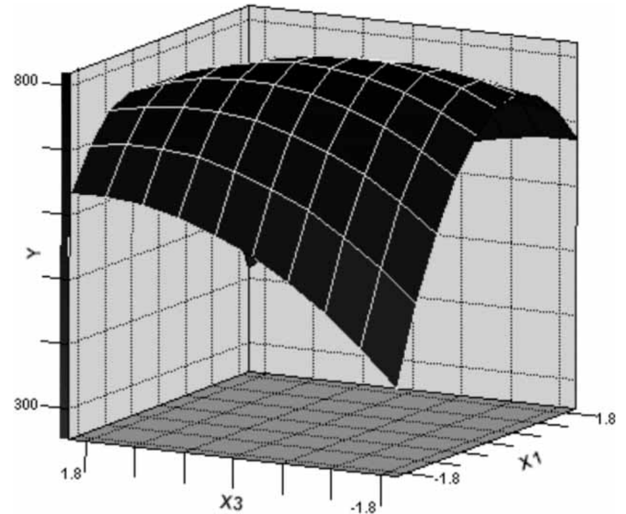

Fig. 2. Effects of glycerol (X_1), yeast extract (X_3) and their interaction on enzyme activity (Y), with other variables set at central level

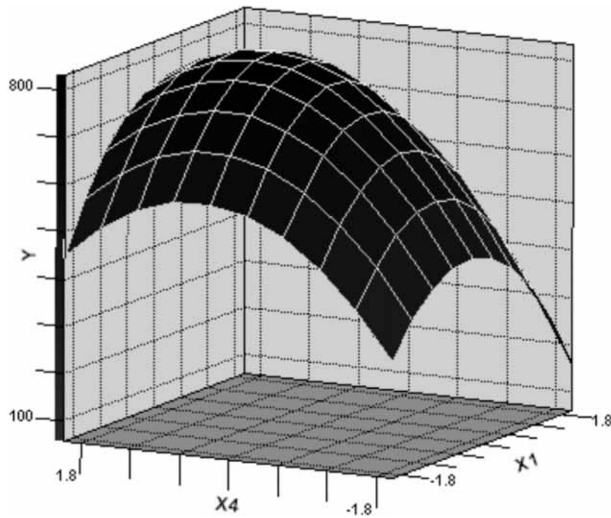

Fig. 3. Effects of glycerol (X_1), pH (X_4) and their interaction on enzyme activity (Y), with other variables set at central level

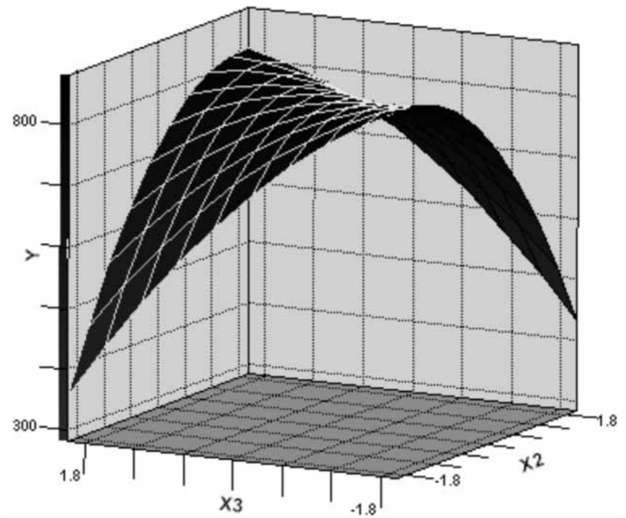

Fig. 4. Effects of DL-ATC (X_2), yeast extract (X_3) and their interaction on enzyme activity (Y), with other variables set at central level

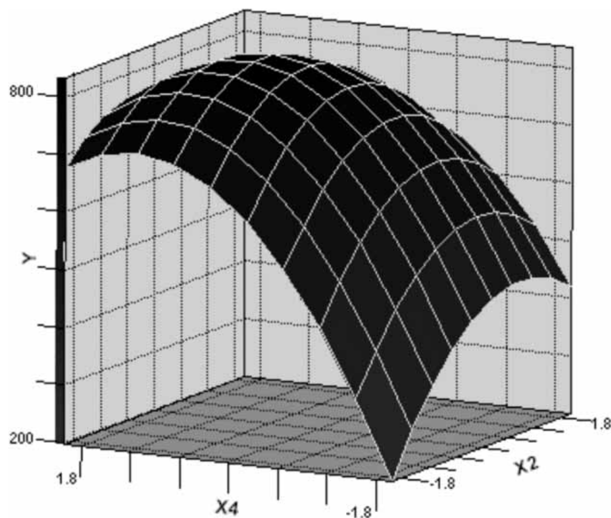

Fig. 5. Effects of DL-ATC (X_2), pH (X_4) and their interaction on enzyme activity (Y), with other variables set at central level

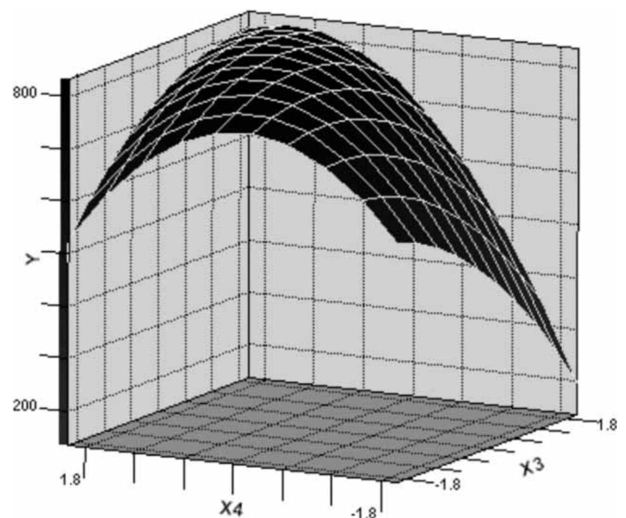

Fig. 6. Effects of yeast extract (X_3), pH (X_4) and their interaction on enzyme activity (Y), with other variables set at central level

higher glycerol concentration could cause the inhibition of enzyme production. The response surface shown in Fig. 3 depicts the combined effects of glycerol concentration and initial pH of the media on enzyme production. These two factors had higher interaction effect. Fig. 4 shows that yeast extract concentration and DL-ATC concentration also had remarkable interaction effect. DL-ATC has been reported to be an inducer of enzymes for L-cysteine production with the optimal concentration of 2–5 g/L (*15*,*18*). As it can be seen from Fig. 5, the minimum response for enzyme production occurs when initial pH of the medium is at its lowest level and the enzyme activity increases considerably with the increase of initial pH. This suggests that initial pH of the medium has a very significant effect on enzyme activity. When initial pH>7.0, higher enzyme activity can be reached, which is in agreement with the previous report (*18*). In Fig. 6 it can be seen that when yeast extract concentration was at a high level, enzyme activity increased with the increase of pH level.

The experimental data were fitted to Eqs. 2 and 3, and the optimum values were found to be: X_1=–0.611 (16.94 g/L), X_2=1.59 (4.59 g/L), X_3=1.99 (6.99 g/L) and X_4=0.881 (7.94). At these optimum levels of nutrients, enzyme activity of 903.6 U/mL was obtained, which was close to the predicted value of 918.7 U/mL. This result corroborated the validity and the effectiveness of this model.

The time course of changes in A_{600} and enzyme activity for *Pseudomonas* sp. Zjwp-14 cultured under the optimal conditions are shown in Fig. 7. During cultivation, fermentation broth was taken out of the Erlenmeyer flasks or the 5-litre fermentor (BIOSTAT 100, B. Braun Co., Germany) for measuring A_{600} and the biotransformation of DL-ATC to L-cysteine with an interval of 2 h. It was found that the enzyme activity increased with the A_{600}, and maximum enzyme activity occurred in the middle stage of bacterial growth. The growth of *Pseudomonas* sp. Zjwp-14 was faster in 5-litre fermentor than in Erlenmeyer flasks. The maximum enzyme activity in the Erlenmeyer flasks was 903.6 U/mL in 12 h. Comparatively, the maximum enzyme activity in the fermentor reached 929.6 U/mL in 6 h. The cultivation time of *Pseudomonas* sp. Zjwp-14 with maximum enzyme activity in the fermentor was shorter for 6 h than in the Erlenmeyer flasks.

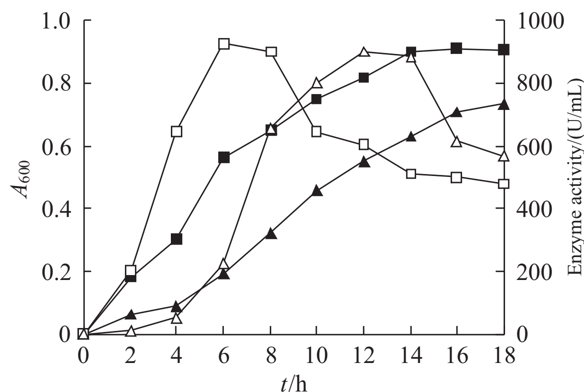

Fig. 7. The time course of changes in A_{600} and enzyme activity for *Pseudomonas* sp. Zjwp-14 cultured under the optimal conditions: ■ A_{600} in 5-litre fermentor, ▲ A_{600} in Erlenmeyer flasks, □ enzyme activity in 5-litre fermentor, △ enzyme activity in Erlenmeyer flasks

The cultured *Pseudomonas* sp. Zjwp-14 cells in the 5-litre automatic fermentor were applied for the biotransformation of DL-ATC to L-cysteine. The molar yield of L-cysteine reached 57.4 %, with the DL-ATC concentration of 10 g/L for 6 h.

In order to confirm that the bioconversion of DL-ATC to L-cysteine can be obtained by the activity of intracellular enzyme of *Pseudomonas* sp. Zjwp-14, further studies were performed. The mixture containing the product L-cysteine was centrifuged. Then, the supernatant obtained after low-speed centrifuge was also used to perform the conversion of DL-ATC to L-cysteine. The result showed that only negligible L-cysteine was detected in the supernatant of the reaction mixture after quenching the biotransformation. We also examined the possibility of the permeation of the mentioned enzyme from the cells during the conversion, but the cells were not broken at the end of the process when observed under the microscope. Thus, our results clearly demonstrate that the charging enzyme is intracellular, and the low molecular mass DL-ATC should diffuse into the cell, interact with the intracellular enzyme, and the product (L-cysteine) is then produced and secreted into the reaction buffer.

Conclusions

The response surface methodology allowed a rapid screening of the important influence factors and development of a polynomial model to optimize culture medium for enzymatic production of L-cysteine by *Pseudomonas* sp. Zjwp-14. The R^2 value of 0.9817 showed a good fit of the model with the experimental data. The model predicted accurately the maximum point of enzyme production. The optimal culture medium contained (in g/L): glycerol 16.94, DL-ATC 4.59, yeast extract 6.99, NaCl 5.0, $MgSO_4 \cdot 7H_2O$ 0.4, peptone 5.0, beef extract 5.0, and pH=7.94. Under the optimum conditions, the maximum enzyme activity reached 903.6 U/mL in Erlenmeyer flasks and 929.6 U/mL in 5-litre fermentor, with an increase of 15.6 and 18.9 % respectively, compared to the original medium components. The cultivation time of *Pseudomonas* sp. Zjwp-14 with maximum enzyme activity in 5-litre fermentor was shorter for 6 h than in Erlenmeyer flasks, and the molar yield of L-cysteine reached 57.4 % after biotransformation for 6 h.

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