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Optimization of enzyme-producing conditions of *Micrococcus* sp. S-11 for L-cysteine production

Yu Huang[a], Lin Zhao[a,b]*, Tao Dong[b] and Xin Tan[a,b]

[a]School of Environment Science and Technology, TianJin University, Tianjin 300072, PR China.
[b]School of Chemical Engineering and Technology ,TianJin University, Tianjin 300072, PR China.

Micrococcus sp.S-11 is capable of converting racemic 2-amino-Δ^2-thiazoline-4-carboxylic acid (DL-ATC) to L-cysteine. The enzyme-producing conditions were optimized by using response surface methodology to maximize enzyme activity. Glucose, DL-ATC·3H$_2$O and rotational speed have significant effects on enzyme activity. Their optimal values were 21.7 g/l glucose, 7.3 g/l DL-ATC·3H$_2$O and 141 rpm rotational speed. Then validation experiments were conducted under optimal conditions. The enzyme activity was up to 2291 U/g, which was close to the predicted maximum enzyme activity (2284 U/g) and 31.4% higher than the initial activity (1743 U/g). A two-step culture was performed with the optimal conditions for cell growth in the first step and enzyme activity in the second step. The results suggested that high carbon/nitrogen (C/N) mass ratio was favorable to enzyme activity and the two-step culture strategy was proposed to be adopted for achieving both high biomass and enzyme production simultaneously.

Key words: L-Cysteine, enzyme activity, Plackett-Burman design, response surface methodology, two-step culture.

INTRODUCTION

L-Cysteine is widely applied in many fields, for instance, food additives, pharmaceutical industry, feedstuff and cosmetic additives. Currently, four manufacturing methods have been developed to produce L-cysteine. These are acid or alkali hydrolysis of hair, chemical synthesis, microbe fermentation (Maier and Winterhalter, 2001) and bioconversion of 2-amino-Δ^2-thiazoline-4-carboxylic acid (DL-ATC) (Sano and Mrrsugi, 1978). Bioconversion of DL-ATC by whole-cell biocatalyst, a competent method with advantages of low energy requirement and high molar yield, has being substituted for acid or alkali hydrolysis of hair as a main method for the production of L-cysteine on industrial scale (Hee et al., 1997).

With respect to enzymatic production of L-cysteine from DL-ATC, a lot of work has been done on strains screening and genes cloning (Tamura et al., 1998; Ohmachi et al., 2002, 2004; Shiba et al., 2002; Yu et al., 2006). Early 1977, the isolated strain *Pseudomonas thiazolinophilum*

AJ 3584 which could convert DL-ATC into L-cysteine was obtained (Sano et al., 1977) and had been used in the commercial production of L-cysteine (Yamamoto et al., 1999). *Pseudomonas* sp.M-38, a mutant from *Pseudomonas* sp.CU6, had weaker activity of L-cysteine desulfhydrase which was an intrinsic decomposing enzyme existing in the genus *Pseudomonas* (Moon et al., 1992). However, all the isolated L-cysteine-producing strains referred in previous literature with high molar yield of L-cysteine exclusively belong to the genus *Pseudomonas* (Hiroshi et al., 2002).

In our previous work, a germ *Micrococcus* sp.S-11 was isolated, which has a comparable hydrolytic activity with the *Pseudomonas* in producing L-cysteine from DL-ATC. Besides, it does not have desulfhydrase activity which means a possible higher molar yield of L-cysteine. The good performances make *Micrococcus* sp. S-11 a promising L-cysteine producer (Dong, 2009).

Obviously, optimization of cultivation conditions strongly enhances the productivity of the bioconversion or bio-catalysis (Djekrif et al., 2006). The conventional optimization methods, for example, one-factor-at-a-time, is time-consuming and fail to predict response under untested

*Corresponding author. E-mail: tjudongtao@tju.edu.cn. Tel: +86 22 27405495. Fax: +86 22 27890259.

sets of parameters (Liu and Tzeng, 1998). Furthermore, they cannot guarantee the determination of the optimal conditions and depict the frequent interactions occurring between two or more factors (Hye et al., 2008; Kammoun et al., 2008; Lotfy et al., 2007). Statistic experimental design and analysis methods consider the interactions between two or more parameters and provide reliable results (Kumar and Satyanarayana, 2007; Song et al., 2007). Especially, it is suitable for a complicated system with multiple variables (Xu et al., 2008). One of such method is Plackett-Burman design, which has been frequently applied in bioprocess optimization.

Plackett-Burman design is capable of rapidly identifying the significant factors which have strong influence on the response from a multi-parameters system in a quite small number of experiments. Then, these significant factors were further investigated by Box-behnken experimental design in details. The experimental results were analyzed and the optimal levels of significant parameters were determined by response surface methodology.

Recently, the statistical optimization design on L-cysteine production from ATC by *Pseudomonas* has been reported. Liu et al. (2004) has optimized the conditions of enzyme production by *Pseudomonas* sp. TS1138 and obtained a 25.4% increase in enzyme activity. Previously, the conditions for cell growth of *Micrococcus* sp.S-11 have been optimized (Dong, 2009). The optimization on biomass significantly improved the cell growth of *Micrococcus* sp. S-11 but had limited enhancement on enzyme activity for L-cysteine production, which suggested that the productions of biomass and enzymes involved in L-cysteine production by *Micrococcus* sp. S-11 had different nutrient requirements.

In this work, a screening experimental design on eleven parameters was conducted through Plackett-Burman design, and three factors were chosen as significant with strong effects on enzyme activity of *Micrococcus* sp.S-11. Then the three variables were optimized by Box-behnken experimental design and response surface methodology. Compared with original culture conditions, the optimal conditions strongly enhanced enzyme activity.

MATERIALS AND METHODS

All experiments were performed at least in triplicate and the results were presented as their mean value. Experimental error was not over 5%.

Microbe, medium and cultivation

Micrococcus sp.S-11 was isolated and stored in our laboratory. The basal enzyme-producing medium was composed of 20 g glucose, 3 g urea, 5 g DL-ATC·3H$_2$O, 1.5 g NaCl, 3g K$_2$HPO$_4$, 0.12 g MnSO$_4$, 0.5 g MgSO$_4$·7H$_2$O, 0.01 g FeSO$_4$·7H$_2$O and 1000 mL distilled water. The pH was adjusted to 7.0 with NaOH. For preparation of the inoculum, *Micrococcus* sp.S-11 was transferred from a slant culture into an Erlenmeyer flask (250 ml) containing 50 ml optimized growth medium which was composed of glucose 21.98 g/l, urea

4.75 g/l, 1.5 g NaCl, 3 g K$_2$HPO$_4$, 0.12 g MnSO$_4$, 0.5 g MgSO$_4$·7H$_2$O and 0.01 g FeSO$_4$·7H$_2$O in 1000 ml distilled water, and incubated at 35°C on a rotary shaker at 124 rpm for 20 h.

All the experiments were accomplished in 250 ml flask containing 50 ml medium. Each of the flasks was inoculated with 10% (v/v) of the inoculum.

Estimation of biomass and enzyme activity

The biomass of *Micrococcus* sp.S-11 grown under different conditions was expressed as dry cell weight. The cultivation broth was centrifuged (8000 g × 15 min) at 4°C and washed twice with distilled water to get rid of the residual medium, and then freeze-dried to constant weight at -40°C for 24 h.

Enzyme activity of the whole-cell catalysts was assessed by adding the prepared dry cells 0.1 g into 50 ml transformation solution containing 2% (w/v) DL-ATC·3H$_2$O, 1% (w/v) K$_2$HPO$_4$, 0.14% (w/v) NH$_2$OH. HCl (pH 8. 0) and incubation at 40°C for 1 h with a rotation speed of 130 rpm. The producing L-cysteine was determined by Ellman's test (Ellman et al., 1961).One unit of enzyme (1 U) was defined as the amount of enzyme that produced 1 μmol L-cysteine per minute under the standard assay conditions.

Experimental designs and data analysis

Plackett-Burman experimental design and analysis

Plackett-Burman experimental design, a two-level factorial design, offers the screening of a large number of parameters (*n*) with a small number of experiments (n+1). In order to screen out the key factors which have significant influence on enzyme production by *Micrococcus* sp.S-11, a Plackett-Burman experimental design was formulated for eleven parameters as follows: Glucose (X$_1$), urea (X$_2$), DL-ATC·3H$_2$O (X$_3$), K$_2$HPO$_4$ (X$_4$), MnSO$_4$ (X$_5$), FeSO$_4$·7H$_2$O (X$_6$), MgSO$_4$·7H$_2$O (X$_7$), NaCl (X$_8$) concentration, initial pH (X$_9$), rotational speed (X$_{10}$) and cultural temperature (X$_{11}$). Each factor was investigated at two levels, high (+) and low (-). A design of a total of 12 experiments was generated by using the software Minitab (Minitab. Inc., USA). The average of enzyme activity obtained was taken as the response (Table 1). The effect of individual factors on enzyme activity was calculated according to following equation:

$$E_i = \frac{(\sum P_{i+} - \sum P_{i-})}{N} \tag{1}$$

Where, E_i is the effect of parameter *i* under study, P_{i+} and P_{i-} are responses (enzyme activity) of trials at which the parameter was at it's high and low levels, respectively, and *N* is the total number of trials.

Box-behnken design and response surface methodology

Box-behnken design is a fractional factorial design. The response surface methodology was used to analyze the experimental design data. The three significant variables chosen from the Plackett-Burman design were optimized through Box-behnken experimental design and response surface methodology, while the others were identified as insignificant factors and fixed at constant level (same as in the basal medium). The response was fitted by a second order model to be correlated with the independent parameters. The correlation between the three parameters and the response (enzyme activity) was described by the following predictive quadratic

Table 1. Experimental design and response of Plackett-Burman (N = 12).

Run Order	Urea (g/L)	(g/L)	DL-ATC·3H$_2$O (g/L)	K$_2$HPO$_4$ (g/L)	MnSO$_4$ (g/L)	FeSO$_4$·7H$_2$O (g/L)	MgSO$_4$·7H$_2$O (g/L)	NaCl (g/L)	pH (g/L)	Rotational speed (rpm)	Temperature (°C)	Enzyme activity (U/g)
	X$_1$	X$_2$	X$_3$	X$_4$	X$_5$	X$_6$	X$_7$	X$_8$	X$_9$	X$_{10}$	X$_{11}$	
1	15(-)	4.5(-)	7.5(+)	3.75(+)	0.15(+)	0.01(-)	0.625(+)	5(+)	7(-)	135(+)	25(-)	1914.39
2	20(+)	6(+)	5(-)	3.75(+)	0.15(+)	0.01(-)	0.625(+)	2.5(-)	7(-)	110(-)	35(+)	1666.97
3	15(-)	4.5(-)	Glucose(g/L)	3(-)	0.12(-)	0.01(-)	0.5(-)	2.5(-)	7(-)	110(-)	25(-)	1580.64
4	20(+)	4.5(-)	X$_1$	3(-)	0.15(+)	0.0125(+)	0.625(+)	2.5(-)	8(+)	135(+)	25(-)	1860.99
5	15(-)	6(+)	7.5(+)	3(-)	0.15(+)	0.01(-)	0.5(-)	2.5(-)	8(+)	135(+)	35(+)	1939.31
6	15(-)	6(+)	7.5(+)	3.75(+)	0.12(-)	0.0125(+)	0.625(+)	2.5(-)	8(+)	110(-)	25(-)	1754.19
7	20(+)	4.5(-)	7.5(+)	3.75(+)	0.12(-)	0.0125(+)	0.5(-)	2.5(-)	7(-)	135(+)	35(+)	2210.76
8	20(+)	6(+)	5(-)	3.75(+)	0.12(-)	0.01(-)	0.5(-)	5(+)	8(+)	135(+)	25(-)	1841.41
9	15(-)	6(+)	5(-)	3(-)	0.12(-)	0.0125(+)	0.625(+)	5(+)	7(-)	135(+)	35(+)	1594.88
10	15(-)	4.5(-)	5(-)	3.75(+)	0.15(+)	0.0125(+)	0.5(-)	5(+)	8(+)	110(-)	35(+)	1411.54
11	20(+)	6(+)	7.5(+)	3(-)	0.15(+)	0.0125(+)	0.5(-)	5(+)	7(-)	110(-)	25(-)	2044.33
12	20(+)	4.5(-)	7.5(+)	3(-)	0.12(-)	0.01(-)	0.625(+)	5(+)	8(+)	110(-)	35(+)	2025.64

(+), High level; (-) low level.

polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=1}^{3} \beta_{ij} X_i X_j$$

(2)

Where, Y is the predicted response, β_0 is the constant term, β_i the linear coefficients, β_{ii} the squared coefficients and β_{ij} the interaction coefficients. The quality of fitting by the polynomial model equation was expressed using co-efficient of determination R^2. Equation 2 was used to construct contour plots.

The experimental plan consisted of 15 trials and the three variables were studied at three different levels, low (-1), medium (0) and high (+1). The experimental design and results used for the study are shown in Table 2. The optimal values of the three parameters were achieved by solving the obtained polynomial equation. In addition, two-dimensional contour plots were constructed for visual observation of the trend of the maximum response and the interactive effects of the significant variables on the response.

One-step and two-step cultivation

The culture in the optimized growth medium was referred to as the one-step culture. In the two-step culture experiments, the cells were first cultivated in the optimized growth medium for 20 h, and then in the basal enzyme-producing medium (or in the optimized enzyme-producing medium as optimized two-step culture) until harvest. In order to replace culture medium between the two steps, the growth medium was removed by centrifuging at 4°C (8000×g, 15 min) and the cell pellet was resuspended in fresh medium. During the culture process, samples were taken from the flasks every certain hour for the measurement of biomass concentration and enzyme activity.

RESULTS

Enzyme production characteristics of Micrococcus sp.S-11

Effects of different carbon sources and nitrogen sources on enzyme activity were investigated (data not shown). The results showed that the optimal carbon and nitrogen source for enzyme activity of *Micrococcus* sp.S-11 was respectively, glucose and urea, which were also the optimal carbon and nitrogen source for its biomass production (Dong, 2009). So glucose and urea were chosen as the carbon and nitrogen source of the basal enzyme-producing medium, respectively.

In order to determine the appropriate harvest time, enzyme-producing curve of *Micrococcus* sp. S-11

Table 2. Results and design table of box-behnken.

Run Order	Glucose (g/L)	DL-ATC·3H$_2$O (g/L)	Rotational speed (rpm)	Enzyme activity (U/g)
	X$_1$	X$_3$	X$_{10}$	
1	20(0)	10(+)	160(+)	1831.50
2	20(0)	5(-)	110(-)	1759.50
3	20(0)	7.5(0)	135(0)	2257.00
4	25(+)	5(-)	135(0)	1960.75
5	25(+)	7.5(0)	160(+)	2160.75
6	20(0)	10(+)	110(-)	1811.00
7	15(-)	7.5(0)	160(+)	1955.75
8	20(0)	5(-)	160(+)	1905.00
9	15(-)	10(+)	135(0)	1744.25
10	20(0)	7.5(0)	135(0)	2261.00
11	25(+)	7.5(0)	110(-)	2064.25
12	20(0)	7.5(0)	135(0)	2261.00
13	15(-)	7.5(0)	110(-)	1856.25
14	25(+)	10(+)	135(0)	1782.25
15	15(-)	5(-)	135(0)	1562.41

(+), High level; (0), medium level; (-), low level.
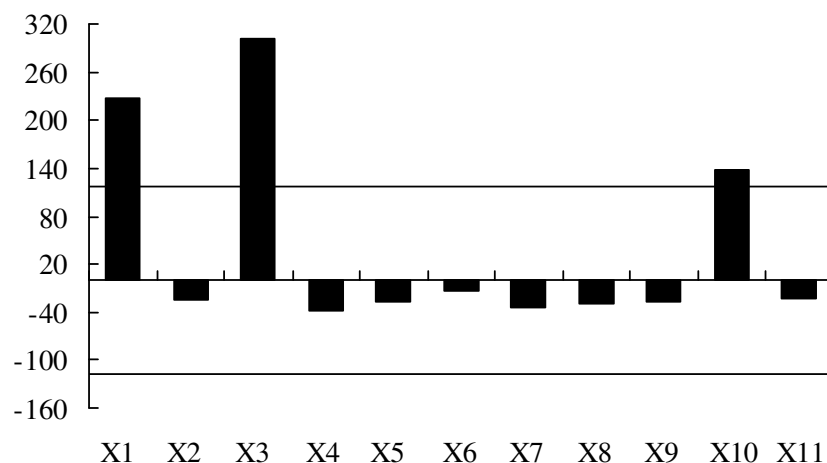
Figure 1. The influence of the parameters on enzyme activity.

was investigated. It was found that enzyme activity reached its peaks during culture time from 18 to 24 h, so activity at 20 h was chosen as the maximal value.

Optimization of enzyme-producing conditions of *Micrococcus* sp.S-11

Screening the significant parameters on enzyme activity

The influence of the parameters on enzyme activity was estimated and graphically presented in Figure 1 to illustrate the relative magnitude and the statistical significance of the impact. Length of the column represented significance of the influence of studied factors on enzyme activity. The reference line was drawn at a significant α-level of 0.05 and used to determine which factor was significant. From Figure 1, it was obvious that glucose (X$_1$), DL-ATC·3H$_2$O (X$_3$) and rotational speed (X$_{10}$) had significant impact on enzyme activity. In these three factors, DL-ATC·3H$_2$O had the greatest influence on enzyme activity, and glucose had greater effects than rotational speed. When the sign of the effect E_i of the tested parameter was positive, the enzyme activity was greater at a high level of the parameter. However, if the sign was negative, the enzyme activity is greater at a low level of the parameter. The three factors had positive

Table 3. Analysis of variance for the fitted quadratic model.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	645805	645805	71756	1321.35	0.000
Linear	3	106596	363274	121091	2229.83	0.000
Square	3	502839	502839	167613	3086.50	0.000
Interaction	3	36370	36370	12123	223.24	0.000
Residual Error	5	272	272	54		
Lack-of-Fit	3	261	261	87	16.30	0.058
Pure Error	2	11	11	5		
Total	14	646076				
R-Sq = 100.0% R-Sq (adj) = 99.9%						

influence on enzyme activity which indicated that the enzyme activity would be enhanced by these factors at their high level. Therefore, in order to locate the maximal enzyme activity in the tested ranges of these parameters, the higher concentration of glucose and DL-ATC·3H$_2$O and the higher rotational speed would be investigated in the following optimization.

Optimization of enzyme production conditions

Based on the above screening test, DL-ATC·3H$_2$O, glucose and rotational speed were identified as significant factors in following investigation at three levels each. Therefore, a Box-behnken design was formulated to investigate the optimum levels of these three factors. The experimental design and the response (enzyme activity) are presented in Table 2. By applying multiple regression analysis on experimental data, a predictive quadratic polynomial equation was constructed to describe the correlation between enzyme activity and the three significant parameters as follows:

$$Y = -8295.79 + 327.90X_1 + 1023.41X_3 + 45.87X_{10} - 6.30X_1^2 - 54.38X_3^2 - 0.15X_{10}^2 - 7.21X_1X_3 - 0.01X_1X_{10} - 0.50X_3X_{10}$$

Where, Y is the predicted enzyme activity, X_1, X_3, and X_{10} were the coded values of glucose concentration, DL-ATC·3H$_2$O concentration and rotational speed, respectively. The analysis of variance for response surface quadratic model is summarized in Table 3. The p-values (0.000) indicated the linear, the interactive and the square terms, all had quite significant influence on enzyme activity. The lack-of-fit measured the failure of the model to represent data in the experimental domain at points which were not included in the regression. The p-value for lack of fit was 0.058, indicating that this full quadratic model adequately fitted the data. The determination coefficient, R^2, (100%) indicated that the predicted and experimental value had perfect coherence with each other. The value of adjusted R^2 (99.9%) suggested that the total variation of 99.9% for

enzyme activity was attributed to the independent variables and only 0.1% of the total variation could not be explained by the model.

By solving the predictive quadratic equation, the maximum enzyme activity was 2284 U/g when the optimal culture conditions are 21.7 g/l glucose, 7.3g/l DL-ATC·3H$_2$O and 141 rpm rotational speed. The contour curves (plotted in Figure 2) were graphic representations of the regression equation. The figures showed the interaction of two variables varying within the experimental ranges with the third variable fixed at its optimal level. It was observed from contour curves that the maximum response located inside the design boundary validated the properness of the tested ranges of the parameters. The maximum enzyme activity was obtained with glucose at its higher level and DL-ATC·3H$_2$O and rotational speed at their middle levels. The shapes of the contours indicated whether the interactions between the factors were significant or not. The elliptic contours in Figure 2A and C indicated that the interactions between glucose and DL-ATC·3H$_2$O, DL-ATC·3H$_2$O and rotational speed were more significant than that between glucose and rotational. The density of the contours indicated whether the influence of the variables on the response was significant. According to Figure 2, the relative significance of the impact of the parameters on enzyme activity was in the following order: DL-ATC·3H$_2$O> glucose>rotational speed, which was identical with the result from Figure 1. In order to obtain enzyme activity at about 2250 U/g, the ranges of the tested parameters were as follows: Glucose 18.5 - 24.0g/l, DL-ATC·3H$_2$O 6.7 - 8.2g/l and rotational speed 125 - 155 rpm. With increase of glucose concentration from 15 to 22 g/l, DL-ATC·3H$_2$O concentration from 5 to 7.5g/l and rotational speed from 110 to 140 rpm, the enzyme activity had a striking rise and then declined a little when the three factors continued to increase.

Validation of the mathematic model

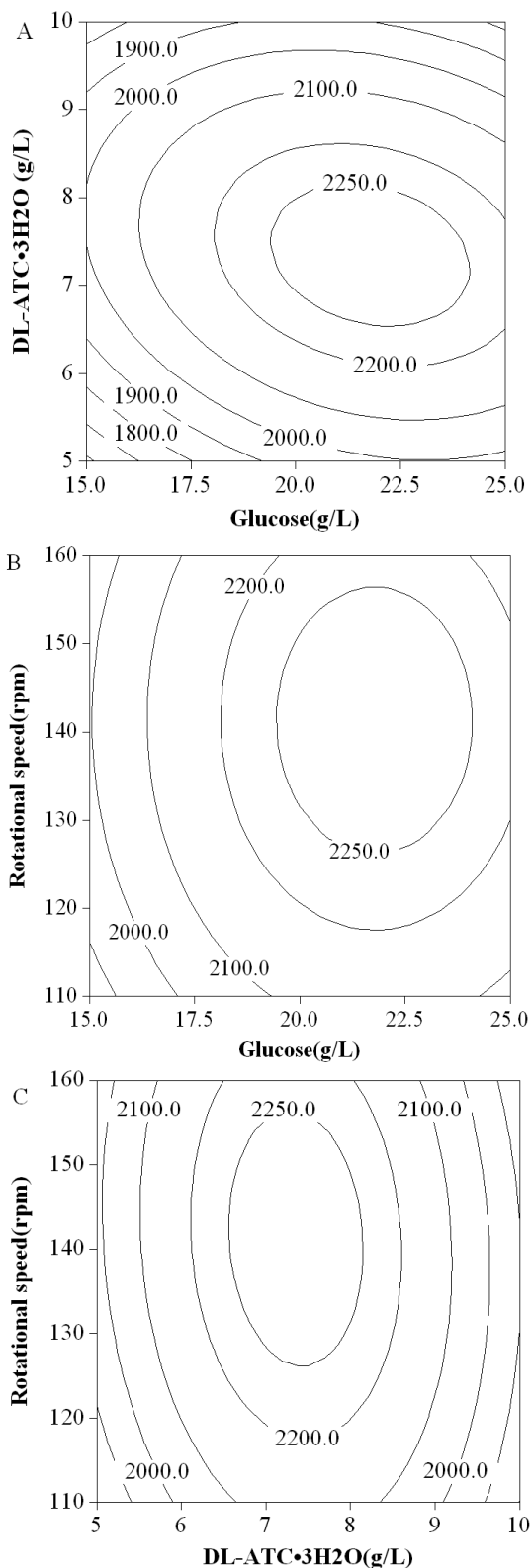In order to determine the predicted results of the quadratic

Figure 2. Contour plots of the enzyme activity. (A) Glucose concentration vs. DL-ATC·3H$_2$O concentration at optimal rotational speed; (B) glucose concentration vs. rotational speed at optimal DL-ATC·3H$_2$O concentration; (C) DL-ATC·3H$_2$O concentration vs. rotational speed at optimal glucose concentration.

model, the cultivation of *Micrococcus* sp. S-11 under both initial and optimized conditions was conducted in triplicate. The average enzyme activity of 2291 U/g was obtained under optimized conditions, which was very close to the predicted maximum enzyme activity (2284 U/g) and was 31.4% higher than the one 1743 U/g obtained under initial conditions. The coherence between the experimental and estimated responses verified the validity and accuracy of the model in terms of depicting the enzyme production by *Micrococcus* sp.S-11.

One-step and two-step culture

The time courses of the production of biomass and enzymes of *Micrococcus* sp. S-11 were investigated under different culture processes: one-step, two-step and optimized two-step culture (Figure 3).

With the increasing cultivation time, the cell concentration would increase firstly and then enter into the stationary phase. In one-step, the cells experienced a rapid growth period between 6-18 h followed by the stationary phase, and the maximum biomass was 11.3 g/l. The cell concentration in the two-step cultures continued to go up after the medium replacement until 28 h, reaching a higher cell concentration, as 13.7 g/l in the two-step culture and as 14.8 g/l in the optimized two-step culture, than that in the batch culture (Figure 3A). The medium replacement did not cause obvious delay in the growth, but the increasing rate of cell growth obviously slowed down. The enzyme activity of cells in the batch culture increased slightly and reached its peak value at 320 U/g during 22 – 26 h, then decreased a little. The enzyme activity in the two-step cultures increased slightly and slowly before medium replacement (0 - 18 h) and then rose more rapidly and steadily. The maximum values were 1752 and 2295 U/g in the two-step culture and the optimized two-step culture, respectively (Figure 3B).

DISCUSSION

The enzyme activity showed a remarkable rise trend with the increasing concentration of DL-ATC•3H$_2$O, which indicated that DL-ATC was an inductor to enzyme activity of *Micrococcus* sp.S-11 (data not shown). It was reported that DL-ATC also induced the enzyme activity of L-cysteine producers belonging to *Pseudomonas* (Yoshiharu et al., 1998). Hence, the enzymes involved in the conversion of DL-ATC to L-cysteine in *Micrococcus* sp.S-11 might be similar to those in the *Pseudomonas*. Therefore, DL-ATC•3H$_2$O was selected as a component of the basal enzyme-producing medium. Addition of DL-ATC was one of the most important reasons for enzyme production enhancement.

The increase of the carbon/nitrogen (C/N) mass ratio might be another most important reason for enzyme production enhancement. Glucose in the culture medium
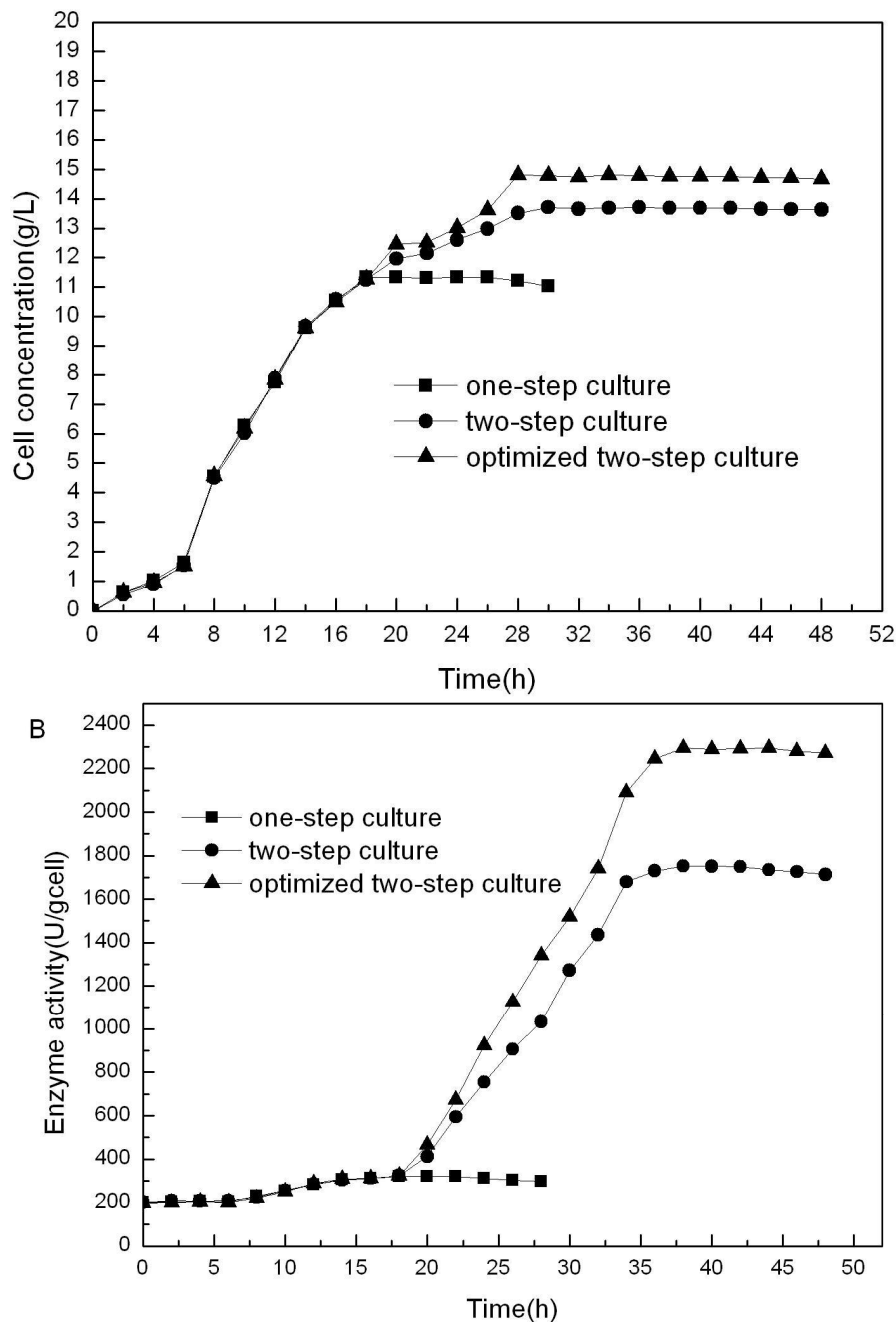
Figure 3. Time courses of one-step and two-step cultures. (A) Cell concentration;
(B) enzyme activity.

provided both the major energy source for cell metabolism and the carbon element for cell growth. Optimal carbon content in enzyme-producing medium was 21.7 g/l, which was very close to that in biomass-producing medium (21.98 g/l). Urea as nitrogen source was another major nutrient, which had more influence on the cell growth than the enzyme activity of *Micrococcus* sp. S-11. The optimal nitrogen content in the two mediums quite differed from each other, as 3 g/l for enzyme activity and

4.75g/l for cell growth. The glucose and urea concentrations determined the C/N ratio in the culture medium. At the optima, the C/N mass ratio was about 4.6 for cell growth and 7.2 for enzyme activity in this study. Numerous previous studies have shown that C/N ratio has significant influence on cell growth and biosynthesis in some microorganisms and that high C/N ratio is more favorable for the biosynthesis in some organisms. The enzymes involve in the transformation from ATC to L-

cysteine in *Micrococcus* sp. S-11 might be generated in the secondary metabolism. During nutritional imbalance, cell growth was suppressed and entered into the sub-metabolism, leading to enzyme activity enhancement. In the two-step cultivation, cell biomass from culture medium was transferred to a second step in which the medium was usually nitrogen-limited or nitrogen-free (Sudesh and Doi, 2000; Lee et al., 2004). Both the timing of enzyme production after the rapid cell growth phase and its low nutrient requirement were apt for two-step production process.

Micrococcus sp. S-11 being an aerobe, shaking culti-vation prompted its cells sufficient contact not only with nutrition but with oxygen. During proper range, the increase of rotational speed would enhance oxygen transfer and complete mixing of further enzyme generation. But the higher rotational speed would bring higher shearing strength which had considerable harm to microbe cells. It was supposed that strong shearing strength might account for the remarkable decrease of enzyme activity with the further rise of rotational speed.

The proportion of the cost of enzymes is a major index for the cost of bioprocess and a higher enzyme activity means a lower request of enzyme quantity and lower cost. The medium replacement at the end of the growth stage not only furnished the limiting nutrients with an optimal production medium but also removed the inhibitory metabolites accumulated during the previous growth stage. Compared with the batch culture, the two-steps cultures showed relatively limited increase in cell concen-trations, but dramatically improved the enzyme activity. It indicated that the two-step strategy, that is, firstly making the cell increase to a high density in the optimal cell growth medium and then switching to conditions favorable for enzyme generation, is effective in obtaining the maximum biomass and enzyme production at the same time.

Conclusions

Glucose and urea were the optimal carbon and nitrogen sources for both enzyme activity and biomass of *Micro-coccus* sp. S-11. DL-ATC was an inducer to its enzyme activity. Glucose, DL-ATC·$3H_2O$ and rotational speed had significant influence on its enzyme activity. The optimal conditions for enzyme activity were 21.7g/l glucose, 7.3 g/l DL-ATC·$3H_2O$ and 141 rpm rotational speed. Obvious enhancement of enzyme activity from 1743 to 2291 U/g had been obtained. The two-step process strategy was successfully adopted to enhance the L-cysteine yield in the first step for biomass growth and the second step for enzyme generation.

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