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Enzymatic synthesis enhancement of mutagenised strain *Pseudomonas* sp. C-25 for *L*-cysteine production

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ABSTRACT

Enzymatic synthesis of L-cysteine from DL-2-amino-△²-thiazoline-4-carboxylic acid (DL-ATC) by a mutagenised strain, Pseudomonas sp. C-25, was studied in this paper. Low-energy ion beam implantation was applied in the original strain Pseudomonas sp. B-3 and a mutant strain designated as Pseudomonas sp. C-25 was obtained, which enzyme activity achieved 2747±3.7 U/mL, an increase of 26.2% compared to that of the initial one (2175±5.1 U/mL). The components of enzyme-producing medium for Pseudomonas sp. C-25 were subsequently optimized by response surface methodology. With Plackett-Burman design (PBD) method, DL-ATC·3H₂O, glycerol and beef extract were found to be the significant factors among eight tested variables that influenced the enzyme activity. Further research on the interaction among the significant elements was then conducted by means of steepest ascent, Box-Behnken design and response surface methodology. Consequently, it turned out that the optimum medium was composed of (in g/L): DL-ATC·3H₂O 4.7, glycerol 16.0, beef extract 6.5, yeast extract 4.0, peptone 5.0, NaCl 3.0, MnSO₄·H₂O 0.06, MgSO₄·7H₂O 0.5. Under such conditions, the enzyme activity reached 3301±6.4 U/mL in shake culture and 3925±5.7 U/mL in 7 L fermentor, with an increase of 20.1% and 42.8% respectively, compared to that of the original medium components.

Key words: *L*-cysteine, *Pseudomonas* sp. C-25, enzymatic synthesis enhancement, response surface methodology, fermentation medium

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INTRODUCTION

L-cysteine, the only sulphydryl-containing amino acid, is crucial to protein folding and synthesis of other organism components, such as thiamine, glutathione, biotin, etc. Owing to its special structure, it has been widely applied in the areas of pharmaceuticals, food additives and cosmetics. Currently, *L*-cysteine has mainly been produced by acid hydrolysis of human hairs and duck feathers [1]. However, this process not only has low yield with poor quality [2, 3], but also pollutes environment. A bioconversion process, instead of extracting from hairs and feathers for producing *L*-cysteine, has been developed[4]. Some bacteria, particularly in the genus of *Pseudomonas*, can hydrolyze *DL*-2-amino-Δ^2- thiazoline-4-carboxylic acid (*DL*-ATC) to *L*-cysteine [5, 6]. *DL*-ATC is a desirable substrate for product *L*-cysteine due to simplicity of the process for chemical synthesis of *DL*-ATC and the favorable cost for *L*-cysteine production using *DL*-ATC, because the price of *DL*-ATC is lower than that of *L*-cysteine [7-9]. It is valuable for large-scale *L*-cysteine production in industry.

Our lab isolated a strain *Pseudomonas* sp. Zjwp-14 (CCTCC M 206104) from soil, which can biosynthesize *L*-cysteine from *DL*-ATC, and the maximum enzyme activity reached 929.6 U/mL [10]. Subsequently, a mutant strain *Pseudomonas* sp. B-3 was obtained by ultraviolet mutagenesis, which enzyme activity reached 2175±5.1 U/mL. In order to further improve the capacity of *L*-cysteine production by *Pseudomonas* sp. B-3, low-energy ion implantation was applied in this study, and the components of enzyme-producing medium for the mutant strain were

subsequently optimized.

Low-energy ion implantation, as a new breeding technique, was widely used to some biology areas, such as microbes and plants[11]. Compared with traditional physical or chemical mutagenesis approaches, it has wider mutation spectrum and higher mutation rate [12, 13]. Compared to genetic modification methods, such as site-directed mutagenesis, low-energy ion implantation did not require full information derived from crystallographic studies of the active site, which was very suitable for an enzyme that was not known enough about its structure and active site [14].

Response surface methodology (RSM), an efficient mathematical statistics approach, which was more economical and precise than that of conventional methods [15-17], and has been successfully applied in optimization of fermentation processes and medium compositions[18]. It can measure various factors at the same time, and gain information about the interaction among variables to obtain a reasonable optimization result [19].

The objective of this study was to obtain some potential strains with improved ability of bioconversion from *DL*-ATC to *L*-cysteine and determine the optimal medium components for enhancing the enzyme-producing by the mutant strain of *Pseudomonas* sp. through RSM, and then increase the production of *L*-cysteine. Subsequently, scaled-up fermentation was carried out with the optimal medium in a 7 L fermentor.

EXPERIMENTAL SECTION

Microorganism and materials

Original strain *Pseudomonas* sp. B-3 was preserved in our lab. Chemically synthesized *DL*-ATC·3H_2O used for *L*-cysteine production was provided by Tianjin Chemical Co. Ltd., China. All other reagents were of analytical grade. The seed medium consisted of following components: glycerol, 10 g/L; yeast extract, 5 g/L; beef extract, 5 g/L; peptone, 5 g/L; NaCl, 5 g/L; and *DL*-ATC·3H_2O, 2 g/L; pH 7.0. The original fermentation medium contained as follows: glycerol, 10 g/L; yeast extract, 5 g/L; peptone, 5 g/L; beef extract, 5 g/L; NaCl, 5 g/L; *DL*-ATC·3 H_2O, 3 g/L; and $MnSO_4 \cdot H_2O$, 0.0072 g/L; pH 7.0. The conversion solution was composed of: *DL*-ATC·3H_2O, 30 g/L; *D*-sorbitol, 50 g/L; tween-60, 0.5 g/L; hydroxylammonium chloride, 0.24 g/L; $MnSO_4 \cdot H_2O$, 0.025 g/L, dissolved in 0.1 M PBS buffer (pH 8.0).

Culture method

Transferred a loop of cells from a slant into a 250 mL flask containing 50 mL seed medium and cultivated at 30°C on a rotary shaker at 200 rpm for 12 h, with A_{600} (10-fold dilution) reaching 0.4-0.5. Subsequently, transferred the seed culture (1.4 mL) to a 250 mL flask containing 70 mL fermentation medium and cultivated at 30°C on a rotary shaker at 200 rpm for 16 h, with A_{600} (10-fold dilution) in the range of 0.6-0.7.

Microbial transformation conditions

The incubated cells were harvested from 14 mL fermentation broth by centrifugation (5000 rpm, 5 min, 4°C) and washed twice with 0.1 M PBS buffer (pH 8.0). Transferred obtained cells into 10 mL centrifuge tube and added 3 mL conversion solution. Enzymatic production of *L*-cysteine from *DL*-ATC·3H_2O was preformed at 42°C in a water bath kettle for 2 h. The reaction mixture was centrifuged at 10000 rpm for 5 min, and the obtained supernatant was assayed for *L*-cysteine.

Determination of bacterial growth

Diluting of 0.5 mL cultured broth with 4.5 mL distilled water, and absorbance was measured at 600 nm with UV-4802 spectrophotometer.

Enzymatic activity assay

The concentration of *L*-cysteine was determined with Gaitonde's ninhydrin method. Each test was repeated three times to obtain a mean value. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μg of *L*-cysteine from *DL*-ATC·3H_2O per hour according to above react condition, enzyme activity was expressed as units per mL of enzyme solution.

Mutagenesis method

Strain *Pseudomonas* sp. B-3 was mutated by low-energy ion beam with the energy of 10 keV and different doses ranging from $3\times2.6\times10^{15}$ to $15\times2.6\times10^{15}$ ions/cm^2.

Plackett-Burman design (PBD)

Single- factor experiment was used to determine the levels of each medium component, and then utilized PBD for

screening significant effect factors on enzymatic activity for *L*-cysteine production. The various medium components as follow: *(*X_1) *DL*-ATC·$3H_2O$, (X_2) glycerol, (X_3) yeast extract, (X_4) peptone, (X_5) beef extract, *(*X_6) NaCl, (X_7) $MgSO_4 \cdot 7H_2O$ and (X_8) $MnSO_4 \cdot H_2O$. Each factor was examined on 2 levels: lower level (-1) and higher level (+1), enzyme activity of *L*-cysteine as the response value.

Path of steepest ascent

The method of the steepest ascent can move rapidly towards to the neighborhood of optimum response. The experiments steps along the direction according to the results of PDB until the response showed no further increase. This point could be used as center point for Box-Behnken design. The experimental design and results of the steepest ascent were shown in **Table 3**.

Box-Behnken design (BBD) and statistical analysis

RSM was employed to optimize the concentrations of most significant variables and find the relationship between the factors and enzymatic activity. The most significant factors (*DL*-ATC·$3H_2O$, glycerol, and beef extract) were determined by PBD **(Table 2)**. These independent factors were further studied by BBD; the design and results were shown in **Table 4**. Statistical analysis of the model was evaluated by analysis of variance (ANOVA) using Design Expert version 7.0.0 statistical software (Stat-Ease, Minneapolis, USA). The quality of the model was determined by the coefficient of determination R^2; the significance of the fitness polynomial equation for the response variables was evaluated by *p* value.

Enzyme-producing process in 7 L fermentor

Scaled-up fermentation is critical to the potential industrial strain *Pseudomonas* sp. C-25 for the biosynthesis of *L*-cysteine from *DL*-ATC·$3H_2O$. Optimum fermentation medium components were utilized to culture *Pseudomonas* sp. C-25 in 7 L fermentor (NBS, BioFlo 415, USA) for the sake of large-scale fermentation. Transferred the seed culture (60 mL) to a 7 L fermentor containing 3 L fermentation medium and cultivated at 30°C, pH 7.0-7.5, the dissolved oxygen was kept in the range of 30% to 40%, the agitate speed and ventilatory capacity were controlled by dissolved oxygen.

RESULTS AND DISCUSSION

Screening of high enzyme activity mutant strain

After mutated**,** a mutant strain designated as *Pseudomonas* sp. C-25 with highest enzyme activity was obtained. The results were shown in **Fig. 1**, *L*-cysteine enzyme activity of mutant strain C-25 achieved 2747±3.7 U/mL, an increase of 26.2% compared to that of initial strain B-3 (2175±5.1 U/mL).

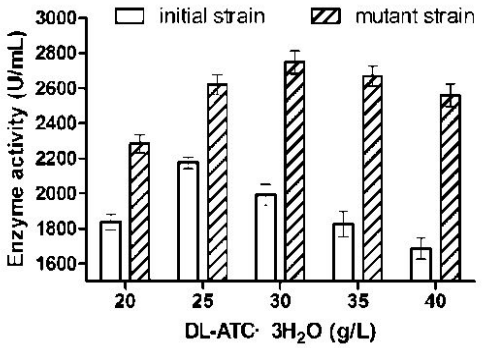

Fig. 1 Enzyme activity for L-cysteine bioconversion of initial strain *Pseudomonas* sp. B-3 and mutant strain *Pseudomonas* sp. C-25 under different substrate(*DL*-ATC·$3H_2O$) concentrations

Plackett-Burman design

The components of fermentation medium for enzyme activity were assessed by PBD (**Table 1**). It was found that there was a wide variation of enzyme activity from 1626±2.8 U/mL to 3075±6.9 U/mL in the 12 trials. This variation indicated that the optimization of fermentation medium was important to enhance enzyme activity. The experimental results were fitted with a fist-order polynomial equation:

$$Y=+2432.5+248.50X_1+287.17X_2-22.33X_3+46.17X_4+112.17X_5-59.83X_6+41.00X_7+80.50X_8 \quad (1)$$

Table 1 Plackett-Burman design for 8 variables with coded values along with the observed results for enzyme activity

Run	X_1 (g/L)	X_2 (g/L)	X_3 (g/L)	X_4 (g/L)	X_5 (g/L)	X_6 (g/L)	X_7 (g/L)	X_8 (g/L)	Enzyme activity (U/mL)	
									Observed	Predicted
1	4 (1)	8 (-1)	4 (-1)	5 (1)	6 (1)	5 (1)	0.5 (1)	0.02 (1)	2563±8.4	2635
2	1 (-1)	14 (1)	7 (1)	3 (-1)	4 (-1)	5 (1)	0.5 (1)	0.02 (1)	2318±6.9	2352
3	1 (-1)	8 (-1)	7 (1)	3 (-1)	6 (1)	3 (-1)	0.5 (1)	0.02 (1)	2156±7.5	2121
4	1 (-1)	14 (1)	4 (-1)	5 (1)	6 (1)	3 (-1)	0.3 (-1)	0.02 (1)	2792±8.1	2751
5	4 (1)	8 (-1)	7 (1)	3 (-1)	6 (1)	5 (1)	0.3 (-1)	0.01 (-1)	2297±4.9	2256
6	1 (-1)	14 (1)	7 (1)	5 (1)	6 (1)	5 (1)	0.3 (-1)	0.01 (-1)	2385±6.1	2425
7	1 (-1)	8 (-1)	4 (-1)	5 (1)	4 (-1)	5 (1)	0.5 (1)	0.01 (-1)	1827±5.2	1753
8	4 (1)	14 (1)	7 (1)	5 (1)	4 (-1)	3 (-1)	0.5 (1)	0.01 (-1)	2902±3.7	2900
9	4 (1)	14 (1)	4 (-1)	3 (-1)	4 (-1)	5 (1)	0.3 (-1)	0.02 (1)	2846±8.5	2811
10	4 (1)	14 (1)	4 (-1)	3 (-1)	6 (1)	3 (-1)	0.5 (1)	0.01 (-1)	3075±6.9	3076
11	1 (-1)	8 (-1)	4 (-1)	3 (-1)	4 (-1)	3 (-1)	0.3 (-1)	0.01 (-1)	1626±2.8	1699
12	4 (1)	8 (-1)	7 (1)	5 (1)	4 (-1)	3 (-1)	0.3 (-1)	0.02 (1)	2403±5.7	2404

Table 2 Statistical analysis of the model

Source	SS	DF	MS	*F* Value	*p* (Prob > F)
Model	2.05E+006	8	2.568E+005	31.34	0.0083*
X_1	7.41E+005	1	7.41E+005	90.46	0.0025*
X_2	9.89E+005	1	9.896E+005	120.81	0.0016*
X_3	5985.33	1	5985.33	0.73	0.4555
X_4	25576.33	1	25576.33	3.12	0.1754
X_5	1.51E+005	1	1.51E+005	18.43	0.0232*
X_6	42960.33	1	42960.33	5.22	0.1059
X_7	20172.00	1	20172.00	2.46	0.2146
X_8	77663.00	1	77763.00	9.493	0.0541
Residual	24574.33	3	8191.44		
Cor Total	2.079E+006	11			

*SS, sum of squares; DF, degree of freedom; MS, mean square; *indicates significant*

There, Y is the response value (enzyme activity), X_1, X_2, X_3, X_4, X_5, X_6, X_7, and X_8 are the coded values of *DL*-ATC·3H_2O, glycerol, yeast extract, peptone, beef extract, NaCl, $MgSO_4·7H_2O$, and $MnSO_4·H_2O$, respectively. The data of regression analysis for PBD were shown in **Table 2**. The value of coefficient of determination R^2 was 0.9882, implied that 98.82% variability in the data was explained by the model. The p value of this model was less than 0.05 (p=0.0083), which indicated that the model fitness was significant. The data in **Table 2** suggested that the *DL*-ATC·3H_2O, glycerol and beef extract were significant variables (p<0.05). *DL*-ATC·3H_2O as an inducer, which can induce cells produce enzymes to bioconversion from *DL*-ATC to *L*-cysteine, glycerol and beef extract as a C-source and N-source, respectively. They are vital to the growth and enzyme production of cells. So, these three factors were selected for further optimization to gain a maximum response.

Path of steepest ascent

Results of PBD indicated that *DL*-ATC·3H_2O (X_1), glycerol (X_2) and beef (X_3) had positive effects on enzyme activity. Thus, proportional increases in the concentrations of *DL*-ATC·3H_2O (X_1), glycerol (X_2) and beef (X_3), based on **Eq. 1**, with concentrations of the rest of medium components fixed at the center of PBD, should lead to higher enzyme activity. The center point of PBD was determined as the origin of the path. The experimental design and corresponding results were shown in **Table 3**. The highest enzyme activity was 3209±9.4 U/mL at run 2, suggesting that this point might be near the region of the maximum enzyme activity response, and can be chose for further optimization.

Table 3 Experiment design and results of steepest ascent

Run	*DL*-ATC·3H_2O (g/L)	Glycerol (g/L)	Beef extract (g/L)	Enzyme activity (U/mL)
1	4.0	14.0	6.0	2897±6.8
2	4.5	15.0	6.5	3209±9.4
3	5.0	16.0	7.0	2967±7.6
4	5.5	17.0	7.5	2743±6.9
5	6.0	18.0	8.0	2474±5.7

Optimization by response surface analysis

RSM using Box-Behnken design was applied to determine the optimal levels of the three selected variables (*DL*-ATC·3H_2O, glycerol and beef extract) that significantly affected enzyme activity. **Table 4** showed the

experimental design and corresponding results. The results were fitted with a second-order polynomial equation:
Y=3230.20+137.75X_1+42.63X_2-46.63X_3+61.00X_1X_2-27.50X_1X_3+56.25X_2X_3-207.98X_1^2-29.73X_2^2-74.72X_3^2 (2)

Table 4 Box-Behnken experiments design and results

Run	X_1 (*DL*-ATC·3H$_2$O) g/L	X_2 (Glycerol) g/L	X_3 (Beef extract) g/L	Enzyme activity (U/mL)	
				Observed	Predicted
1	4.5 (0)	15 (0)	6.5 (0)	3252±5.9	3230
2	4.5 (0)	15 (0)	6.5 (0)	3215±7.2	3230
3	5 (1)	16 (1)	6.5 (0)	3254±8.1	3233
4	4.5 (0)	16 (1)	7 (1)	3184±3.4	3178
5	4 (-1)	15 (0)	7 (1)	2802±8.7	2790
6	4 (-1)	14 (-1)	6.5 (0)	2853±5.2	2873
7	4.5 (0)	14 (-1)	7 (1)	2989±4.8	2980
8	4 (-1)	15 (0)	6 (-1)	2855±7.6	2828
9	5 (1)	15 (0)	7 (1)	2985±8.4	3011
10	4.5 (0)	15 (0)	6.5 (0)	3228±6.2	3230
11	5 (1)	15 (0)	6 (-1)	3148±3.7	3159
12	4 (-1)	16 (1)	6.5 (0)	2819±2.9	2836
13	4.5 (0)	15 (0)	6.5 (0)	3241±4.2	3230
14	5 (1)	14 (-1)	6.5 (0)	3044±2.7	3026
15	4.5 (0)	15 (0)	6.5 (0)	3215±3.7	3230
16	4.5 (0)	14 (-1)	6 (-1)	3180±5.4	3186
17	4.5 (0)	16 (1)	6 (-1)	3150±6.8	3158

Table 5 Analysis of variance for quadratic model for enzyme activity

Sources	S S	M S	F value	p (Prob > F)
Model	4.361E+005	48460.32	78.50	< 0.0001**
X_1	1.518E+005	1.518E+05	245.88	< 0.0001**
X_2	14535.13	14535.13	23.54	0.0019*
X_3	17391.13	17391.13	28.17	0.0011*
X_1X_2	14884.00	14884.00	24.11	0.0017*
X_1X_3	3025.00	3025.00	4.90	0.0625
X_2X_3	12656.25	12656.25	20.50	0.0027*
X_1^2	1.821E+005	1.821E+05	295.00	< 0.0001**
X_2^2	3720.32	3720.32	6.03	0.0438*
X_3^2	23510.84	23510.84	38.08	0.0005*
Residual	4321.55	617.36		
Lack of Fit	3262.75	1087.58	4.11	0.1029 (not significant)
Pure Error	1058.80	264.70		
Cor Total	440530.4			
	R^2=0.9902			
	Adj R^2=0.9776		Adeq Precision=23.26	

*SS, sum of squares; DF, degree of freedom; MS, mean square; *indicates significant; **indicates extremely significant*

There Y is the response value (enzyme activity), X_1, X_2 and X_3 were represented *DL*-ATC·3H$_2$O, glycerol and beef extract, respectively. The ANOVA for the model was shown in **Table 5**. The value of coefficient of determination R^2 was 0.9902. Normally, a regression model of R^2 higher than 0.9 is shown a high correction [4,20], it was suggested that 99.02% of the total variance could be explained by the model. The adjusted determination coefficient (Adj R^2=0.9776) was also high enough to confirm the significance of the model. The p value of this model was less than 0.0001, which indicated that the model fitness was extremely significant **(Table 5)**. Moreover, the lack of fit value of the model was non-significant (0.1029), suggesting that the model was adequate to predict the response and explain the effect of variables on the response.

The three-dimensional (3D) response surface plots were employed to illustrate the interaction among the variables on enzyme activity **(Fig. 2)**. Each 3D plot represented combinations of the two test variables, while the other variables maintained at the center level. An elliptical contour or saddle nature of the contour plots suggested that there has a significant interaction between two variables [21-23], such as *DL*-ATC·3H$_2$O and glycerol **(Table 5 and Fig. 2a)**, as well as *DL*-ATC·3H$_2$O and beef extract **(Table 5 and Fig. 2b)**. The interaction of glycerol and beef extract was non-significant on enzyme activity **(Table 5 and Fig. 2c)**. The quadratic model predicted the maximum enzyme activity was 3290 U/mL, when the coded level of *DL*-ATC·3H$_2$O, glycerol and beef extract were 4.7 g/L, 16.0 g/L and 6.5 g/L, respectively. The concentration of other medium components were fixed at the center of PBD (yeast extract, 4.0 g/L; peptone, 5.0 g/L; NaCl, 3.0 g/L; $MgSO_4·7H_2O$, 0.5 g/L and $MnSO_4·H_2O$, 0.02 g/L, respectively). In order to confirm the model adequacy for predicting the maximum response, a validation experiment was conducted with five tests. Under such optimum medium, the experimentally generated mean value of 3301±6.4 U/mL, it was excellent agreement with the model prediction value. The enzyme activity was increased by 20.1% in

comparison with that of original medium.

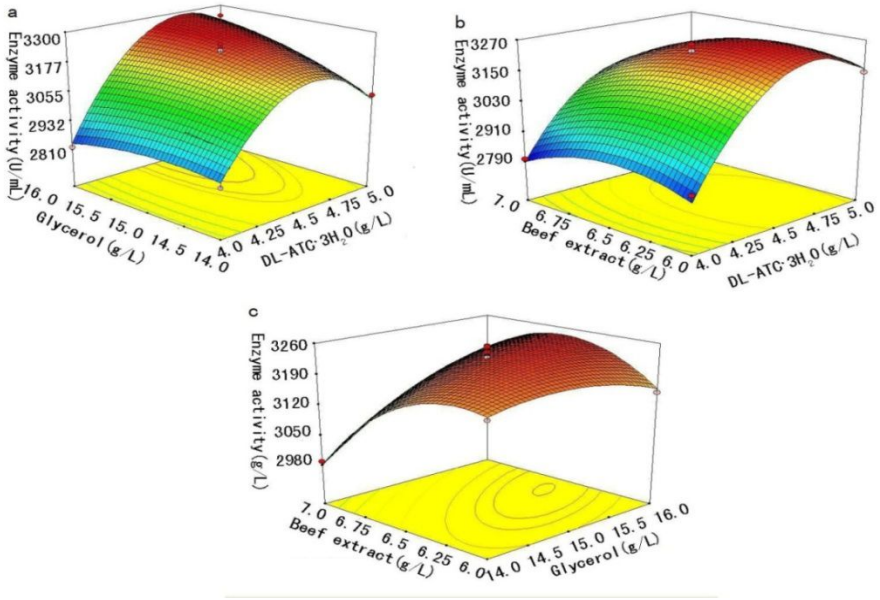

Fig. 2 Response surface plots of enzyme activity responses to *DL*-ATC·3H$_2$O, glycerol, and beef extract

Comparisons of enzyme production of Pseudomonas sp. C-25 in 250 mL flask and in 7 L fermentor

The time courses of biomass and enzyme activity by *Pseudomonas* sp. C-25 under the optimum medium conditions in 250 mL shake flask and in 7 L fermentor (BioFlo 415, USA) were shown in **Fig. 3**. It was found that the enzyme activity increased with cell growth. The maximum enzyme activity (3925±5.7 U/mL) occurred in the stable phase of cell growth, it's higher than cultured in the shake flask (3301±6.4 U/mL), and the enzyme-production time cut down from 16 h in shake culture to 5 h in 7 L fermentor.

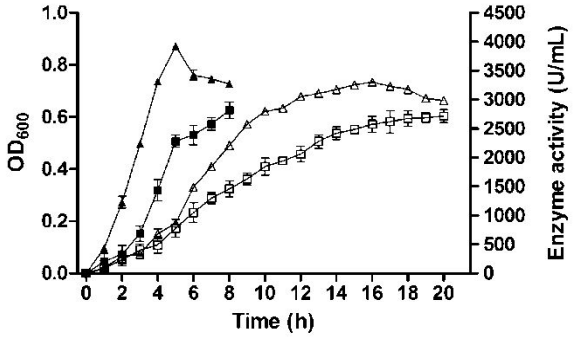

Fig. 3 Time courses of enzyme activity and biomass of *Pseudomonas* sp. C-25 under optimum conditions

▲ *enzyme activity in 7 L fermentor;* ■ *OD600 in 7 L fermentor;* △ *enzyme activity in shake flasks;* □ *OD600 in shake flasks*

CONCLUSION

A positive mutant strain *Pseudomonas* sp. C-25 with high enzyme activity (2747±3.7 U/mL) was obtained by low-energy ion beam implantation, which increased by 26.2% compared with that of the initial strain. The high quality of a mathematical model indicated that RSM could be used to optimize the components of the fermentation medium for enhance enzyme activity for *L*-cysteine production by *Pseudomonas* sp. C-25. The optimum fermentation medium contained: *DL*-ATC·3H$_2$O 4.7 g/L, glycerol 16.0 g/L, beef extract 6.5 g/L, yeast extract 4.0 g/L, peptone 5.0 g/L, NaCl 3.0 g/L, $MnSO_4 \cdot H_2O$ 0.06 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L. Under such fermentation conditions, the maximum enzyme activity attained 3301±6.4 U/mL in shake flask and 3925±5.7 U/mL in 7 L fermentor, with an increase of 20.1% and 42.8% respectively, compared with the original fermentation medium components. The results demonstrated that the enzyme activity was significantly increased through scaled-up fermentation in 7 L fermentor, compared with fermented in the shake flask. Furthermore, the fermentation time of highest enzyme activity cut

down from 16 h to 5 h. Consequently, the optimized fermentation medium obtained in this work possessed potential application in the industrial biosynthesis of *L*-cysteine from *DL*-ATC·$3H_2O$ by *Pseudomonas* sp. C-25.

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