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Optimization of medium for phenylalanine ammonia lyase production in *E. coli* using response surface methodology

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Abstract−A culture medium for phenylalanine ammonia lyase (PAL) production in *E. coli* was developed following preliminary studies by means of response surface methodology (RSM). The medium components having significant effect on the production were first identified by using a fractional factorial design. Then, central composite design (CCD) was used to optimize the medium constituents and explain the combined effects of four medium constituents: glucose, yeast extract, $(NH_4)_2HPO_4$ and $MgSO_4$. A quadratic model was found to fit the PAL production. CCD revealed that the optimum values of the test variables for PAL production were glucose 28.2 g/L, yeast extract 5.01 g/L, $(NH_4)_2HPO_4$ 7.02 g/L and $MgSO_4$ 1.5 g/L. PAL production of 62.85 U/g, which was in agreement with the prediction, was observed in the verification experiment. In comparison to the production of basal medium, 1.8-fold increase was obtained.

Key words: Enzyme Activity, Phenylalanine Ammonia Lyase, Response Surface Method, Fermentation, Medium, Batch Culture

INTRODUCTION

Phenylalanine ammonia lyase (E.C.4.3.1.5-PAL) is widely distributed in higher plants, some fungi, yeasts and *Streptomyces* species [1-4]. It has been used chiefly in the manufacture of L-phenylalanine by reversing the enzyme reaction with high concentration of trans-cinnamic acids and ammonia at an elevated pH. There is a great demand for the production of L-phenylalanine, since it is one of the two precursors required for the synthesis of the artificial sweetener aspartame. Therefore, the enzyme is of particular interest to researchers in the biotechnology industry. At present, the industrial production of PAL mainly utilizes the genus *Rhodotorula* [3]. The levels of enzyme in these wild-type strains are relatively low; thus, the production of L-phenylalanine from trans-cinnamic acids was of limited success. A recombinant strain capable of producing a large amount of PAL is therefore highly desirable in order to improve L-phenylalanine from trans-cinnamic acids. Although some efforts have been made to construct recombinant strains with high PAL activity [5,6], the yields of recombinant enzyme obtained were disappointingly low. In terms of large-scale production of PAL for industrial and medical uses, recombinant PAL production needs to be improved substantially. In our earlier reports, a recombinant strain capable of producing a large amount of PAL was also constructed [7]. However, few results have been reported that the effects of medium composition on production of PAL in recombinant *E. coli*. The traditional one-factor-at-a-time is an operation frequently used in medium optimization to obtain high yields of the desired metabolic products in a microbial system. But that method disregards the complex interactions among various physicochemical parameters [8]. Statistical experimental designs such as factorial design and response surface methodology (RSM) particularly fulfill this requirement. As a powerful statistical and mathematical tool, RSM helps identify the effective factors, study interactions, select optimum conditions and quantify the relationships between one or more measured responses and the vital input factors in limited number of experiments [9,10]. This method has been successfully applied in many areas of biotechnology such as bioconversion of cheese whey to mycelia of *Ganoderma lucidum* [11], enzyme production [12], and extra cellular glucansucrase production [13]. In this study, PAL production from recombinant *E. coli* as a result of the interaction between four variables, glucose, yeast extract, $(NH_4)_2HPO_4$ and $MgSO_4$, which had played a significant role in enhancing the production of PAL, was optimized with RSM.

MATERIALS AND METHODS

1. Microorganism and Grown Conditions

The plasmid pBV-PAL was constructed according to the method reported by Jia et al. [7]. The gene *pal* of *R. toruloides* was cloned and expressed in plasmid pBV-PAL (Amp^r). The expression of PAL was controlled by a combined promoter of *tac* and P_LP_R. The plasmid pBV-PAL was transformed in *E. coli* JM109. The recombinant *E. coli* JM109 was used throughout this work. The medium used for batch fermentation contained the following components [14]: glucose 20 g/L, yeast extract 5 g/L, KH_2PO_4 13.3 g/L, $(NH_4)_2HPO_4$ 4 g/L, $MgSO_4 \cdot 7H_2O$ 1.2 g/L, citric acids 1.7 g/L. Trace elements solution was comprised of EDTA 8.4 mg/L, $CoCl_2 \cdot 6H_2O$ 2.5 mg/L, $MnCl_2 \cdot 4H_2O$ 15 mg/L, $CuCl_2 \cdot 4H_2O$ 1.5 mg/L, H_3BO_4 3 mg/L, $Na_2MoO_4 \cdot 2H_2O$ 2.5 mg/L, $Zn(CH_3COO)_2 \cdot 2H_2O$ 13 mg/L. For cell activation, 1 mL frozen stock culture was added to 50 mL LB medium containing ampicillin (100 μg/mL) in a 500 mL Erlenmeyer flask at 200 r/m for 12 h at 30 ℃. The inoculum culture was then prepared by adding 2% (V/V) of this reactivated culture to 50 mL

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LB medium containing ampicillin (100 μg/mL) in a 500 mL Erlenmeyer flask, followed by culturing under the same conditions for 12 h [15]. For shaker-flask culture, 10 mL of inoculum culture was added to 90 mL fermentation medium in a 500 mL Erlenmeyer flask. The culture were incubated at 30 °C in a rotary set at 200 r/m until the optical density (OD_{600}) reached 0.6, at this point, 0.5 mmol/L isopropyl β-D-thiogalactoside (IPTG) was added to fermentation medium, and the cells were cultured at 42 °C for 4 h for induction of the PAL.

2. Assay of PAL Activity

PAL activity of recombinant *E. coli* whole cells was determined by a modification of the procedure [7] with permeabilized viable-cell suspension. First, cells were harvested from the culture at 4,000 g for 10 min. The collected cells were washed twice by 0.85% sodium chloride solution and recovered at 4,000 g for 10 min. The washed cells were then suspended in 25 mmol/L Tris-HCl buffer solution (pH 8.8). The suspension was added to an enzymatic reaction medium comprising 25 mmol/L Tris-HCl buffer solution (pH 8.8) supplemented with 25 mmol/L L-phenylalanine and 0.005% (W/V) cetyl trimethyl ammonium bromide (CTAB). The resultant reaction medium was incubated at 30 °C for 20 min. The reaction was terminated by addition of 1 mol/L HCl. After centrifugation, the rate of trans-cinnamic acid formation was determined by measuring the increase in A_{280} with a 752 spectrophotometer (Shanghai Precision and Scientific Instrument Co., China). One unit of PAL activity was defined as the amount of enzyme required to convert one μmole of L-phenylalanine to trans-cinnamic acids per min. PAL activity (specific activity) is expressed as units of enzyme/gram (dry cell weight) of cells. Dry cell weight (DCW) was measured by a modification of the procedure of Jung et al. [16]. DCW was estimated by using a calibration curve obtained from the relationship between OD_{600} and the DCW. Higher OD samples were diluted suitably to have an absorbance at the range of 0.1-0.6. The cell pellets which resulted from the centrifugation were washed with distilled water and dried to constant weight at 80 °C. One OD_{600} unit corresponds to 0.46±0.05 g DCW/L.

3. Experimental Design and Optimization

The culture medium was optimized by a combination of traditional non-statistical technology and statistical technology based experimental design. First, the selection of medium components (one-factor-at-a-time experiments) was elucidated by non-statistical technology. The results revealed that the carbon sources glucose, the nitrogen sources yeast extract, $(NH_4)_2HPO_4$ and $MgSO_4$ were supposed to have effects on PAL activity (data not shown). Then, the variables having significant effects on PAL production were identified by a 2-level fractional design. The results revealed that the mean of the center points had no significant difference from the mean of the factorial points (P=0.1014), which implied that the optimum was outside the experimental design space and CCD should be applied (data not shown). Thus, CCD was used in the optimization of PAL production. The carbon sources glucose (X_1, g/L), the nitrogen sources yeast extract (X_2, g/L), $(NH_4)_2HPO_4$ (X_3, g/L) and $MgSO_4$ (X_4, g/L) were chosen as the independent variables shown in Table 1. PAL activity (Y, U/g) was used as dependent output variable. A 2^4 full-factorial CCD for four independent variables at five levels was employed and the total number of experiments was 30 ($=2^k+2k+6$), where k is the number of independent variables [17].

Table 1. Process variables used central composite design with actual factor levels corresponding to coded factor levels

Variables	Symbol	Coded levels				
		−2	−1	0	1	2
Glucose (g/L)	X_1	5	15	25	35	45
Yeast extract (g/L)	X_2	2	3.5	5	6.5	8
$(NH_4)_2HPO_4$ (g/L)	X_3	0	2.5	5	7.5	10
$MgSO_4$ (g/L)	X_4	0.5	1	1.5	2	2.5

And each variable was designated as −2, −1, 0, 1 and 2, respectively. The interrelationships of the variables were determined by fitting the second degree polynomial equation to data obtained from 30 experiments by using mean values of the triplicates of each experiment conducted trice at different occasions. The maximum values of the yield were taken as the responses of the design experiment. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The minimum and maximum range of variables investigated and the full experimental plan with respect to their actual and coded values are listed in Table 1. A multiple regression analysis of the data was carried out with a statistical package (Stat-Ease Inc., Minneapolis, MN, USA) and the second-order polynomial equation that defines predicted response (Y) in terms of the independent variables (X_1, X_2, X_3 and X_4) was obtained:

$$Y=B_0+B_1X_1+B_2X_2+B_3X_3+B_4X_4+B_{12}X_1X_2+B_{13}X_1X_3+B_{14}X_1X_4 +B_{23}X_2X_3+B_{24}X_2X_4+B_{34}X_3X_4+B_{11}X_1^2+B_{22}X_2^2+B_{33}X_3^2+B_{44}X_4^2 \quad (1)$$

Where X_1, X_2, X_3 and X_4 are input variables; B_0 is constant; B_1, B_2, B_3 and B_4 are linear coefficients; B_{12}, B_{13}, B_{14}, B_{23}, B_{24} and B_{34} are cross-product coefficients; B_{11}, B_{22}, B_{33} and B_{44} are quadratic coefficients. Combinations of factors (such as X_1X_2) represent an interaction between the individual factors in that term. Then the response is a function of the levels of factors.

RESULTS AND DISCUSSION

1. Response Surface Analysis for the Optimization of the Three Factors

Some reports showed that PAL gene from *R. toruloides* was expressed in *E. coli* [5,6]. Furthermore, a recombinant *E. coli* strain producing a significant amount of PAL has been constructed in our earlier report; recombinant PAL activity reached 35 U/g [7]. However, few results have been reported about optimizing culture medium for PAL production in *E. coli*. In this study, RSM was used to improve the composition of the medium by comparing different levels of several factors that were found to have more influence on the production of PAL in *E. coli*. The results of CCD experiments consisted of experimental data for studying the effects of four independent variables; that is, glucose, yeast extract, $(NH_4)_2HPO_4$ and $MgSO_4$ on the recombinant PAL activity are presented in Table 2. The analysis of variance (Table 3) indicated that the model terms of X_1, X_3, X_1^2, X_2^2 and X_3^2 were highly significant ("probe>F" less than 0.001), and the interactive effects of X_1X_2, X_1X_3, X_1X_4, X_2X_3 and X_3X_4 were not significant, but the interactive effect of X_2X_4 was significant ("probe>F" less than 0.05). It means that glucose, yeast

Table 2. Central composite design and response value

Run	X_1	X_2	X_3	X_4	Y (U/g)
1	−1	−1	−1	1	42.79
2	−1	−1	1	−1	46.37
3	−1	1	−1	−1	45.89
4	−1	1	1	1	52.68
5	1	−1	−1	−1	49.79
6	1	−1	1	1	53.64
7	1	1	−1	1	52.98
8	1	1	1	−1	48.93
9	0	0	0	0	65.48
10	0	0	0	0	65.96
11	−1	−1	−1	−1	45.42
12	−1	−1	1	1	48.12
13	−1	1	−1	1	42.36
14	−1	1	1	−1	40.57
15	1	−1	−1	1	43.76
16	1	−1	1	−1	57.98
17	1	1	−1	−1	46.35
18	1	1	1	1	55.78
19	0	0	0	0	65.43
20	0	0	0	0	65.89
21	−2	0	0	0	33.32
22	2	0	0	0	40.79
23	0	−2	0	0	49.48
24	0	2	0	0	57.49
25	0	0	−2	0	42.21
26	0	0	2	0	62.36
27	0	0	0	−2	63.89
28	0	0	0	2	62.98
29	0	0	0	0	65.29
30	0	0	0	0	65.68

extract and $(NH_4)_2HPO_4$ have important effects on PAL production, and the quadratic effects of glucose, yeast extract and $(NH_4)_2HPO_4$ are more significant than other factors. Furthermore, the interactive effect of yeast extract and $MgSO_4$ may be significant to some extent ("probe>F" less than 0.05). Multiple regression analysis of the experimental data gave the following second-order polynomial equation:

$$Y=65.32+2.50X_1+0.57X_2+3.13X_3+0.37X_4-7.68X_1^2+0.0044X_1X_2 +0.76X_1X_3-0.28X_1X_4-3.57X_2^2-0.87X_2X_3+2.08X_2X_4 -3.87X_3^2+1.37X_3X_4-1.08X_4^2 \quad (2)$$

Where Y is the response, that is, PAL activity, and X_1, X_2, X_3 and X_4 are the coded values of the test variables, glucose, yeast extract, $(NH_4)_2HPO_4$ and $MgSO_4$, respectively. After the neglect of insignificant terms (on the basis of "probe>F" which are more than 0.05) (Table 3), model Eq. (2) was modified to reduced fitted model Eq. (3):

$$Y=65.32+2.50X_1+3.13X_3-7.68X_1^2-3.57X_2^2+2.08X_2X_4-3.87X_3^2 \quad (3)$$

The regression equation obtained from analysis of variance (ANOVA) indicated that the multiple correlation coefficient of R^2 is 0.9478. The model can explain 94.78% variation in the response. The value of the adjusted determination coefficient (Adj. R^2=0.9123) is also very high to advocate for a high significance of the model [18,19]. The model *F*-value of 14.76 implied that the model was significant. From the statistical results obtained, it was shown that the above models were adequate to predict the recombinant PAL activity within the range of variables studied.

2. Interactions Among the Factors

Fig. 1 shows the predicted values versus the experimental values for recombinant PAL activity. The points are clustered around the diagonal line, which indicates the good fit of the model.

The 2D contour plots are generally the graphical representations of the regression equation, and 2D contour plots are presented in

Table 3. Parameter estimates and analysis of variance

Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	Probe>F
X_1	1	149.7501	149.7501	13.9185	0.0025**
X_2	1	7.8090	7.8090	0.7258	0.4096
X_3	1	234.5625	234.5625	21.8015	0.0004**
X_4	1	3.3675	3.3675	0.3129	0.5853
X_1^2	1	1617.584	1617.584	150.3469	0.0001**
X_1X_2	1	0.0003	0.0003	0.000028	0.9958
X_1X_3	1	9.2568	9.2568	0.8603	0.3705
X_1X_4	1	1.3167	1.3167	0.1223	0.7320
X_2^2	1	349.9621	349.9621	32.5273	0.0001**
X_2X_3	1	12.1975	12.1975	1.1337	0.3063
X_2X_4	1	69.3472	69.3472	6.4455	0.0247*
X_3^2	1	411.2153	411.2153	38.2205	0.0001**
X_3X_4	1	30.0581	30.0581	2.7937	0.1185
X_4^2	1	32.2586	32.2586	2.9982	0.1069
Model	16	2541.176	158.8235	14.7619	0.0001
Error	13	139.8671	10.7590		
Total	29	2681.044			

**Indicate highly significant, *Indicate signficant

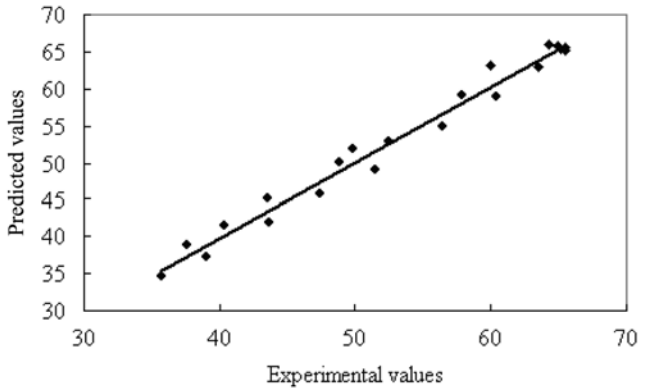

Fig. 1. Parity plots showing the distribution of experimental vs. predicted values of recombinant PAL activity.

Fig. 2. Each contour curve plot represents an infinite number of combinations of two test variables with the other two maintained at their respective zero level. From the contour plots, it is easy and convenient to understand the interactions between two factors and also locate their optimum levels. The circular contour plots of response surfaces suggest that the interaction is negligible between the corresponding variables. An elliptical or saddle nature of the contour plots indicates the significance of the interactions between the corresponding variables [10,13]. The contour plots in Fig. 2(e), 2(c) and 2(f) show that there was significant mutual interaction between yeast extract and $MgSO_4$ (Fig. 2(e)), glucose and $MgSO_4$ (Fig. 2(c)), and $(NH_4)_2HPO_4$ and $MgSO_4$ (Fig. 2(f)). However, there was almost no interaction between glucose and yeast extract (Fig. 2(a)), glucose and $(NH_4)_2HPO_4$ (Fig. 2(b)), yeast extract and $(NH_4)_2HPO_4$ (Fig. 2(d)), as was evident from the relatively circular nature of the

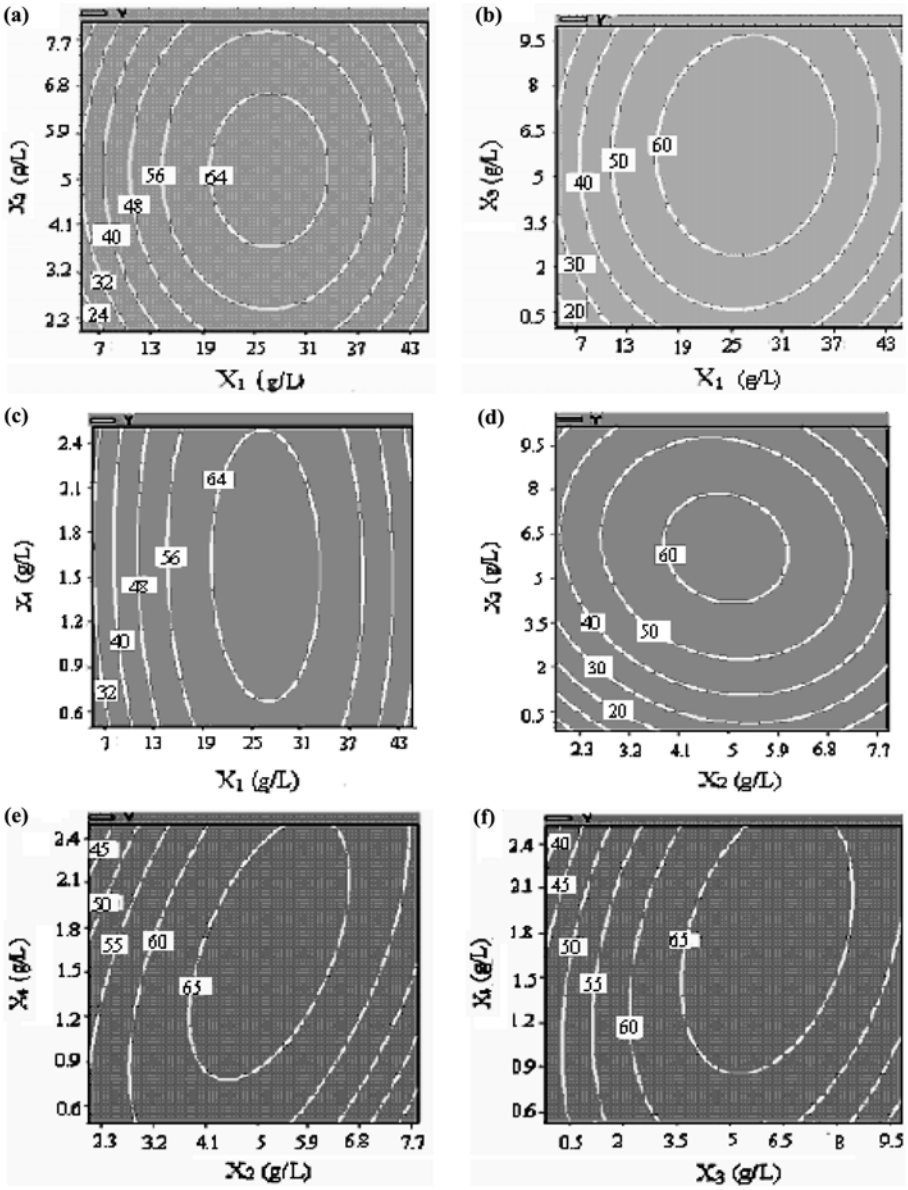

Fig. 2. Contour plots of recombinant PAL activity: the effect of glucose (X_1) and yeast extract (X_2) (a), glucose (X_1) and $(NH_4)_2HPO_4$ (X_3) (b), glucose (X_1) and $MgSO_4$ (X_4) (c), yeast extract (X_2) and $(NH_4)_2HPO_4$ (X_3) (d), yeast extract (X_2) and $MgSO_4$ (X_4) (e), $(NH_4)_2HPO_4$ (X_3) and $MgSO_4$ (X_4) (f) on PAL production. Other varibles are held at zero level.

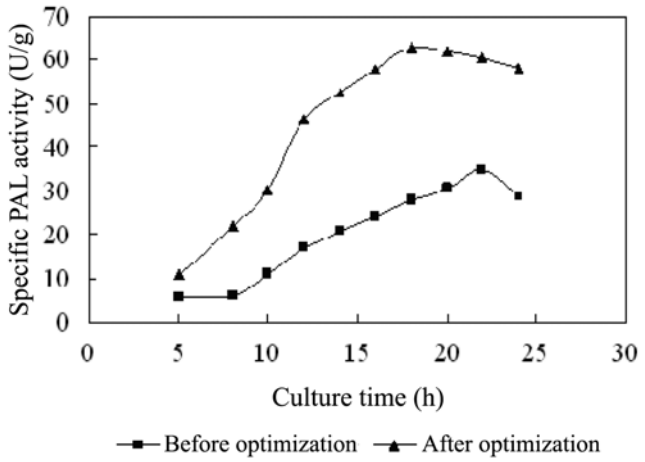

Fig. 3. The time-course of PAL production before and after the optimization.

contour curves.

The optimal conditions were extracted by Design Expert Software with its optimization menus: X_1=0.32, X_2=0.01, X_3=0.81, X_4=0. The real values were glucose concentration at 28.2 g/L, yeast extract concentration at 5.01 g/L, $(NH_4)_2HPO_4$ concentration at 7.02 g/L and $MgSO_4$ concentration at 1.5 g/L. The maximum recombinant PAL activity of 65.36 U/g was predicted by the model. The validation experiment showed that the experimentally determined production values were in close agreement with the statistically predicted ones, confirming the model's authenticity. Fig. 3 shows the time-course of PAL production before and after the optimization. The maximum recombinant PAL activity obtained experimentally was found to be 62.85 U/g under optimized culture conditions; the recombinant PAL activity was above 1.8-fold in comparison with that of original culture conditions (Fig. 3). In particular, under optimized culture conditions, the maximum recombinant PAL activity was obtained when culture time was 18 h. However, under original culture conditions, the maximum recombinant PAL activity was obtained when culture time was 22 h (Fig. 3). Namely, the culture time was decreased. It indicated that the optimized culture conditions obtained in this work possessed a high potential for the industrial production of PAL.

CONCLUSION

The traditional one-factor-at-a-time process requires a complete series of experiments for important factors of interest, which is laborious and time consuming. Furthermore, such methods could not provide information for the interactions of the factors. RSM could locate the most important factors levels with minimum effort and time; moreover, it could reveal the interaction among the factor. The study using RSM based on CCD established an efficient model to describe the process. In this study, the high similarity between the observed value and the predicted ones showed that the RSM was an accurate and applicable tool to optimize culture medium for PAL production in *E. coli*. By utilizing the statistical methodology, the optimum PAL activity was determined to be 62.58 U/g, with glucose concentration of 28.2 g/L, yeast extract concentration of 5.01 g/L, $(NH_4)_2HPO_4$ concentration of 7.02 g/L and $MgSO_4$ concentration of 1.5 g/L. Under optimized culture conditions, the recombinant PAL activity was above 1.8-fold in comparison with that of original culture conditions. In particular, the culture time was decreased under optimized culture conditions. It indicates that the optimized culture conditions for improving the yield of PAL have much applied value in the industry.

REFERENCES

1. H. Y. Yue, Q. P. Yuan and W. Ch. Wang, *Biochem. Eng. J*., **37**, 231 (2007).
2. M. J. Fiske and J. F. Kane, *J. Bacteriol*., **160**, 676 (1984).
3. J. D. Cui and Y. Li, *Korean J. Chem. Eng*., In press (2009).
4. J. Rosler, F. Krekel and N. Amrhein, *Plant Physiol*., **113**, 175 (1997).
5. H. Orum and O. F. Rasmussen, *Appl. Microbiol. Biotechnol*., **36**, 745 (1992).
6. J. D. B. Faulkner, J. G. Anson and M. F. Tuite, *Gene*., **143**, 13 (1994).
7. Sh. R. Jia, J. D. Cui and Y. Li, *Biochem. Eng. J*., **42**, 193 (2008).
8. Y. R. Abdel-Fattah, H. M. Saeed and Y. M. Gohar, *Process Biochem*., **40**, 1707 (2005).
9. D. C Montgomery, *Design and analysis of experiments*, Singapore, Wiley Press, **125** (1984).
10. C. Liyana-Pathirana and F. Shahidi, *Food Chem*., **9**, 347 (2005).
11. H. Lee, M. Song and S. H. Wang, *Process Biochem*., **38**, 1685 (2003).
12. D. A. Bocchini, H. F. Alves-Prado and L. C. Baida, *Process Biochem*., **38**, 727 (2002).
13. M. Avishek and G. Arun, *Bioresource Tech*., **99**, 3685 (2008).
14. M. P. Delisa, G. Rao and W. A. Weigand, *Biotech. Bioeng*., **6**, 554 (1999).
15. J. H. Lee, Y. J. Yoo and K. H. Par, *Korean J. Chem. Eng*., **8**, 39 (1991).
16. J. Y. Jung, T. Khan and J. K. Park, *Korean J. Chem. Eng*., **24**, 1 (2007).
17. R. Sen, *J. Chem. Technol. Biotechnol*., **68**, 263 (1997).
18. S. Akhnazarova and V. Kefarov, *Experiment optimization in chemistry and chemical engineering*, Moscow, Mir Publishers, **135** (1982).
19. A. I. Khuri and J. A. Cornell, *Response surfaces: design and analysis*, New York, Marcel Dekker, **211** (1987).