Food Sci. Biotechnol. Vol. 18, No. 2, pp. 494 ~ 499 (2009)

Food Science and Biotechnology

Enhanced Activity of Phenylalanine Ammonia Lyase in Permeabilised Recombinant *E. coli* by Response Surface Method

Jian-dong Cui*, Yan Li, and Shi Ru Jia[1]

Hebei Fermentation Engineering Research Center, College of Bioscience and Bioengineering, Hebei University of Science and Technology, Shijiazhang 050018, PR China

[1]Key Laboratory of Industry Microbiology, Ministry of Education, Tianjin University of Science and Technology, Tai Da Development Area, Tianjin 300457, PR China

Abstract To improve phenylalanine ammonia lyase (E.C.4.3.1.5-PAL) activity in recombinant *Escherichia coli*, Some approaches for improving phenylalanine ammonia lyase (PAL) activity in recombinant *E. coli* were developed following preliminary studies by means of response surface method. The results shown that permeabilization with combination of Triton X-100, cetyl trimethyl ammonium bromide (CTAB), and acetone enriched cellular recombinant PAL activity significantly, which improved over 10-fold as compared with the control (untreat cell), as high as 181.37 U/g. The optimum values for the tested variables were Triton X-100 0.108 g/L, CTAB 0.15 g/L, and acetone 45.2%(v/v). Furthermore, a second-order model equation was suggested and then validated experimentally. It was indicated that addition of surfactants and organic solvents made the cells more permeable and therefore allowed easier access of the substrate to the enzyme and excretion of the product, which increased the rate of transport of L-phenylalanine and *trans*-cinnamic acids. These improved methods of PAL activity enrichment could serve as a rich enzyme source, especially in the biosynthesis of L-phenylalanine.

Keywords: enzyme activity, recombinant *Escherichia coli*, optimization, response surface method, permeabilization

Introduction

Phenylalanine ammonia lyase (E.C.4.3.1.5-PAL) is widely distributed in higher plants, some fungi, yeasts, and *Streptomyces* sp. (1,2). 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. L-Phenylalanine is an important amino acid and widely used in pharmaceutical and food industries. The commercial source of enzyme has been mainly obtained from the genus *Rhodotorula* (3). The production of L-phenylalanine from *trans*-cinnamic acids was of limited success, partly because of the relatively low specific activity and rapid turnover of PAL during the bioconversion. There is a growing interest in improving PAL activity of *Rhodotorula* by different methods. Nakamichi *et al.* (3) reported that PAL could be induced by L-phenylalanine, L-isoleucine, L-leucine, L-valine, L-methionine, L-tryptophan, and L-tyrosine. Srinivasan *et al.* (4) reported that PAL activity was increased about 6-fold, when *Rhodotorula glutinis* cells were treated with cetyl trimethyl ammonium bromide (CTAB). D'Cunha (5) found that PAL activity of *R. glutinis* could be enhanced about 9-fold with a synergistic effect of Triton-X-100 and glucuronidase. Furthermore, PAL gene from *Rhodosporidium toruloides* was expressed in *Escherichia coli* (6,7). However, few results have been reported about enhancing PAL activity in recombinant *E. coli*. Moreover, most of the studies on the PAL activity changed one separate factor at a time (COST). However, reaction system influenced simultaneously by more than one factor can be poorly understood with the COST-approach. As a powerful statistical and mathematical tool, response surface methodology (RSM) helps to 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 (8,9). In our earlier reports, permeabilization with Triton X-100, CTAB, or acetone enriched cellular recombinant PAL activity (10). However, the relationship between the factors and PAL activity was not investigated. Thus, the objective of the present work was to study the combined effect of the CTAB, acetone, and Triton X-100 for PAL activity of recombinant *E. coli* according to central composite design (CCD) and RSM, and to understand the relationship between the factors and PAL activity.

*Corresponding author: Tel: +86-311-88632170; Fax: +86-311-88632170
E-mail: cjd007cn@163.com
Received October 10, 2008; Revised December 13, 2008;
Accepted December 16, 2008

Materials and Methods

Microorganism and grown conditions The recombinant *Escherichia coli* JM109 (carrying recombinant expression plasmid pBV220-PAL) was constructed and cultured according to our earlier report (11). The gene *pal* of *Rhodotorula toruloides* was polymerase chain reacrion (PCR) amplified from the plasmid pSW-PAL carrying *pal* gene of *R. toruloides*, then, the gene *pal* was cloned and expressed in plasmid pBV220-PAL (Amp^r, kindly provided by Jian-Xin Li, 306 Hospitals, Beijing, China). The expression of PAL was controlled by a promoter of P_LP_R. The plasmid pBV220-PAL was transformed in *E. coli* JM109. The recombinant *E. coli* JM109 was used throughout this work. The fermentation medium was prepared according to the previous report (12). One mL

frozen stock culture was added to LB medium (50 mL working volume in 500-mL Erlenmeyer flask) with ampicillin at a concentration of 100 μg/mL at 30°C and 200 rpm for 12 hr (13). Subsequently, one further preculture (50 mL LB medium with ampicillin at a concentration of 100 μg/mL in a 500-mL Erlenmeyer flask) was inoculated with the first preculture (2%, v/v) and incubated on a rotary shaker at 30°C and 200 rpm for 12 hr. Ten mL of inoculum culture was added to 90 mL fermentation medium in a 500-mL Erlenmeyer flask. The culture was incubated at 30°C and 200 rpm for 12 hr. after 12 hr cultivation; cells were cultured at 42°C for 4 hr for induction of the recombinant proteins.

Treatment of recombinant *E. coli* cells by the surfactants and organic solvents Ten mL of recombinant *E. coli* JM109 culture were centrifuged at 4,000×g for 5 min, and the harvested cells were permeabilized by treatment with Triton X-100, CTAB, and acetone at 30°C for 30 min. Then, cells were centrifuged at 12,000×g for 5 min and washed once with 25 mM Tris-HCl buffer (pH 8.8). PAL activity was determined according to analytical methods.

Analytic methods PAL activity of recombinant *E. coli* whole cells was determined by the procedure of Orndorff *et al.* (14). The reaction mixture (5 mL) containing 25 mM Tris-HCl buffer (pH 8.8), 25 mM L-phenylalanine and 20 mg (wet weight) treated recombinant *E. coli* JM109 cells with Triton X-100 CTAB and acetone was incubated at 30 °C for 20 min. The reaction was terminated by addition of 1 mol/L HCl (0.2 mL). After centrifugation, the absorbance of the clear supernatant was measured at 280 nm with a 752 spectrophotometer (Shanghai Precision and Scientific Instrument Co., Shanghai, China). One unit of PAL activity was defined as the amount of enzyme required to convert one mmol of L-phenylalanine to *trans*-cinnamic acids/min. PAL specific activity is expressed as units of enzyme/g (dry cell weight). Ten mL of recombinant *E. coli* JM109 culture were centrifuged at 4,000×g for 10 min. Dry cell weight (DCW) was measured after the harvested cells were washed twice with distilled water and dried to a constant weight at 80°C (15). Morphologic changes of whole cells of recombinant *E. coli* by treatment with Triton X-100, CTAB, and acetone were examined by a atomic force microscopy (AFM) (JSPM-5200; Jeol Ltd., Tokyo, Japan). The Coulter® Epics® Xl™ Flow Cytometer (Beckman Coulter, Fullerton, CA, USA) was employed to analyze the fluorescence intensities of recombinant *E. coli* cell.

Experimental design and optimization Based on the results obtained in preliminary experiments, Triton X-100, acetone, and CTAB were found to be the major variables in the recombinant PAL activity (10). The CCD was used to find the optimal concentrations of these 3 factors. Triton X-100 (X_1), acetone (X_2), and CTAB (X_3) were chosen as the independent variables shown in Table 1. PAL activity (Y) was used as dependent output variables. A set of 20 experiments consisting of 8 factorial points, 6 axial points (α=1.68), and 6 replicates at the center points were employed (Table 3). All experiments were carried out in triplicates. A multiple regression analysis of the data was carried out with the 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, and X_3) was obtained:

$$Y=B_0+B_1X_1+B_2X_2+B_3X_3+B_{11}X^2_1+B_{22}X^2_2+B_{33}X^2_3+B_{12}X_1X_2+B_{23}X_2X_3+B_{13}X_1X_3 \quad (1)$$

Where B_0 is constant, B_1, B_2, and B_2 are linear coefficients, B_{11}, B_{22}, and B_{33} are squared coefficients and B_{12}, B_{23}, and B_{13} are interaction 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.

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

Variables	Symbol	Coded levels				
		-1.68	-1	0	1	1.68
Triton-X-100 (g/L)	X_1	0.032	0.1	0.2	0.3	0.368
CTAB (g/L)	X_2	0.066	0.1	0.15	0.2	0.234
Acetone (v/v, %)	X_3	6.4	20	40	60	93.6

Table 2. Central composite design and response value

Run	X_1	X_2	X_3	Specific enzyme activity (U/g)
1	-1	-1	-1	80.45
2	+1	-1	-1	92.86
3	-1	+1	-1	67.32
4	+1	-1	+1	94.83
5	-1	+1	-1	73.24
6	+1	-1	+1	95.62
7	-1	+1	+1	72.98
8	+1	+1	+1	98.49
9	-1.68	0	0	124.97
10	+1.68	0	0	138.42
11	0	-1.68	0	112.88
12	0	+1.68	0	110.38
13	0	0	-1.68	132.59
14	0	0	+1.68	134.67
15	0	0	0	186.53
16	0	0	0	187.64
17	0	0	0	184.35
18	0	0	0	185.25
19	0	0	0	183.58
20	0	0	0	182.12

Results and Discussion

Some reports showed that the PAL activity of *Rhodotorula* whole cells could be improved by improving cell membrane permeability with single Triton X-100, CTAB, or acetone (4,5,16). However, above-mentioned reports mainly studied the effect of single Triton X-100, CTAB, or acetone on PAL activity of *Rhodotorula* whole cells, few results have been reported about effect of Triton X-100, CTAB, or acetone on PAL activity in recombinant *E. coli*. In particular, the combined effect of the CTAB, acetone, and Triton X-100 for PAL activity of recombinant *E. coli* hardly has been

Table 3. Parameter estimates and analysis of variance

Source of variation	Degree of freedom (df)	Sum of squares (SS)	Mean squares (MS)	*F*-value	Prob>*F*
X_1	1	55.33736	55.33736	0.111951	0.744846
X_2	1	11.91054	11.91054	0.024096	0.87973
X_3	1	610.4826	610.4826	1.235042	0.292434
X_1^2	1	10,309.01	10,309.01	20.85572	0.001031**[1)]
X_1X_2	1	23.70161	23.70161	0.04795	0.831075
X_1X_3	1	7.940112	7.940112	0.016063	0.901658
X_2^2	1	16,502.97	16,502.97	33.38646	0.000178**
X_2X_3	1	41.54161	41.54161	0.084041	0.777816
X_3^2	1	9,788.379	9,788.379	19.80246	0.001235**
Model	9	31,434.67	31,434.67	7.066018	0.002605
Error	10	4,943.012	4,943.012		
Total	19	36,377.68			

[1)]**Highly significant.

reported. In this study, the CCD was used to find the suitable concentrations of the variables (Triton X-100, CTAB, and acetone) on the recombinant PAL activity, and understand the relationship between the factors and recombinant PAL activity.

RSM for the optimization of 3 factors The actual PAL activity in CCD is shown in Table 2. The regression coefficients and signficance levels were given in Table 3. Table 3 indicated that the quadratic term (X^2_1, X^2_2, and X^2_3) were highly significant ('prob>*F*' less than 0.001), but the interactions of 3 variables were not significant. It means that there were no interactions among 3 independent variables. Multiple regression analysis of the experimental data gave the following second-order polynomial equation:

$$Y=186.1252+2.0129X_1-0.9338X_2+6.6859X_3-26.7459X^2_1+1.7212X_1X_2+0.9962X_1X_3-33.8399X^2_2+2.2787\ X_3X_2-26.0618X^2_3 \quad (2)$$

where X_1, X_2, and X_3 are Triton X-100, CTAB, and acetone concentrations, respectively. The regression equation obtained from analysis of variance (ANOVA) indicated that the multiple correlation coefficient of R^2 is 0.9341. The value of the determination coefficent (R^2=0.9341) indicates that the model can explain 93.41% variation in the response, and only 6.59% of the total variations are not explained by the model. The value of adjusted R^2 (adj. R^2=0.9058) is very high that indicated a high significance of the model (17,18). The model *F*-value of 7.066 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.

Interactions among the factors The predicted values versus the experimental values for recombinant PAL activity are shown in Fig. 1. The points clustered around the diagonal line which indicated the good fit of the model. The 3D response surface and the 2D contour plots are generally the graphical representations of the regression equation. Each contour curve represents an infinite number of combinations of 2 test variables with the other 2

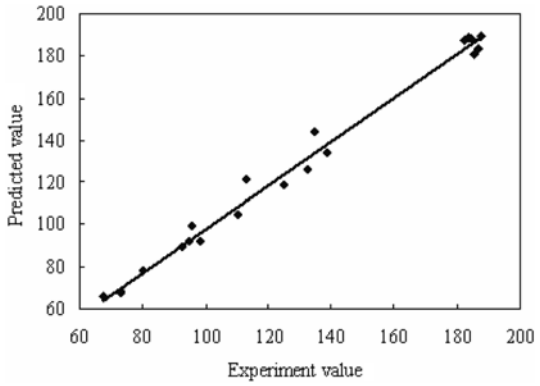

Fig. 1. Parity plot showing the distribution of experimental vs. predicted values of recombinant PAL activity.

maintained at their respective zero level. From the contour plots, it is easy and convenient to understand the interactions between 2 factors and also locate their optimum levels. Thus, in this study, 2D contour plots of the response and 3D response surface curve were plotted to show the interactions of the additive (various concentrations of Triton X-100, CTAB, and acetone) and the optimum concentrations of determined components on recombinant PAL activity (Fig. 2). The contour plots in Fig. 2B, 2D, and 2F were circular contour plots. It indicated that there was not relatively significant interaction between Triton X-100 and CTAB, Triton X-100, and acetone, CTAB and acetone corresponding to the response surface, which was consistent with the results of the ANOVA for quadratic model. The protuberant shapes of the 3D response surfaces (Fig. 2A, 2C, and 2E) showed that there was a maximum value for this model. The optimal conditions were extracted by Design Expert Software with its optimization menus: X_1=0.085, X_2=−0.0057, X_3=0.26, The real values were Triton X-100 concentration at 0.108 g/L, CTAB concentration at 0.15 g/L, and acetone concentration at 45.2%(v/v). The maximum recombinant PAL activity obtained by using the above optimized concentrations of the variables is 186.10 U/g. The maximum recombinant PAL activity obtained experimentally was found to be

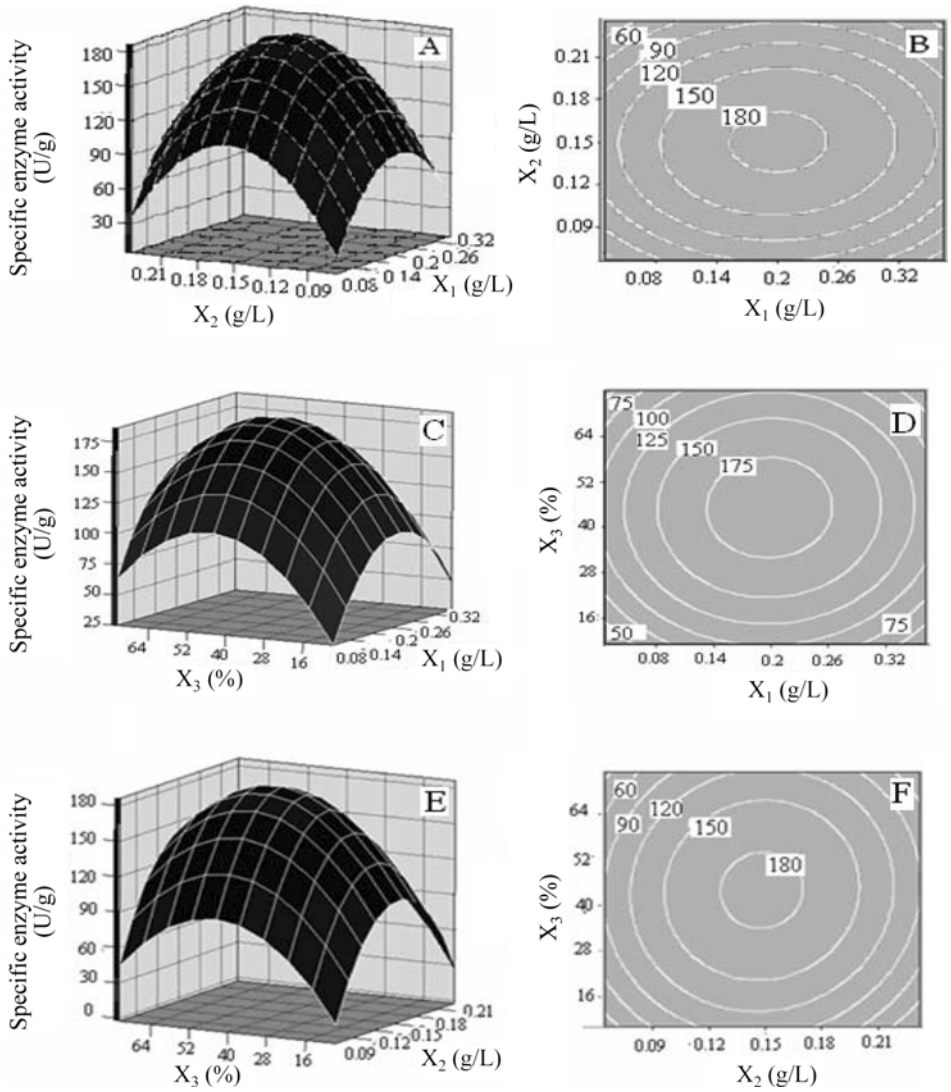

Fig. 2. Response surfaces and contour plots for the effects of Triton X-100 (X_1), CTAB (X_2), and acetone (X_3) on recombinant PAL activity.

181.37 U/g. This is obviously in close agreement with the model prediction.

Comparison of recombinant PAL activity between treatment cells and untreatment cells Comparison of recombinant PAL activity between treatment cells and untreatment cells (control) were showed in Fig. 3. The results showed that the treatment strategy to cells under optimum conditions was effective to enhance PAL activity significantly. The maximum PAL activity was obtained when the whole cells of recombinant *E. coli* was treated under optimum conditions (0.108 g/L Triton X-100, 0.15 g/L CTAB, and 45.2%(v/v) acetone), the recombinant PAL activity was above 10-fold in comparison to the control (untreat cell), reached 181.37 U/g. To determine whether permeabilization of *E. coli* cells was improved, AFM was used to observe the morphologic changes of whole cells treated under optimum conditions. As shown in Fig.4, the control cells (untreated cells) had distinct outlines and smooth surfaces, whereas the cell shapes treated under optimum conditions had altered and the cell membranes and walls seemed to be folded, which maybe indicated the improvement of cell membrane permeability. The control cells and treated cells under optimum conditions were colored with propidium iodide (PI) and the fluorescence intensities were analyzed by flow cytometry. The results were shown in Table 4. As an ordinary fluorescence dye, PI gives fluorescence from the combination of DNA. In the

Table 4. Flow cytometry of whole cells of recombinant *E. coli*

Treat method	Control	0.15 (g/L) CTAB	60%(v/v) Acetone	Optimum treatment conditions
MnX[1)]	7	37	45.1	52.2

[1)]Mean of fluorescent intensity (MnX) indicated the average fluorescence intensities.

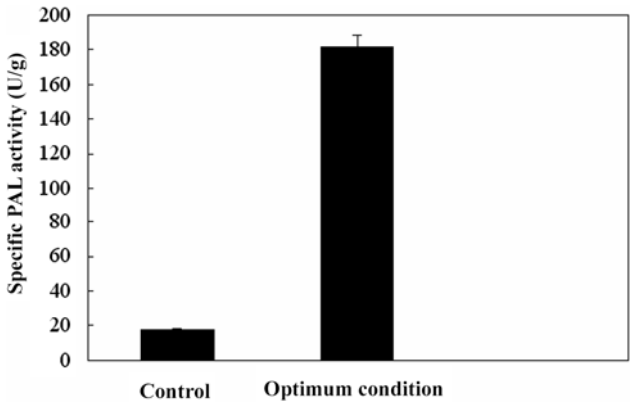

Fig. 3. Comparison of recombinant PAL activity between treatment cells and untreatment cells.

coloring process, PI penetrates through the cell membrane into the cell and combines with DNA molecules. If the cell membrane is damaged and the permeability will be increased, PI can enter the cell more easily and the fluorescence intensified. Thus, the changes of the fluorescence intensity of the colored cells can be used to determine the permeability changes of cells. If the fluorescence intensity of treated cells increases under optimum conditions, it indicates that the permeability of recombinant *E. coli* cells is increased. Table 4 showed that the mean of fluorescence intensity of treated cells were higher than that of the control. The mean of fluorescence intensities of treated cells under optimum conditions were 7.4-fold in comparison with that of the control. Above results showed that permeabilization of treated recombinant *E. coli* cells under optimum conditions was improved significantly. The more likely explanation was that the treatment with combined of Triton X-100, CTAB, and acetone made recombinant *E. coli* cells more permeable and therefore allowed easier access of the substrate to the enzyme and excretion of the product, which increased the rate of transport of L-phenylalanine and *trans*-cinnamic acids. Some reports showed that the PAL activity of *Rhodotorula* whole cells could be improved by improving cell membrane permeability with surfactants and organic solvents. Srinivasan *et al.* (4) found that whole cells of the yeast *R. glutinis* IFO 0559 had low PAL activity due to a limited membrane permeability barrier for phenylalanine. They reported that PAL activity could be increased about 6-fold, when *R. glutinis* cells were treated with CTAB. EI-Batal (19) found that the conversion rate of *trans*-cinnamic acids was increased 56% as compare to the control when *R. glutinis* cells were treated with Triton X-100. In our previous study, permeabilization with Triton X-100, CTAB, or acetone enriched cellular recombinant PAL activity (10). However, above-mentioned reports mainly studied the effect of one factor on PAL activity; reaction system influenced simultaneously by more than one factor on PAL activity was not investigated. Some reports showed that RSM is a suitable technique for designing experiment, building models, evaluating the effects of several factors, and searching optimum conditions for desirable responses and reducing number of experiments. And this process has been successfully applied to optimize various kinds of biotechnology production. Wang *et al*. (20) utilized RSM to optimize the conditions of simultaneous saccharification and fermentation (SSF) for ethanol production from kitchen garbage. The ethanol yield could reach 0.23 g ethanol/dry g garbage under the optimum conditions. Oskouie *et al*. (21) utilized RSM to optimize a culture medium for production of bacterial alkaline protease; under the optimum conditions, the maximum alkaline protease production was over 6-fold as compare with the basal medium, reached 1,520 U/mL. In our study, the combined effect of the CTAB, acetone, and Triton X-100 for PAL activity of recombinant *E. coli* was investigated by using RSM, the results showed that the maximum recombinant PAL activity under optimum conditions was above 10-fold in comparison to the control (untreat cell), reached 181.37 U/g. To confirm the validity of the statistical experimental strategies, confirmation experiments were repeated thrice. The measured PAL activity was close to the predicted values for recombinant PAL activity using RSM (data not shown). These results confirm the predictability of the model for the improvement of recombinant PAL activity in the experimental condition used.

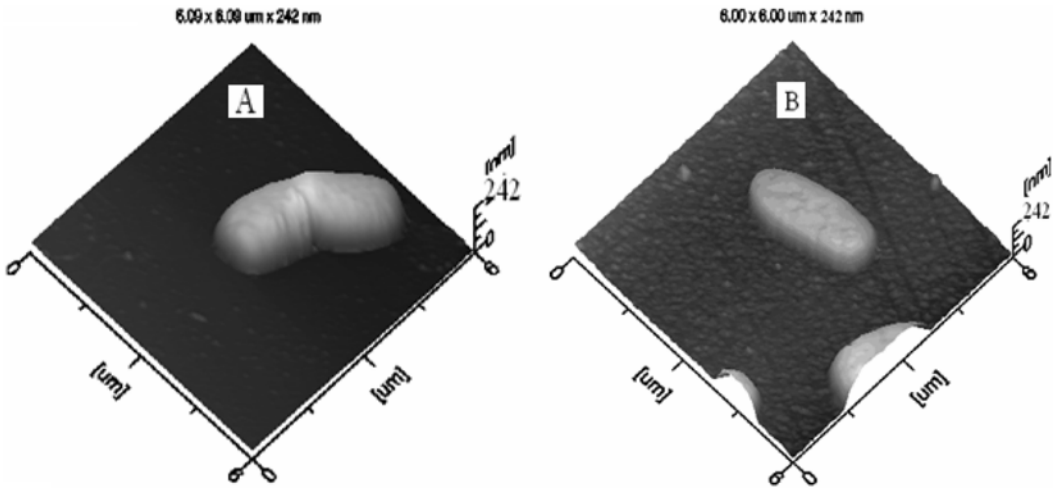

Fig. 4. Atomic force microscopy of whole cells of recombinant *E. coli*. A, control (untreated cells); B, cells treated under optimum conditions.

This work has demonstrated the use of a central composite design by determining conditions leading to the maximum PAL activity. Permeabilization with combinations of Triton X-100, CTAB, and acetone enriched cellular recombinant PAL activity significantly. Central composite experimental design maximizes the amount of information that can be obtained, while limiting the numbers of individual experiments required. The enzyme activity predicted by the model at optimal conditions agreed fittingly with experimental data, thus confirming the model validity. These improved methods of PAL activity enrichment could serve as a rich enzyme source, especially in the biosynthesis of L-phenylalanine. To the best of our knowledge, they reached an about 2-fold increase as compared to the state of the art.

References

1. Fiske MJ, Kane JF. Regulation of phenylalanine biosynthesis in *Rhodotorula glutinis*. J. Bacteriol. 160: 676-681 (1984)
2. Rosler J, Krekel F, Amrhein N. Maize phenylalanine ammonia-lyase has tyrosine ammonia-lyase activity. Plant Physiol. 113: 175-179 (1997)
3. Nakamichi K, Yamada NS, Chibata I. Induction and stabilization of L-phenylalanine ammonia lyase activity in *Rhodotorula glutinis*. Eur. J. Appl. Microbiol. 18: 158-162 (1983)
4. Srinivasan AR, Nagajyothi R, Gowda LR. Phenylalanine ammonia lyase activity enhancement in permeabilized yeast cells (*Rhodotorula glutinis*). Biotechniques 8: 729-732 (1994)
5. D'Cunha GB. Enrichment of phenylalanine ammonia lyase activity of *Rhosotorula* yeast. Enzyme. Microb. Technol. 36: 498-502 (2005)
6. Orum H, Rasmussen OF. Expression in *E. coli* of the gene encoding phenylalanine ammonia lyase from *Rhodosporidium toruloides*. Appl. Microbiol. Biot. 36: 745-748 (1992)
7. Faulkner JDB, Anson JG, Tuite MF. High-level expression of the phenylalanine ammonia lyase-encoding gene from *Rhodosporidium toruloides* in *Sacchpromyces cerevisiae* and *Escherichia coli* using a bifunctional expression system. Gene 143: 13-20 (1994)
8. Avishek M, Arun G. Enhanced prodution of exocellular glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 using response surface methodology. Bioresource Technol. 99: 3685-3691 (2008)
9. Liyana-Pathirana C, Shahidi F. Optimization of extraction of phenolic compounds from wheat using surface response methodology. Food. Chem. 9: 347-356 (2005)
10. Cui JD, Jia SR, Sun AY. Influence of amino acids, organic solvents, and surfactants for phenylalanine ammonia lyase activity in recombinant *E. coli*. Lett. Appl. Microbiol. 46: 631-635 (2008)
11. Jia SR, Cui JD, Li Y. Production of L-phenylalanine from *trans*-cinnamic acids by high-level expression of phenylalanine ammonia lyase gene from *Rhodosporidium toruloides* in *Escherichia coli*. Biochem. Eng. J. 42: 193-197 (2008)
12. Delisa MP, Rao G, Weigand WA. Monitoring GFP-operon fusion protein expression during high cell density cultivation of *Escherichia coli* using an on-line optical sensor. Biotechnol. Bioeng. 65: 54-64 (1999)
13. Paik H-D, Kim I-G, Lee J-H, Park K-Y, Ji G-E, Jin T-E, Rhim S-L. Heterologous expression of α-amylase gene of *Bifidobacterium adolescentis* Int57 in *Bacillus polyfermenticus* SCD. Food Sci. Biotechnol. 16: 655-658 (2007)
14. Orndorff SA, Costantino N, Stewart D. Strain improvement of *Rhodotorula graminis* for production of a novel L-phenylalanine ammonia-lyase. Appl. Environ. Microb. 54: 996-1002 (1988)
15. Song JY, Keum I-K, Jin Q, Park J-M, Kim BS, Jung BH, Kim T-J, Han NS. Batch and fed-batch production of hyperthermostable α-L-arabinofuranosidase of *Thermotoga maritima* in recombinant *E. coli* by using constitutive and inducible promoters. Food Sci. Biotechnol. 17: 990-995 (2008)
16. Yuan Q, Yue H. PAL activity and productivity of L-phenylalanine in the production of L-phenylalanine. J. Beijing. Univ. Chem. Technol. China 30: 1-3 (2003)
17. Myers RH, Montgomery DC. Response Surface Methodology: Process and Product Optimization Using Designed Experiments. 2nd ed. John Wiley and Sons, New York, NY, USA. pp. 146-149 (2002)
18. Jung SJ, Jang MS. Effects of *wasabi* stalk on the fermentation of *baechukimchi*. Food Sci. Biotechnol. 17: 692-699 (2008)
19. EI-Batal AI. Optimization of reation conditions and stabilization of phenylalanine ammonia lyase-containing *Rhosotorula glutinis* cells during bioconversion of *trans*-cinnamic acid to L-phenylalanine. Acta Microbiol. Pol. 51: 139-152 (2002)
20. Wang QH, Ma H, Xu W, Gong L, Zhang W, Zou D. Ethanol production from kitchen garbage using response surface methodology. Biochem. Eng. J. 39: 604-610 (2008)
21. Oskouie SFG, Fatemeh T, Bagher Y, Fereshteh E. Response surface optimization of medium composion for alkaline protease production by *Bacillus clausii*. Biochem. Eng. J. 39: 37-42 (2008)