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Modeling and Optimization of Phenylalanine Ammonia Lyase Stabilization in Recombinant *Escherichia coli* for the Continuous Synthesis of L-Phenylalanine on the Statistical-Based Experimental Designs

Bing-Zhu Zhang,[†] Jian-Dong Cui,*[,‡,§] Gui-Xia Zhao,[∥] and Shi-Ru Jia[§]

[†]Department of Scientific Research, [‡]Hebei Fermentation Engineering Research Center, College of Bioscience and Bioengineering, and [∥]Labour Union, Hebei University of Science and Technology, 70 Yuhua East Road, Shijiazhang 050018, People's Republic of China, and [§]Key Laboratory of Industry Microbiology, Ministry of Education, Tianjin University of Science and Technology, 29 Thirteenth Street, Tai Da Development Area, Tianjin 300457, People's Republic of China

Some approaches for improving recombinant phenylalanine ammonia lyase (PAL) stability in *Escherichia coli* during the enzymatic methods of L-phenylalanine (L-Phe) production were developed following preliminary studies by means of statistical-based experiment designs (response surface method). The traditional non-statistical technology was used to screen four critical factors for PAL stability during the bioconversion process, viz., glycerin, sucrose, 1,4-dithiothreitol (DTT), and $MgSO_4$. The central composite design (CCD) was applied to optimize the combined effect of critical factors for recombinant PAL stability and understand the relationship between the factors and PAL stability. The optimum values for testing variables were 13.04 mM glycerin, 1.87 mM sucrose, 4.09 mM DTT, and 69 mM Mg^{2+}. A second-order model equation was suggested and then validated experimentally. The model adequacy was very satisfactory because the coefficient of determination was 0.88. The maximum PAL activity was retained as 67.73 units/g after three successive cycles of bioconversion. In comparison to initial PAL activity, the loss of PAL activity was only 22%. PAL activity was enhanced about 23% in comparison to the control (without any stabilizer additives). PAL stability was significantly improved during successive bioconversion. The results obtained here verified the effectiveness of the applied methodology and may be helpful for L-Phe production on an industrial scale.

KEYWORDS: Phenylalanine ammonia lyase; stability; recombinant *Escherichia coli*; optimization; statistical-based experimental design

INTRODUCTION

Phenylalanine ammonia lyase (EC 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 (L-Phe) by reversing the enzyme reaction with high concentrations of *trans*-cinnamic acid (*t*-CA) and ammonia at an elevated pH. There is a great demand for the production of L-Phe, because it is one of the two precursors required for the synthesis of artificial sweetener aspartame. Therefore, the enzyme is of particular interests to researchers in the biotechnology industry. At present, the industrial production of PAL mainly uses the genus *Rhodotorula* (*3*). The levels of enzyme in these wild-type strains are relatively low; thus, the production of L-Phe from *t*-CA was of limited success. A recombinant strain capable of producting a large amount of PAL is therefore highly desirable to improve L-Phe from *t*-CA. 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 the large-scale production of PAL for industrial and medical uses, recombinant PAL productions need to be improved substantially. In our earlier reports, a recombinant strain capable of producing a large amount of PAL has also been constructed (*7*) and more than 80% conversion of *t*-CA was achieved under the optimized conditions (*8*). However, recombinant PAL activity in the cells declined rapidly during the bioconversion; thus, improving stabilization of recombinant PAL is necessary to enhance the L-Phe yield.

The traditional "one-factor-at-a-time" is an operation frequently used in bioprocess optimization to obtain high yields of the desired products in a microbial system. However, this method disregards the complex interactions among various physicochemical parameters (*9*). Statistical experimental designs, such as factorial design and response surface methodology (RSM), particularly fulfill this requirement. As a powerful statistical

*To whom correspondence should be addressed: College of Bioscience and Bioengineering, Hebei University of Science and Technology, 186 Yuhua East Road, Shijiazhuang 050018, People's Republic of China. Telephone: +86-311-88632170. Fax: +86-311-88632170. E-mail: cjd007cn@163.com.

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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 a limited number of experiments (*10*, *11*). This method has been successfully applied in many areas of biotechnology, such as optimization of the medium (*12*), enzyme production (*13*), and extracellular glucansucrase production (*14*). In our earlier reports, inclusion of several activity modifiers and stabilizer additives in biotransformation reaction mixtures were shown to enhance the yield of L-Phe and maintained recombinant PAL stability over several successive incubations during the bioconversion process (*8*). However, the relationship between the above factors and PAL stability and the best combination influencing factors was not investigated. Thus, the objective of the present work was to study the combined effect of factors for recombinant PAL stability according to central composite design (CCD) and RSM and to understand the relationship between the factors and PAL stability during the bioconversion process.

MATERIALS AND METHODS

Chemicals. L-Phe was purchased from Biodev (Shanghai, China). High-performance liquid chromatography (HPLC)-grade methanol was supplied by Carlo Erba (Rodano, Milan, Italy). All other chemicals were analytical-grade and commercially available.

Microorganism and Growth Conditions. The plasmid pBV-PAL was constructed according to our earlier reports (*7*). The gene *pal* of *Rhodosporidium toruloides* was cloned and expressed in plasmid pBV-PAL (Amp^r). The expression of PAL was controlled by a combine promoter of *tac* and P_LP_R. The plasmid pBV-PAL was transformed in *Escherichia coli* JM109. The strain was maintained as 40% (v/v) glycerol stock at −80 °C in Luria−Bertani (LB) medium and used throughout this work. Medium used for batch fermentation contained the following components (*15*): 20 g/L glucose, 5 g/L yeast extract, 13.3 g/L KH_2PO_4, 4 g/L $(NH_4)_2HPO_4$, 1.2 g/L $MgSO_4 \cdot 7H_2O$, and 1.7 g/L citric acid. The trace element solution comprises 8.4 mg/L ethylenediaminetetraacetic acid (EDTA), 2.5 mg/L $CoCl_2 \cdot 6H_2O$, 15 mg/L $MnCl_2 \cdot 4H_2O$, 1.5 mg/L $CuCl_2 \cdot 4H_2O$, 3 mg/L H_3BO_4, 2.5 mg/L $Na_2MoO_4 \cdot 2H_2O$, and 13 mg/L $Zn(CH_3COO)_2 \cdot 2H_2O$. For cell activation, 1 mL of frozen stock culture was added to LB medium (50 mL working volume in a 500 mL Erlenmeyer flask) with ampicillin at a concentration of 100 μg/mL at 37 °C and 200 rpm for 12 h. Subsequently, one further preculture (50 mL of 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 37 °C and 200 rpm for 12 h. For the shaker-flask culture, 10 mL of inoculum culture was added to 90 mL of fermentation medium in a 500 mL Erlenmeyer flask. The culture was incubated at 37 °C in a rotary set at 200 rpm until the optical density (OD_{600}) reached 0.6. At this point, 0.2 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG) was added and the cells were cultured at 42 °C for 4 h for induction of the PAL.

PAL Activity Assay. The PAL forward activity of recombinant *E. coli* JM109 cells was measured spectrophotometrically by following the formation of *t*-CA from L-Phe at A_{280} according to a modification of the procedure by Orndorff et al. (*16*). First, cells were recovered from the culture by centrifugation. The harvested cells were permeabilized by treatment with 0.3 g/L cetyl trimethyl ammonium bromide (CTAB) at 30 °C for 30 min. The collected cells were washed by suspending them in a 0.85% sodium chloride solution and recovered by centrifugation. The washed cells were then suspended in 25 mM Tris-HCl buffer solution (pH 8.8) (as a cell suspension). The suspension was added to an enzymatic reaction medium comprising a 25 mM Tris-HCl buffer solution (pH 8.8) supplemented with 25 mM L-Phe and incubated at 30 °C for 20 min. The reaction was terminated by the addition of 1 M HCl (0.2 mL). After centrifugation, the rate of formation of *t*-CA 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 1 μmol of L-Phe to *t*-CA per minute. PAL activity (specific activity) is expressed as units of enzyme per gram (dry cell weight) of cells.

Table 1. Process Variables Used CCD with Actual Factor Levels Corresponding to Coded Factor Levels

		coded levels				
variables	symbol	−2	−1	0	1	2
glycerin (mM)	X_1	5.4	10.8	21.6	32.4	43.2
sucrose (mM)	X_2	0	0.73	1.46	2.19	2.92
DTT (mM)	X_3	0	1.62	3.25	4.86	6.5
$MgSO_4$ (mM)	X_4	0	25	50	75	100

Bioconversion Experiments. The formation of L-Phe from *t*-CA was carried out according to the procedure by Jia et al. (*7*). For the preparation of the substrate solution, 740 mg of *t*-CA was dissolved in 45 mL of 28% ammonium hydroxide. The resulting solution was adjusted to pH 10.0 with HCl and diluted with distilled water in a final volume of 80 mL. A reaction mixture of 5.0 mL of the cell suspension and 20.0 mL of substrate solution was incubated at 30 °C for 20 h. At the end of 20 h, the reaction mixture was centrifuged to separate the cells. After the residual PAL activity of the used cells was determined, the cells were resuspended in fresh reaction mixture and the reaction was continued. The supernatant obtained after each cycle was processed as before, and the yield of L-Phe was determined by HPLC. The reaction was discontinued after three continuous cycles. HPLC analysis was performed on a reverse-phase C18 column (4.6 × 250 nm, ODS-100S, Shimadzu, Japan). The highly purified L-Phe (>99%) was dissolved in HPLC-grade methanol to make a 5 g/L L-Phe stock solution. A standard curve was established for L-Phe using 0.5, 1.0, 2.0, 3.0, 4.0 g/L L-Phe versus the corresponding area number of spectra. Samples were eluted isocratically with methanol/water (20:80, v/v) at a flow rate of 1.0 mL/min and detected at 250 nm using a UVD 170U detector (Dionex, P680, Bannockburn, IL). The retention time for L-Phe was 6.5 min. The standard curve for L-Phe on HPLC was as follows:

$$C = 0.1579A + 0.0344 \quad (r^2 = 0.9951)$$

where C is the L-Phe concentration and A is the corresponding area number of spectra.

Experimental Design and Optimization. The influence of the presence of various compounds (activity modifiers and stabilizer additives) on recombinant PAL stability during L-Phe synthesis in the bioconversion system was investigated by traditional non-statistical technology (*8*). The results revealed that sucrose, glycerin, 1,4-dithiothreitol (DTT), and $MgSO_4$ were supposed to have effects on PAL stability (data not shown). Then, the effect of various concentrations of glycerin, sucrose, DTT, and $MgSO_4$ on the stability of recombinant *E. coli* PAL during the bioconversion reaction was investigated separately by traditional non-statistical technology. After critical factors were identified via screening and a significant factor range had been detected in the design space, the CCD was proceeded to obtain a quadratic model, consisting of trials plus a star configuration to estimate quadratic effects and central points to estimate the pure process variability and reassess gross curvature, with active substance production as a response. The CCD used was generated by "Design-Expert" software (version 7.0, Stat-Ease, Inc., Minneapolis, MN) (*17*). According to this design, The CCD was used to find the optimal concentrations of these four factors and the relationship between the factors and PAL stability. Thus, glycerin (X_1, mM), sucrose (X_2, mM), DTT (X_3, mM), and $MgSO_4$ (X_4, mM) were chosen as the independent variables shown in **Table 1**. PAL activity (Y, units/g) was used as dependent output variables. 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. In addition, each variable was designated as −2, −1, 0, 1, and 2, respectively. The inter-relationships of the variables were determined by fitting the second-order polynomial equation to data obtained from 30 experiments using mean values of the triplicates of each experiment conducted twice on 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 were listed in **Table 1**. A multiple regression analysis of the data was carried out with the statistical package (Stat-Ease, Inc., Minneapolis, MN), and the second-order

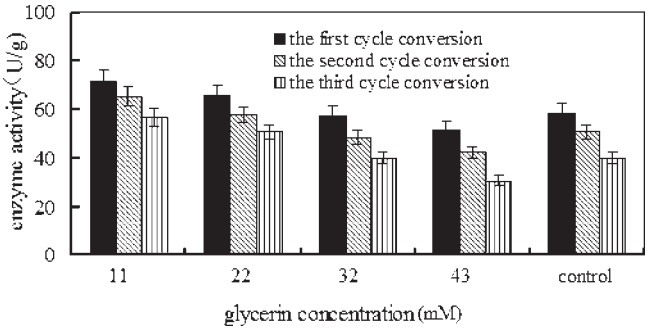

Figure 1. Effect of various concentrations of glycerin on the recombinant PAL stability during L-Phe synthesis in the bioconversion system.

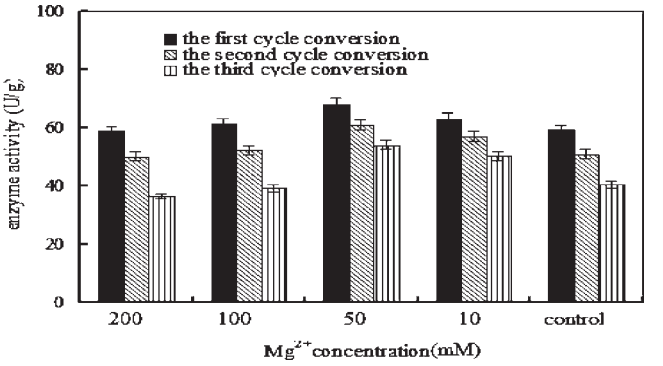

Figure 2. Effect of various concentrations of $MgSO_4$ on the recombinant PAL stability during L-Phe synthesis in the bioconversion system.

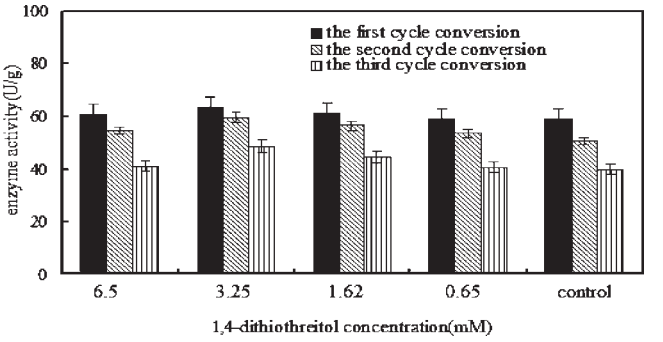

Figure 3. Effect of various concentrations of DTT on the recombinant PAL stability during L-Phe synthesis in the bioconversion system.

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, and 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 level of factors.

RESULTS AND DISCUSSION

Effect of Various Concentrations of Glycerin on the Recombinant PAL Stability during L-Phe Synthesis in the Bioconversion System. As shown in **Figure 1**, glycerin gave measurable protection to recombinant PAL and prolonged the synthesis of L-Phe up to the third cycle conversion. The stability of PAL decreased at glycerin concentrations greater than 32 mM. The maximum stabilization of PAL was obtained at the glycerin concentration of 11 mM, and L-Phe synthesis continued up to 60 h (the third cycle). In comparison to initial PAL activity (before the first cycle conversion, PAL activity reached 90.2 units/g), the loss in PAL activity was only 37%. However, the loss in PAL activity of the absence of glycerin was 56%. In the other words, PAL activity was increased by 19% in comparison to the control (without stabilizer additives) when 11 mM glycerin was added to the L-Phe synthesis system. D'Cunha et al. reported that 11−16 mM glycerol concentrations could improve PAL stability of *Rhodotorula glutinis* (*3*). However, at glycerol concentrations greater than 22 mM, the yield of L-Phe decreased (*3*). In this study, PAL stability decreased at glycerol concentrations greater than 32 mM. The results differed with those of D'Cunha et al. (*3*). Some reports showed that the conformation of enzyme was stabilized because of the preferential hydration of protein in the presence of glycerol (*19*, *20*). Thus, glycerol somehow might be stabilizing the PAL active conformation inside the cells in a similar manner. In addition, the introduction of glycerin into the reaction mixture, apart from solubilizing the substrate, will also serve to eliminate/reduce inhibition by substrate. However, at glycerin concentrations greater than 32 mM, PAL activity decreased because of the high viscosity of the aqueous medium, which prevented proper mixing of the two layers and hardly allowed access of the substrate to the enzyme and excretion of the product.

Effect of Various Concentrations of $MgSO_4$ on the Recombinant PAL Stability during L-Phe Synthesis in the Bioconversion System. The effect of various concentrations of Mg^{2+} on PAL stability of recombinant *E. coli* was shown in **Figure 2**. Mg^{2+} had elicited an appreciable protective effect, and L-Phe synthesis was extended up to the third cycle. The protective effect of Mg^{2+} increased as its concentration was raised from 10 to 50 mM and decreased thereafter at higher metal ion concentrations. The maximum stabilization of PAL was obtained at the Mg^{2+}concentration of 50 mM. In comparison to initial PAL activity, the loss in PAL activity was only 40%. PAL activity was enhanced about 16% in comparison to the control, when 50 mM Mg^{2+} was regarded as the stabilizer additives during L-Phe synthesis. The possible biochemical mechanism of PAL stabilization in recombinant *E. coli* by Mg^{2+} is not known but could be due to the specific stabilization of a more active conformation of the enzyme.

Effect of Various Concentrations of DTT on the Recombinant PAL Stability during L-Phe Synthesis in the Bioconversion System. The results were given in **Figure 3**. DTT gave a positive effect on the stabilization of PAL activity. The protective effect of DTT increased as its concentration was raised from 0.65 to 3.25 mM and decreased thereafter at higher DTT concentrations. The maximum stabilization of PAL was obtained at the DTT concentration of 3.25 mM. In comparison to initial PAL activity, the loss in PAL activity was 46%. Some reports showed that the negative effects of O_2 in the solution for PAL activity of *Rhodotorula* were decreased when the reducers were added to the reaction mixture during conversion (*21*). In this study, the results showed that reducers could improve PAL stability of recombinant *E. coli*. It has been indicated that reducers reduced the effects of oxygen on the PAL catalyst life of recombinant *E. coli*.

Effect of Various Concentrations of Sucrose on the Recombinant PAL Stability during L-Phe Synthesis in the Bioconversion System. The effect of the sucrose concentration on the stabilization of PAL during L-Phe synthesis was shown in **Figure 4**. The sucrose could significantly improve stability of PAL and prolong the synthesis of L-Phe up to the third cycle. The protective effect of

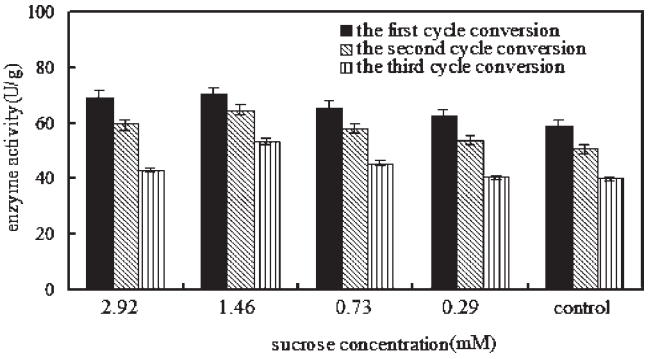

Figure 4. Effect of various concentrations of sucrose on the recombinant PAL stability during L-Phe synthesis in the bioconversion system.

sucrose increased as its concentration was raised from 0.29 to 1.46 mM and decreased thereafter at higher sucrose concentrations. The maximum stabilization of PAL was obtained at the sucrose concentration of 1.46 mM. In comparison to initial PAL activity, the loss in PAL activity was only 40%. PAL activity was enhanced about 16% in comparison to the control, when 1.46 mM sucrose was regarded as the stabilizer additives during L-Phe synthesis. D'Cunha et al. reported that sucrose gave measurable protection to PAL of *R. glutinis* and prolonged the synthesis of L-Phe up to the fifth or sixth cycle (*3*). In this work, the sucrose could significantly improve the PAL stability of recombinant *E. coli*. This result was similar to those of D'Cunha et al. (*3*). Some reports suggested that polyhydric alcohols could covalently attach to the yeast PAL enzyme; the enzyme has increased resistance to proteolytic degradation. In addition, substrate inhibition of PAL was alleviated by polyhydric alcohols (*22*).

Response Surface Analysis for the Optimization of Four Factors. After critical factors were identified via screening and a significant factor range had been detected in the design space, the CCD was used to find the suitable concentrations of the variables on the recombinant PAL stability and understand the relationship between the factors and recombinant PAL stability. The results of CCD experiments consisting of experimental data for studying the effects of four independent variables, viz., glycerin, sucrose, DTT, and Mg^{2+}, on the recombinant PAL stability are presented in **Table 2**. The ANOVA (**Table 3**) indicated that the model terms of X_4, X_1^2, X_3^2, X_2^2, and X_4^2 were significant ("probability > F" less than 0.05) and the interactive effects of X_1X_2, X_1X_3, X_2X_4, X_2X_3, and X_3X_4 were not significant; however, the interactive effect of X_1X_4 was significant ("probability > F" less than 0.05). It means that glycerin and Mg^{2+} have important effects on PAL stability and the quadratic effects of glycerin and Mg^{2+} are more significant than other factors. Furthermore, the interactive effect of glycerin and Mg^{2+} may be siginificant to some extent ("probability > F" less than 0.05). After the neglect of insignificant terms (on the basis of "probability > F" more than 0.05) (**Table 3**), multiple regression analysis of the experimental data gave the following second-order polynomial equation:

$$Y = 72.74 + 3.25X_4 - 4.84X_1^2 + 2.93X_1X_4 - 1.95X_2^2 - 1.60X_3^2 - 2.87X_4^2 \quad (2)$$

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, glycerin, sucrose, DTT, and Mg^{2+}, respectively.

The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R^2 is 0.8813. The model can explain 88.13% variation in the response. The value of the adjusted regression coefficient (adjusted R^2 = 0.8556) is also very high, to advocate for a high significance of the model (*18*). The model F value of 14.76 implied that the model was significant. Values of probability > F less than 0.05 (probability > F of 0.0001) indicated that the model terms were 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. Figure 5 showed the predicted values versus the experimental values for recombinant PAL activity. The points clustered around the diagonal line, which indicated the good fit of the model.

Table 2. CCD and Response Value

run	X_1	X_2	X_3	X_4	Y (units/g)
1	−1	−1	−1	−1	57.42
2	−1	−1	1	−1	58.30
3	−1	−1	1	−1	57.89
4	−1	−1	1	1	61.68
5	−1	1	−1	−1	58.79
6	−1	1	−1	1	59.64
7	−1	1	1	−1	60.98
8	−1	1	1	1	67.93
9	1	−1	−1	−1	53.48
10	1	−1	−1	1	65.96
11	1	−1	1	−1	52.42
12	1	−1	1	1	66.12
13	1	1	−1	−1	52.36
14	1	1	−1	1	67.37
15	1	1	1	−1	61.76
16	1	1	1	1	69.98
17	−2	0	0	0	55.35
18	2	0	0	0	56.78
19	0	−2	0	0	67.43
20	0	2	0	0	67.89
21	0	0	−2	0	69.32
22	0	0	2	0	68.79
23	0	0	0	−2	62.48
24	0	0	0	2	65.49
25	0	0	0	0	72.71
26	0	0	0	0	72.66
27	0	0	0	0	72.89
28	0	0	0	0	72.98
29	0	0	0	0	73.29
30	0	0	0	0	71.98

Table 3. Parameter Estimates and ANOVA

source of variation	degree of freedom	sum of squares	mean squares	F value	probability > F
X_1	1	0.004267	0.004267	0.000378	0.9847
X_2	1	11.2888	11.2888	1.001081	0.3319
X_3	1	8.6160	8.6160	0.7640	0.3949
X_4	1	252.8504	252.8504	22.4225	0.00022[a]
X_1^2	1	671.5294	671.5294	59.5505	0.0001[a]
X_1X_2	1	4.5796	4.5796	0.4061	0.5329
X_1X_3	1	10.923	10.923	0.9686	0.3396
X_1X_4	1	137.7102	137.7102	12.212	0.00299[a]
X_2^2	1	108.4269	108.4269	9.6151	0.006868[a]
X_2X_3	1	5.6882	5.6882	0.5044	0.4877
X_2X_4	1	6.4770	6.4770	0.5743	0.4595
X_3^2	1	73.0662	73.0662	6.4794	0.0216[b]
X_3X_4	1	11.2896	11.2896	1.0011	0.3319
X_4^2	1	234.8813	234.8813	20.829	0.00031[a]
model	14	1339.784	95.6988	14.7619	0.0001[a]
error	16	180.4261	11.2766	8.4864	
total	30	1520.211			

[a] Highly significant. [b] Signficant.

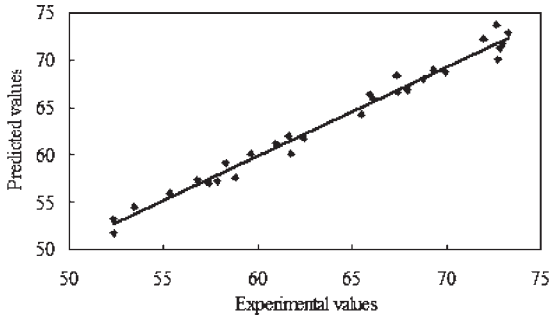

Figure 5. Parity plots showing the distribution of experimental versus predicted values of recombinant PAL activity.

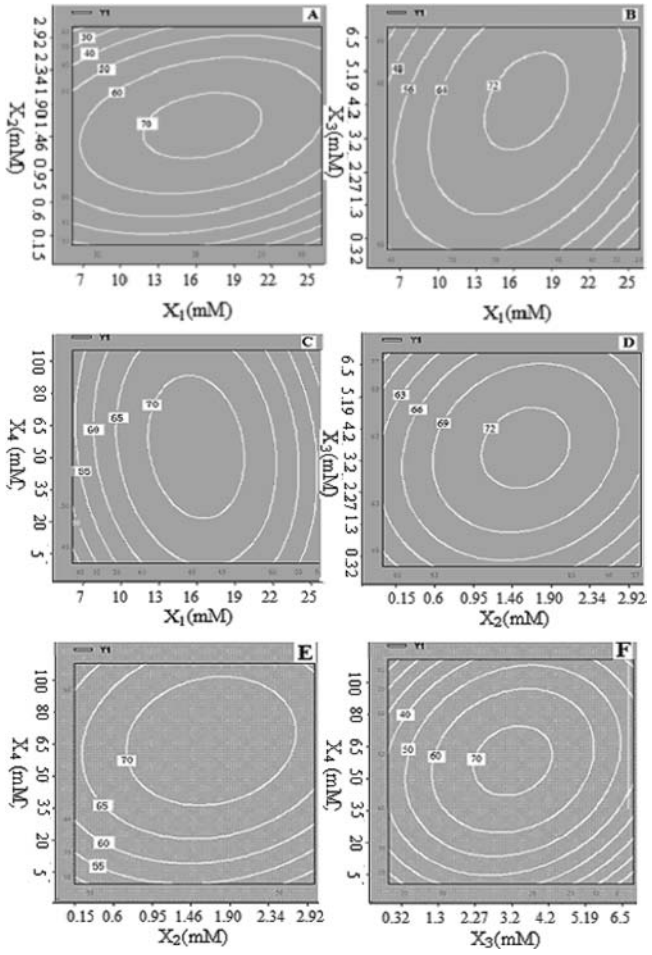

Figure 6. Contour plots of recombinant PAL activity. The effect of (A) glycerin (X_1) and sucrose (X_2), (B) glycerin (X_1) and DTT (X_3), (C) glycerin (X_1) and Mg^{2+} (X_4), (D) sucrose (X_2) and DTT (X_3), (E) sucrose (X_2) and Mg^{2+} (X_4), and (F) DTT (X_3) and Mg^{2+} (X_4) on PAL activity. Other varibles are held at zero level.

The 2D contour plots are generally the graphical representations of the regression equation and are presented in **Figure 6**. 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. The contour plots in panels **A**−**C** of **Figure 6** showed that there was significant mutual interaction between glycerin and sucrose (**Figure 6A**), glycerin and DTT (**Figure 6B**), and glycerin and Mg^{2+} (**Figure 6C**). However, there was almost no interaction between sucrose and DTT (**Figure 6D**), sucrose and Mg^{2+} (**Figure 6E**), and DTT and Mg^{2+} (**Figure 6F**), as evident from the relatively circular nature of the contour curves.

The optimal values of the test variables in code unit are as follows: $X_1 = -0.79$, $X_2 = 0.56$, $X_3 = 0.52$, and $X_4 = 0.76$, with the corresponding $Y = 67.73$. The natural values obtained from the respective values of X_i are concentrations of glycerin at 13.04 mM, sucrose at 1.87 mM, DTT at 4.09 mM, and Mg^{2+} at 69 mM. The model predicts that the maximum recombinant PAL activity that can be obtained under the above optimum conditions of the variables is 67.73 units/g. In comparison to initial PAL activity, the loss in PAL activity was only 22%. PAL activity was enhanced about 23% in comparison to the control. PAL stability was significantly improved during continuous bioconversion. The validation experiment showed that the experimentally determined production values were in close agreement with the statistically predicted ones, confirming the authenticity of the model. The maximum recombinant PAL activity obtained experimentally was found to be 68.85 units/g under optimum conditions.

L-Phe Production under Optimum Conditions. The yield of L-Phe in the bioconversion reaction mixture was determined by HPLC after three continuous cycles of conversion. Under optimum conditions, synthesis of L-Phe continued up to 60 h and the total amount of L-Phe produced in three cycles came to about 14.86 g/L, which is about 2.4 times the amount produced by the control (without any stabilizer additives) during the repeated batch bioconversion.

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