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Optimization of Medium Composition for L-phenylalanine Production from Glycerol using Response Surface Methodology

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ABSTRACT

L-phenylalanine was produced by genetically modified bacterium, *Escherichia coli* BL21(DE3), using glycerol as an alternative carbon source. Response surface methodology (RSM) involving central composite design (CCD) was adopted to evaluate the amount of L-phenylalanine produced. In this work, the optimum concentrations were determined of the major nutrients in the fermentation medium, which included glycerol, $(NH_4)_2SO_4$, $MgCl_2$, K_2HPO_4, KH_2PO_4, yeast extract and thiamine-HCl. Analysis using biomass weight (gL^{-1}) and amino acid production (gL^{-1}), indicated that the optimum medium composition and concentration for the biomass production were: glycerol 10 gL^{-1}, $(NH_4)_2SO_4$ 10 gL^{-1}, $MgCl_2$ 0.98 gL^{-1}, K_2HPO_4 2.94 gL^{-1}, KH_2PO_4 2.94 gL^{-1}, yeast extract 0.878 gL^{-1} and thiamine-HCL 0.0878 gL^{-1}, with a maximum biomass weight of 5.0 $gDCWL^{-1}$. In addition, the optimum medium composition for L-phenylalanine production was: glycerol 10 gL^{-1}, $(NH_4)_2SO_4$ 100 gL^{-1}, $MgCl_2$ 0.64 gL^{-1}, K_2HPO_4 1.91 gL^{-1}, KH_2PO_4 1.91 gL^{-1}, yeast extract 0.823 gL^{-1} and thiamine-HCl 0.0823 gL^{-1}. The highest L-phenylalanine weight at the optimum nutrient concentration was 6.2 gL^{-1}.

Key words: *Escherichia coli* BL21(DE3), glycerol, L-phenylalanine, fermentation, response surface methodology (RSM)

INTRODUCTION

Thailand was one of the countries that responded to the worldwide challenge to find alternative energy sources to petroleum, with its increasing price. Biodiesel was selected to be the first alternative energy because of the abundant resources within the country. Presently, hundreds of thousands of liters of biodiesel are produced daily, not only for commerce, but also for the household consumption. In the production process of transesterification, which is a popular dominant reaction, 10-25% of the byproduct, glycerol, is produced (Mu *et al*., 2006), depending on the completion of the reaction. However, glycerol production is expected to be more than 300,000 to 400,000 liters per day based on world dairy biodiesel product capacity (The Department of Alternative Energy Development and Efficiency (DEDE)).

The study of using glycerol as an alternative source of carbon in microbial

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fermentation is one of the remaining areas of glycerol reduction as a value-adding process (Barbiorato *et al*., 1997). Microbial and chemical conversion of various compounds of glycerol have been investigated recently, with particular focus on the production of amino acids, which can be used in medicine, cosmetics and food industries (Ohshima and Soda, 1989; Khamduang, 2004). The fermentation of glycerol to produce amino acids has been studied using *Escherichia coli* groups. Interestingly, the genetically modified *Escherichia coli* BL21(DE3), a high extracellular L-phenylalanine producer that can convert various carbohydrates to L-phenylalanine, was investigated (Nelson and Michael, 2000; Packdibamrung *et al*., 2007). Nevertheless, it was found that the recombinant *E. coli* mainly produced L-phenylalanine, when glycerol was used as the carbon source (Packdibamrung *et al*., 2007).

Statistical experimental design of L-phenylalanine production in batch fermentation was performed in this study to optimize the medium composition. Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions. This technique has been used successfully in the optimization of bioprocesses (Kwak *et al*., 2006; Nikerel *et al*., 2006). RSM mainly consists of central composite design, the Box-Behnken design, the one factor design, the D-optimal design, the user-defined design and the historical data design. The central composite design (CCD) and the Box-Behnken design (BBD) are the most popular techniques in RSM. For a particular design, different levels of one numeric factor are assigned, with five and three levels of one numeric factor being assigned for CCD and BBD, respectively (Imandi *et al*., 2006; Zain *et al*., 2007; Zheng *et al*., 2008).

The present study adopted RSM using CCD methods to optimize the medium components that affected the L-phenylalanine production and biomass concentration of the recombinant *E. coli* in batch fermentation.

MATERIALS AND METHODS

Microorganism

Escherichia coli BL2(DE3) (genotype: F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *gal dcm* (DE3)) was the host strain (Invitrogen Corporation, Carlsbad, CA, USA) harboring gene-encoding phenylalanine dehydrogenase from *Acinetobacter lwoffii*. The phenylalanine dehydrogenase gene was closed using pET-17b (Novagen; Merck KGaA, Darmstadt, Germany) as an expression vector. The expression of phenylalanine dehydrogenase by this strain was not affected by IPTG (isopropyl-β-D-thiogalactopyranoside) relative to the control (Sitthai, 2004; Packdibamrung *et al*., 2007). This recombinant strain had been constructed at the Department of Biochemistry, Faculty of Science, Chulalongkorn University, Thailand, and was used throughout the study (Sitthai, 2004).

Growth medium and culture conditions

The culture was maintained on Luria-Bertani (LB) agar slant containing 50 mgL^{-1} ampicillin. The pH of the medium was adjusted to 7.4 and the culture was incubated at 37°C for 24 h. Sub-culturing was carried out once every 4 weeks and the culture was stored at 4°C.

The basic culture medium for L-phenylalanine production contained trace elements of $FeSO_4$, $MnSO_4$, $CaCl_2$ and $ZnSO_4$ at concentrations of 0.002, 0.002, 0.05 and 0.01 gL^{-1}, respectively. Glycerol and $(NH_4)_2SO_4$ were used as carbon and nitrogen sources. $MgCl_2$, KH_2PO_4 and K_2HPO_4 were used as salts at a mixing ratio of 14.30% $MgCl_2$, 42.85% KH_2PO_4 and 42.85% K_2HPO_4). Yeast extract and thiamine-HCl were used as vitamins at a mixing ratio of 90.91% yeast extract and 9.09% thiamine-HCl. The $MgCl_2$ solution was sterilized separately. Prior to the inoculation, the pH of the sterilized

(121°C, 15 min) and cooled medium was adjusted to 7.4 by 3 M NaOH.

Recombinant cells were cultivated in 250 mL Erlenmeyer flasks containing 50 mL medium, the composition of which was specified according to the experimental design, with 50 mgL^{-1} ampicillin in an orbital shaker. Inoculum volume was 5% (v/v) of the 50 mL medium. The culture was incubated at 37°C at a rotational speed of 200 rpm for 32 h.

Analytical methods

Biomass concentration was determined by optical density at 600 nm (OD_{600}) and a calibration curve relating to the dry cell weight (DCW) to OD_{600} (1 unit of OD_{600} was equivalent to 1.72 g DCW L^{-1}). A culture broth sample was centrifuged at 10,000 × g for 10 min. The supernatant was then filtered through a syringe filter (0.2 μm pore size). L-phenylalanine was measured in the filtered supernatant.

L-phenylalanine in the culture supernatant was derivatized as follows: 50 μL of 1.5 M $NaHCO_3$ (pH 9.0) was added to a 110 μL aliquot of the supernatant. A 100 μL solution of dabsyl-chloride (2 $mg.mL^{-1}$ in acetone) was then added. The mixture was vortexed, then heated at 70°C for 10 min. The solution was then dried under vacuum and the solids were resuspended in 200 μL of 70 % ethanol. The resulting solution was centrifuged for 2 min at 14,000 × g, filtered through a syringe filter (0.2 μm pore size) and analyzed by HPLC (SUPELCO, LC-DABS column, 15 cm × 4.6 mm ID, 3 μm particles) at room temperature. The mobile phase consisted of a 70:30 v/v mixture of a phase A (25 mM potassium dihydrogen phosphate, pH 6.8) and a phase B (acetonitrile and 2-propanol, 75:25 v/v). The flow rate of the mobile phase was 1.0 mL. min^{-1}. The detection wavelength was 436 nm (Stocchi *et al*., 1985).

Experimental design

The growth medium contained the carbon source (glycerol), the inorganic nitrogen source ($(NH_4)_2SO_4$)), salts ($MgCl_2$, K_2HPO_4 and KH_2PO_4) and vitamins (yeast extract and thiamine-HCl). For the experimental design, three levels of each nutrient composition (low, medium and high) were specified, as shown in Table 1.

A 2^4 full factorial central composite design (CCD) with eight star points and seven replicates at the center points leading to 31 runs was employed for the optimization of the culture conditions (Table 2). For statistical calculations, the relationship between the coded values and actual values are described in Equation 1 (Prakash *et al*., 2007).

$$x_i = \frac{(X_i - X_0)}{\Delta X};\ i = 1,2,\ldots,\ k \qquad (1)$$

Where x_i is the code value of a variable, X_i the real value of a variable, X_0 the value of X_i at the center point, and ΔX is the step change of variable.

The 31 experiments were performed in triplicate.

A second-order polynomial, Equation 2, which included all interaction terms, was used to calculate the predicted response (Prakash *et al*., 2007).

Table 1 Levels of variables used in the experimental design.

Variables	levels		
	-1	0	+1
G, Glycerol (gL^{-1})	10.0	55.0	100.0
N, $(NH_4)_2SO_4$ (gL^{-1})	10.0	55.0	100.0
S, Salts (gL^{-1})	1.750	4.375	7.000
V, Vitamins (gL^{-1})	0.550	1.375	2.200

$$\hat{Y}_i = \beta_0 + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i,j=1}^{4} \beta_{ij} x_i x_j \qquad (2)$$

Where $\hat{Y}_i$ is the predicted response, β_0 the offset term, β_i the linear effect, β_{ii} the squared effect, β_{ij} the interaction effect and x_i, x_j are independent variables.

The proportion of variance explained by the polynomial models, is given by the multiple coefficient of determination, R^2. Analysis of variance, (ANOVA) was performed using the MINITAB software, version 15.0 (trial version).

Table 2 Experimental plan of the optimization design.

Runs	Concentration (gL^{-1})			
	Glycerol	$(NH^4)_2SO_4$	Salts	Vitamins
1	100.0	10.0	1.750	2.200
2	100.0	100.0	7.000	2.200
3	55.0	55.0	4.375	1.375
4	10.0	10.0	7.000	2.200
5	10.0	100.0	1.750	2.200
6	55.0	55.0	7.000	1.375
7	55.0	100.0	4.375	1.375
8	10.0	55.0	4.375	1.375
9	10.0	100.0	1.750	0.550
10	10.0	10.0	1.750	0.550
11	55.0	55.0	4.375	0.550
12	55.0	55.0	4.375	1.375
13	55.0	55.0	4.375	1.375
14	55.0	55.0	1.750	1.375
15	100.0	10.0	7.000	2.200
16	100.0	100.0	7.000	0.550
17	100.0	55.0	4.375	1.375
18	55.0	10.0	4.375	1.375
19	55.0	55.0	4.375	1.375
20	55.0	55.0	4.375	1.375
21	10.0	100.0	7.000	0.550
22	55.0	55.0	4.375	1.375
23	10.0	100.0	7.000	2.200
24	10.0	100.0	1.750	2.200
25	10.0	10.0	7.000	0.550
26	10.0	10.0	1.750	2.200
27	100.0	10.0	7.000	0.550
28	100.0	10.0	1.750	0.550
29	55.0	55.0	4.375	2.200
30	55.0	55.0	4.375	1.375
31	100.0	100.0	1.750	2.200

RESULTS AND DISCUSSION

Construction of the models

The effects of four variables on the L-phenylalanine and biomass productions were studied. The L-phenylalanine and biomass productions were selected as the responses due to the different cycles of the runs. The experimental design matrix is presented in Table 3.

By applying multiple regression analysis, Equation 3 and Equation 4 were proposed for the optimum nutrient compositions of the biomass and L-phenylalanine productions, respectively.

Table 3 Experimental and predicted values for biomass and L-phenylalanine production of recombinant *E.coli* BL21(DE3) cells in different media.

Run	Biomass (gL^{-1})		L-phenylalanine (gL^{-1})	
	Experimental	Predicted	Experimental	Predicted
1	4.350	4.341	1.020	1.038
2	4.445	4.494	3.466	2.761
3	4.305	4.438	3.248	3.674
4	4.897	5.000	4.090	3.597
5	4.659	4.736	5.979	5.812
6	4.504	4.542	2.542	3.218
7	4.504	4.291	3.777	4.342
8	4.798	4.721	5.007	5.504
9	4.038	4.153	5.731	5.554
10	4.794	4.746	2.169	2.465
11	4.397	4.360	3.141	3.280
12	4.440	4.438	4.180	3.674
13	4.452	4.438	3.405	3.674
14	4.452	4.354	3.468	3.204
15	4.487	4.390	0.931	1.414
16	4.073	4.158	3.320	3.592
17	4.288	4.304	3.528	3.443
18	4.383	4.536	2.278	2.124
19	4.340	4.438	4.489	3.674
20	4.366	4.438	3.983	3.674
21	4.469	4.480	5.632	5.206
22	4.383	4.438	3.703	3.674
23	4.987	4.915	5.876	6.219
24	4.357	4.414	1.814	2.026
25	5.082	5.043	1.664	1.758
26	4.920	4.852	3.512	3.548
27	4.607	4.531	1.662	1.420
28	4.245	4.334	1.836	1.799
29	4.604	4.581	3.133	3.406
30	4.589	4.438	3.944	3.674
31	4.031	3.929	3.529	3.613

$$Y_{Biomass}\ (gL^{-1}) = 4.82395 - 0.08181G - 0.07404N + 0.01905S - 0.00723V + 0.00372G^2 - 0.00119N^2 + 0.00018S^2 + 0.00059V^2 + 0.00233GN - 0.00073GS - 0.00073GV + 0.00023NS + 0.00354NV - 0.00066SV \quad \ldots\ldots (3)$$

$$Y_{L\text{-}phe}\ (gL^{-1}) = 0.830645 - 0.436399G + 0.615735N + 0.136773S + 0.222248V + 0.039486G^2 - 0.021768N^2 - 0.00823S^2 - 0.005883V^2 - 0.015750GN - 0.002428GS - 0.013661GV + 0.002653NS - 0.00611NV - 0.003358SV \quad \ldots\ldots\ldots (4)$$

When the values of G, N, S and V were substituted in Equations 3 and 4, these equations could be used to predict the biomass and L-phenylalanine concentrations as shown in Table 3.

The significance of each coefficient was determined by *p*-values, which are listed in Table 4. The smaller the *p*-value ($p \leq 0.05$), the more significant is the corresponding coefficient. Table 4 shows that the interaction effect of (NH4)2SO4 and vitamins (NV) was significant in the biomass production, while for L-phenylalanine, the two first orders (G and N), one second order (G2) and two interactions (GN and GV) were found to be significant. The parity plot (Figure 1) showed a satisfactory correlation between the experimental and the predicted values of the biomass and L-phenylalanine productions. The pointsed cluster around the diagonal line indicated a good relationship between the experimental and predicted values.

The results of the second order response-surface model in the form of analysis of variance (ANOVA) are given in Table 5. The lack of fit was tested by comparing the value of $F = MS_{lack\ of\ fit}/MS_{pure\ error}$ in Table 5 to a suitable upper percentage point of $F\ (0.05, DF_{lack\ of\ fit}, DF_{pure\ error})$ in the distribution table. A larger value of *F* in the distribution table indicates the model provides a good fit (Box and Draper, 2007). In this case, the *F*-value from the distribution table for biomass and L-phenylalanine was 4.06, while the calculated values were 2.06 and 1.63. So the models provide a good fit with the results from the experiment. In addition, Table 5 shows that the least square values for the experimental and predicted data (R^2) for biomass and L-phenylalanine productions were 0.89 and 0.93, respectively. The values of the

Table 4 Model coefficients estimated by multiple linear regression.

Parameters	Coefficient of biomass	Coefficient of L-phenylalanine	*P*-value of biomass	*P*-value of L-phenylalanine
constant	4.82395	0.830645	0.000	0.390
G	-0.08181	-0.436399	0.088	0.035
N	-0.07404	0.615735	0.120	0.005
S	0.01905	0.136773	0.594	0.368
V	-0.00723	0.222248	0.839	0.152
G^2	0.00372	0.039486	0.332	0.023
N^2	-0.00119	-0.021768	0.753	0.184
S^2	0.00018	-0.008230	0.892	0.164
V^2	0.00059	-0.005883	0.668	0.312
GN	0.00233	-0.015750	0.139	0.024
GS	-0.00073	0.002428	0.426	0.530
GV	-0.00073	-0.013661	0.426	0.002
NS	0.00023	0.002653	0.801	0.493
NV	0.00354	-0.006110	0.001	0.126
SV	-0.00066	0.003358	0.239	0.159

G = glycerol; N = $(NH_4)_2SO_4$; S = salts; V = vitamins.

adjusted determination coefficient (adjusted R^2 = 0.78 for biomass and adjusted R^2 = 0.86 for L-phenylalanine) also supported the significance of the goodness of fit of the models. The high values of the correlation coefficient (R^2 = 0.89 for biomass and R^2 = 0.93 for L-phenylalanine) indicated a good correlation between the independent variables. The predicted optimum levels for the glycerol, $(NH_4)_2SO_4$, salts and vitamins were obtained by applying the regression analysis to Equations 3 and 4. The same equations were also used to predict the biomass and L-phenylalanine productions at the optimum level of each medium's components.

Optimization of medium

The full quadratic model equations were optimized using simultaneous optimization technique (Myers and Montgomery, 2002) that were included in the Response Optimizer function in the MINITAB program to maximize the biomass and L-phenylalanine concentrations. The optimum composition of the medium for biomass production was found to be: 10 gL^{-1} glycerol, 10 gL^{-1} $(NH_4)_2SO_4$, 0.98 gL^{-1} $MgCl_2$, 2.94 gL^{-1} K_2HPO_4, 2.94 gL^{-1} KH_2PO_4, 0.878 gL^{-1} yeast extract and 0.0878 gL^{-1} thiamine-HCl with a prediction of 5.0 g $DCWL^{-1}$ for biomass production. The optimum composition of the

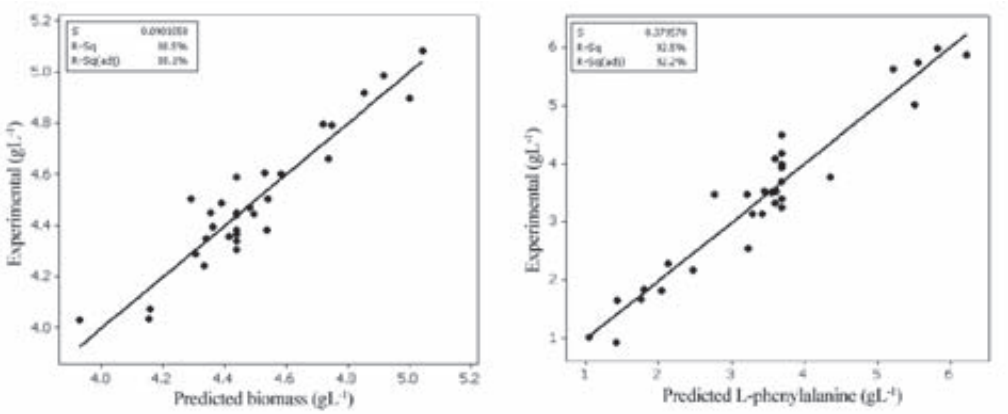

Figure 1 Plotted values of predicted versus experimental biomass and L-phenylalanine.

Table 5 ANOVA for full quadratic models.

Source	DF	SS	MS	*F*-value	*P*-value
Model *(Biomass)*	14	1.80347	0.128819	8.75	0.000
Residual Error *(Biomass)*	16	0.23542	0.014714		
Lack-of-Fit *(Biomass)*	10	0.18233	0.018233	2.06	0.195
Pure Error *(Biomass)*	6	0.05309	0.008849		
Total *(Biomass)*	30	2.03889			
Model *(L-phe)*	14	51.220	3.6586	14.01	0.000
Residual Error *(L-phe)*	16	4.178	0.2611		
Lack-of-Fit *(L-phe)*	10	3.052	0.3052	1.63	0.285
Pure Error *(L-phe))*	6	1.126	0.1877		
Total *(L-phe)*	30	55.398			

Biomass R^2 = 0.89; Biomass adjusted R^2 = 0.78

L-phenylalanine R^2 = 0.93; L-phenylalanine adjusted R^2 = 0.86

medium for L-phenylalanine production was found to be: 10 gL^{-1} glycerol, 100 gL^{-1} $(NH_4)_2SO_4$, 0.64 gL^{-1} $MgCl_2$, 1.91 gL^{-1} K_2HPO_4, 1.91 gL^{-1} KH_2PO_4, 0.823 gL^{-1} yeast extract and 0.0823 gL^{-1} thiamine-HCl with a prediction for L-phenylalanine production of 6.2 gL^{-1}.

Response surface analysis

The effects of the four medium components on the biomass and L-phenylalanine concentrations are given in Figures 2 and 3. Figure 2 represents the model and Equation 3 implies for biomass production. The decrease in glycerol and $(NH_4)_2SO_4$ increased the biomass production; however, a further decrease of salts and vitamins concentrations reversed the trend. Figure 3, representing the model and Equation 4, shows the relative effect of glycerol, $(NH_4)_2SO_4$, salts and vitamins on L-phenylalanine production. The L-phenylalanine weight increased with an increase in $(NH_4)_2SO_4$ concentration. On the other hand, high concentration of glycerol reduced the L-phenylalanine production. An increase of salts with the concentration of the vitamins up to the optimum point increased the L-phenylalanine production to a maximum level and any further

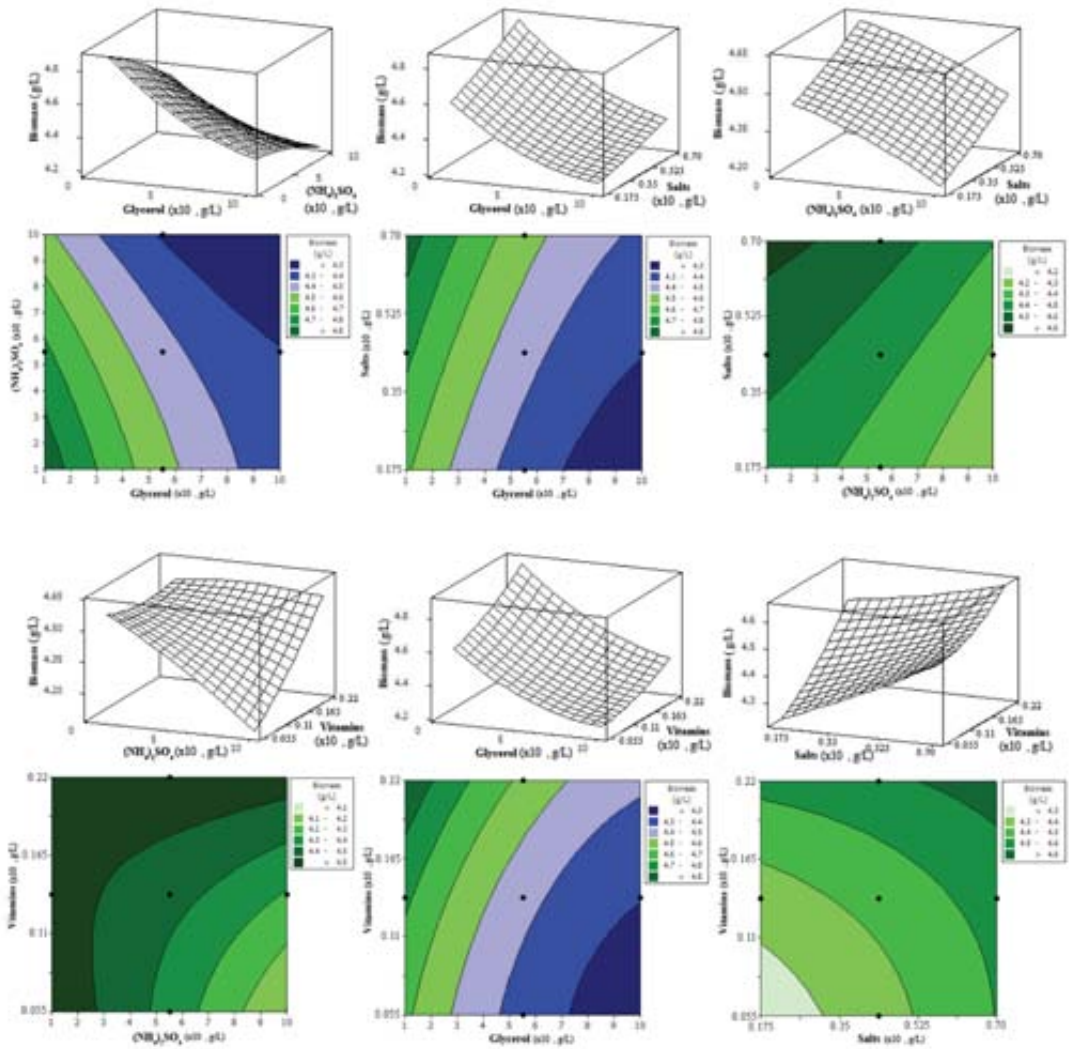

Figure 2 Response surface and contour plot of independent variables on biomass production.

increase of salts with vitamin concentration decreased the L-phenylalanine production.

CONCLUSIONS

The submerged fermentation process seemed to be the preferred mass production method for obtaining large quantities of the recombinant *E. coli* required for commercial application. The present study using RSM with CCD enabled the determination of the optimal medium constituents for the productions of biomass and L-phenylalanine. The validity of the model was proven by fitting the values of the variables in a second order polynomial equation and by actually carrying out the experiment at those predicted values for the four independent variables of glycerol, $(NH_4)_2SO_4$, salts and vitamin concentrations. All four variables tested for the correlation between their concentrations and the productions of biomass and L-phenylalanine showed significant influence on the production. The maximum amounts of biomass and L phenylalanine produced from glycerol were predicted to be 5.00 gL^{-1} and 6.20 gL^{-1}, respectively when the optimized medium

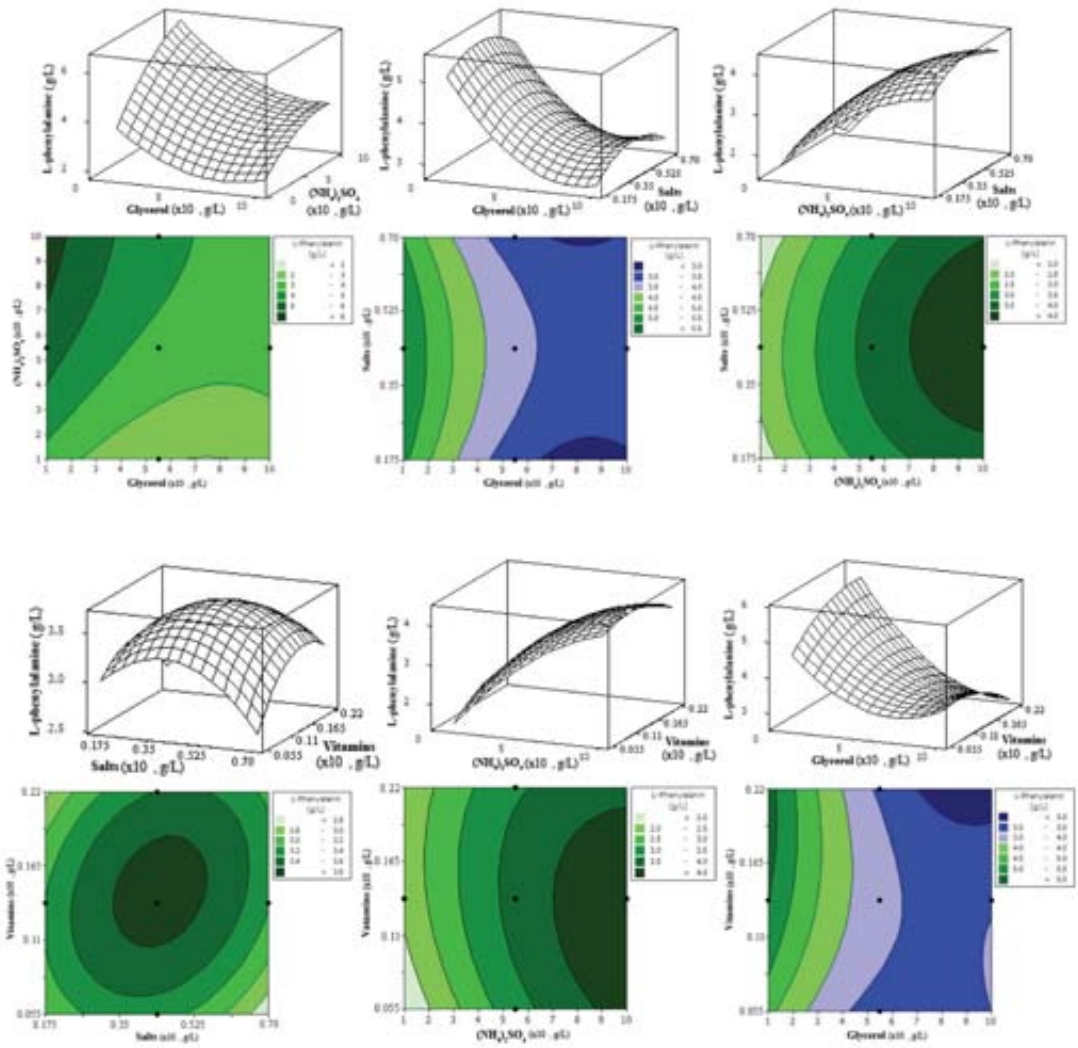

Figure 3 Response surface and contour plot of independent variables on L-phenylalanine production.

constituents of the fermentation medium were set at: glycerol 10 gL^{-1}, $(NH_4)_2SO_4$ 10 gL^{-1}, salts 6.867 gL^{-1}, vitamins 0.966 gL^{-1} and glycerol 10 gL^{-1}, $(NH_4)_2SO_4$ 100 gL^{-1}, salts 4.460 gL^{-1} and vitamins 0.905 gL^{-1}, respectively. The methodology as a whole proved to be adequate for the design and optimization of the fermentation process.

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