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Optimized Production of L-phenylalanine by Fermentation Using Crude Glycerol

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ABSTRACT

Crude glycerol, a by-product of the commercial production of biodiesel, was used directly as the carbon source to produce the essential amino acid L-phenylalanine in an optimized fermentation process involving the novel recombinant bacterium *Escherichia coli* BL21(DE3). In developing the optimal process, a Plackett-Burman experimental design was used to identify the components of the medium that most affected the production of the cell mass and L-phenylalanine. For the specified carbon source and with ammonium sulfate as the nitrogen source, the media components that were the most influential were: KH_2PO_4, K_2HPO_4, NaCl, $MgCl_2$, $CaCl_2$, $CoCl_2$, $CuSO_4$, Na_2MoO_4, $ZnSO_4$ and yeast extract. A three-level and five-factor central composite experimental design was then used to identify the optimal concentrations of the most influential components of the medium and the optimal duration of the fermentation. Under the optimal combination of conditions for biomass production, a biomass concentration of 1.23 g/L was achieved in 32.43 h. Similarly, under the optimal conditions for production of L-phenylalanine, a metabolite concentration of 1.03 g/L was achieved in 30.29 h. A high correlation between the models' predicted responses and the actual responses achieved in independent validation experiments confirmed the validity of the models.

Keywords: *Escherichia coli* BL21(DE3), glycerol, L-phenylalanine, response surface methodology, medium optimization

INTRODUCTION

The cost of the carbon source is a significant contributor to the cost of production of many fermentation products (Unden and Bongaerts, 1997; Peters, 2007). Molasses, a by-product of the production of sugar, is one of the most commonly used carbon sources in microbial fermentation media. Glycerol, a potential alternative carbon source, is recognized as an inexpensive material that is becoming available in huge quantities as a consequence of the rapid growth in production of biodiesel from vegetable oils (Demirbas, 2009; Moser, 2009). For each tonne of biodiesel produced from vegetable oil, nearly 100 kg of crude glycerol is produced (Chisti, 2007; da Silva *et al.*, 2009; Khamduang *et al.*, 2009a; Jun *et al.*, 2010). Therefore, the utilization

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of glycerol is needed to be explored. In some fermentation processes, crude glycerol can potentially displace molasses, sucrose and glucose. Glycerol has indeed been used as a carbon source in several fermentation processes (Andrade and Vasconcelos, 2003; Khamduang *et al*., 2009a; da Silva *et al*., 2009; Jun *et al*., 2010; Moon *et al*., 2010; Wu *et al*., 2010), but its purification significantly adds to the cost. The present study discusses the optimized production of the essential amino acid L-phenylalanine ($HO_2CCH(NH_2)CH_2C_6H_5$) by a recombinant *Escherichia coli* using crude glycerol as produced in large-scale commercial processes for making biodiesel.

L-phenylalanine is a nutritional supplement and a precursor for the production of various catecholamines and the artificial sweetener aspartame. Commercially, L-phenylalanine is mostly produced by fermentation processes involving recombinant strains of the bacterium *E. coli* (Leuchtenberger *et al*., 2005; Khamduang *et al*., 2009b). The carbon source used in commercial production of L-phenylalanine is sucrose, although a genetically modified *E. coli* has been shown to produce this amino acid using purified glycerol (Khamduang *et al*., 2009b).

Crude glycerol (derived from the alkali-catalyzed production of biodiesel from palm oil and methanol) was used without any prior purification to produce L-phenylalanine using the recombinant *E. coli* BL21(DE3) in shake flasks. A statistical design of the culture experiments was used to effectively optimize this fermentation process. First, a Plackett-Burman screening design was applied to identify the most significant components of the medium that affected the production of L-phenylalanine and the bacterial biomass (Krishnan *et al*., 1998). In the second stage, a central composite design was applied to determine the optimum level or magnitude of each significant influential factor (Li *et al*., 2008). Mathematical models were devised for the relationship between the influencing factors and the production of L-phenylalanine and the biomass. A novel recombinant *E. coli* was used (Khamduang *et al*., 2009b) to validate this model.

MATERIALS AND METHODS

Microorganism

Escherichia coli BL21(DE3) (genotype: F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *gal dcm* (DE3)) was the host strain (Invitrogen Corporation, Carlsbad, CA, USA) used to express the phenylalanine dehydrogenase gene of *Acinetobacter lwoffii* (Khamduang *et al*., 2009a,b).

Crude glycerol

Crude glycerol (approximately 75–80% w/w glycerol) was obtained from the Patum Vegetable Oil Co. Ltd (Bangkok, Thailand), a producer of biodiesel. This glycerol was a by-product of the alkali-catalyzed (NaOH) transesterification of palm oil with methanol to make biodiesel. The impurities in the crude glycerol were methanol (approximately 1–2% w/w), soap (approximately 13–15% w/w) and ash (approximately 3–4% w/w).

Growth medium and culture conditions

A stock culture of recombinant *E. coli* was maintained on Luria-Bertani (LB) agar slants containing 50 mg/L ampicillin. The pH of the medium was adjusted to 7.4 prior to autoclaving (121 °C, 15 min). Slants were incubated at 37 °C for 24 h and then stored at 4 °C. Sub-culturing was carried out once every 4 wk (Khamduang *et al*., 2009a,b).

A loopful of culture from the LB medium slant was transferred aseptically to 50 mL of LB broth and incubated for 18 h in a rotary shaker at 200 rpm and 37 °C. The cells were harvested by centrifugation (10,000×g, 10 min) and washed three times with sterile distilled water (100 mL per wash). A 0.5 mL portion of the washed cell suspension was used to inoculate 50 mL of sterile fermentation medium dispensed in a 250 mL Erlenmeyer flask. Unless otherwise indicated,

prior to inoculation, the flasks had been autoclaved with the medium (121 °C, 20 min) and cooled to the incubation temperature (37 °C). The culture flasks were incubated for 24 h. The culture medium used in the flasks varied in accordance with the experimental design matrix, as discussed later. All experiments were performed in triplicate and the data were averaged.

Analytical methods

The biomass concentration in the culture broth was determined by gravimetry (Khamduang *et al*., 2009a, b).

L-phenylalanine was measured by high performance liquid chromatography (HPLC; ACE, C18 column, 150×4.6 mm, 5 mm particles) at room temperature. The mobile phase consisted of a 50:50 v/v mixture of a phase A (10 mM potassium dihydrogen phosphate, pH 6.55) and a phase B (acetonitrile and 2-propanol, 75:25 v/v). The mobile phase flow rate was 1.0 mL/min. The detection wavelength was 436 nm (Khamduang *et al*., 2009a,b).

Plackett-Burman design

A Plackett-Burman experimental design was used to screen the components of the medium that had the greatest impact on production of the biomass and L-phenylalanine. For screening experiments, the concentrations of the carbon source (that is, crude glycerol) and the nitrogen source ($(NH_4)_2SO_4$) were fixed at 10 g/L each (Khamduang *et al*., 2009b). Concentrations of the other fifteen components ($X_{1–15}$) varied as in Table 1 in accordance with a Plackett-Burman design (Plackett and Burman, 1946). All trials were performed in triplicate. Average responses, that is, the concentrations of L-phenylalanine and the biomass, are reported for each run in Table 1. The main effect of each variable (or factor) was calculated as the difference between the average of measurements made at the high setting (+1 in Table 1) and the average of the measurements made at the low setting (-1 in Table 1) of that factor.

A Plackett-Burman experimental design is based on a first-order model (Equation 1):

$$Z = b_0 + \sum b_i x_i \quad (1)$$

where Z is the response (that is, L-phenylalanine and biomass concentrations), b_0 is the model intercept, b_i is the linear coefficient of the model and x_i is the level of an independent variable.

This model does not account for interactions among factors and it is used simply to screen and evaluate the important factors that influence the response. The variables whose confidence levels were higher than 85% were considered to significantly influence the production of L-phenylalanine and biomass.

Central composite design

The factors that were identified by the Plackett-Burman design to significantly influence the production of L-phenylalanine and the biomass were optimized using the central composite design, which is a response surface method (Myers and Montgomery, 2002; Box and Draper, 2007). Two additional factors were included in the optimization as mentioned later. A total of five factors were therefore optimized. These were: the concentration of crude glycerol; the concentration of ammonium sulfate; the concentration of the mixed salts (the mixture had a fixed composition by weight percent of 26.32% KH_2PO_4, 26.32% K_2HPO_4, 38.59% NaCl, and 8.77% $MgCl_2$); the concentration of the vitamins and trace elements mixture (the mixture had a fixed composition by weight percent of 3.75% $CaCl_2$, 0.24% $CoCl_2$, 0.76% $ZnSO_4$, 0.43% $CuSO_4$, 0.46% Na_2MoO_4, and 94.35% yeast extract); and the length of the fermentation period. For these five factors, the experiments were conducted using a 2^{5-1} factorial design with ten star points and four replicates at the center points (2.0000 for α rotatability and 1.5467 for α orthogonality). This resulted in a total of 30 experimental runs as identified in Table 2. The responses, that is, the concentrations of L-

Table 1 Plackett-Burman design for screening the media components affecting the production of L-phenylalanine and the bacterial biomass[a].

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	L-phenylalanine (g/L)	Biomass (g/L)
1	-1	-1	-1	-1	1	1	1	1	1	1	-1	-1	-1	-1	1	0.88	0.83
2	1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	1	-1	-1	0.25	0.78
3	-1	1	-1	-1	-1	1	1	-1	-1	1	1	1	-1	1	-1	0.21	0.58
4	1	1	-1	-1	1	-1	-1	-1	-1	1	-1	-1	1	1	1	0.92	0.68
5	-1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	1	-1	0.21	0.52
6	1	-1	1	-1	-1	1	-1	-1	1	-1	-1	1	-1	1	1	1.25	0.67
7	-1	1	1	-1	-1	-1	1	1	-1	-1	-1	1	1	-1	1	0.88	0.83
8	1	1	1	-1	1	1	-1	1	-1	-1	1	-1	-1	-1	-1	0.54	0.78
9	-1	-1	-1	1	1	1	-1	1	-1	-1	-1	1	1	1	-1	0.20	0.35
10	1	-1	-1	1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1.00	0.88
11	-1	1	-1	1	-1	1	-1	-1	1	-1	1	-1	1	-1	1	0.96	0.82
12	1	1	-1	1	1	-1	1	-1	1	-1	-1	1	-1	-1	-1	0.46	0.76
13	-1	-1	1	1	1	-1	-1	-1	-1	1	1	1	-1	-1	1	1.38	0.64
14	1	-1	1	1	-1	1	1	-1	-1	1	-1	-1	1	-1	-1	0.34	0.65
15	-1	1	1	1	-1	-1	-1	1	1	1	-1	-1	-1	1	-1	0.52	0.82
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.04	1.05
17	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.23	0.35
18	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.21	0.41

[a] (-1) indicates a zero concentration; (+1) indicates the following concentrations: 1.875 g/L KH_2PO_4 (X_1), 1.875 g/L K_2HPO_4 (X_2), 0.625 g/L $MgCl_2$ (X_3), 2.75 g/L NaCl (X_4), 0.002 g/L $FeSO_4$ (X_5), 0.002 g/L $MnSO_4$ (X_6), 0.05 g/L $CaCl_2$ (X_7), 0.01 g/L $ZnSO_4$ (X_8), 0.003 g/L $CoCl_2$ (X_9), 0.0055 g/L $CuSO_4$ (X_{10}), 0.006 g/L Na_2MoO_4 (X_{11}), 0.00525 g/L H_3BO_3 (X_{12}), 0.125 g/L thiamine-HCl (X_{13}), 0.0125 g/L biotin (X_{14}) and 1.25 g/L yeast extract (X_{15}).

Table 2 Central composite design for five independent variables[a].

Run	Variables					Results	
	Crude glycerol (g/L)	$(NH_4)_2SO_4$ (g/L)	Salts[b] (g/L)	Vitamins and trace elements[c] (g/L)	Incubation time (h)	L-phenylalanine (g/L)	Biomass (g/L)
1	1.0 (-1)	1.0 (-1)	1.778 (-1)	0.331 (-1)	36 (1)	0.80	0.14
2	1.0 (-1)	1.0 (-1)	1.778 (-1)	2.318 (1)	12 (-1)	0.80	0.09
3	1.0 (-1)	1.0 (-1)	12.467 (1)	0.331 (-1)	12 (-1)	0.61	0.18
4	1.0 (-1)	1.0 (-1)	12.467 (1)	2.318 (1)	36 (1)	0.58	0.15
5	1.0 (-1)	50.0 (1)	1.778 (-1)	0.331 (-1)	12 (-1)	0.71	0.11
6	1.0 (-1)	50.0 (1)	1.778 (-1)	2.318 (1)	36 (1)	0.86	0.18
7	1.0 (-1)	50.0 (1)	12.467 (1)	0.331 (-1)	36 (1)	0.63	0.35
8	1.0 (-1)	50.0 (1)	12.467 (1)	2.318 (1)	12 (-1)	0.68	0.21
9	50.0 (1)	1.0 (-1)	1.778 (-1)	0.331 (-1)	12 (-1)	0.78	0.71
10	50.0 (1)	1.0 (-1)	1.778 (-1)	2.318 (1)	36 (1)	0.94	1.43
11	50.0 (1)	1.0 (-1)	12.467 (1)	0.331 (-1)	36 (1)	0.76	1.21
12	50.0 (1)	1.0 (-1)	12.467 (1)	2.318 (1)	12 (-1)	0.64	0.84
13	50.0 (1)	50.0 (1)	1.778 (-1)	0.331 (-1)	36 (1)	0.52	1.07
14	50.0 (1)	50.0 (1)	1.778 (-1)	2.318 (1)	12 (-1)	0.45	0.79
15	50.0 (1)	50.0 (1)	12.467 (1)	0.331 (-1)	12 (-1)	0.40	0.84
16	50.0 (1)	50.0 (1)	12.467 (1)	2.318 (1)	36 (1)	0.49	1.15
17	25.5 (0)	25.5 (0)	7.125 (0)	1.325 (0)	24 (0)	0.94	1.07
18	25.5 (0)	25.5 (0)	7.125 (0)	1.325 (0)	24 (0)	0.99	0.97
19	1.0 (-1)	25.5 (0)	7.125 (0)	1.325 (0)	24 (0)	0.80	0.42
20	50.0 (1)	25.5 (0)	7.125 (0)	1.325 (0)	24 (0)	0.80	1.45
21	25.5 (0)	1.0 (-1)	7.125 (0)	1.325 (0)	24 (0)	0.61	1.27
22	25.5 (0)	50.0 (1)	7.125 (0)	1.325 (0)	24 (0)	0.58	1.24
23	25.5 (0)	25.5 (0)	1.778 (-1)	1.325 (0)	24 (0)	0.71	1.16
24	25.5 (0)	25.5 (0)	12.467 (1)	1.325 (0)	24 (0)	0.86	1.34
25	25.5 (0)	25.5 (0)	7.125 (0)	0.331 (-1)	24 (0)	0.63	1.13
26	25.5 (0)	25.5 (0)	7.125 (0)	2.318 (1)	24 (0)	0.68	1.09
27	25.5 (0)	25.5 (0)	7.125 (0)	1.325 (0)	12 (-1)	0.78	0.97
28	25.5 (0)	25.5 (0)	7.125 (0)	1.325 (0)	36 (1)	0.94	1.18
29	25.5 (0)	25.5 (0)	7.125 (0)	1.325 (0)	24 (0)	0.76	1.16
30	25.5 (0)	25.5 (0)	7.125 (0)	1.325 (0)	24 (0)	0.64	1.10

[a] The variables were: glycerol concentration, ammonium sulfate concentration, concentration of the salt mixture, concentration of the vitamins and trace elements mix, and the duration of the fermentation; (-1) indicates the low level; (1) indicates the high level; (0) indicates the base level.

[b] Included KH_2PO_4, K_2HPO_4, NaCl and $MgCl_2$.

[c] Included $CaCl_2$, $CoCl_2$, $ZnSO_4$, $CuSO_4$, Na_2MoO_4 and yeast extract.

phenylalanine and the biomass, were assessed using a second-order polynomial equation as shown in Equation 2:

$$Z = b_0 + \sum_{i=1}^{j} b_i x_i + \sum_{i}^{j} b_{ii} x^2 + \sum_{i<j}^{j} b_{ij} x_i x_j \quad (2)$$

where Z is the predicted response, b_0 the intercept term, b_i is the linear coefficient, b_{ii} is the squared coefficient, and b_{ij} is the interaction coefficient.

The data were fitted to Equation 2 using the multiple regression procedure.

The values of the various coefficients and the optimum concentrations were calculated using the Statistica software (StatSoft Inc., Tulsa, OK, USA). The quality of fit of the model equations was characterized by values of the coefficient of determination, R^2. Experiments were performed in triplicate and the mean values were reported.

RESULTS AND DISCUSSION

Identification of the most significant medium constituents

The basal medium (Table 1) had the following composition (g/L): 10 crude glycerol, 10 $(NH_4)_2SO_4$, 1.875 KH_2PO_4, 1.875 K_2HPO_4, 0.625 $MgCl_2$, 2.75 NaCl, 0.002 $FeSO_4$, 0.002 $MnSO_4$, 0.05 $CaCl_2$, 0.01 $ZnSO_4$, 0.003 $CoCl_2$, 0.0055 $CuSO_4$, 0.006 Na_2MoO_4, 0.00525 H_3BO_3, 0.125 thiamine-HCl, 0.0125 biotin, and 1.25 yeast extract. The initial pH was adjusted to 7.4. Eighteen different media compositions based on the basal medium, were experimented with as identified in Table 1. In different runs, the media had the various pure components (all components except crude glycerol) except the nitrogen source set at a concentration of zero, represented as the -1 level in Table 1.

The final concentrations of L-phenylalanine and the biomass obtained in these Plackett-Burman screening experiments are shown in Table 1. The concentrations varied widely from 0.2 to 1.38 g/L for L-phenylalanine and from 0.35 to 1.05 g/L for the biomass. These variations reflect the importance of medium optimization for attaining high productivity. The calculated main effects (Table 3) of the examined factors on the production of L-phenylalanine and the biomass are shown in Figure 1. A *t*-test was used to identify the factors with positive main effects (Table 3).

The factors with a positive main effect refer to higher yield. The main components affecting the production of L-phenylalanine were: KH_2PO_4, K_2HPO_4, $MgCl_2$, NaCl, $FeSO_4$, $CoCl_2$, $CuSO_4$, Na_2MoO_4, H_3BO_3 and the yeast extract. Of these, KH_2PO_4, $MgCl_2$, NaCl and the yeast extract were the most significant components in enhancing the production of L-phenylalanine. The factors that had a substantial main effect on the production of biomass were: KH_2PO_4, K_2HPO_4, NaCl, $MgCl_2$, $CoCl_2$, $ZnSO_4$, $CaCl_2$, $CuSO_4$, Na_2MoO_4 and the yeast extract.

The *t*-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. In other studies, confidence levels greater than 70% have been considered as acceptable (Stowe and Mayer, 1966); however, in the present work only the factors with a confidence level greater than 85% were considered as having a significant effect on the outcome. The insignificant factors were neglected and the concentration of L-phenylalanine ($Y_{L\text{-phe}}$, g/L) was correlated only to the significant factors, as shown in Equation 3:

$$Y_{L\text{-phe}} = 22.811 + 1.336[KH_2PO_4] + 2.795[MgCl_2] - 1.941[CaCl_2] + 1.767[NaCl] - 2.819[\text{thiamine-HCl}] + 11.783[\text{yeast extract}] \quad (3)$$

Similarly, the concentration of the biomass ($Y_{biomass}$, g/L) was correlated to the significant factors, as shown in Equation 4:

$$Y_{biomass} = 0.727 + 0.055[KH_2PO_4] + 0.064[KH_2PO_4] + 0.02[NaCl] + 0.025[FeSO_4] + 0.036[CaCl_2] + 0.064[ZnSO_4] + 0.055[CoCl_2] + 0.027[CuSO_4] + 0.03[Na_2MoO_4] - 0.019[H_3BO_3] - 0.033[\text{biotin}] + 0.074[\text{yeast extract}] \quad (4)$$

The results of the Plackett-Burman

Table 3 Statistical analysis of Plackett-Burman design[a] .

Factor	Effect		Coefficients		*t*-value		*P*-value ($P \leq 0.15$)	
	L-phenylalanine	Biomass	L-phenylalanine	Biomass	L-phenylalanine	Biomass	L-phenylalanine	Biomass
Mean/Intercept	22.8115	0.7265	22.8115	0.7265	39.426	89.161	0.0006	0.0001
KH_2PO_4	2.6712	0.1096	1.3356	0.0546	2.309	6.725	0.1473	0.0214
K_2HPO_4	0.3399	0.1271	0.1700	0.0635	0.294	7.799	0.7967	0.0160
$MgCl_2$	5.5844	0.0371	2.7922	0.0185	4.826	2.276	0.0404	0.1506
NaCl	3.5335	0.0396	1.7668	0.0198	3.054	2.429	0.0926	0.1358
$FeSO_4$	1.2664	-0.0504	0.6332	-0.0252	1.094	-3.094	0.3880	0.0905
$MnSO_4$	-0.4783	-0.0204	-0.2391	-0.0102	-0.413	-1.253	0.7195	0.3369
$CaCl_2$	-3.8825	0.0721	-1.9412	0.0360	-3.355	0.424	0.0886	0.0475
$ZnSO_4$	-1.4627	0.1271	-0.7314	0.0635	-1.264	7.799	0.3336	0.0160
$CoCl_2$	0.8187	0.1096	0.4094	0.0548	0.708	6.725	0.5526	0.0214
$CuSO_4$	0.4656	0.0546	0.2328	0.0273	0.402	3.350	0.7264	0.0787
Na_2MoO_4	0.8960	0.0596	0.4480	0.0298	0.774	3.656	0.5198	0.0673
H_3BO_3	1.6791	-0.0379	0.8395	-0.0190	1.451	-2.327	0.2839	0.1455
Thiamine-HCl	-5.6377	-0.0329	-2.8188	-0.0165	-4.872	-2.020	0.0396	0.1808
Biotin	-1.1190	-0.0654	-0.5595	-0.0327	-0.967	-4.014	0.4356	0.0568
Yeast extract	23.5660	0.1471	11.7830	0.0735	20.365	9.026	0.0024	0.0121

[a] R^2 (adjusted) for L-phenylalanine was 0.968; R^2 (adjusted) for biomass was 0.969.

experiment revealed the main components of the medium that influenced the production of L-phenylalanine and the biomass. Subsequently, a response surface analysis based on a central composite design was performed to optimize the levels of the factors that had the most influence on the outcomes of this fermentation.

Optimization by central composite design

Based on the Plackett-Burman design, the five most influential variables (including the crude glycerol level and the duration of the fermentation) were the concentrations of ammonium sulfate, the mixed salts (KH_2PO_4, K_2HPO_4, NaCl, $MgCl_2$) and the vitamins and trace elements mixture ($CaCl_2$, $CoCl_2$, $ZnSO_4$, $CuSO_4$, Na_2MoO_4, yeast extract). The interactions of these variables were studied using a central composite design within the ranges of the concentrations used in the Plackett-Burman design. The design matrix and the corresponding experimental data are shown in Table 2. Each run was performed in triplicate and the average values were reported (Table 2).

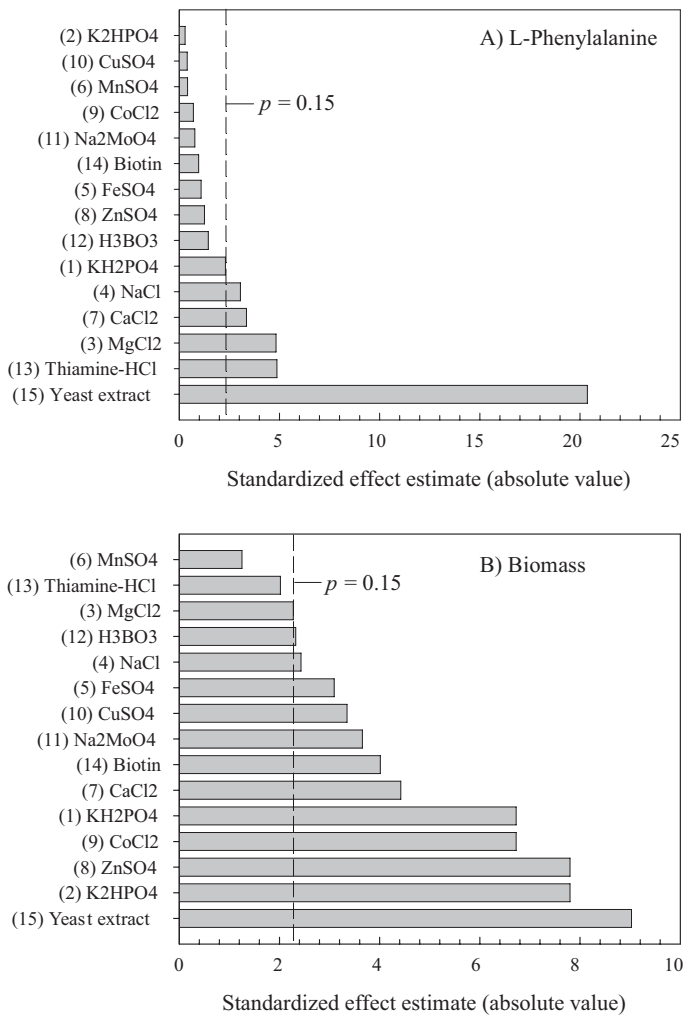

Figure 1 Effect of nutritional factors on L-phenylalanine (A) and biomass (B) production based on the results of Plackett-Burman design.

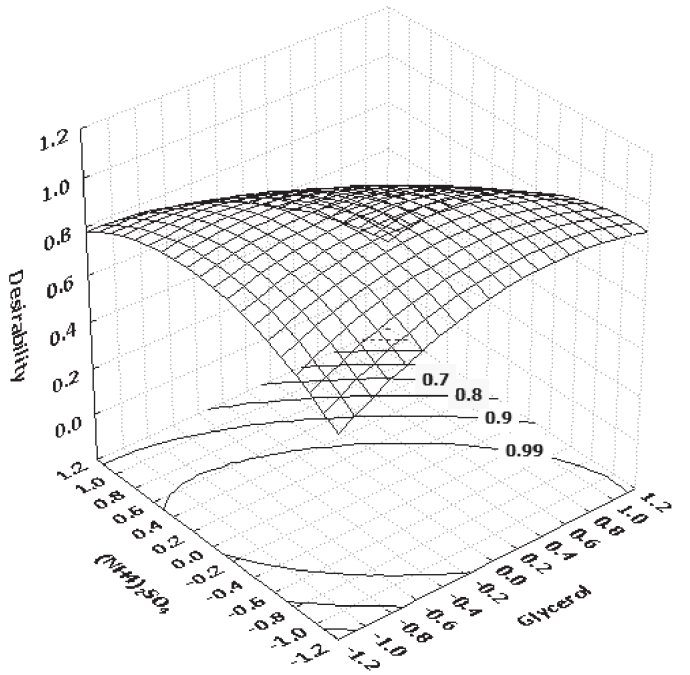

Figure 2 L-phenylalanine production response surfaces significantly affected ($P \leq 0.05$) by the crude glycerol and ammonium sulfate concentration.

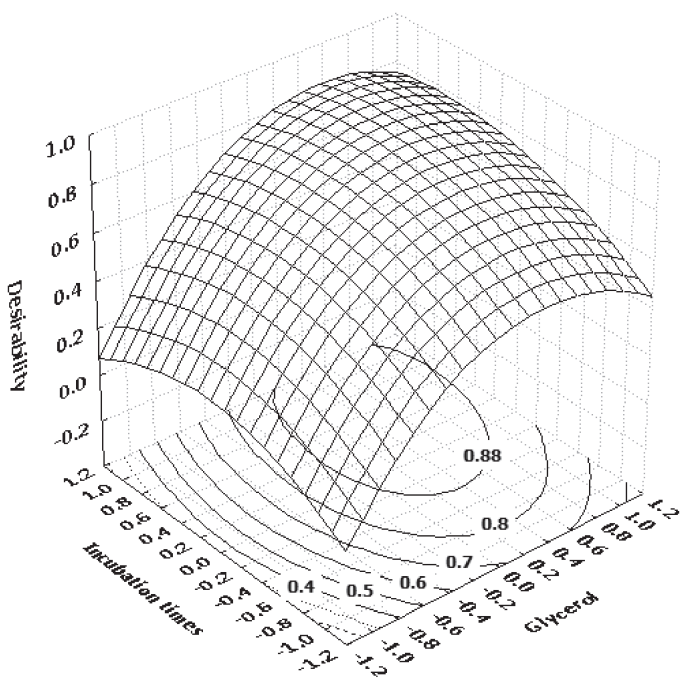

Figure 3 Biomass production response surfaces significantly affected ($P \leq 0.05$) by the crude glycerol concentration and the incubation time.

Surface plots of the significant data are shown in Figures 2 and 3 for the concentration of L-phenylalanine and the biomass, respectively. The plots (Figures 2 and 3) revealed that moderate levels of crude glycerol, ammonium sulfate and incubation time support a high level of production of L-phenylalanine and the biomass. For predicting the optimal point within the experimental space, a second-order polynomial function was fitted to the experimental results. The best-fit equations were Equations 5 and 6:

$$Y_{L\text{-phe}} = 30.987 - 1.447C - 2.334N - 2.334S + 0.559T + 0.850I - 2.805CN + 0.592CS - 0.226CT + 0.751CI + 0.782NS + 0.449NT + 0.001NI - 0.559ST - 0.539SI + 0.155TI - 1.992C^2 - 2.577N^2 - 1.732S^2 - 2.507T^2 - 1.302I^2 \quad (5)$$

and

$$Y_{biomass} = 1.101 + 0.426C + 0.004N + 0.033S + 0.011T + 0.118I - 0.039CN - 0.021CS + 0.033CT + 0.091CI + 0.024NS - 0.019NT - 0.019NI - 0.043ST - 0.021SI + 0.003TI - 0.273C^2 + 0.047N^2 + 0.042S^2 - 0.098T^2 - 1.133I^2 \quad (6)$$

where C is the concentration of crude glycerol (g/L), N is the concentration of ammonium sulfate (g/L), S is the concentration of the mixed salts (g/L), T is the concentration of the mixture of the vitamins and trace elements (g/L), and I is incubation time (h).

The p-values for the significance of the various coefficients in the above equations are given in Table 4. A small p-value ($p \leq 0.05$) for a coefficient implies that it is highly significant.

Table 4 Model coefficients estimated by multiple linear regressions.

Parameters[a]	Coefficient		P-value ($P \leq 0.05$)	
	L-phenylalanine	Biomass	L-phenylalanine	Biomass
Mean/Intercept	30.9870	1.1010	0.0002	0.0005
C	-1.4467	0.4256	0.0222	0.0010
C^2	-1.9916	-0.2730	0.0810	0.0187
N	-2.3309	-0.0044	0.0087	0.7777
N^2	-2.5766	0.0471	0.0508	0.3400
S	-2.3344	0.0328	0.0087	0.1399
S^2	-1.7316	0.0421	0.1032	0.3824
T	0.5994	0.0106	0.1118	0.5228
T^2	-2.5066	-0.0980	0.0535	0.1226
I	0.8500	0.1178	0.0606	0.0133
I^2	-1.3016	-0.1330	0.1640	0.0724
CN	-2.8050	-0.0394	0.0068	0.1141
CS	0.5925	0.0207	0.1257	0.2927
CT	-0.2262	0.0331	0.4333	0.1510
CI	0.7513	0.0907	0.0840	0.0249
NS	0.7825	0.0244	0.0781	0.2365
NT	0.4468	0.0194	0.1935	0.3152
NI	.0013	-0.0194	0.9962	0.3152
ST	-0.5588	-0.0431	0.1383	0.0978
SI	-0.5388	-0.0207	0.1465	0.2927
TI	0.1550	0.0031	0.5737	0.8501

[a] C = crude glycerol; N= $(NH_4)_2SO_4$; S = salts; T = vitamins and trace elements; and I = incubation time.

Based on the *p*-values (Table 4), crude glycerol, ammonium sulfate and the mixed salts had a significant effect on the production of L-phenylalanine. The interactive effect of the crude glycerol and ammonium sulfate also significantly affected the production.

The significant effects for production of biomass, were crude glycerol and the duration of the fermentation. The second order effect of crude glycerol was also significant as was the interactive effect of crude glycerol and the fermentation time. The parity plots in Figure 4 confirmed a generally satisfactory correlation between the measured data and the values predicted using the model equations (Chisti, 2007; Jun *et al*., 2010).

The analysis of variance (ANOVA) results for the second order response models are shown in Table 5. The quality of the fitness of the model to the data was established by the R^2 coefficient and the statistical significance of the R^2 value was determined by the *F*-test value. R^2 represents the proportion of variation in the response data that can be explained by the fitted models. The lack-of-fit term for the residuals

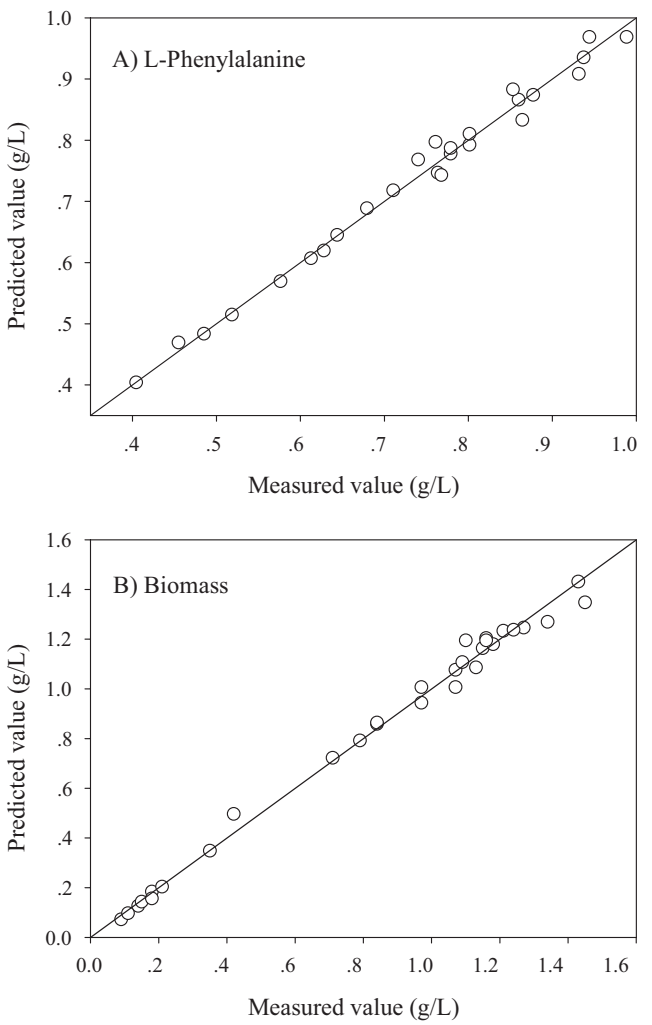

Figure 4 Predicted data versus experimental values for L-phenylalanine concentration (A) and biomass concentration (B).

indicates the variation due to inadequacies in the model. Although for both the concentrations of L-phenylalanine and the biomass, the lack-of-fit statistics were significant (Table 5), models with a significant lack-of-fit can be used confidently whenever a large amount of data is included in the analysis (Taragono and Pilosof, 1999). The fit of the models was checked by determining the R^2 values which were 0.943 and 0.973 for L-phenylalanine and biomass, respectively (Table 5). Therefore, 94.3 and 97.3% of the total variability in the responses was explained by the respective models.

The optimal levels of the five factors were calculated using the Statistica software from the maximum points of the polynomial models. The optimum factor values for producing L-phenylalanine (at desirability = 1.0) were: 25.27 g/L crude glycerol, 11.53 g/L ammonium sulfate, 2.225 g/L mixed salts, 1.511 g/L of vitamins and trace elements mix and 30.29 h of incubation time. For this combination of conditions, the predicted L-phenylalanine concentration was 1.03 g/L.

For the production of the biomass, the optimum factor levels (at desirability = 1.0) were : 46.71 g/L crude glycerol, 40.55 g/L ammonium sulfate, 6.617 g/L mixed salts, 1.495 g/L vitamins and trace elements mix, and 32.43 h of incubation time. For this set of conditions, the predicted concentration of the biomass was 1.23 g/L.

Verification of the models

The optimal conditions identified in the optimization experiments were verified by independent experiments performed in triplicate. For the above noted optimal conditions, the verification experiments gave values (mean ± SD) of 1.09 ± 0.05 g/L of L-phenylalanine and 1.31 ± 0.05 g/L of biomass. These values compared exceedingly well with the model predicted values of 1.03 g/L for L-phenylalanine and 1.23 g/L for the biomass. The verification revealed the high degree of accuracy (more than 94%) of the models.

CONCLUSION

Derived crude glycerol from commercial processes and used for making biodiesel was shown to be a satisfactory carbon source for producing L-phenylalanine using the novel recombinant bacterium *E. coli* BL21(DE3). The production of L-phenylalanine was most influenced by the concentrations of the carbon source, the nitrogen source, the mixed salts and the vitamins and trace elements mix. In addition, the duration of the fermentation was an influential factor. Under the optimal conditions (25.27 g/L crude glycerol, 11.53 g/L ammonium sulfate, 2.225 g/L mixed salts, 1.511 g/L of vitamins and trace elements mix, and 30.29 h of incubation time), the predicted concentration of L-phenylalanine

Table 5 ANOVA for full quadratic models [a] for production of L-phenylalanine and the biomass of *E. coli* BL21(DE3)

Source	Df[b]	SS[b]	MS[b]	*F*-value	*P*-value
Residual error *(L-phenylalanine)*	8	12.2796	1.5349		
Lack-of-fit *(L-phenylalanine)*	6	10.5482	1.7480	2.0308	0.3662
Pure error *(L-phenylalanine)*	2	1.7314	0.8657		
Residual error *(Biomass)*	8	0.0454	0.0057		
Lack-of-fit *(Biomass)*	6	0.0386	0.0064	1.8919	0.3854
Pure error *(Biomass)*	2	0.0068	0.0034		

[a] The adjusted R^2 values were 0.943 for L-phenylalanine and 0.973 for the biomass.

[b] Df = degrees of freedom; SS = sum of squares; MS = mean square.

was 1.03 g/L and agreed closely with the experimentally observed concentration of 1.09 ± 0.05 g/L. Similarly, the model developed for predicting the concentration of the biomass closely agreed with the independently measured data.

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