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Full Length Research Paper

Optimization of critical medium components for higher phycocyanin holo-α subunit production in *Escherichia coli* using statistical approach

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To improve phycocyanin holo-α subunit (cpcA) production from *Escherichia coli* BL21 (DE3) cells, culture medium was screened and optimized using the statistical experimental designs of Plackett-Burman and response surface methodology. In the first step, one-factor-at-a-time method was used to evaluate the effect of carbon sources and nitrogen sources on the yield of cpcA. A two-level Plackett-Burman design was then adopted to select the most important nutrients influencing the cpcA production, which showed that beef extract, KH_2PO_4 and $K_2HPO_4{\cdot}3H_2O$ were the most significant ingredients ($P< 0.05$). Finally, response surface Box-Behnken design was employed to develop a mathematical model to identify the optimum concentrations of the key components for higher cpcA production, which revealed these as follows: beef extract (2.22%, w/v), KH_2PO_4 (0.52%, w/v), $K_2HPO_4{\cdot}3H_2O$ (0.94%, w/v). The high correlation between the predicted and observed values indicated the validity of the model. CpcA yield increased significantly with optimized medium (47.62 mg/L) when compared with original medium (2.72 mg/L).

Key words: Phycocyanin holo-α subunit, Plackett-Burman design, optimization, response surface methodology.

INTRODUCTION

Phycocyanin is one of the phycobiliproteins which are light-harvesting antenna proteins found in cyanobacteria and certain eukaryotic algae belonging to the Rhodophyta, Cryptophyta and Glaucophyta. Phycocyanin is water-soluble and provides intense blue color. Many research achievements indicate phycocyanin has various biological and pharmacological characteristics such as anti-inflammatory, scavenging peroxyl radical, anti-tumor, and immune enhancement (Bhat and Madyastha, 2000; Subhashini et al., 2004; Li et al., 2005). Moreover, phycocyanin is widely used as nutrient and natural dye for food and cosmetics, and as fluorescent probe in fluorescence immunoassay, fluorescence-activated cell sorting and histochemistry.

Extraction of phycocyanin from algae is expensive, complicated and raw material wasting. So using genetically engineered strain to producing phycocyanin at large scale is very attractive and promising (Yu et al., 2002). Phycocyanin holo-α subunit consists of apoprotein and chromophoric group phycocyanobilin (PCB) and the covalently linked PCB at α84 site via thioether bond is imperative to the pharmacological activity. In previous work, five genes involved in the biosynthesis of phycocyanin holo-α subunit was amplified from *Arthrospira platensis* and *Synechocystis* sp. PCC6803 genomic DNA and was cloned into one expression vector; then it was transformed into the competent *Escherichia coli* BL21 (DE3).

Thus, it is of primary importance to develop an effective medium for higher cpcA production. Nutritional requirement can be manipulated by the conventional or statistical methods. Conventional method involves changing one independent variable at a time, while keeping others at a fixed level. This method is often used to screen suitable carbon source and nitrogen source (Bajaj et al.,

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Table 1. Levels of the variables and statistical analysis of Plackett-Burman design.

Code	Variable	Low level (-1)	High level (+1)	Effect	*t*-Value	*P*- Value
X1	Glucose (g/L)	10	40	-6.597	-3.857	0.061
X2	Dummy	0	0	-0.378	-0.221	0.846
X3	Beef extract (g/L)	5	20	7.904	4.621	0.044
X4	NaCl (g/L)	1.25	5	6.868	4.015	0.057
X5	Dummy	0	0	2.131	1.246	0.339
X6	$K_2HPO_4 \cdot 3H_2O$ (g/L)	2	8	7.416	4.335	0.049
X7	KH_2PO_4 (g/L)	1	4	9.493	5.550	0.031
X8	$MgSO_4 \cdot 7H_2O$ (g/L)	1.25	5	3.852	2.252	0.153
X9	Dummy	0	0	-0.057	-0.033	0.977

2009). However, it is not quite effective to determine the optimum level of each medium component for it is not only time consuming but also easily misses the interactions between the components. On the contrary, statistical approaches which have been extensively applied to medium optimization can eliminate the above-mentioned limitations (Ren et al., 2008; Pan et al., 2008; Xu et al., 2008). Plackett-Burman design is well established and widely used statistical technique for selecting the most effective components with high significance levels for further optimization, while ignoring interactions among variables (Plackett and Burman, 1946). Response surface methodology may be summarized as a collection of experimental strategies, mathematical methods and statistical inference for exploring the functional relationship between a response value and a set of design variables (Han et al., 2008; Kumar and Gupta, 2008). Furthermore, the optimum of each variable will be obtained by differential approximation. Here, we report the medium optimization for maximum cpcA production in the engineered strain *E. coli* BL21 (DE3) by one-factor-at-a-time and statistical approaches.

MATERIALS AND METHODS

Microorganism and medium

The engineered strain *E. coli* BL21 (DE3), which bears an expression vector containing all the necessary five genes to produce phycocyanin holo-α subunit, was constructed in our laboratory and used throughout the study. The seed medium contained (g/L): peptone, 10; yeast extract, 5; sodium chloride, 10. The basal medium contained (g/L): yeast extract, 10; sodium chloride, 5; peptone, 16; $K_2HPO_4 \cdot 3H_2O$, 8; KH_2PO_4, 4; $MgSO_4 \cdot 7H_2O$, 5; NH_4Cl, 2. The medium was sterilized in an autoclave for 20 min at 121°C.

Cultivation and induction

One loopful of cells from the seed medium slant was transferred aseptically to 50 mL of the seed medium in a 250 mL conical flask and incubated for 10 h on a rotary shaker operating at 160 and 37 r/min. This was used as the inoculum. Fermentation and induction was carried out in 100 mL Erlenmeyer flask containing 20 mL of the modified basal medium. The medium was inoculated with 5% (v/v) of 10 h old *E. coli* culture and incubated at 37°C and 160 rpm for 2 h. Then, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM to exponentially growing cells. Induced cells were grown for 12 h at 20 and 160 rpm. All experiments were performed in triplicate and results represented the mean values of three independent experiments.

Assay of cpcA production

After induction, 10 mL of the culture broth was centrifuged at 5000 and 4 r/min for 5 min and pellet was resuspended in 1 mL of the phosphate buffer (20 mM; pH 7.2), and then disrupted ultrasonically. Cell debris was removed by centrifugation at 16000 and 4 rpm for 20 min. The optical density of the supernatant was measured at 623 and 650 nm. CpcA concentration, according to Bennett and Bogorad (1973), was defined as:

$$\mathrm{cpcA} = \frac{(\mathrm{OD}_{623} - 0.474(\mathrm{OD}_{650}))}{5.34} \quad (1)$$

Where cpcA is the phycocyanin holo-α subunit concentration (g/L), OD_{623} is the optical density of the sample at 623 nm, and OD_{650} is the optical density of the sample at 650 nm.

Evaluation of carbon and nitrogen sources for the production of cpcA

Carbon and nitrogen sources were screened by one-factor-at-a-time method, and the best nutrient was included for the next design. The effect of different carbon sources such as glucose, citric acid, sucrose, malic acid, glycerol, soluble starch and maltose was studied as an additive 1% (w/v) in the basal medium. Similarly, the effect of different nitrogen sources; peptone, yeast extract, beef extract, $NaNO_3$, NH_4Cl, glutamic acid and urea was individually evaluated by replacing 2.8% (w/v) peptone, yeast extract and NH_4Cl in the basal medium.

Plackett-Burman design

Based on Plackett-Burman design (Yuan et al., 2008; Volontè et al., 2008; Hao et al., 2007), six factors glucose, sodium chloride, $K_2HPO_4 \cdot 3H_2O$, KH_2PO_4, beef extract, $MgSO_4 \cdot 7H_2O$ were used to determine the key ingredients significantly affecting the cpcA production. Each factor was examined at two levels: -1 for low level and +1 for high level. Table 1 illustrates the levels of each factor used in the experimental design, whereas, Table 2 represents the

Table 2. Plackett-Burman design for 9 variables with coded values along with the observed results.

Run	X1	X2	X3	X4	X5	X6	X7	X8	X9	cpcA(mg/L)
1	1	-1	1	-1	-1	-1	1	1	1	15.95
2	1	1	-1	1	-1	-1	-1	1	1	3.84
3	-1	1	1	-1	1	-1	-1	-1	1	8.76
4	1	-1	1	1	-1	1	-1	-1	-1	14.75
5	1	1	-1	1	1	-1	1	-1	-1	12.88
6	1	1	1	-1	1	1	-1	1	-1	16.68
7	-1	1	1	1	-1	1	1	-1	1	33.60
8	-1	-1	1	1	1	-1	1	1	-1	30.40
9	-1	-1	-1	1	1	1	-1	1	1	21.57
10	1	-1	-1	-1	1	1	1	-1	1	12.54
11	-1	1	-1	-1	-1	1	1	1	-1	19.54
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	2.36

design matrix. The effect of each variable on cpcA production was calculated by the following equation:

$$E(Xi) = \frac{2(\sum Ri_{(+)} - \sum Ri_{(-)})}{N} \quad (2)$$

Where E(Xi) is the concentration effect of the variable under study, $Ri_{(+)}$ and $Ri_{(-)}$ are responses of trials where the variable (Xi) measured was at its high and low levels, respectively, and N is the total number of trials. Experimental error was evaluated by calculating the variance among the dummy variables as follows:

$$V_{eff} = \sum \frac{(E_d)^2}{n} \quad (3)$$

Where V_{eff} is the variance of the concentration effect, n is the number of dummy variables, and E_d is the concentration effect for the dummy variables. The significance of each variable was measured via Student's *t*-test. The variables with confidence levels greater than 95% ($P< 0.05$) were considered to have significant impact on cpcA production.

Response surface methodology

The next step in the formulation of the medium was to determine the optimum levels of significant variables for cpcA production. Therefore, the response surface methodology, using a three-variable Box-Behnken design (Box and Behnken, 1960) with three replicates at the center point, was adopted for maximizing cpcA production. For statistical calculations, coding of the variables was done according to the following equation:

$$X_i = \frac{N_i - N_0}{\triangle N_i} \quad (4)$$

Where X_i is the coded value of an independent variable, N_i is the actual value of an independent variable, N_0 is the actual value of an independent variable at the center point, and $\geq N_i$ is the step change. In this study, the experimental plan consisted of 15 trials and the value of the dependent response was the average of three replications. For predicting the optimal point, a second-order polynomial equation was fitted to correlate the relationship between variable and response. The model equation used for the analysis is given below:

$$y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (5)$$

Where y is the predicted response, β_0 is the intercept term, β_i is the linear coefficient, β_{ii} is the squared coefficient, and β_{ij} is the interaction coefficient. Experimental designs were generated using the software Design Expert 7.0 (Stat-Ease Inc., Minneapolis, USA). Statistical analysis of the data was conducted to evaluate the analysis of variance (ANOVA) to determine the significance of each term in the fitted equation.

RESULTS AND DISCUSSION

Effect of carbon and nitrogen sources on cpcA production

Different carbons were tested for cpcA production from the engineered strain *E. coli* BL21 (DE3). It was observed that glucose was the best carbon source for maximum production of cpcA (Figure 1) followed by glycerol and citric acid. However, maltose and sucrose were found to slightly decrease the yield of cpcA compared with control. It was reported that glucose being a simple carbon source was preferred by several microorganisms.

Of the seven nitrogen sources evaluated, beef extract resulted in the highest yield of cpcA (Figure 2). Ammonium chloride and sodium nitrate, as inorganic nitrogen source, were not suitable for cpcA production, which was mainly due to very low biomass (data not shown). Beef extract has abundant amino acid of all kinds, so it is beneficial to both cell growth and cpcA production.

Screening of important medium constituents using Plackett-Burman design

As shown in Table 2, the experiment was conducted in 12

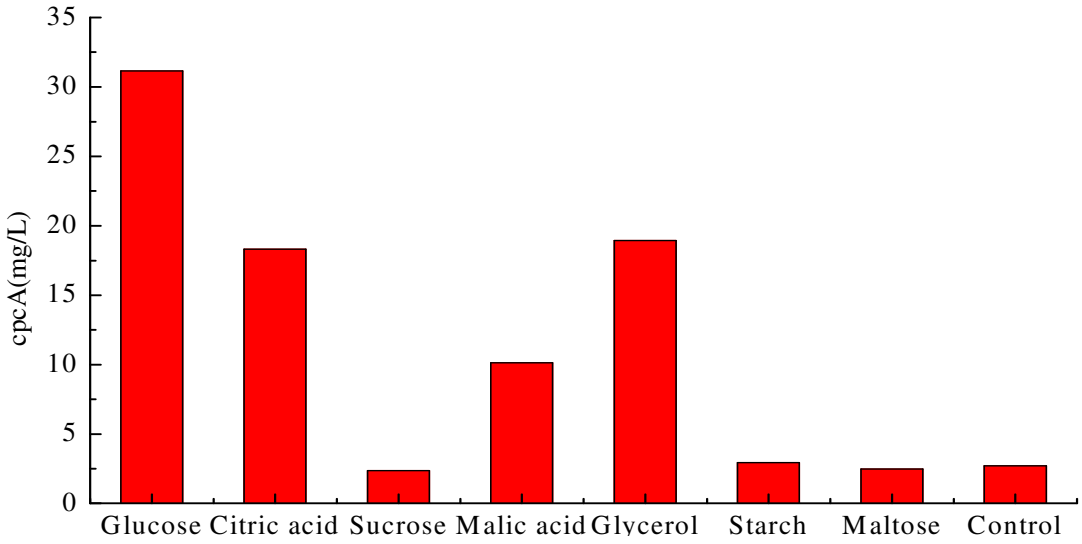

Figure 1. Effect of different carbon sources on cpcA production in *Escherichia coli*.

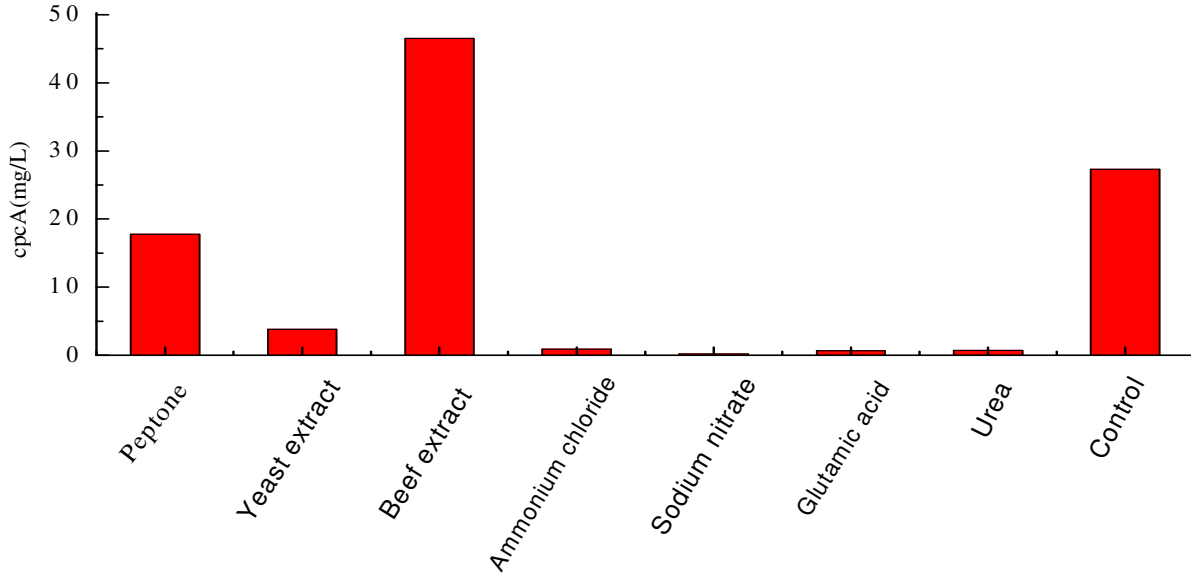

Figure 2. Effect of different nitrogen sources on cpcA production in *Escherichia coli*.

runs to investigate the effect of 9 variables (including three dummy variables). The dummy variables were used as the measure of variability for Plackett-Burman design and they gave a direct estimate of the standard error of a factor effect. Statistical analysis of the responses were performed which is represented in Table 1. Glucose was found to have negatively effect to cpcA production. If the effect of the tested variable is positive, the influence of the variable on response is greater at a high level; in contrast, if it is negative, the influence of the variable is greater at a low level. Analysis of *P*-value showed that beef extract, $K_2HPO_4 \cdot 3H_2O$ and KH_2PO_4 had significant effect on cpcA production ($P < 0.05$) and hence they were selected for further optimization. Variables with insignificant effect ($P > 0.05$); glucose, sodium chloride and $MgSO_4$ were not included in the next optimization experiment, but used in all trials at their high or low level, according to the positive or negative effect, respectively.

Box-Behnken design and response surface analysis

The Box-Behnken design was employed to study the interactions among the significant variables and also their optimal levels. The range and levels of three variables, viz. beef extract, $K_2HPO_4 \cdot 3H_2O$ and KH_2PO_4 are presen-

Table 3. Experiment range and levels of independent variables.

Variable	Symbol	Range and levels		
		-1	0	1
Beef extract (%,w/v)	A	10	20	30
$K_2HPO_4 \cdot 3H_2O$ (%,w/v)	B	1	8	15
KH_2PO_4 (%,w/v)	C	1	4	7

Table 4. Box-Behnken design matrix of three variables in coded units and the experimentally observed responses.

Run	A	B	C	cpcA (mg/L)
1	-1	-1	0	18.996
2	-1	1	0	33.078
3	1	-1	0	27.019
4	1	1	0	42.097
5	0	-1	-1	10.068
6	0	-1	1	39.452
7	0	1	-1	42.243
8	0	1	1	35.889
9	-1	0	-1	34.174
10	1	0	-1	29.865
11	-1	0	1	36.1961
12	1	0	1	43.517
13	0	0	0	45.900
14	0	0	0	44.087
15	0	0	0	46.767

Table 5. ANOVA for quadratic model.

Source	Sum of squares	DF	Mean square	*F*-Value	*P*-Value
Model	1473.02	9	163.67	23.28	0.0015
A	50.27	1	50.27	7.15	0.0441
B	417.2	1	417.2	59.35	0.0006
C	187.25	1	187.25	26.64	0.0036
AB	0.25	1	0.25	0.035	0.8582
AC	33.81	1	33.81	4.81	0.0798
BC	319.32	1	319.32	45.43	0.0011
A^2	117.08	1	117.08	16.66	0.0095
B^2	344.26	1	344.26	48.97	0.0009
C^2	59.54	1	59.54	8.47	0.0334
Residual error	35.15	5	7.03		
Lack of fit	31.41	3	10.7	5.6	0.1553
Pure error	3.74	2	1.87		
Total	1508.17	14			

ted in Table 3. The design matrix of the variables in coded units is shown in Table 4 along with experimental responses. The results of ANOVA of quadratic regression model are given in Table 5. The model presented a high determination coefficient (R^2 = 0.9767) explaining 97.67% of the total variability in the response. The coefficients of regression were calculated and the following second-order polynomial equation was obtained:

Y = 45.58449 + 2.506706A + 7.221518B + 4.837994C –

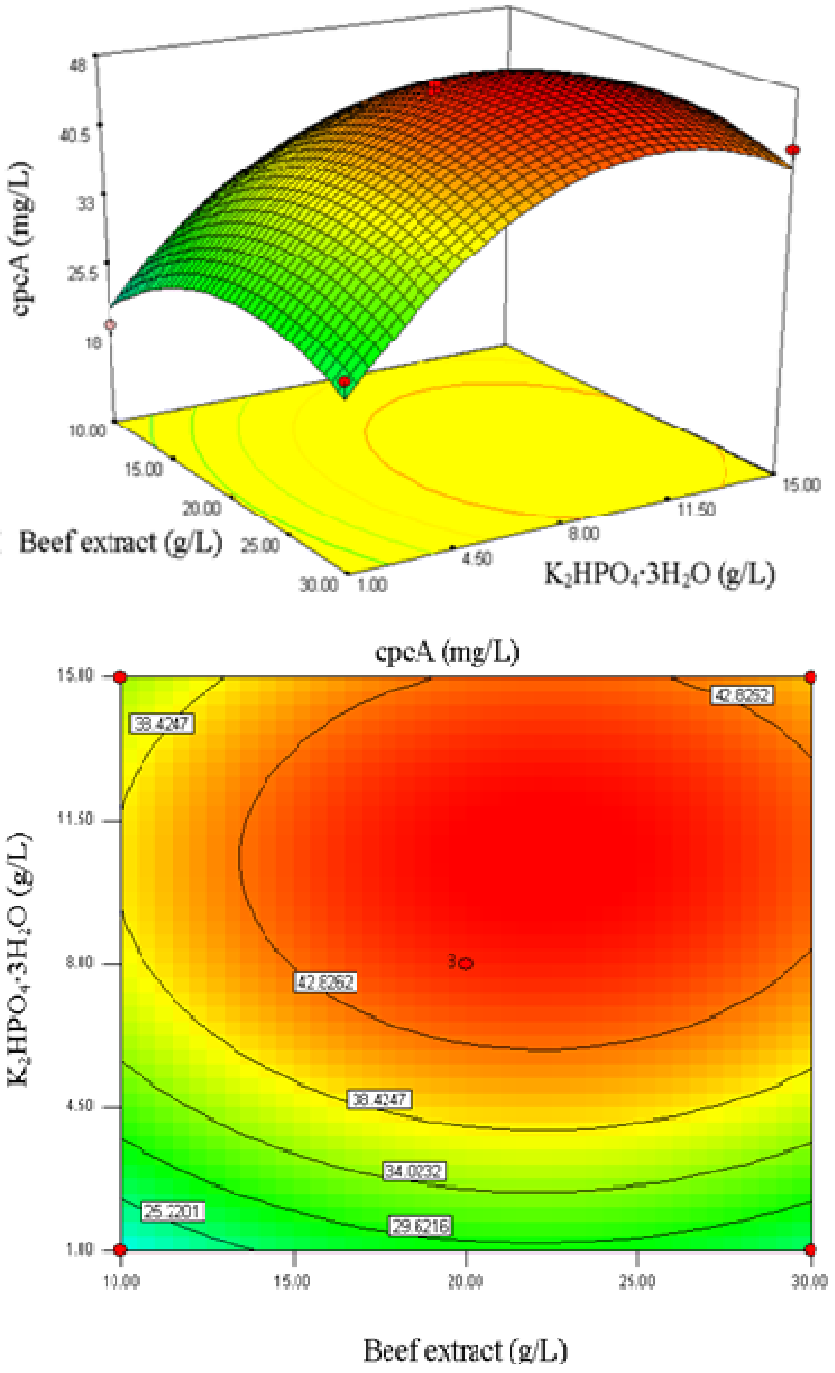

Figure 3. The response surface plot and corresponding contour plot showing the effect of beef extract and $K_2HPO_4{\cdot}3H_2O$ on cpcA production in *Escherichia coli*.

$$5.631069A^2 - 9.655972B^2 - 4.015599C^2 - 8.934718BC \quad (6)$$

Where Y is the predicted response; A, B and C are the coded values of beef extract, $K_2HPO_4{\cdot}3H_2O$ and KH_2PO_4, respectively.

The ANOVA of quadratic regression model demonstrates that the model is highly significant, as is evident from the *F*-test with a very low probability value (0.0015). The *P*-values less than 0.05 indicate model terms are significant. In this case, A, B, C, A^2, B^2, C^2, BC are significant model terms. The 3D response surface and the 2D contour plots are the graphical representations of the second-order polynomial equation. Both plots are presented in Figures 3-5.

From equations derived by differentiation of Equation

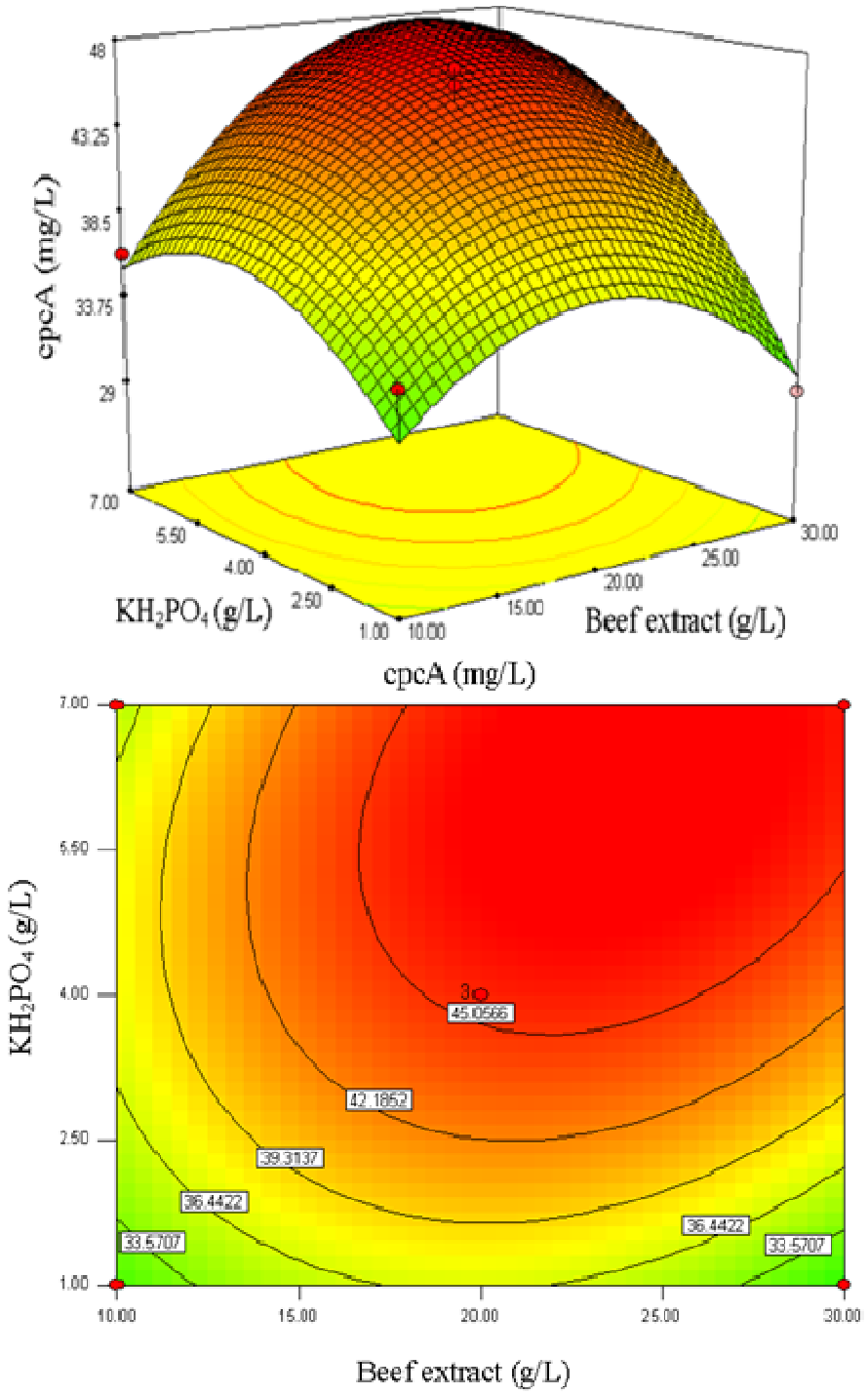

Figure 4. The response surface plot and corresponding contour plot showing the effect of beef extract and KH_2PO_4 on cpcA production in *Escherichia coli*.

(6), the optimal values of A, B and C in the coded units were found to be 0.2226, 0.1965 and 0.3841, respectively. Correspondingly, beef extract was 22.23 g/L, $K_2HPO_4{\cdot}3H_2O$ was 9.37 g/L and KH_2PO_4 was 5.15 g/L. The model predicted a maximum cpcA production of 47.50 mg/L. In order to validate the optimization results, six experiments were conducted using the optimum medium composition. CpcA yield of 47.62 mg/L was obtained at this medium composition. The excellent correlation between experimental value and predicted value confirmed the validity of the response model (Gangadharan et al., 2008; Silva and Roberto, 2001).

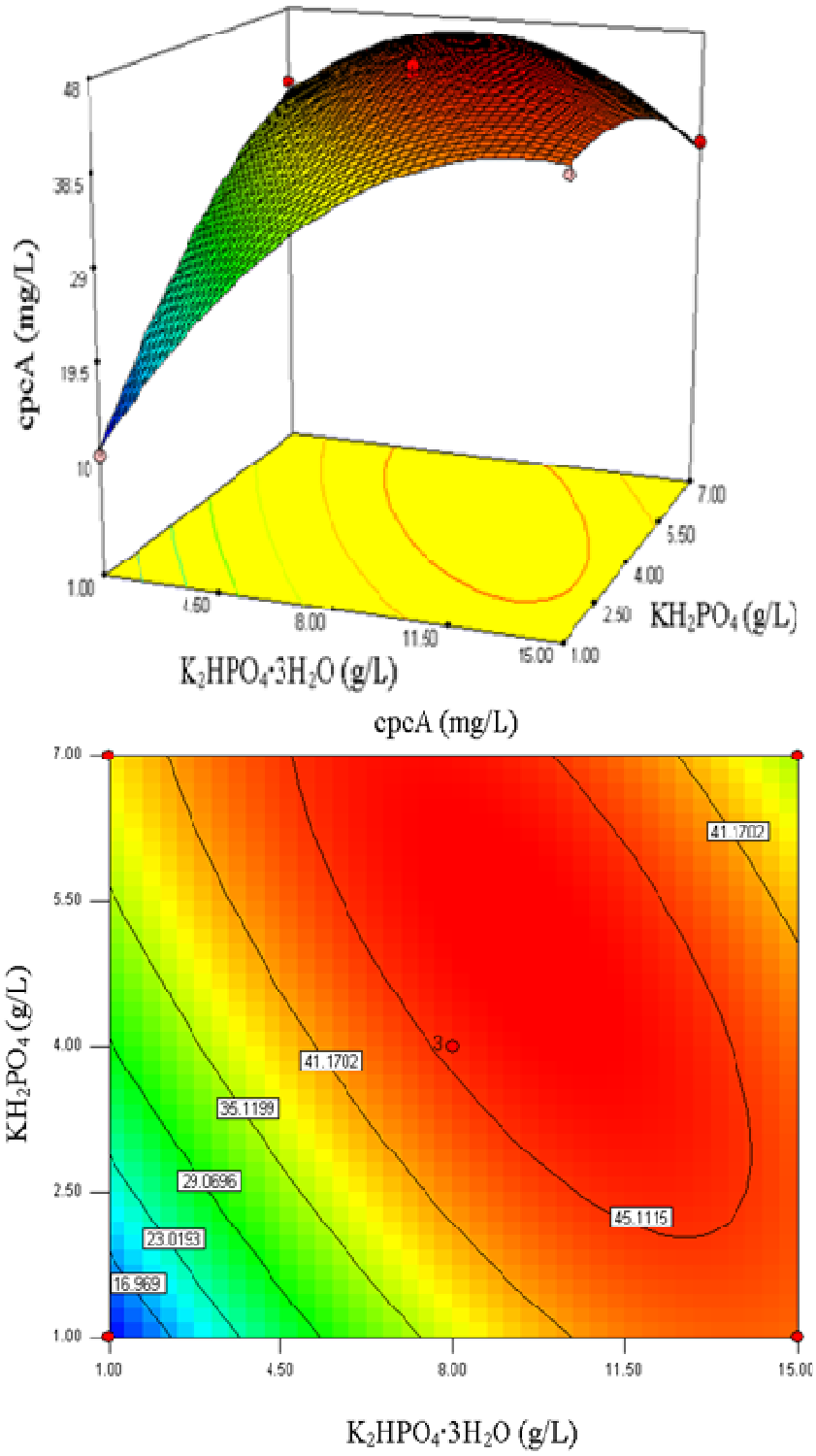

Figure 5. The response surface plot and corresponding contour plot showing the effect of $K_2HPO_4·3H_2O$ and KH_2PO_4 on cpcA production in *Escherichia coli*.

Conclusion

To date, no study has been reported to optimize medium components using statistical approaches for the enhancement of cpcA production from engineered strain. In our work, the effect of different nutrients on cpcA production was investigated. Glucose and beef extract were selected as the best carbon source and nitrogen source, respec-

tively. Plackett-Burman design demon-strated the effect of beef extract, $K_2HPO_4 \cdot 3H_2O$ and KH_2PO_4 in the culture medium to be significant. The interaction between the significant variables and their op-timum levels for maximum cpcA production were deter-mined by using a Box-Behnken design. The final com-position of the optimized medium was as follows (g/L): glucose, 10; NaCl, 5; $MgSO_4 \cdot 7H_2O$, 5; beef extract, 22.23; $K_2HPO_4 \cdot 3H_2O$, 9.37; KH_2PO_4, 5.15. The optimized medium established in this work provides a reference for industrial production of cpcA.

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