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Original Article

Nutrient optimization of polyhydroxyalkanoate production from palm oil fiber by *Ralstonia eutropha* TISTR 1095 using response surface methodology

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\Abstract

Polyhydroxyalkanoate (PHA) is polyesters formed in several bacteria as a carbon or energy storage under some nutrient limitation. *Ralstonia eutropha* has potential to produce PHA from various substrates, including carboxylic acids. In this research, the nutrient optimum to produce PHA from carboxylic acid was studied by using response surface methodology. The carboxylic acids were produced by anaerobic palm oil fiber fermentation for 7 days. Then *Ralstonia eutropha* TISTR 1095 was cultured in fermented broth containing 840 mg/l of acids. The interaction of two factors at the same time was investigated. The effects of propionic acid, butyric acid, $(NH_4)_2SO_4$ and K_2HPO_4 addition were examined. The result showed that the nutrient optimum for PHA production was fermented broth with nutrient addition (2.50 g/l propionic acid, 6.53 g/l butyric acid, 1.53 g/l, $(NH_4)_2SO_4$ and 0.03 g/l K_2HPO_4). The cell concentration, PHA concentration, and PHA content were 1.53 g/l, 0.70 g/l and 46.5%, respectively.

Keywords: polyhydroxyalkanoate, response surface method, carboxylic acid, *Ralstonia eutropha*, palm oil fiber

1. Introduction

Nowadays, the synthetic plastics are largely used in almost every manufacturing industry. However, the synthetic plastics are not degradable after disposal, resulting in environmental impact. Recently, the development of biodegradable plastics has become attractive because of its potential as biodegradable thermoplastic and elastomer (Holmes, 1990). Polyhydroxyalkanoate (PHA) is biopolymer that can be used as biodegradable plastics. PHA is energy and carbon storage material accumulated by a wide variety of microorganisms under unfavorable growth conditions (Anderson and Dawes, 1990). *Ralstonia eutropha* (formerly know as *Alcaligenes eutrophus*) is one of the PHA-producing bacteria that has the potential for commercial production of PHA due to its high polymer content depending on the carbonaceous substrates. Moreover, *R. eutropha* attracted scientific investigation because of its ability to grow on various nutrients. Many researchers verified that *R. eutropha* could use short chain fatty acid as carbon source to synthesize PHA (Lee and Yu, 1997; Jin *et al.*, 1999).

Although PHA was found to be advantage comparing to non-biodegradable plastics, its application is inevitably limited due to high production costs. Thus, optimization of cultivation medium is vital in the cost reduction of this biotechnological process. Optimization of any bioprocesses can be conducted either by changing one factor at a time or by varying several factors at the same time and examining their effects and interactions using statistical analysis. To date, response surface methodology (RSM) has been used mostly for the optimization of process parameters in bacterial fermentation. By adopting this technique, factors and interactions, which affect the desired response, are tested for optimizing the fermentation process parameters in a limited

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number of experimentation (Lakshman *et al*., 2004).

Many researchers studied statistical optimization for PHA production using synthetic media (Lakshman *et al*., 2004; Khanna and Srivastava, 2005). In this study, the liquid from anaerobic palm oil fiber fermentation was used as culture broth. Also, RSM was applied to investigate the optimal condition of culture medium for PHA accumulation in bacterial cells by central composite design (CCD) experimental design.

2. Materials and Methods

2.1 Microorganism

Ralstonia eutropha TISTR 1095 used in this study was stored on nutrient agar slants containing peptone 5 g/l, meat extract 3 g/l and agar 15 g/l. The microorganism was cultured in nutrient broth medium containing peptone 5 g/l and meat extract 3 g/l and incubating at 30°C and 200 rpm for 12 h. Then, the culture was adjusted to OD_{660} of 0.5 as the starter culture.

2.2 Fermentation medium and culture condition

The fermentation was conducted two steps; anaerobic palm oil fiber fermentation and aerobic PHA production. Palm oil fiber fermentation was fermented under anaerobic condition at 30°C for 7 days. The 20 g dry biomass of palm oil fiber, 200 ml deoxygenated water, 100 ml iodoform (methane inhibitor), 2 g/l $CaCO_3$ (buffer reagent) and 50 ml sludge obtained from palm oil wastewater pond were added in fermentor. Fermented broth was centrifuged at 8,000 rpm for 15 min and 4°C to remove particulates. The composition of supernatant was shown following : total acid of 840 mg/l (containing 28.3% acetic acid, 8.8% propionic acid, 4.4% butyric acid and 58.5% other acids), reducing sugar of 14.0 mg/l, nitrogen of 34.0 mg/l, phosphate of 2,009 mg/l and total organic carbon of 2,655 mg/l.

Then, supernatant of palm oil fiber fermented broth was used as fermentation medium for PHA production. After the 10% (v/v) of starter culture was inoculated, the cells were cultivated in aerobic condition at 30°C and 200 rpm for 60 h.

2.3 PHA optimization using statistical analysis

A central composite design (CCD) with four variables including concentrations of propionate, butyrate, $(NH_4)_2SO_4$ and K_2HPO_4 was used to study the response pattern and optimum combination of the variables. For each variable, five levels were tested (Table 1). Table 2 shows the 30 treatments for four-factor CCD and the responses for biomass concentration, PHA concentration and PHA content. CCD was arranged to fit the regression model using second order polynomial equation. Also, the CCD combines the vertices of the hypercubes whose coordinates are given by a $2n$ factorial design to provide for the estimation of curvature of the model. In Table 2, six replicates (treatments 25-30) were integrated for evaluation of a pure error of sum of squares (Lakshman *et al*., 2004).

All designed experiments were conducted in triplicate. Statistical and numerical analyses were carried out by means of the analysis of variance (ANOVA). Responses obtained from trial experiments were compared with the predicted regression models. Furthermore, the fitted polynomial equation was presented in 3 D-response surface plots that expressed the interaction between the response and the experimental levels of each factor used in the experiments (Lakshman *et al*., 2004).

2.4 Estimation of biomass and PHA production

The 10 ml culture samples were centrifuged at 8,000 rpm for 15 min and 4°C. The pellets were resuspended in distilled water and centrifuged again for washing. The washed cells were dried at 105°C for 24 h in a hot air oven then cooled down in desiccators. The drying was repeated until obtaining a constant weight. Cell concentration was expressed as dry cell weight (DCW) (g/l). PHA was extracted by solvent treatment with sodium hypochlorite as described previously and determined gravimetrically (Grothe *et al*., 1999). PHA content is defined as the percentage of PHA based on DCW, i.e., 100 x (g PHA / g DCW).

3. Results and Discussion

Table 2 shows the design layout and results from each trial treatment. The responses measured in the experiments were DCW, PHA concentration and PHA content. The results show that the addition of 10 g/l of propionate, 10 g/l of butyrate, 2 g/l of $(NH_4)_2SO_4$ and 0.1 g/l of K_2HPO_4 (treatment 18, 20, 22 and 24) can increase PHA contents about 44%, 30%, 55% and 46%, respectively, comparing to the experiment without nutrient supplement (treatment 17, 19, 21 and 23). However, propionate, butyrate and $(NH_4)_2SO_4$ caused biomass inhibition.

A second order polynomial equation was employed to fit the experimental data presented in Table 2. The proposed model for the responses was described by Lakshman *et al*. (2004). The responses under different combinations were analyzed using analysis of variance (ANOVA). The equations obtained for biomass concentration (DCW), PHA concentration and PHA content were:

$$\text{DCW (g/l)} = 2.98 - 0.112x_1 + 0.278x_2 - 1.62x_3 - 0.032x_2^2 + 0.311x_3^2 - 0.016x_1x_2 + 0.099x_1x_3$$

$$\text{PHA (g/l)} = 0.926 - 0.037x_1 + 0.040x_2 - 0.358x_3 + 0.194x_4 + 0.111x_3^2 - 0.009x_1x_2 + 0.023x_1x_3 + 0.710x_1x_4 - 0.650x_1x_4$$

$$\text{PHA content (\%)} = 37.6 + 2.59x_1 - 9.13x_2 + 7.47x_3 + x_2^2 - 0.222x_1x_2 - 1.07x_1x_3 + 1.62x_2x_3$$

Table 1. Variables and their levels for CCD.

Variables	Levels				
	-2	-1	0	1	2
Propionic acid (g/l) (x_1)	0	2.5	5	7.5	10
Butyric acid (g/l) (x_2)	0	2.5	5	7.5	10
$(NH_4)_2SO_4$ (g/l) (x_3)	0	0.5	1	1.5	2
KH_2PO_4 (g/l) (x_4)	0	0.025	0.05	0.075	0.1

Table 2. Treatment schedule for four-factor CCD and the response for biomass concentration, PHA concentration and PHA yield by cultivation of *R. eutropha* TISTR 1095.

Trial treatment	x_1	x_2	x_3	x_4	Response		
					DCW (g/l)	PHA (g/l)	PHA content (%)
1	-1	-1	-1	-1	2.07	0.70	33.6
2	1	-1	-1	-1	2.12	0.69	32.4
3	-1	1	-1	-1	2.36	0.86	36.4
4	1	1	-1	-1	0.81	0.31	38.5
5	-1	-1	1	-1	1.96	0.84	42.8
6	1	-1	1	-1	1.53	0.66	43.1
7	-1	1	1	-1	1.10	0.67	60.6
8	1	1	1	-1	0.87	0.44	50.4
9	-1	-1	-1	1	2.39	0.78	32.7
10	1	-1	-1	1	1.99	0.75	37.5
11	-1	1	-1	1	1.85	0.61	33.1
12	1	1	-1	1	1.00	0.38	37.8
13	-1	-1	1	1	2.12	0.77	36.6
14	1	-1	1	1	1.90	0.82	43.4
15	-1	1	1	1	0.87	0.46	52.5
16	1	1	1	1	0.96	0.43	44.4
17	-2	0	0	0	2.49	0.72	28.9
18	2	0	0	0	1.56	0.65	41.6
19	0	-2	0	0	1.37	0.70	51.5
20	0	2	0	0	0.84	0.56	66.9
21	0	0	-2	0	2.91	0.81	28.0
22	0	0	2	0	1.53	0.66	43.5
23	0	0	0	-2	1.86	0.48	25.8
24	0	0	0	2	1.87	0.71	37.7
25	0	0	0	0	1.37	0.60	43.6
26	0	0	0	0	2.07	0.74	36.0
27	0	0	0	0	1.69	0.54	31.8
28	0	0	0	0	1.77	0.57	32.5
29	0	0	0	0	1.71	0.55	32.1
30	0	0	0	0	2.02	0.63	31.2

x_1: Propionic acid (g /l); x_2: Butyric acid (g/l); x_3: $(NH_4)_2SO_4$ (g/l) and x_4: KH_2PO_4 (g/l)

Figures 1-3 shows the comparison between the predicted and actual biomass concentration (DCW), PHA concentration and PHA content with the average errors were 14.5%, 10.3% and 8.03%, respectively. The 3 D-response surface is the graphical representations of the regression equation and presented in Figures 4-6. Response surface plots were performed as a function of two factors at a time, maintaining all other factors at fixed levels.

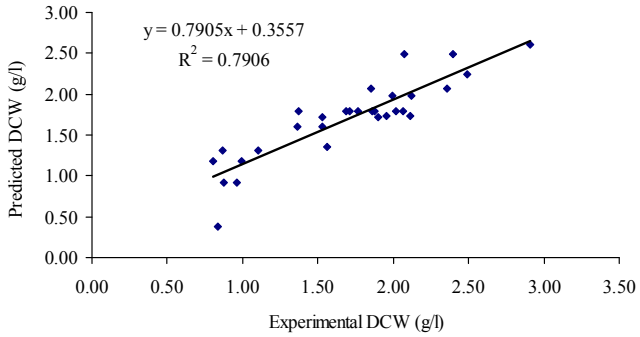

Figure 1. Correlation of predicted and experimental biomass concentration (DCW).

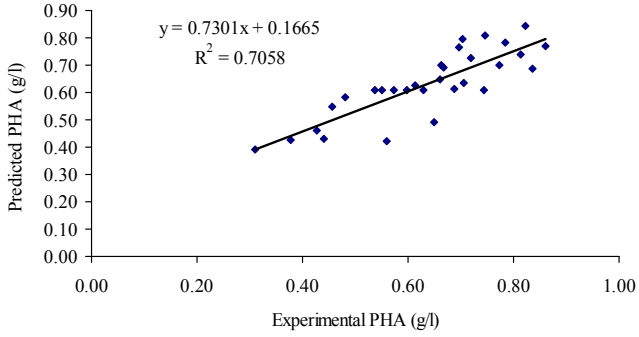

Figure 2. Correlation of predicted and experimental PHA concentration.

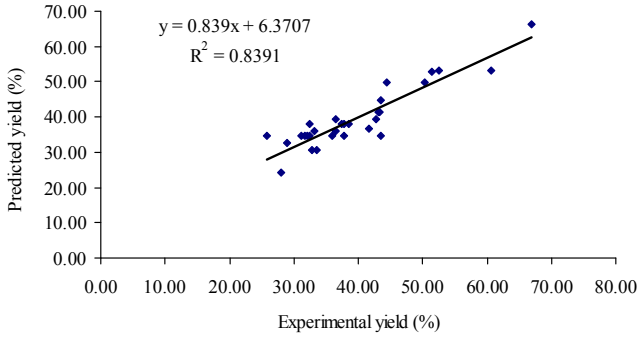

Figure 3. Correlation of predicted and experimental PHA content.

3.1 The interaction of propionic acid and butyric acid concentration on PHA production

The results show that acid supplements resulted in reduction of biomass and PHA concentration (Figures 4a, and 5a), but enhancement of PHA content (Figure 6a). Organic acids could inhibit the growth of bacteria because they would lead to the collapse of the transmembrane pH gradient of bacteria cells, especially at high acid concentrations (Yan *et al*., 2003). In addition, the effect of propionic acid was less sensitive on PHA content than butyric acid (Figure 6a and 6b). In contrast, Akiyama *et al.* (1992) presented the dry cell yield and PHA content were no difference between using *n*-alkanoic acids of carbon numbers C_3 (propionic acid) and C_4 (butyric acid). However, *n*-alkanoic acids with chain lengths ranging from C_{11} to C_{19} yielded higher dry cell matter. Also, the longer chain alkanoates of C_7 to C_{16} gave higher PHA contents over 50%.

The optimum levels of propionic and butyric acids for PHA accumulation in cells were 2.50 and 6.53 g/l, respectively at $(NH_4)_2SO_4$ of 1.53 g/l and K_2HPO_4 of 0.03 g/l. Under these conditions, the C:N ratio of 19.4 was obtained. At the optimum condition, the biomass and PHA concentrations were 1.52 g/l and 0.64 g/l, respectively with PHA content of 46.5%.

3.2 The interaction of $(NH_4)_2SO_4$ and acid concentration on PHA production

The interaction of $(NH_4)_2SO_4$ and acid addition (propionic and butyric acids) for biomass concentration, PHA concentration and PHA content was presented in Figures 4b, 4c, 5b, 5c, 6b, and 6c, respectively. At the optimum level of acids, a maximum quantity of PHA yield was found at 1.5 g/l of $(NH_4)_2SO_4$ (Figures 6b and 6c). The PHA content rather increased with increasing $(NH_4)_2SO_4$ concentration. However, the biomass production decreased with increasing $(NH_4)_2SO_4$ concentration (Figures 4b, 4c, 5b and 5c). According to these results, the nitrogen could be inhibitory to microbial growth, but stimulated PHB production.

According to Grothe *et al*. (1999), the biomass concentration increased with increasing $(NH_4)_2SO_4$ until reaching the optimum $(NH_4)_2SO_4$ concentration. Grothe *et al*. (1999) reported that the maximum biomass and PHA concentration were obtained with 1.4 g/l $(NH_4)_2SO_4$ addition (C:N ratio of 28.3). At higher concentration of $(NH_4)_2SO_4$ biomass production decreased. Nevertheless, PHA content accumulated in cell was slightly sensitive to $(NH_4)_2SO_4$ concentration. Also, Wang *et al*. (2007) found that the biomass concentration decreased and PHA content increased with increasing C:N ratio. Therefore, the inhibition of cell growth

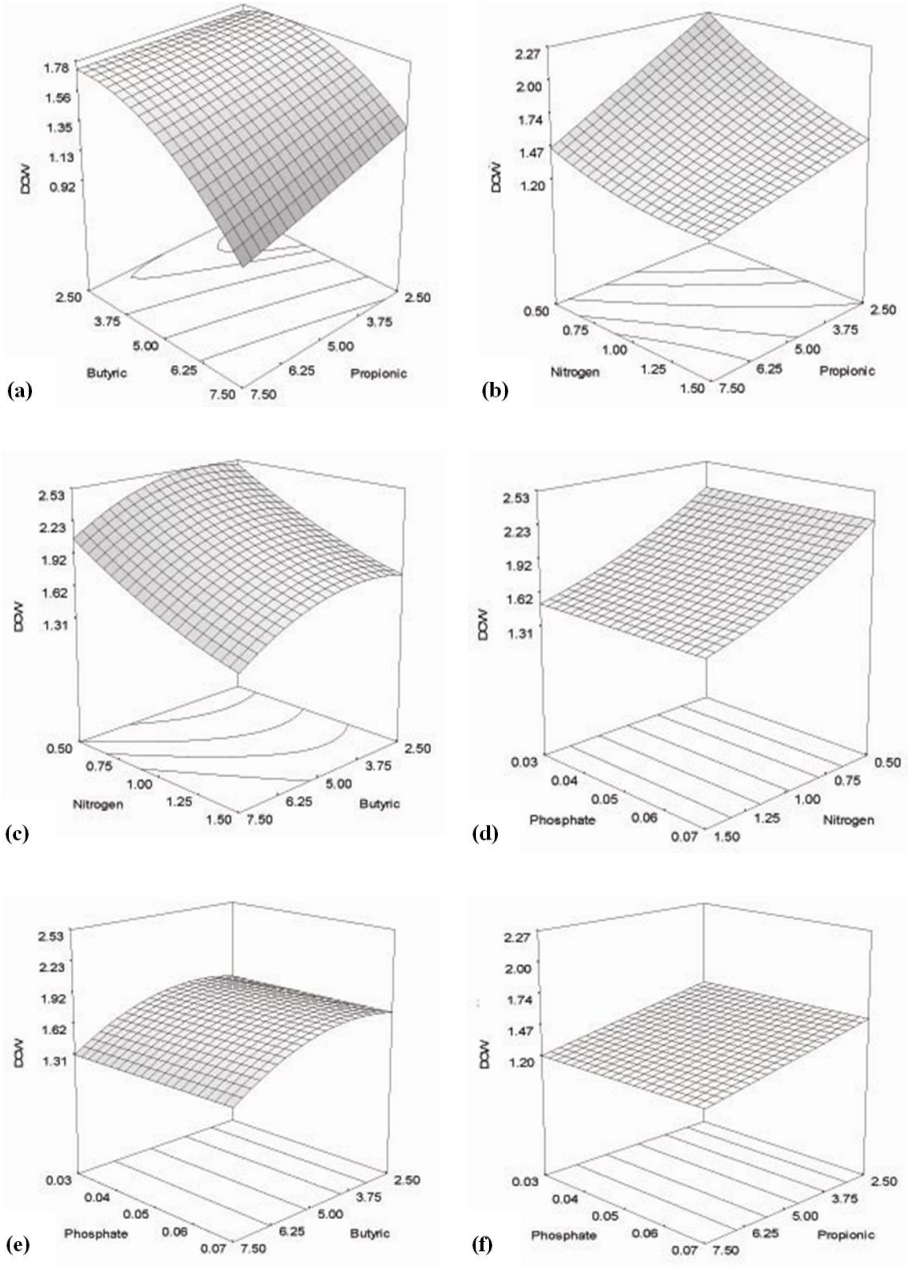

Figure 4. Response surfaces showing the effect of propionic acid and butyric acid (a); $(NH_4)_2SO_4$ concentration and acid concentration (b and c); $(NH_4)_2SO_4$ concentration and K_2HPO_4 concentration (d); K_2HPO_4 concentration and acid concentration (e and f) on biomass.

in this experiment may cause from too high nitrogen concentration in the medium. Moreover, the sensitivity of nitrogen concentration likely depended on acid concentration (Figures 3b and 3c).

3.3 The interaction of KH_2PO_4 and acid concentration and the interaction of KH_2PO_4 and $(NH_4)_2SO_4$ concentration on PHA production

The medium with 0.03 g/l phosphate and the optimum level of acid and $(NH_4)_2SO_4$ addition gave the maximum biomass production, PHA concentration and PHA content. However, the interaction of phosphate on biomass, PHA concentration and PHA content was insignificantly ($p>0.05$) (Figures 4 d-f, 5d-f and 6d-f). In addition, the effect of acid and $(NH_4)_2SO_4$ addition was more sensitive. This result may cause from high phosphorus content in culture broth (2.66 g/l). Therefore, the reduction of the initial phosphate concentration in the medium has possibility to increase PHA concentration in the cell (Lakshman *et al*., 2004).

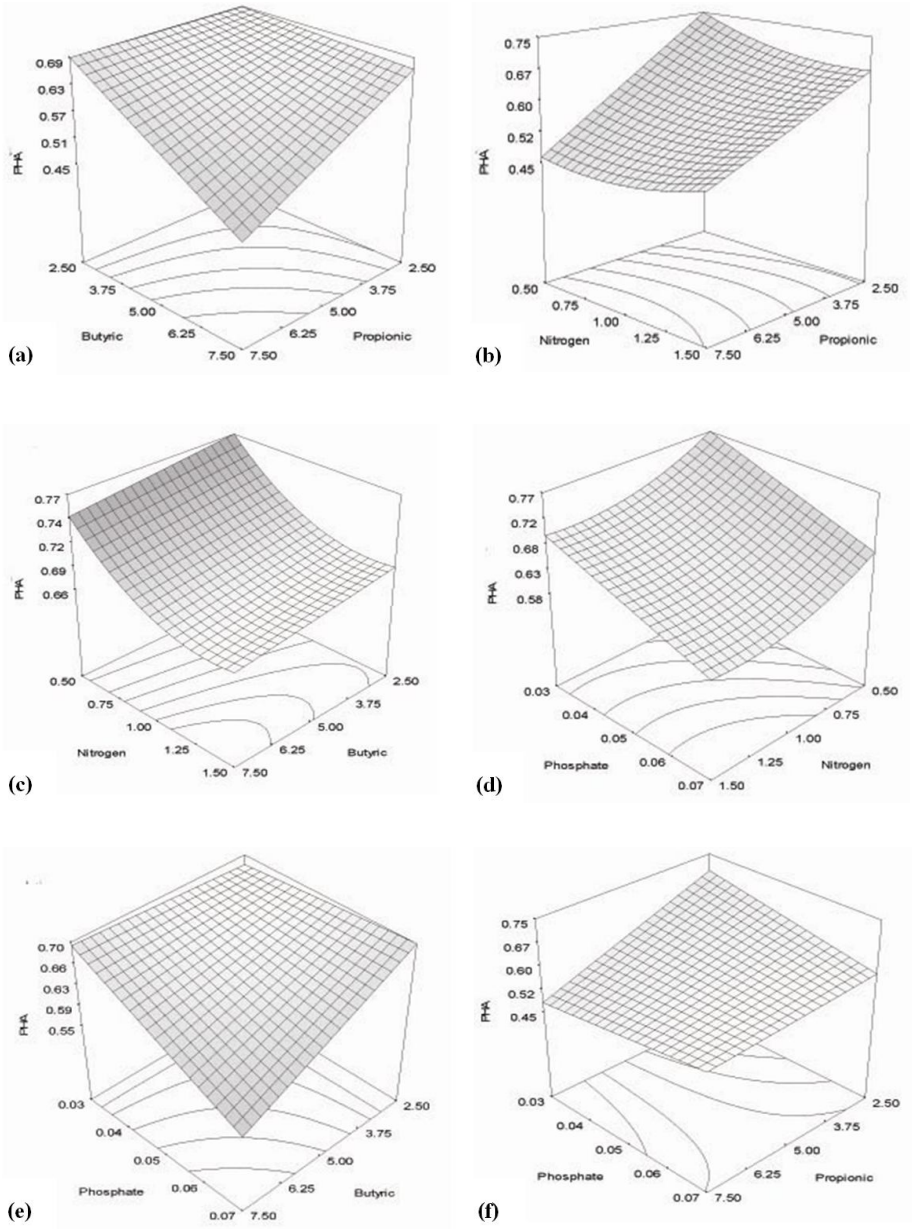

Figure 5. Response surfaces showing the effect of propionic acid and butyric acid (a); $(NH_4)_2SO_4$ concentration and acid concentration (b and c); $(NH_4)_2SO_4$ concentration and K_2HPO_4 concentration (d); K_2HPO_4 concentration and acid concentration (e and f) on PHA concentration.

4. Conclusion

With a central composite design (CCD), the optimal medium for PHA production using *R. eutropha* TISTR 1095 contained 2.50 g/l propionic acid, 6.53 g/l butyric acid, 1.53 g/l $(NH4)_2SO_4$ and 0.03 g/l K_2HPO_4. However, the growth of *R. eutropha* decreased, but PHA content increased with increasing propionic acid, butyric acid and $(NH4)_2SO_4$ concentration. Furthermore, phosphate supplement has no influence on growth and PHA production. Additionally, the regression model obtained for biomass concentration (DCW), PHA concentration and PHA content can predict the optimum conditions with the average errors less than 15.0%. However, the predicted optimum conditions should be verified in a pilot plant before proceeding to industrial scales.

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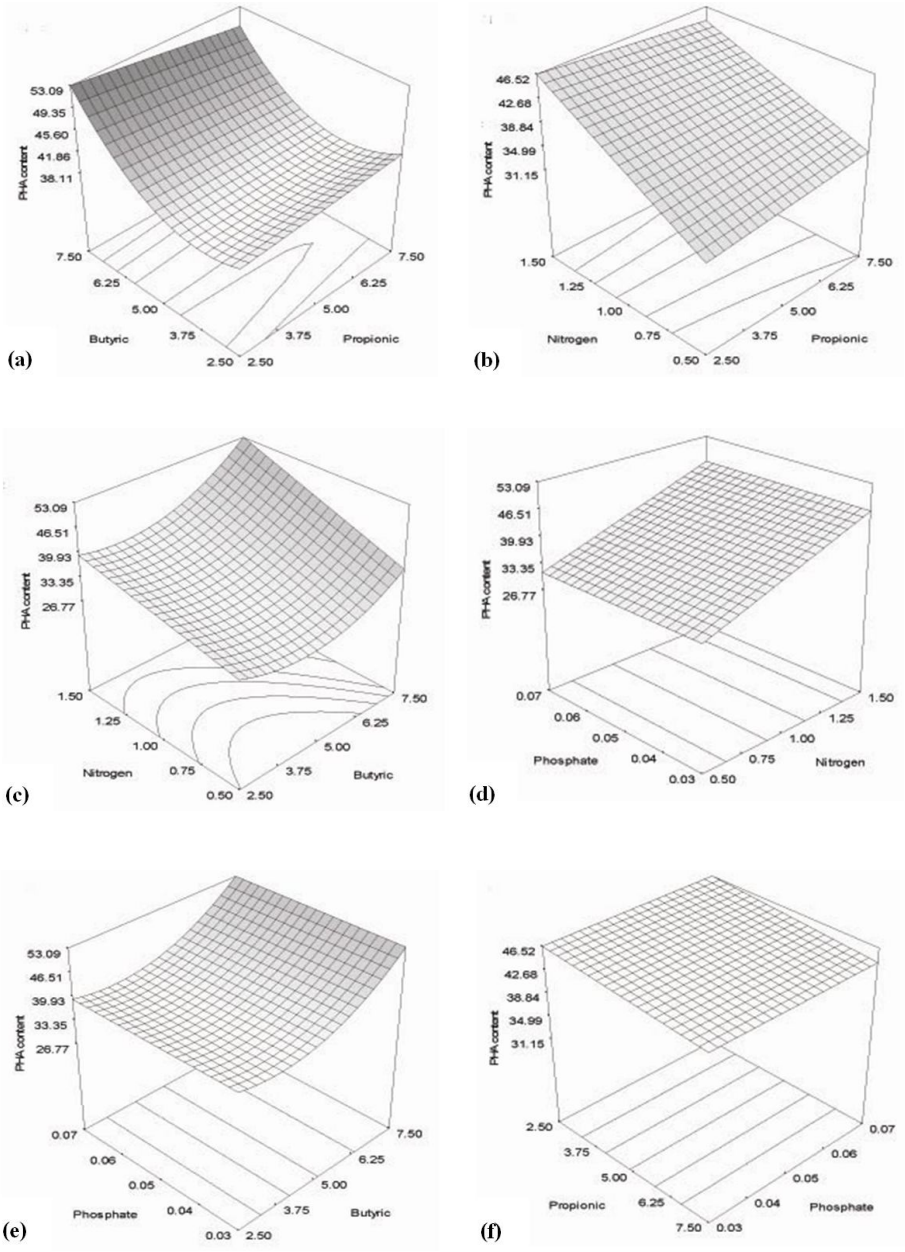

Figure 6. Response surfaces showing the effect of propionic acid and butyric acid (a); $(NH_4)_2SO_4$ concentration and acid concentration (b and c); $(NH_4)_2SO_4$ concentration and K_2HPO_4 concentration (d); K_2HPO_4 concentration and acid concentration (e and f) on PHA content.

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