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Evaluation of Factors Affecting Polyhydroxyalkanoates Production by *Comamonas* sp. EB172 Using Central Composite Design

Noor Azman Mohd Johar[1], Mohd Ali Hassan[1,2], Mohd Rafein Zakaria[1], Phang Lai Yee[1], Yoshihito Shirai[3] and Hidayah Ariffin[1]*

[1]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences,Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Department of Process and Food Engineering, Faculty of Engineering, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[3]Department of Biological Functions and Engineering, Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu-ku, Kitakyushu, Fukuoka 808-0196, Japan
E.mail: hidayah_a@biotech.upm.edu.my

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ABSTRACT

Aims: Statistical approach, central composite design (CCD) was used to investigate the complex interaction among temperature (25-37 °C), initial medium pH (5-9), inoculum size (4-10 % (v/v)), concentration of $(NH_4)_2SO_4$ (0-1 g/L) and concentration of mixed organic acids (5-10 g/L) in the production of polyhydroxyalkanoates by *Comamonas* sp. EB172.
Methodology and Results: Mixed organic acids derived from anaerobically treated palm oil mill effluent (POME) containing acetic:propionic:butyric (ratio of 3:1:1) were used as carbon source in the batch culture of *Comamonas* sp. EB172 to produce polyhydoxyalkanoates (PHAs). The analysis of variance (ANOVA) showed that all five factors were significantly important in the batch fermentation by shake flask with a P value of less than 0.001. The optimal temperature, initial medium pH, inoculum size, concentration of $(NH_4)_2SO_4$ and concentration of mixed organic acids were 30 °C, 7.04, 4.0 % (v/v), 0.01 g/L and 5.05 g/L respectively.
Conclusion, significance and impact of study: Optimization of the production medium containing mixed organic acids has improved the PHA production for more than 2 folds. Under optimal condition in the shake flask fermentation, the predicted growth is 2.98 g/L of dry cell weight (DCW) with 47.07 wt % of PHA content. The highest yield of PHA was 0.28 g of PHA per g mixed organic acids.

Keywords: optimization, central composite design, *Comamonas* sp. EB172, polyhydroxyalkanoate, response surface methodology

INTRODUCTION

Studies on biodegradable plastics derived from microbes have been carried out for many years. However, the production cost is still a barrier to the use of biodegradable plastics, eg. polyhydroxyalkanoates (PHAs). Hence, the solution will lie upon low-cost options such as using cheaper carbon sources, efficient fermentation, and economical recovery process for PHA production (Grothe *et al.* 1999, Patwardhan *et al.* 2004). The cost for substrate may contribute 30 – 60 % of the overall PHAs production cost (Zakaria *et al.* 2010a). Therefore, there have been several studies on the utilization of industrial by-producrs and agricultural wastes as alternative carbon sources for PHAs production (Hassan *et al.* 1997, Mumtaz *et al.* 2008). Utilization of biomass or renewable resource for PHAs production would be viable depending on the availability of the biomass and the technology involved in converting the complex materials into PHAs.

Palm oil mill effluent (POME) has been the most abundant and polluting agricultural wastewater in Malaysia (Alam *et al.* 2008). Hassan *et al.* (1997) studied on the production of organic acids from partial anaerobically treated POME, and it was revealed that the organic acids derived from POME could be use as carbon source for PHAs production by *Rhodobacter sphaeroides*. There were some other reports on the use of organic acids as substrate for PHA production by single and mixed culture (Chakraborty *et al.* 2009, Albuquerque *et al.* 2011). The feeding strategies of organic acids in the fermentation contributed to the variations of PHAs accumulation in the cell. Apart from feeding strategy, PHA accumulation can also be triggered under nutrient-limited conditions such as limited nitrogen, oxygen, sulphur, magnesium or phosphorus in the presence of excess carbon (Annuar *et al.* 2007; Sharma *et al.* 2007). Besides, the ration of carbon to nitrogen (C/N ratio) in medium formulation is an important factor with respect to the nutritional needs for both microbial biomass and PHA accumulation. . The effects of other factors such as temperature, initial medium pH, inoculum size and concentration of $(NH_4)_2SO_4$ on PHAs production by using various types of

*Corresponding author

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microorganisms have been well studied (Sharma *et al.* 2007, Kemavongse *et al.* 2008, Mokhtari-Hosseini *et al.* 2009, Yang *et al.* 2010). Carbon sources such as sucrose (Grothe *et al.* 1999), glucose (Sharma *et al.* 2007), saponified palm kernel oil (Annuar *et al.* 2007) and methanol (Mokhtari-Hosseini *et al.* 2009) were also used to enhance PHAs production.

Recently, our group reported on the PHA production by *Comamonas* sp. EB172 utilizing mixed organic acids derived from POME. The strain accumulated PHAs up to 59 wt % of DCW in fed batch cultivation (Zakaria *et al.* 2010a). However, the PHAs production by this strain from mixed organic acids was not optimized yet in batch fermentation. In the present study, we evaluate the factors affecting PHA production by *Comamonas* sp. EB172 with the use of statistical design. Preliminary screening was done by two-level factorial method. Two-level factorial design was used since it is possible to determine the influence of the multivariable factors and lessen the number of experiments by this method (Rasdi *et al.* 2009). Subsequently, the significant factors were optimized by using CCD whereby the experimental results were fitted with a second order polynomial equation in order to correlate the response variables to the independent variables.

MATERIALS AND METHODS

Microorganism and Inoculums Preparation

A locally isolated bacterium identified as *Comamonas* sp.EB172 was used in this study (Zakaria et al. 2010a). Enrichment medium used in this study was modified from Zakaria et al. (2010a), contained (g/L in distilled water): nutrient broth 8; yeast extract 5; peptone 5; and sodium acetate 5.The pH was adjusted to pH 7.0 by 2M NaOH. The strain was cultivated at 30 °C and agitated at 200 rpm until the OD_{600nm} was more than 2.0. The cultures were then transferred into the production medium with 10 % (v/v) inoculum.

Production Medium

In optimization study, the medium contained (g/L in distilled water) KH_2PO_4, 5; K_2HPO_4 2; $MgSO_4.7H_2O$ 0.4; $CaCl_2.2H_2O$ 0.1; $FeSO_4.7H_2O$ 0.01 and trace elements solution of 0.1 mL was used. The trace elements solution was prepared according to Hassan *et al.* (1997). Mixed organic acids derived from anaerobically treated POME with acid composition of acetatic:propionic:butyric at ratio 3:1:1 as carbon source in the fermentation. The production of clarified organic acid was discussed elsewhere (Hassan *et al.* 1997; Mumtaz *et al.* 2008). $(NH_4)_2SO_4$ was used as the nitrogen source since it had been shown to be a suitable nitrogen source for bacterial growth and PHA production (Zakaria *et al.* 2008). Temperature, initial medium pH, inoculum size, concentration of $(NH_4)_2SO_4$ and concentration of mixed organic acid were adjusted according to the experimental design protocols. The cells were cultivated for 36 h and agitated at 200 rpm in 250 mL shake flask containing 50 mL of the medium described above. After fermentation, the cells were harvested by centrifugation at a speed 10,000 rpm and freeze dried for further analysis.

Analytical Method

Cell growth was monitored by measuring the absorbance of the culture broth at 600 nm using a spectrophotometer (Hitachi, U-2900). Dry cell weight (DCW) was determined by standard plot between OD_{600nm} and dry biomass. The organic acids concentration was analyzed by high performance liquid chromatography (HPLC) (Shimadzu, LC-10 AS) with Aminex 87H column (BIORAD, U.S.A) with 4 mM H_2SO_4 as mobile phase (Hassan *et al.* 1997). The PHA content of the biomass was determined by using gas chromatography (Agilent, 7890A) with benzoic acid as the internal standard by following the standard method (Braunegg *et al*. 1978).

Statistical Design

Two level factorial design

The two-level factorial design is a tool for this initial screening since it is possible to determine the influence of the multivariable factors and lessen the number of experiments (Rasdi *et al.* 2009). The variables tested were temperature, initial medium pH, inoculum size, concentration of $(NH_4)_2SO_4$ and concentration of mixed organic acids. **Table 1** shows the factors in the design and 20 experiments consisting of 16 runs and 4 center points were formulated for 5 factors using the software according to the 2^{5-1} fractional factorial design. Each factor was investigated at high (+1) and low (-1) levels. Concentration range for the variables was decided based on the reports for PHA production by *Comamonas* sp. EB172 (Zakaria *et al*. 2010a). The significances of the factors were identified by confidence level above 95 % ($P < 0.05$). Response was measured for the DCW and PHA content. Factors which gave $P < 0.05$ were selected for further studies.

Response surface methodology (RSM)

The optimal condition of growth and PHA production was investigated by statistical experiments using the Design Expert Software V7.0.0 (Stat-Ease Corporation, USA). The experiments were carried out according to the central composite design (CCD) (**Table 2**). A 2^5-half fractional factorial central composite experimental design, ten axial points ($\alpha = 2$) and six replication of center points leading to a total number of 32 experiments was employed for the optimization of the factors (developed by design expert V7.0). The experimental results were fitted with a second order polynomial equation in order to correlate the response variables to the independent variables. This second order polynomial equation was obtained after the elimination of the insignificant parameters (Khanna *et al*.

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Table 1: Experimental design and responses of two-fractional factorial study

Expt. No.	X_1(°C)	X_2	X_3 % (v/v)	X_4 (g/L)	X_5 (g/L)	Response	
						DCW g/l	PHA content%
1	25	5	4	1	5	0.412	2.16
2	37	9	4	1	5	0.362	3.31
3	37	5	4	0	5	0.417	9.10
4	37	5	10	0	15	0.659	3.90
5	37	9	4	0	15	0.264	5.30
6	31	7	7	0.5	10	0.505	11.68
7	25	5	10	0	5	0.634	3.79
8	31	7	7	0.5	10	0.519	12.33
9	25	9	10	0	15	0.514	5.00
10	25	9	10	1	5	0.662	8.31
11	25	5	4	0	15	0.443	2.50
12	31	7	7	0.5	10	0.499	13.21
13	25	9	4	0	5	0.387	3.80
14	31	7	7	0.5	10	0.514	11.90
15	25	5	10	1	15	0.647	4.00
16	37	5	10	1	5	0.643	3.90
17	37	9	10	1	15	0.501	7.19
18	37	5	4	1	15	0.337	4.20
19	37	9	10	0	5	0.503	13.32
20	25	9	4	1	15	0.337	3.26

X_1: temperature, X_2: initial medium pH, X_3: inoculums size, X_4: concentration of $(NH_4)_2SO_4$, X_5: concentration of mixed organic acids

2005). Lack of fit obtained from the analysis will determine the significance of the model while student *t*-test will determine the significance of each parameter (Mrudula *et al.,* 2011).

RESULTS AND DISCUSSION

Two Level Factorial Design

A screening process is necessary to determine the most influential factors affecting the cultivation process. The selection of factors involved in this screening process was based on previous studies reported by Zakaria *et al*. (2010a, 2010b) in which temperature, initial medium pH, inoculum size, concentration of $(NH_4)_2SO_4$ and concentration of mixed organic acids were important parameters in regulating bacterial growth and PHA accumulation. The experimental range for each of the factors were as follows: temperature (25-37 °C), initial medium pH (5-9), inoculum size (4-10 % v/v), concentration of $(NH_4)_2SO_4$ (0-1 g/L) and concentration of mixed organic acids (5-10 g/L). **Table 1** shows the experimental design for 5 factors according to the Design Expert Software and the responses (DCW and PHA content). The patterns of response for low and high growth clearly corresponded to initial medium pH and inoculums size. From **Table 1**, runs 5, 13 and 19 showed that the growth and PHA production were inhibited at high initial pH. This finding was supported by the results reported by Zakaria *et al.* (2010b) that *Comamonas* sp. EB172 could not grow and produce PHA at pH more than 8. Yang *et al*. (2010) reported that the growth of *Ralstonia eutropha* was inhibited in initial medium pH of more than 7.5. Besides, growth and PHA production were greatly affected by changes of temperature. Temperatures of 37 and 25 °C were not suitable for PHA production from *Comamonas* sp. EB172). As observed at the center point of the design (runs 6, 8, 12, and 14), there was interaction between the concentration of $(NH_4)_2SO_4$ and other factors such as inoculums size, acid concentration and pH. Apparently the growth of *Comamonas* sp. EB172 was affected by all the factors except for $(NH_4)_2SO_4$ concentration since the P-value for the $(NH_4)_2SO_4$ concentration was 0.1630 which was greater than 0.05. In two level factorial design, an independent variable with p-value >0.05 can be taken as being not significant (Rasdi *et al*. 2009) and hence, it can be eliminated. However, PHA production was influence by $(NH_4)_2SO_4$ concentration as shown by analysis of variance with P-value of 0.02. Moreover, all of the other factors were significant since their P-values were <0.05. The screening of factors for growth and PHA production showed that all the factors were found to have significant effects and should be further investigated by using the central composite design (CCD) for optimization.

Central Composite Design (CCD)

Based on the screening test of variables by the two-fractional factorial design, all five variables mentioned above were set in the ranges as shown in **Table 2**. *Comamonas* sp. EB172 was found to be an effective PHA producer by using mixed organic acids as carbon source. *Comamonas* sp. EB172 was identified as a growth-associated bacteria which produce PHA during their growth. One-step cultivation was used in this experiment.

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Table 2: The experimental design and results for the cell dry weight and PHAs production for the central composite design (CCD)

						Experimental		Predicted	
Run	X_1(°C)	X_2	X_3 (% v/v)	X_4 (g/L)	X_5 (g/L)	DCW (g/L)	PHA content (wt. %)	DCW (g/L)	PHA content (wt. %)
1	34	8	5.5	0.25	8.75	3.14	29.98	1.91	36.93
2	28	6	5.5	0.75	6.25	2.55	5.77	0.97	4.17
3	31	7	7	0.5	7.5	4.2	34.5	3.98	27.70
4	31	7	7	0.5	7.5	4.98	24.66	3.98	27.70
5	34	6	8.5	0.75	6.25	3.92	21.11	2.68	14.96
6	28	8	8.5	0.75	6.25	7.88	15.94	5.80	16.57
7	34	6	8.5	0.25	8.75	2.67	18.75	1.81	20.11
8	31	7	7	1	7.5	6.98	10.75	3.98	19.97
9	25	7	7	0.5	7.5	3.92	1.55	2.29	0.38
10	34	6	5.5	0.75	8.75	2.75	34.44	1.12	30.33
11	28	8	5.5	0.75	8.75	6.33	1.48	4.24	3.66
12	31	7	7	0.5	5	4.71	38.76	3.50	37.52
13	37	7	7	0.5	7.5	1	20.44	0.98	27.10
14	31	7	4	0.5	7.5	4.35	28.51	3.28	34.42
15	28	8	5.5	0.25	6.25	4.02	15.32	5.11	18.11
16	31	9	7	0.5	7.5	4.82	9.66	4.71	6.07
17	31	7	7	0.5	7.5	5.3	41.6	3.98	27.70
18	28	6	8.5	0.25	6.25	2.19	9.98	1.66	11.86
19	31	7	7	0	7.5	4.33	44.81	3.98	36.68
20	28	6	8.5	0.75	8.75	1.07	1.44	0.80	1.21
21	28	8	8.5	0.25	8.75	6.3	9.55	4.94	11.00
22	28	6	5.5	0.25	8.75	0.72	1.3	0.10	1.66
23	31	7	10	0.5	7.5	5.53	14.55	4.67	21.70
24	34	8	8.5	0.25	6.25	4.63	15.63	3.47	19.69
25	31	5	7	0.5	7.5	1.31	0.32	0.23	1.61
26	31	7	7	0.5	7.5	4.17	22.2	3.98	27.70
27	34	6	5.5	0.25	6.25	2.88	47.05	1.98	41.97
28	31	7	7	0.5	7.5	4.98	28.7	3.98	27.70
29	34	8	5.5	0.75	6.25	4.67	33.38	2.77	29.82
30	34	8	8.5	0.75	8.75	4.37	17.44	2.61	12.02
31	31	7	7	0.5	7.5	4.85	25.92	3.98	27.70
32	31	7	7	0.5	10	2.22	26.45	1.78	19.36

X_1: temperature, X_2: initial medium pH, X_3: inoculums size, X_4: concentration of $(NH_4)_2SO_4$, X_5: concentration of mixed organic acids

Table 3: Summary of model terms for growth of and PHAs production *Comamonas* sp. Eb172

Terms	P-value	
	CDW	PHA content
X_1	0.0430	< 0.0001
X_2	< 0.0001	0.0501
X_3	0.0331	0.0477
X_4	0.0029	0.0113
X_5	0.0097	0.0067
X_1 X_2	0.0002	0.0285
X_1 X_3	-*	0.0023
X_1 X_4	-*	0.0634
X_1^2	0.0003	0.0002
X_2^2	0.0045	< 0.0001
X_4^2	0.0233	-*

*Interaction with P-value > 0.05 were eliminated in CCD

Table 2 shows that the highest DCW was achieved (7.88 g/L) on run no. 6, with 0.75 g/L of $(NH_4)_2SO_4$ contributing to low PHA accumulation (15.94 wt.%). On the other hand, it could be seen that PHA accumulation was triggered by nitrogen limitation as seen on run no. 27, 47.05 wt % of PHA content was achieved with slightly low DCW (2.88 g/L). $(NH_4)_2SO_4$ concentration played an important role in DCW and PHA accumulation. This was comparable with the study of Annuar *et al*. (2007) who reported that *Pseudomonas putida* needed nitrogen limitation for PHA accumulation. As shown in **Table 3**, the P-value of $(NH_4)_2SO_4$ concentration for growth was 0.0029 and for PHA accumulation at was 0.0113. P-value lower than 0.05 (P <0.05) indicated high influence of the factors to the response. The results also showed that initial medium pH was important for bacterial growth and PHA production with P-values <0.0001 and 0.0501 respectively. This finding was supported by Zakaria *et al* (2010b) who reported that increasing the initial medium pH (6.5-8) would increase the biomass and reduce the PHA production. We found that, initial medium pH 7 was the best condition for both responses.

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Table 4: Analysis of variance (ANOVA): a) for the growth of *Comamonas* sp. EB172, b) for the PHAs production by *Comamonas* sp. EB172.

a.

Source	Sum of Squares	Degree of freedom	Mean Square	F-Value	Prob > F
Model	80.9484	9	8.9943	16.0849	< 0.0001
Residual	12.3018	22	0.5592		
Lack of Fit	11.2447	17	0.6615	3.1285	0.1057
Pure Error	1.0571	5	0.2114		
Cor Total	93.2503	31			
Coefficient of correlation (R^2)			0.8681		
Coefficient of determination			0.8141		
Coefficient of variation			18.7325		

b.

Source	Sum of Square	Degree of freedom	Mean squares	F-value	Prob>F
Model	84.4244	10	8.4424	17.2424	< 0.0001
Residual	10.2823	21	0.4896		
Lack of Fit	8.1968	16	0.5123	1.2282	0.4432
Pure Error	2.0855	5	0.4171		
Cor Total	94.7066	31			
Coefficient of correlation (R^2)			0.8914		
Coefficient of determination			0.8397		
Coefficient of variation			16.7684		

The mathematical models for the DCW (Eq. (1)) and PHA (Eq. (2)) content in response to the process variables are given by:

$$Y_{DCW} = -156.8 + 5.88X_1 + 15.98X_2 + 0.23X_3 + 2.87X_5 - 0.28X_1X_2 - 0.07X_1^2 - 0.43X_2^2 - 0.21X_5^2 \quad (1)$$

$$\sqrt{Y_{PHA}} = -145.99 + 5.65X_1 + 16.44X_2 + 3.97X_3 - 1.59X_4 - 3.18X_5 - 0.14X_1X_2 - 0.14X_1X_3 + 0.09X_1X_5 - 0.07X_1^2 - 0.85X_2^2 \quad (2)$$

Where Y_{DCW} is the dry cell weight (g/L) and $\sqrt{Y_{PHA}}$ is the PHA content (wt. %). X_1, X_2, X_3, X_4 and X_5 are the variables for temperature (°C), initial medium pH, inoculum size (% v/v), concentration of $(NH_4)_2SO_4$ (g/L) and concentration of mixed organic acids (g/L) respectively.

The P-values for the model are shown in **Table 3.** Non-significant terms with P-values of more than 0.05 were eliminated such as the interaction of temperature with inoculums size and temperature with concentration of $(NH_4)_2SO_4$ (g/L) and as a result an equation (1) was generated. The main and quadratic effect (interaction) mentioned in Table 3 with p-value <0.05 were the most important in the growth of the *Comamonas* sp. EB172. A summary of the analysis of variance (ANOVA) for the model Y_{DCW} is presented in **Table 4**. Data of the experiments were fitted to the model and supported by the multiple correlation coefficients (R^2) and the correlation of determination (adjusted R^2). Adjusted R^2 at 0.87 indicated that 87 % of the sample variation for the DCW was attributed to independent variables (**Table 4a**). This means that the model is significant. A large value of F indicates more variance as explained by the model.

The Box Cox plot was used to determine the appropriate power transformation in PHA production (Davenport and Curtis, 2008). The model for PHA production was implemented with square root equation as shown in equation (2). The coefficient of determination (R^2=0.89) showed that 89 % of the variability in the response could be explained by the model and demonstrates that the model was significant for PHA production (**Table 4b**). All the main effects for PHA production were significant. The initial medium pH was found to have the least effect compared to the other factors. Three interaction between the factors with P-values <0.05 were selected to be analysed in the 3D surface graph to determine the optimal value.

Figure 1 shows the 3D surface graph of the model. The results of further analyses on two-factor interaction effects showed that growth was significantly increased

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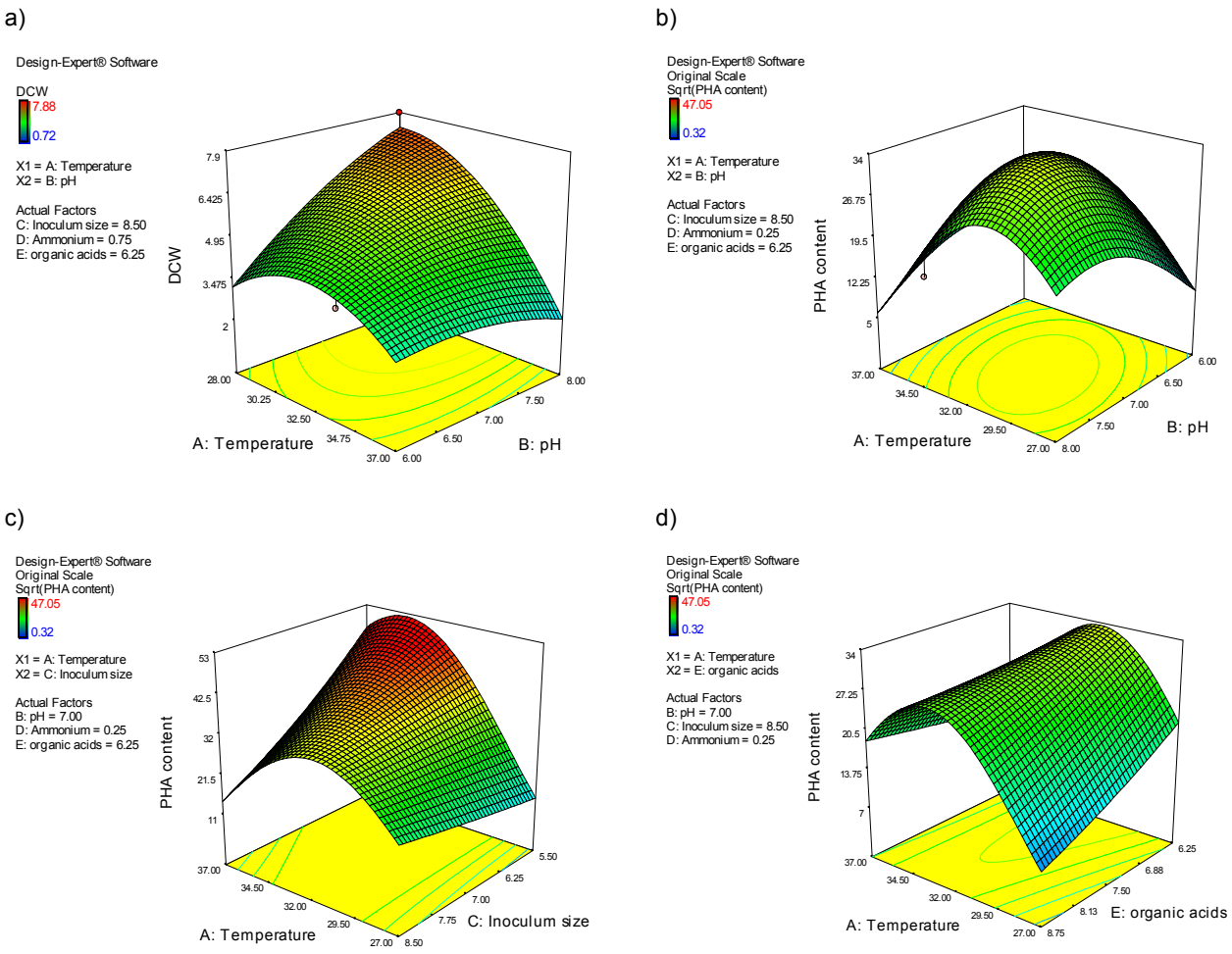

Figure 1: Three dimensional surface graph of the model A) for DCW with interaction of temperature and initial medium pH, b) temperature and initial medium pH, c) temperature and inoculum size and d) temperature and concentration of mixed organic acids.

(P=0.0002) by interactions of temperature with pH as shown in **Figure 1a**. There wassignificant increase in the growth of *Comamonas* sp. EB172 at temperature 30 °C and $(NH_4)_2SO_4$ concentration of 0.75 g/L as the initial medium pH increased. This can be explained when the maximum predicted value is indicated by the surface confined in the smallest ellipse in the graph (Alam *et al*. 2008). However, PHA accumulation was inhibited at pH more than 7.5 (**Figure 1b**). Two-factor interaction effects also showed that PHA accumulation increased by interaction of inoculums size with temperature (P=0.0023) and mixed organic acids with temperature (P=0.0634) (**Figure 1c, d**). The maximumcell growth (5.14 g/L) was recorded at temperature 30 °C; initial medium pH of pH 7.99; inoculum size of 7.06 % (v/v); $(NH_4)_2SO_4$ concentration of 0.52 g/L and mixed organic acids concentration of 9.04 g/L. The optimal values for temperature, initial medium pH, inoculum size, concentration of $(NH_4)_2SO_4$ and concentration of organic acids was 30 °C, 7.04, 0.01 g/L, 4.0 % v/v and 5.05 g/L respectively when both response were considered. The predicted maximum growth of *Comamonas* sp. EB172 was 2.98 g/L and the maximum PHA content was 47.1 wt. % of the DCW in the shake flasks study.

CONCLUSION

Medium composition was optimized by statistical design for bacterial growth and PHA production by *Comamonas* sp. EB172 from mixed organic acids derived from anaerobically treated POME. The mathematical model generated from the CCD for growth and PHA production had high correlation coefficient (R^2), 0.87 and 0.89 respectively after screening and optimization by RSM. The optimized conditions included temperature, initial medium pH, inoculum size, concentration of $(NH_4)_2SO_4$ and concentration of organic acids of 30 °C, 7.04, 0.01 g/L, 4.0 % v/v and 5.05 g/L respectively. In further investigation, the development of a mathematical model for batch fermentation and simulation of fed batch

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fermentation in a bioreactor should be done.

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