African Journal of Biotechnology Vol. 10(77), pp. 17812-17824, 5 December, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2184
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Utilization of biodiesel waste as a feedstock for the production of polyhydroxybutyrate by *Cupriavidus necator*

Kanokphorn Sangkharak[1]* and Poonsuk Prasertsan[2]

[1]COE of Renewable Energy, Department of Chemistry, Faculty of Science, Thaksin University, Phattalung, Thailand.
[2]Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hatyai, Thailand.

Accepted 11 November, 2011

This study aimed to investigate the potential of using wastewater and crude glycerol from biodiesel refinery to produce polyhydroxyalkanoates (PHAs) through fermentation of *Cupriavidus necator* TISTR 1095. The result indicates that crude glycerol yielded high cell growth (35 to 37 g/L) and poly-3-hydroxybutyrate (PHB; 17.85 to 19.98 g/L). However, no cell growth obtained from biodiesel-wastewater due to high Na^+ presented. Among medium and experimental factors influencing PHB accumulation, crude glycerol, $(NH_4)_2SO_4$ and trace element concentration revealed significant effects (P<0.1). Their optimal values were 60 g/L crude glycerol, 1.32 g/L $(NH_4)_2SO_4$ and 2.0 g/L trace element. Under these optimal conditions, the strain TISTR 1095 produced the highest biomass (46.25±2.10 g/L) and PHB concentration of 24.98±1.87 g/L with PHB content of 54.01% of DCW. Effect of experimental conditions including aeration rate and agitation speed as well as sterile condition on PHB accumulation was also studied. The optimal aeration rate (2 vvm) and agitation speed (150 rpm) under septic condition during cultivation gave slightly increase of biomass and PHB. The maximum biomass (46.96±0.28 g/L) and PHB concentration of 25.32±0.20 g/L (53.92% of DCW) was achieved in 20-L fermentor. Moreover, the purified PHB from *C. necator* TISTR 1095 was partially characterized; their properties were similar to commercial PHB.

Key words: Biodiesel, *Cupriavidus necator*, glycerol, optimization, polyhydroxybutyrate, poly-3-hydroxybutyrate (PHB).

INTRODUCTION

Biodiesel is a renewable energy derived from the reaction of vegetable oils or lipids and alcohol with or without the presence of a catalyst. The advantage of biodiesel is that carbon neutral, exhibiting similar features to diesel, biodegradable, less-toxic and significantly cheaper to manufacture than its petroleum equivalent. As the demand of biodiesel increasing exponentially nowadays, the biodiesel process has also generated wastes in a large quantity. An annual production of biodiesel is approximately 150 million gallon per year; an amount of 23 to 35 million gallons of wastes are generated. Three wastes from biodiesel process composed of glycerol, methanol and wastewater. Since previous reports have stated that all wastes should be provided as carbon source in fermentation process to produce various products such as 1,3-propanediol, ethanol, succinate, acetate, hydrogen as well as eco-friendly biopolymer (Grengross and Slater, 2000; Thomson, 2001; Khardenavis et al., 2007). However, this study was

*Corresponding author. E-mail: skanokphorn@yahoo.com. Tel:/Fax: 00 667460 9604.

Abbreviations: PHAs, Polyhydroxyalkanoates; **PHB,** poly-3-hydroxybutyrate.

interested in the improvement of fermentation process for the production of biopolymer from biodiesel wastes due to the current situation about global environment and solid waste management problems.

Among various classes of biopolymer, polyhydroxyalkanoates (PHAs) have played much attention because their properties are similar to synthetic plastics as well as it can be produced from renewable resources and possess complete biodegradability which all of their properties give them an edge over conventional plastics (Steinbüchel and Doi, 2001; Jendrossek and Handrick, 2002; Shah et al., 2007; Sangkharak, 2009; Chaudhry et al., 2010; Mumtaz et al., 2010). PHAs are degraded naturally by PHAs depolymerases at the high rate within 3 to 9 months. However, the major drawback of PHAs is their high costs. The use of different waste materials for PHAs production is a good strategy as production is cost efficient and disposal problems are also over come (Chaudhry et al., 2010). Interestingly, the production of PHAs was also reported in the range of 30 to 62% from biodiesel wastes (Taidi et al., 1994; Cavalheiro et al., 2009; Rebah et al., 2009; Shrivastav et al., 2010). However, only little information has been published.

In the present study, we test the combinatorial effect of the production of PHAs from biodiesel wastes using *Cupriavidus necator* strain TISTR 1095. The specific goals of this study were to improve the PHAs production by optimization study using both statistical and conventional methods to quantify the effects and optimal levels that will maximize the PHAs yield as well as the isolated-PHAs were characterized for their physical properties.

MATERIALS AND METHODS

Microorganisms and cultivation medium

PHAs-producing bacteria was *C. necator* (former *Ralstonia eutropha*) TISTR 1095 which were obtained from Culture collection, Thailand Institute of Scientific and Technological Research, Thailand. The strain was maintained at 30°C in mineral medium contained (g/L) glucose 10, $(NH_4)_2SO_4$ 1, KH_2PO_4 1.5, Na2HPO4·12H2O 9, $MgSO_4·7H_2O$ 0.2 in addition of 1 mL of trace element solution. The trace element solution has the following composition (g/L) $FeSO_4·7H_2O$ 10, $ZnSO_4·7H_2O$ 2.25, $CuSO_4·5H_2O$ 1, $MnSO_4·4H_2O$ 0.5, $CaCl_2·2H_2O$ 2, $Na_2B_4O_7·10H_2O$ 0.23, $(NH_4)_6Mo_7O_{24}$ 0.1, and 1 mL of 35% HCl. The medium was adjusted to pH 6.8 using 5 mol/L NaOH.

Characteristic of biodiesel waste

Crude glycerol and wastewater were obtained from biodiesel refinery (Suratthani, Thailand) and laboratory (Thaksin University). Biodiesel was generated from vegetable oils by using sodium metoxide as catalyst. Each waste sample was measured for pH, Na^+ quantification, methanol and monoacylglycerides (Cavalheiro et al., 2009).

Glycerol and methanol concentrations were determined by a Shimadzu Prominence HPLC System (Shimadzu Scientific Instruments, Inc. Columbia, MD). The detailed procedures were described previously (Chi et al., 2007). The sodium quantification was performed at 589 nm in a flame atomic absorption spectrophotometer (Perkin Elmer) after being diluted in water. Samples were kept at temperature below 4°C in order to prevent the wastes from undergoing microbial biodegradation.

Effect of crude glycerol on PHAs production in shaken flask cultivation

Starter culture of *C. necator* was prepared by cultivating aerobically with shaking (150 rpm) in mineral medium at 30°C for 24 h. Periodically, aliquots were removed to determine for the cell growth by measurement of optical density at 660 nm. The starter culture (5%) was added into the mineral medium (200 mL) and cultivated on a rotary shaker (150 rpm) at 30˚C for 48 h. Samples were taken every 6 h to measure for biomass, the concentrations of PHAs and glycerol concentration.

The mineral medium was prepared in the addition of 1 mL of yeast extract. The effect of crude glycerol on PHAs production was studied compared to the mineral medium containing glucose (control) and pure glycerol (Sigma-Aldrich).

Biomass measurement

10 mL of culture samples were centrifuged at 12000 *x g* for 15 min at 4°C. The pellet was resuspended with distilled water (50 mL) and then centrifuged again for washing. The washed cells were dried at 105°C for 24 h in a hot air oven, then cooled down in dessicator. The drying was repeated until constant weight was obtained (Sangkharak and Prasertsan, 2007).

Determination of PHAs content of bacterial cell

For qualitative determination, PHAs were analyzed in whole-cell samples or after extraction with chloroform and purification by repeated ethanol precipitation from a chloroform solution. The PHAs content and composition was determined by gas chromatography (GC) (Steinbüchel and Wise, 1992; Timm et al., 1990). Final confirmation of structures was performed by gas chromatography-mass spectrometry (GC-MS). PHAs content is defined as the percentage of dry cell weight (DCW), that is, 100 x (g PHAs/g DCW).

Optimization for PHAs production by statistical method

The optimization study for PHAs production was determined by statistical method including a Plackett-Burman design and central composite design (CCD). Firstly, a Plackett-Burman design was used to screen the factors possessing a significant effect on the DCW and PHAs production from crude glycerol. The variables to be evaluated are listed in Table 2, including various medium components, pH and initial temperature. Each independent variable was investigated at a high (+) and a low (-) level. The low levels (-) of medium components were taken as their concentrations in mineral medium.

The design matrix and data analysis were similar as previously reported by Wen and Chen (2001). In summary, there were 20-runs of experiment. The effect of each variable (E_i) on response was determined by subtracting the average response of the low level

from high level. The data were analyzed for significant (P) level through F-test. Here, only parameters contained P<0.10 were accepted as significant factors, which were further optimized in the following CCD (Wen and Chen, 2001; Chi et al., 2007; Sangkharak, 2011).

Three significant factors with (P<0.10) including glycerol, $(NH_4)_2SO_4$ as well as trace element concentration obtained from previous section were used to determined the optimum values for PHAs production using CCD. A 2^n CCD (2^3 = 8 factor points) plus 2^n (2x3 = 6: axial points, with $\alpha = \sqrt{3}$) and six replicates at the center points (n_0 = 6) was used. The variables are coded according to the following equation (Box et al., 1978; Luengo et al., 2003; He et al., 2004; Nikel et al., 2005; Cho and Zoh, 2007; Sangkharak, 2011):

$x_i = (X_i - X_0) / \Delta X_i, \quad i = 1, 2, 3, \ldots\ldots, k$ (Equation 1)

Where, x_i is the coded value of an independent variable; X_i is the real value of an independent variable; X_0 is the real value of an independent variable at the center point and ΔX_i is the step change value.

In this study, X_1 is the glycerol concentration, X_2 is the $(NH_4)_2SO_4$ concentration and X_3 is the trace element concentration. The DCW or PHAs production was considered as the dependent variable or response (Y_i).

The second order polynomial equation was employed to fit the experimental data presented in Table 3. The proposed model for the responses Y_i was given in the following as shown in Equation 2 (Box et al., 1978):

$Y_i = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3$ (Equation 2)

Where, i is the predicted response; x_1, x_2 and x_3 are the independent variables; β_0 is the offset term; β_1, β_2 and β_3 are the linear effects β_{11}, β_{22} and β_{33} are the squared effects, and β_{12}, β_{13} and β_{23} are the interaction term effects. All designed experiments were conducted in triplicate.

Statistical and numerical analyses were carried out by means of the analysis of variance (ANOVA). A software package Design-Expert® 8.0 (trial version, State-Ease, Inc., Minneapolis, USA) were used to generate and analyze the statistical design.

Model validation and confirmation

The predicted condition which was obtained by statistical design was selected and confirmed by *Cupriavidus necator*. The experiments were operated in shake-flask and 5-L fermentor with control of aeration rate and agitation speed at 1 vvm and 150 rpm, respectively.

Biomass and PHAs content were quantified. The percentage of derivation between the predicted and experimental value were investigated.

Optimization of other experimental conditions on PHAs production by conventional method

The starter (5%) was added into a 5-L fermenter containing the optimal medium formula obtained from the statistical method. Samples were taken at time interval and all parameters were analyzed as mentioned in the shake-flask cultivation. The effects of the experimental conditions including aeration rate (0 to 2.0 vvm), agitation speed (100 to 600 rpm) and sterile condition (septic/aseptic) were also investigated.

Time course on PHAs production under the optimal condition

The starter (5%) of *C. necator* added into a 20-L fermentor containing the optimal medium (from above section) for 96 h. The kinetic parameters such as specific growth rate and productivity were determined.

Characterization of PHAs

The polymer from *C. necator* was extracted and subjected to analyze and determine for the melting temperature (Tm), the crystallization temperature (Tc) and the glass transition temperature (Tg) by differential scanning calorimeter (DSC) following the method described by Inoue and Yoshie (1992) as well as the determination of molecular weight of isolated PHAs by ubbelohde viscometer according to Mark-Houwink-Sakura equation (Marchessault et al., 1970; Suthar et al., 2000; Wang and Yu, 2000).

RESULTS AND DISCUSSION

Characterization of biodiesel-refinery wastes

Biodiesel-refinery wastes including crude glycerol and wastewater which obtained from laboratory and biodiesel refinery were characterized for pH, Na^+ quantification, methanol and monoacylglycerides. The crude glycerol from biodiesel refinery had a dark brown color with a pH of 8 to 9. High amount of Na^+, methanol and mono-acylglyceride were detected in a range of 2.8, 1.2 and 2.5%, respectively. Due to the fact that the biodiesel refinery used excess methanol in order to drive the transesterification towards a maximum biodiesel yield. However, a pH level of crude glycerol obtained from laboratory was only 6 to 8 as well as small amount of Na^+ (1.3%), methanol (0.2%) and monoacylglyceride (1.8%) was detected.

Glycerol contents in crude glycerol from both Suratthani refinery and laboratory were around 80 to 85%. Glycerol purity in biodiesel have been reported in a wide range from 55 to 85% (Gonzalez-Pajuelo et al., 2005; Mu et al., 2006), depends on production processes.

Wastewater from biodiesel refinery and laboratory contained high amount of Na^+ (15 to 25%). The presence of sodium ions (>5%) was found to have a particularly negative effect on both the growth rate and the polymer yield (Mothes et al., 2007). Therefore, only crude glycerol from both sources were selected and used as carbon source for PHAs production.

The characteristics of crude glycerol used in this study were similar to those of glycerol as pre-viously reported. The composition of crude glycerol samples from different studies were summarized in Table 1.

Table 1. The composition of crude glycerol from various refineries.

Glycerol sources	Glycerol (%)	Methanol (%)	NaCl /K_2SO_4 (%)	pH	Reference
Hamburg AG (Germany)	80	<0.01	5.5	5.9	
Natural Energy West GmbH (Germany)	85	<0.01	5.0	6.4	
Bio-Diesel Wittenberge GmbH (Germany)	82	<0.50	4.2	6.8	
EOP Biodiesel GmbH (Germany)	85	0.03	0.8	4.1	Mothes et al. (2007)
Campa Energy GmbH (Germany)	90	0.50	1.6	6.0	
TME Methylesterwerke GmbH (Germany)	77	0.0001	6.6	5.3	
PetroTech GmbH (Germany)	88	1.70	1.0	4.5	
Virginia Biodiesel refinery and Seattle Biodiesel refinery (USA)	75	12	NG[a]	NG[a]	Chi et al. (2007)
Novance (France)	65	1	5	NG[a]	González-Pajuelo et al. (2005)
Waste glycerol (Portugal)	>90	0.2	3	NG[a]	Cavalheiro et al. (2009)
Suratthani Biodiesel refinery (Thailand)	80	1.2	2.8	8-9	This study
Laboratory scale biodiesel	85	0.2	1.3	6-8	

NG[a] = Not given.

Effect of crude glycerol on PHAs production in shaken flask cultivation

Time course of cell growth, substrate consumption and PHAs production of *C. necator* TISTR 1095 cultured in mineral medium supplemented with 4% (40 g/L) of glucose, pure glycerol and crude glycerol (laboratory and biodiesel refinery) being used in the medium without controlled pH. Among 4 substrates, *C. necator* exhibited the highest DCW (40±1.58 g/L) and PHAs concentration (22.4±1.09 g/L) when glucose was utilized as a substrate followed by pure glycerol (with the value of 38±1.87 g/L DCW and 19.76±0.96 g/L PHAs) and crude glycerol (35 to 37 g/L DCW and 17.85 to 19.98 g/L PHAs). The lowest DCW (35±1.02 g/L) and PHAs concentration (17.85±1.11 g/L) obtained from crude glycerol (biodiesel refinery). However, the level was not significantly different between cultivation in glucose, pure glycerol or crude glycerol. The cell grew very well and produced the desired amount of PHAs in crude glycerol compared with glucose and pure glycerol and reached the highest level after 36 to 42 h of cultivation corresponded with low amount of substrates within 48 h of cultivation. Interestingly, when 40 g/L of crude glycerol was used, the real glycerol concentration in the medium was presented only 32 to 34 g/L, suggesting that around 15 to 20% of impurity was contained in the crude glycerol. Therefore, pretreatment of crude glycerol by washing with distilled water before cultivation is necessary to improve PHAs yield (Chi et al., 2007).

Isolated PHAs from four substrates used in this study were characterized by gas chromatography. The gas chromatogram for PHAs from each substrate was observed at the retention time 10.07 min corresponding to 3-hydroxybutyric acid. This revealed that the homopolymer was poly-3-hydroxybutyrate [P(3-HB)], accumulated in either pure substrates (glucose and pure glycerol) or crude glycerol.

Optimization for PHB production from crude glycerol by statistical method

PHB production in crude glycerol

As aforementioned, the production of PHB was not significantly different when produced from glucose, pure glycerol and crude glycerol. The result indicates that crude glycerol was a potential good carbon source for the production of PHB. Therefore, crude glycerol from biodiesel refinery was selected as substrate for PHB biosynthesis by *C. necator* TISTR 1095. In addition, optimization to enhance PHB production by statistical method was also investigated. The crude glycerol was pretreated prior to usage according to the method described by Chi et al. (2007). *C. necator* TISTR 1095 was cultivated under modified

Table 2. Treatment schedule for Plackett-Burman design and responses for dry cell weight (DCW) and PHB production by *Cupriavidus necator* TISTR 1095 in glycerol-containing medium.

Run	Variable								Response	
	A	B	C	D	E	F	G	H	DCW (g/L)	PHB (g/L)
+	80	4	3	9	0.4	2	8	30		
-	40	1	1.5	4.5	0.2	1	6	25		
1	+	+	+	-	-	+	+	-	38.14±2.21	22.12±1.21
2	+	-	+	+	-	-	-	-	36.02±1.56	20.59±1.10
3	-	+	-	+	+	+	+	-	33.21±1.82	19.26±0.59
4	-	-	-	+	-	+	-	+	34.89±1.76	20.23±1.25
5	+	+	-	+	+	-	-	-	35.11±3.65	20.36±1.33
6	+	+	+	+	-	-	+	+	35.02±2.14	20.31±1.08
7	-	+	+	-	-	-	-	+	32.32±1.69	18.75±1.11
8	-	+	-	+	-	+	+	+	33.12±2.02	19.21±0.98
9	-	-	-	-	+	-	+	-	34.32±3.04	19.91±0.69
10	-	+	+	+	+	-	-	+	33.09±2.58	19.19±2.01
11	-	-	-	-	-	-	-	-	35.31±2.24	20.48±1.65
12	+	+	-	-	-	-	+	-	34.43±2.69	19.97±1.10
13	-	-	+	+	-	+	+	-	35.33±1.58	20.49±0.65
14	+	+	-	-	+	+	-	+	35.19±1.99	20.41±0.52
15	+	-	+	+	+	+	-	-	38.26±1.29	22.19±1.22
16	+	-	-	-	-	+	-	+	38.56±2.87	22.36±1.31
17	-	-	+	-	+	-	+	+	34.12±3.25	19.79±1.65
18	+	-	-	+	+	-	+	+	37.11±3.26	21.52±1.57
19	-	+	+	-	+	+	-	-	33.69±2.54	19.54±1.11
20	+	-	+	-	+	+	+	+	38.18±2.11	22.14±1.02

Where A = crude glycerol concentration (g/L); B = $(NH_4)_2SO_4$ concentration: (g/L); C = KH_2PO_4 concentration (g/L); D = $Na_2HPO_4 \cdot 12H_2O$ concentration (g/L); E = $MgSO_4 \cdot 7H_2O$ concentration (g/L); F = trace element concentration (g/L); G = initial pH; H = cultivation temperature (^oC)

mineral medium where glucose was supplemented through crude glycerol (40 g/L). Eight parameters including the effect of crude glycerol, $(NH_4)_2SO_4$, KH_2PO_4, $Na_2HPO_4 \cdot 12H_2O$, $MgSO_4 \cdot 7H_2O$ and trace element, as well as the effect of initial pH and cultivation temperature on biomass and PHB production were investigated by statistical analysis.

Screening the significant factors by Plackett-Burman

Plackett-Burman design, an experiment, initiated for screening the significant factors affecting on PHB production. *C. necator* TISTR 1095 was grown under 20 different conditions with DCW and PHB production being taken as the responses for each run (Table 2). The effects of the variables on the responses and their associated *F*-test as well as significant level (*P*) were calculated (data not shown). The resulted effects with $P<0.10$ were accepted as significant. Only three factors among eight investigated parameters including crude glycerol, $(NH_4)_2SO_4$ and trace element concentration showed significant effects on biomass and PHB production. These parameters were further optimized through a central composite design.

Optimization for PHB production in glycerol-containing medium by central composite design

To validate the exact optimum values of crude glycerol, $(NH_4)_2SO_4$ and trace element as well as their interactions using statistical design, the range between the optimum points (from Plackett-Burman) were selected. The CCD experiments were therefore focused on the interaction of crude glycerol (60.0±10.0 g/L) and $(NH_4)_2SO_4$ (3.0±1.0 g/L) in glycerol-containing medium in addition to trace element concentration (2.0±1.0 g/L) as response-dependent variables. The concentrations of non-significant medium components as identified in Table 3 were set at lower levels while the initial pH was set at 6.8. Levels of glycerol concentration, $(NH_4)_2SO_4$ and trace element were significant factors ($P<0.05$) for both biomass and PHB production from *C. necator* TISTR

Table 3. Treatment schedule for three-factors central composite design (CCD) and the response for PHB production by *Cupriavidus necator* TISTR 1095.

Trial	X_1	X_2	X_3	Response (Y_i)	
+1	70	4	3	DCW (g/L)	PHB (g/L)
0	60	3	2		
-1	50	2	1		
1	-1	-1	-1	38.11±1.56	20.20±0.96
2	+1	-1	-1	39.23±1.25	20.79±1.25
3	-1	+1	-1	35.12±1.22	18.61±1.04
4	+1	+1	-1	36.61±1.84	18.31±0.68
5	-1	-1	+1	37.98±0.96	18.99±0.89
6	+1	-1	+1	40.02±1.59	20.01±1.22
7	-1	+1	+1	33.26±1.46	16.63±1.03
8	+1	+1	+1	35.53±1.98	18.48±1.14
9	-1	0	0	33.99±2.02	17.67±0.88
10	+1	0	0	37.89±1.69	19.70±1.00
11	0	-1	0	45.87±2.21	22.94±1.43
12	0	+1	0	35.01±1.87	18.21±0.59
13	0	0	-1	35.23±1.69	18.32±0.91
14	0	0	+1	36.61±0.99	19.77±1.51
15	0	0	0	36.26±1.54	19.58±1.66
16	0	0	0	35.99±1.25	19.43±1.23
17	0	0	0	35.68±1.67	18.20±1.06
18	0	0	0	36.12±1.24	18.42±1.11
19	0	0	0	36.09±1.01	18.41±1.96
20	0	0	0	36.21±1.12	18.47±1.21

Where X_1 = crude glycerol concentration (50-70 g/L); X_2 = $(NH_4)_2SO_4$ concentration (2-4 g/L); X_3 = trace element concentration (1-3 g/L).

1095 (Table 3). Fischer's *F* test demonstrated that the model applied was significant. The significant terms were calculated using *t*-test and the responses under different combinations were analyzed using the analysis of variance (ANOVA). The first and second (quadratic and cross-product) order terms were found to be significant (P>0.5) and lack of fit was not significant (P>0.05). Only significant terms were selected to possess the highest value of coefficient of determination (R^2) (Box et al., 1978) as shown in the Equations 3 and 4. In this study, the values of R^2 varied from 0.93 to 0.98, which suggested that the models gave good fit. The effects of crude glycerol, $(NH_4)_2SO_4$ and trace element on biomass production and PHB production (responses) were analyzed. Plus (+) and minus (-) symbol represented the positive and negative effects on the response as shown in Equations 3 and 4. The interaction of the factors, however, had a pronounced effect on PHB optimization indicating the importance of these factors on the enhancement of PHB yields.Equations obtained for biomass and PHB production by *C. necator* TISTR 1095 were:

$$\text{Biomass (g/L)} = 45.33 + 0.22x_1 - 10.59x_2 + 0.72x_3 - 0.00156x_1^2 + 1.44x_2^2 - 0.16x_3^2 + 0.0075x_1x_2 + 0.021x_1x_3 - 0.45x_2x_3 \quad \text{(Equation 3)}$$

$$\text{PHB (g/L)} = 23.91 + 0.13x_1 - 4.43x_2 - 2.12x_3 - 0.00124x_1^2 + 0.54x_2^2 + 0.00369x_3^2 - 0.00074x_1x_2 + 0.032x_1x_3 + 0.022x_2x_3 \quad \text{(Equation 4)}$$

Where x_1, x_2 and x_3 represent the codified levels of crude glycerol, $(NH_4)_2SO_4$ and trace element, respectively.

C. necator TISTR 1095 produced biomass (33.26 to 45.87 g/L) and PHB (16.63 to 22.94 g/L) in the range of various parameters.

The lowest value of biomass (33.26 g/L) was obtained at minimum glycerol concentration (50 g/L) with the maximum concentration of $(NH_4)_2SO_4$ (4.0 g/L) and trace element (3.0 g/L).

In treatment number 11, the strain required moderate levels of glycerol (60 g/L) and trace element (2.0 g/L) in addition of least amount of $(NH_4)_2SO_4$ (2.0 g/L) to gain the highest concentration of biomass (45.87±2.21 g/L) and PHB (22.94±1.43 g/L) as well as PHB content 50%

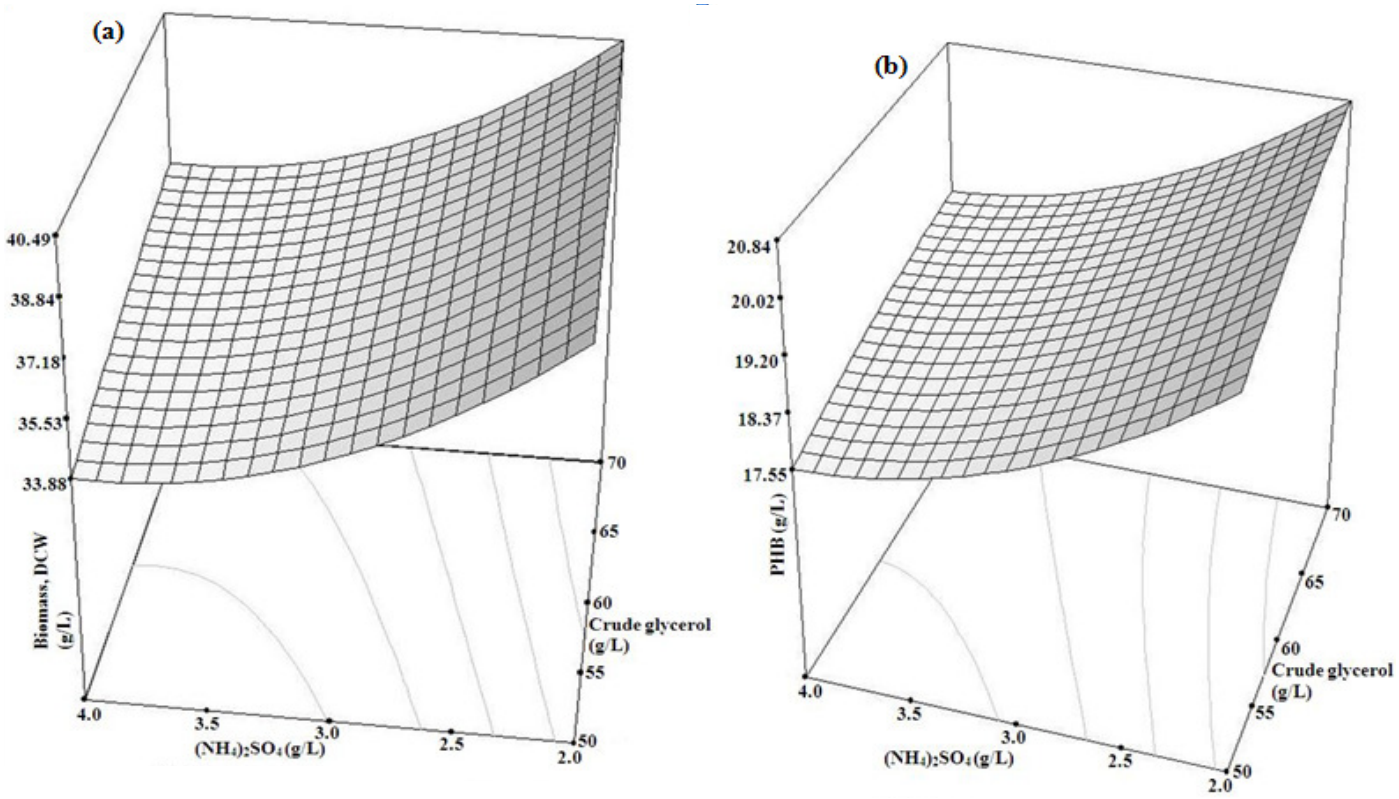

Figure 1. Response surface showing the effect of glycerol and $(NH_4)_2SO_4$ concentration; $(NH_4)_2SO_4$ and trace element; glycerol and trace element on biomass (a, c, e) and PHB production (b, d, f) of *Cupriavidus necator* TISTR 1095.

of DCW.

These results suggested that suitable concentration of carbon and nitrogen by mean of glycerol and $(NH_4)_2SO_4$, respectively, and trace element were required for PHB synthesis, otherwise excess nutrient was diverted towards biomass build up but decreased PHB accumulation (Lakshmar et al., 2004; Sangkharak and Prasertsan, 2007).

Moreover, the highest C/N ratio (30:1) was also observed in this treatment which in turn affected the cell growth and PHB production (Luengo et al., 2003; Reddy et al., 2003). High C/N ratio of glycerol and $(NH_4)_2SO_4$ in this optimal cultivation system had a great influence on PHB accumulation comparable to previous reports (Grothe et al., 1999; Grothe and Chistri, 2000; Reddy et al., 2003).

Interaction of glycerol, $(NH_4)_2SO_4$ and trace element on biomass and PHB production for *C. necator* TISTR 1095 revealed the response surface of biomass production (Figure 1) in which one variable kept at the optimum level and the other two variables varied within the experimental ranges.

Model validation and confirmation

Verification experiments performed at the predicted conditions and other three conditions were also selected to confirm the model using *C. necator* TISTR 1095. The experiment was conducted in 5-L fermentor with controlled condition; pH at 6.8, 1 vvm aeration rate and 150 rpm agitation speed. The results demonstated that the experiment values were higher than predicted values (Table 4) indicating the validity and adequacy of the predicted models. The optimal condition gave higher values in 5-L fermentor (46.25±2.10 g DCW/L and 24.98±1.87 g PHB/L) compared to predicted value and gave the percentages of deviation from predicted values at 1 to 10%. Results obtained from other tested condition in this study also gave higher amount of both biomass and PHB than the predicted value in the range of deviation at 1 to 8%. The higher value was significantly affected by controlling of pH, aeration rate and agitation speed. Therefore, effect of aeration rate and agitation speed were investigated in more detail.

Effects of environmental condition on PHB production

C. necator TISTR 1095 was cultivated in the optimal medium consisting of 60 g/L glycerol, 1.32 g/L $(NH_4)_2SO_4$ and 2 g/L trace element at 30°C. The effect of variation

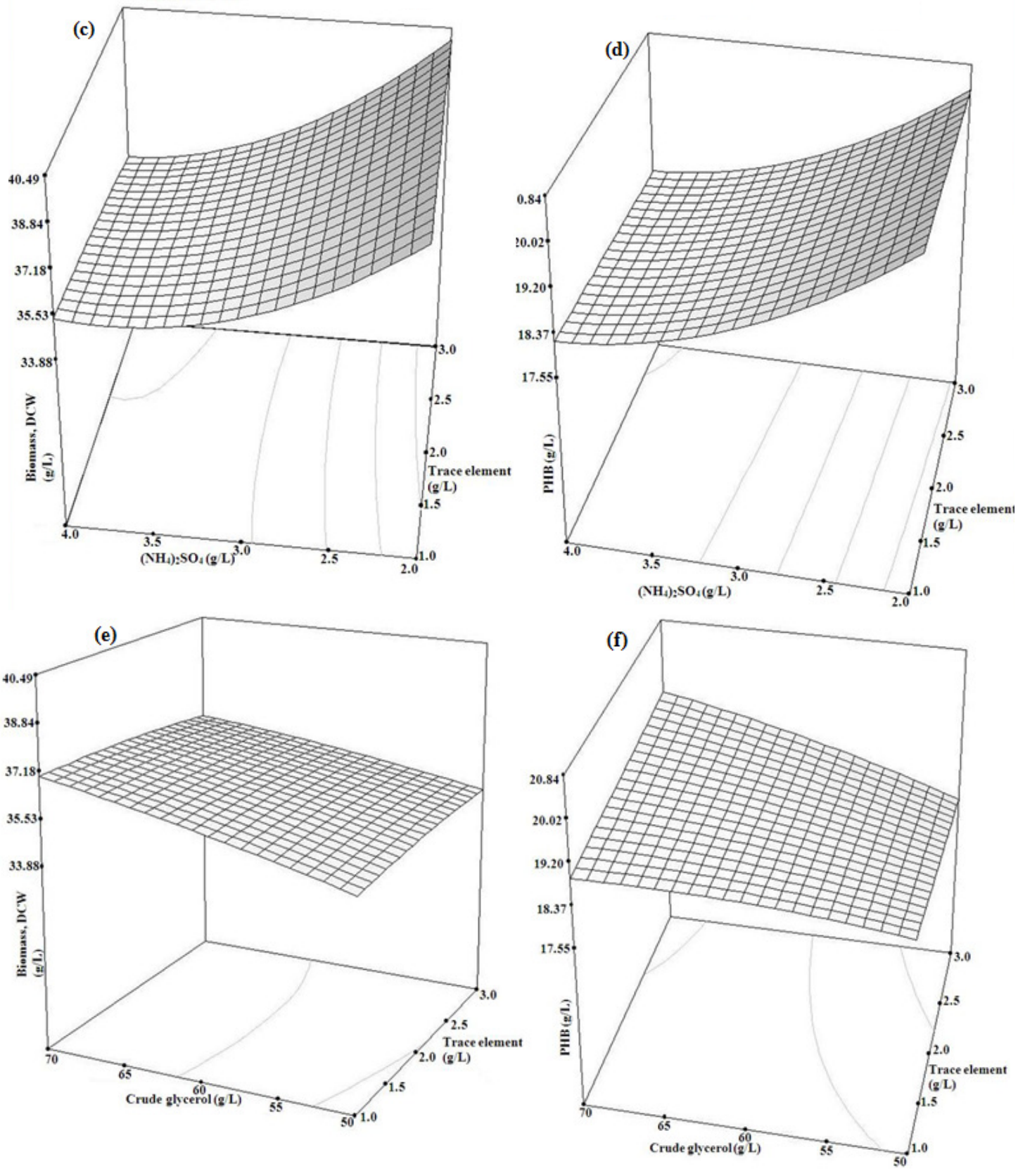

Figure 1. Continue

Table 4. Comparison of predicted and experimental values of two responses (Y_1, Y_2) at the optimal levels predicted and other three conditions from shake-flask model using *Cupriavidus necator* TISTR 1095 cultivated in 5-L fermentors with control of pH at 6.8, 1 vvm aeration rate and 150 rpm agitation speed using response surface method (RSM).

Condition	Parameter (g/L)	Predicted value[a] (g/L)	experimental value[b] (g/L)	Deviation[c] (%)
Optimal condition	X_1 = 60.00	Y_1 = 44.14	Y_1 = 46.25±2.10	4.78
	X_2 = 1.32	Y_2 = 21.95	Y_2 = 24.98±1.87	13.80
Other condition				
	X_3 = 2.00	(49.73% of DCW)	(54.01% of DCW)	
Condition 1	X_1 = 69.30	Y_1 = 41.38	Y_1 = 42.59±1.22	2.92
	X_2 = 2.00	Y_2 = 20.62	Y_2 = 22.57±0.99	9.46
	X_2 = 2.00X_3 = 3.00	Y_2 = 20.57(49.83% of DCW)	Y_2 = 22.38±1.07(53.00% of DCW)	8.80
Condition 2	X_1 = 70.00	Y_1 = 40.95	Y_1 = 42.11±1.04	2.83
	X_3 = 2.52	(50.23% of DCW)	(53.15% of DCW)	
	X_1 = 64.37	Y_1 = 40.65	Y_1 = 41.25±2.06	1.48
Condition 3	X_2 = 2.00	Y_2 = 20.33	Y_2 = 21.78±1.24	
	X_3 = 3.00	(50.01% of DCW)	(52.80% of DCW)	7.13

[a] Predicted value obtained from RSM model, [b] Observed value determined from RSM model, [c] [(observed value-predicted value)*100]/predicted value, Parameters: X_1 = glycerol concentration (g/L), X_2 = $(NH_4)_2SO_4$ (g/L), X_3 = trace element concentration (g/L), Y_1 = biomass (g/L), Y_2 = PHB concentration (g/L).

rate of aeration (0 to 2 vvm) and agitation speed (100 to 600 rpm) on the PHB production was investigated by adjusting the rate of the optimal culture. The result showed that the higher aeration rates, the higher PHB production.

The optimal aeration rate for cell growth (46.88±0.21 g/L) and PHB production (25.02±0.19 g/L) by *C. necator* TISTR 1095 was 2.0 vvm (Figure 2a). When cultivated *C. necator* TISTR 1095 under optimal medium at the aeration rate of 2.0 vvm, with agitation speed at 100 to 600 rpm, the production of PHB decreased as the agitation speed increased over 150 rpm (Figure 2b). This might be due to the cell destruction. Therefore, the maximum biomass (46.96±1.90 g/L) and PHB production (25.09±0.31 g/L) as well as 53.85% of DCW were observed at 150 rpm of agitation speed.

Sterile condition

C. necator strains TISTR 1095 was cultivated in optimal glycerol-containing medium with aeration rate and agitation speed at 2 vvm and 150 rpm, respectively under cultivation temperature at 30°C and pH controlled at 6.8. The effect of sterile condition, septic and aseptic, during cultivation in a 5-L fermentor on PHA production was investigated.

C. necator grew well under both septic and aseptic condition. Cultivation of *C. necator* in septic condition gave lower amount of biomass (45.99±1.68 g/L) and PHB (24.03±1.23 g/L) than aseptic with yielded biomass and PHB at 46.96±1.90 g/L and 25.09±0.31 g/L, respectively. However, the biomass and PHB production were not significantly different. It was found that sterile condition had the least effect on cellular growth and no effect on PHB concentration and content (52.25 to 53.43% of DCW).

The production of PHB under optimal condition in batch culture

According to the results presented above, the optimal cultivation condition of *C. necator* TISTR 1095 was in the medium with controlled pH at 6.8 and incubation at 30°C for 96 h. The cultivation was performed in a 20-L fermentor with aeration rate of 2.0 vvm and agitation speed of 150 rpm. The results are given in Figure 3 and kinetic parameters were calculated. These values were about 0.9-fold higher than those cultivated in mineral medium. The specific growth rate (μ) was 0.023 h^{-1}, yield coefficient ($Y_{p/x}$) was 0.54 g PHB/g cell and productivity (R_m) was 0.52 g/L.h. Cellular growth was 46.96±0.28 g/L, PHB concentration was 25.32±0.20 g/L and PHB content was 53.92% of DCW. These data suggested that *C. necator* TISTR 1095 expressed a good potential for production of PHB by fermentation in a 20-L bioreactor using a cultivation medium containing crude glycerol as

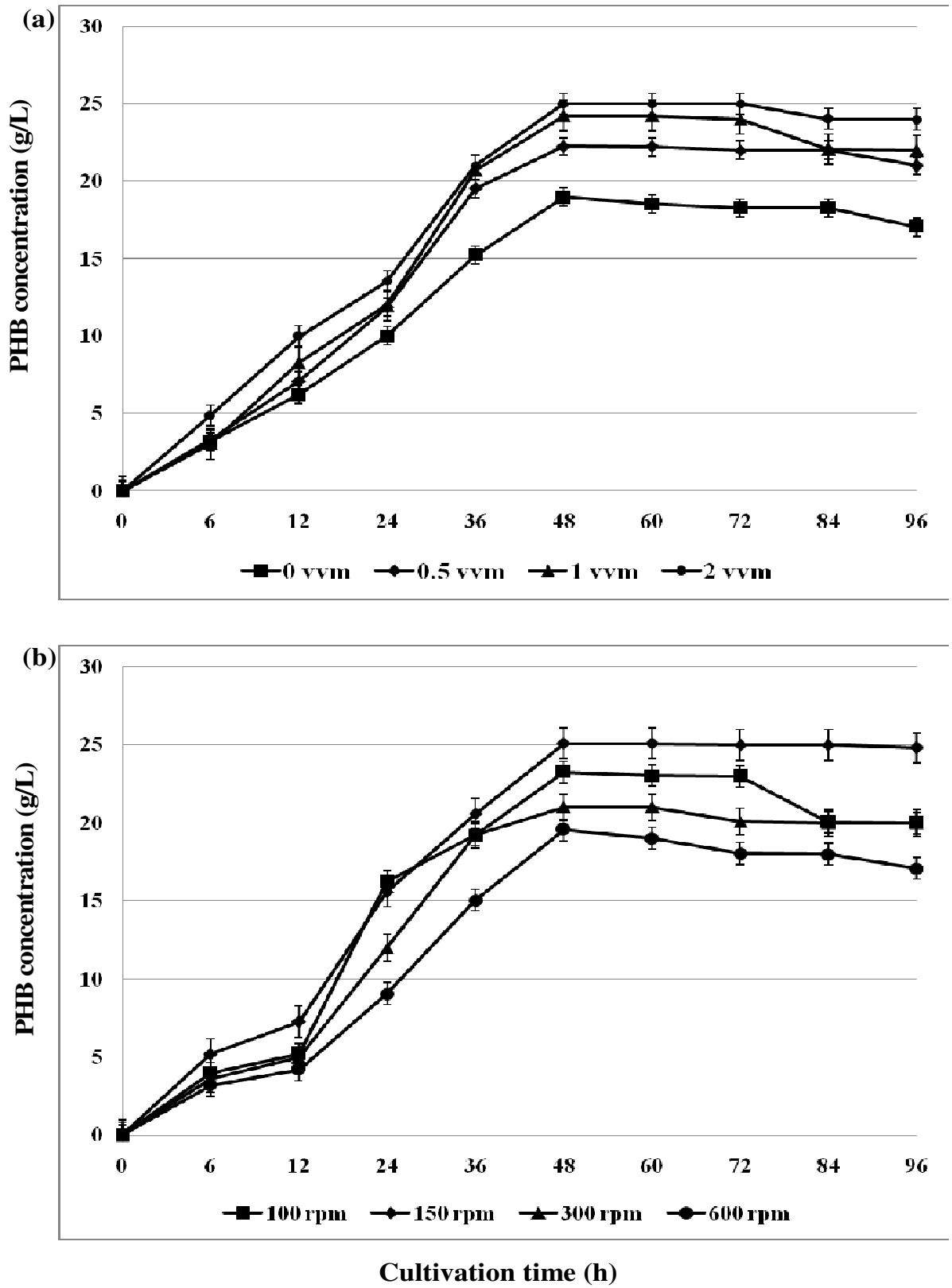

Figure 2. Effect of aeration rate (a) and agitation speed (b) on PHB content from *Cupriavidus necator* TISTR 1095 cultured in glycerol-containing medium consisting of 60 g/L glycerol, 1.32 g/L $(NH_4)_2SO_4$ and 2 g/L trace element at 30˚C under pH control at 6.8.

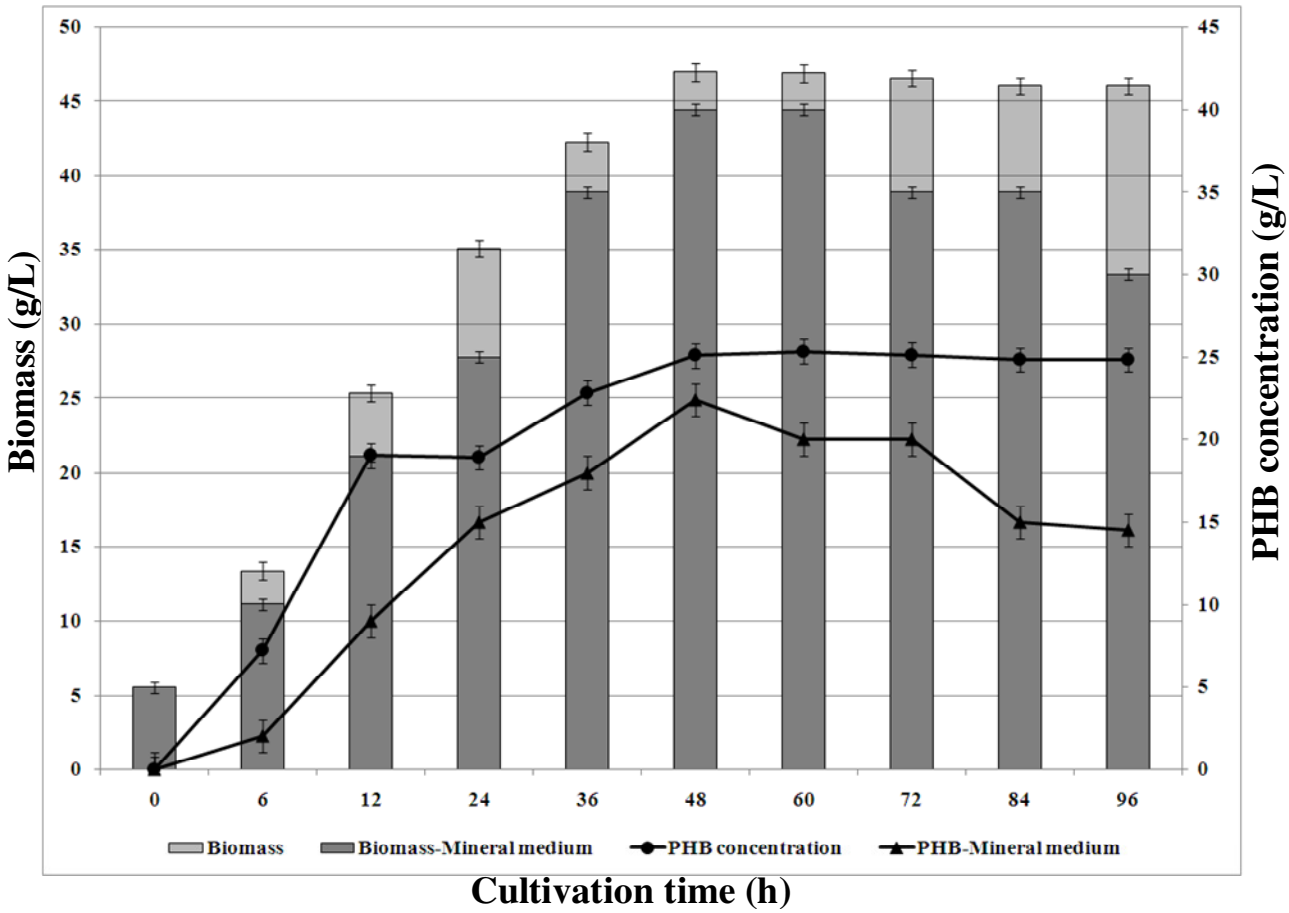

Figure 3. Gr□h (▓ ,) and PHB accumulation (●, ▲) of *Cupriavidus necator* TISTR 1095 on optimal medium containing glycerol (carbon source), 60 g/L; $(NH_4)_2SO_4$ (nitrogen source), 1.32 g/L and trace element 2.00 g/L at 30˚C, under controlled aeration rate (2 vvm), agitation speed (150 rpm), pH (6.8) conditions compared with mineral medium, respectively.

the main substrate.

Characterization of PHB

The thermal behavior of the samples was investigated by differential scanning calorimetry (DSC). The thermal properties determined for the purified samples and for the commercial PHB were compared and there was a good agreement among data from different samples. It is interesting to note that similar value of X_C was obtained from the commercial PHB (53%) and the purified PHB obtained from the optimal glycerol-containing medium (55%). In the DSC determination, the melting temperature of the PHB homopolymer from *C. necator* TISTR 1095 and from a commercial source was 160 to 170˚C and 175˚C, respectively. The molar mass of the isolated PHB was determined by ubbelohde viscometer, since molar mass is an important factor to determine physical properties of polymers and is known to vary with substrate and culture conditions. The results show that final Mv at 30℃ of PHB produced by *C. necator* TISTR 1095, cultivated with optimal glycerol-containing medium, was 400,000 Dalton (Da). The Mv of PHB from several microorganisms has been reported in the range of 300,000 to 500,000 Da depending on microorganisms and carbon source (Quagliano et al., 2001).

Conclusion

An objective of this research were to reduce the production cost of PHAs and to develop a high PHAs production procedure by selecting the prominent renewable carbon source, increase the PHB production by optimization studies as well as characterize partial properties of isolated PHAs. Crude glycerol from biodiesel refinery showed the great ability for PHB production by *C. necator* TISTR 1095. Using both statistically and conventional method, the results indicated that the

highest amount of biomass and PHB production (46.96±0.28 g/L biomass, 25.32±0.20 g/L PHB, 53.92% of DCW and $Y_{p/x}$ 0.54) was achieved with controlled aeration rate and agitation speed at 2 vvm and 150 rpm, respectively under septic condition. Partial properties of PHB produced by *C. necator* TISTR 1095 were similar to those of commercial PHB. The data suggested that crude glycerol, a by-product from biodiesel plant is a promising feedstock for PHB production from *C. necator*.

Pure glycerol is an important industrial feedstock with various applications. However, by-product glycerol from biodiesel process is impure and low economic value, it has been suggested that the open market of crude glycerol was stabilized at low price of $0.02/kg, but the cost to refine will cost approximately $0.10/kg. Although, the alternative uses of the crude glycerol from biodiesel will need to be created (Choi and Lee, 1997; Castilho et al., 2009).

In an economical point of view, *C. necator* TISTR 1095 could grow and produce PHB from biodiesel waste under septic condition. This fact indicated that this strain offer a good prospect for utilizing crude glycerol and converting into a high value product while the market price of PHB was $16.25/kg (Choi and Lee, 1997; Castilho et al., 2009).

Furthermore, this fermentation process can be applied as a part of industrial waste-treatment system. It can indeed provide double benefits because environmentally polluting waste is converted into environmentally friendly biodegradable polymer (Choi and Lee, 1997; Sangkharak and Prasertsan, 2007, 2008; Kemavongse, 2008; Castilho et al., 2009).

ACKNOWLEDGEMENTS

The authors would like to thank for the financial support From Research and Development Institute, Thaksin University, Thailand.

REFERENCES

Box GEP, Hunter WG, Hunter JS (1978). Statistics for experimenters. John Wiley Sons, New York.

Castilho LR, Mitchell DA, Freire DMG (2009). Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. Bioresour. Technol. 10: 5996-6009.

Cavalheiro JMBT, de Alimeida MCMD, Grandfils C, da Fonseca MMR (2009). Poly(3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol. Process Biochem. 44: 509-515.

Chaudhry WN, Jamil N, Ali I, Ayaz MH, Hasnain S (2010). Screening for polyhydroxyalkanoates (PHA)-producing bacterial strains and comparison of production from various inexpensive carbon sources. Ann. Microbiol. 61: 623-629.

Chi Z, Pyle D, Wen Z, Frear C, Chen S (2007). A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerol by microalgal fermentation. Process Biochem. 42: 1537-1545.

Cho IH, Zoh KD (2007). Photocatalytic degradation of azo dye (*Reactive Red 120*) in TiO_2/UV system: optimization and modeling using a response surface methodology (RSM) based on the central composite design. Dyes Pigments, 75: 533-543.

Choi J, Lee SY (1997). Process analysis and economic evaluation for poly(3-hydroxybutyrate) production by fermentation. Bioprocess Eng. 17: 335-342.

Grengross TU, Slater SC (2000). How green are green plastics?. Sci. Am. 8: 37-41.

Grothe E, Chistri Y (2000). Poly(β-hydroxybutyric acid) thermoplastic production by *Alcaligenes latus*: Behavior of fed-batch cultures. Bioprocess Eng. 22: 441-449.

Grothe E, Young MM, Chisti Y (1999). Fermentation optimization for the production of poly(β-hydroxybutyric acid) microbial thermoplastic. Enzyme Microb. Tech. 25: 132-141.

Gonzalez-Pajuelo M, Meynial-Salles I, Mendes F, Andrade JC, Vasconcelos I, Soucaille P (2005). Metabolic engineering of *Clostridium acetobutylicum* for the industrial production of 1,3-propanediol from glycerol. Metab. Eng. 7: 329-336.

He GQ, Kong Q, Ding LX (2004). Response surface methodology for optimization the fermentation medium of *Clostridium butyricum*. Lett. Appl. Microbiol. 39: 363-368.

Inoue Y, Yoshie N (1992). Structure and physical properties of bacterially synthesized polyesters. Prog. Polym. Sci. 17: 571-610.

Jendrossek D, Handrick R (2002). Microbial degradation of polyhydroxyalkanoates. Annu. Rev. Microbiol. 56: 403-432.

Kemavongse K, Prasertsan P, Upaichit A, Methacanon P (2008). Poly-β-hydroxyalkanoate production by halotolerant *Rhodobacter sphaeroides* U7. World J. Microb. Biot. 24: 2073-2085.

Khardenavis AA, Kumar MS, Mudliar SN, Chakrabarti T (2007). Biotechnological conversion of agro-industrial wastewaters into biodegradable plastic, poly-β-hydroxybutyrate. Bioresour. Technol. 98: 3579-3584.

Lakshmar K, Rastogi NK, Shamala TR (2004). Simultaneous and comparative assessment of parent and mutant strain of *Rhizobium meliloti* for nutrient limitation and enhance polyhydroxyalkanoate (PHA) production using optimization studies. Process Biochem. 39: 1977-1983.

Luengo JM, Garcia B, Sandoval A, Naharro G, Oliver ER (2003). Bioplastics from microorganisms. Curr. Opin. Biotechnol. 6: 251-260.

Marchessault RH, Okamura K, Su CJ (1970). Physical properties of poly(β-hydroxy butyrate). II. conformational aspects in solution. Macromolecules, 3: 735-740.

Mothes G, Schnorpfeil C, Ackermann JU (2007). Production of PHB from crude glycerol. Eng. Life Sci. 7: 475-479.

Mu Y, Teng H, Zhang DJ, Wang W, Xiu ZL (2006). Microbial production of 1,3-propandiol by *Klebsiella pneumoniae* using crude glycerol from biodiesel preparation. Biotechnol. Lett. 28: 1755-1759.

Mumtaz T, Yahaya NA, Abd-Aziz S, Rahman NAA, Yee PL, Shirai Y, Hassan AM (2010). Turning waste to wealth-biodegradable plastics polyhydroxyalkanoates from palm oil mill effluent-a Malaysian perspective. J. Clean Prod. 18: 1393-1402.

Nikel PI, Pettinari MJ, Mendez BS, Galvagno MA (2005). Statistical optimization of a culture medium for biomass and poly(3-hydroxybutyrate) production by recombinant *Escherichia coli* strain using agroindustrial by products. Int. Microbiol. 8: 243-250.

Quagliano JC, Amarila F, Fernandes EG, Mata D, Miyazaki SS (2001). Effect of simple and complex carbon sources, low temperature culture and complex carbon feeding polices on poly-3-hydroxybutyric acid (PHB) content and molecular weight (mw) from *Azotobacter chroococcum* 6B. World J. Microb. Biot. 17: 9-14.

Rebah FB, Prévost D, Tyagi RD, Belbahri L (2009). Poly-beta-hydroxybutyrate production by fast-growing *Rhizobia* cultivated in sludge and in industrail wastewater. Appl. Biochem. Biotech. 158(1): 155-163.

Reddy CSK, Ghai R, Rasmi, Kalia VC (2003). Polyhydroxyalkanoates: an overview. Bioresour. Technol. 87: 137-146.

Sangkharak K (2009). The production of polyhydroxyalkanoates by microorganisms. Thaksin University Journal [online] 12: 75-89. http://www.pt.tsu.ac.th/rdi/Ejournals/EJournals/TSUJournal2009-2.pdf.

Sangkharak K (2011). Optimization studies on enzymatic hydrolysis for ethanol production by simultaneous saccharification and fermentation from wastepaper. Waste Manage. Res. [online].doi:10.1177/0734242X10387656.
Sangkharak K, Prasertsan P (2007). Optimization of polyhydroxybutyrate production from wild type and two mutant strains of *Rhodobacter sphaeroides* using statistical method. J. Biotechnol. 132: 331-340.
Sangkharak K, Prasertsan P (2008). Nutrient optimization for production of polyhydroxybutyrate from halotolerant photosynthetic bacteria cultivated under aerobic-dark condition. Electron. J. Biotechn. [online] 11 http://www.ejbiotechnology.info/content/vol11/issue3/full/2/2.pdf.
Shah AA, Hasan F, Hameed A, Ahmed S (2007). Isolation and characterisation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) degrading actinomycetes and purification of PHBV depolymerase from newly isolated *Streptoverticillium kashmirense* AF1. Ann. Microbiol. 57: 583-588.
Shrivastav A, Mishra SK, Shethia B, Pancha I, Jain D, Mishra S (2010). Isolation of promising bacterials strains from soil and marine environment for polyhydroxyalkanoates (PHAs) production utilizing *Jatropha* biodiesel byproduct. Int. J. Biol. Macromol. 47: 283-287.
Steinbüchel A, Doi Y (2001). Biopolymer, volume 3b, polyesters II, properties and chemical synthesis. Wiley-VCH, Weinheim.
Steinbüchel A, Wiese S (1992). A *Pseudomonas* strain accumulating polyesters of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids. Appl. Microbiol. Biot. 37: 691-697.
Suthar V, Pratap A, Raval H (2000). Studies on poly (hydroxy alkanoates)/(ethylcellulose) blends. B. Mater. Sci. 23: 215-219.
Thomson H (2001). Kife in plastic. Engineering-London, 242: 59-60.
Taidi B, Anderson A, Dawes EA, Byrom D (1994). Effect of carbon sources and concentrational on the molecular mass of poly(3-hydroxybutyrate) produced by *Methylobacterium extorquens* and *Alcaligenes eutrophus*. Appl. Microbiol. Biot. 40: 786-790.
Timm A, Byrom D, Steinbüchel A (1990). Formation of blends of various poly(3-hydroxyalkanoic acids) by a recombinant strain of *Pseudomonas oleovorans*. Appl. Microbiol. Biot. 33: 296-301.
Wang J, Yu J (2000). Kinetic analysis on inhibited growth and poly(3-hydroxybutyrate) formation of *Alcaligenes eutrophus* on acetate under nutrient-rich conditions. Process Biochem. 36: 201-207.
Wen ZY, Chen F (2001). Application of statistically-based experimental designs for the optimization of eicosapentaenoic acid production by the diatom *Nitzschia laevis*. Biotechnol. Bioeng. 75: 159-169.