Electronic Journal of Biotechnology 19 (2016) 50–55

HOSTED BY

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research Article

Bioconversion of mixed free fatty acids to poly-3-hydroxyalkanoates by *Pseudomonas putida* BET001 and modeling of its fermentation in shake flasks

Khalil Munawar Makhdum Munawar, Khanom Simarani *, Mohamad Suffian Mohamad Annuar

Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

ARTICLE INFO

Article history:
Received 16 April 2015
Accepted 14 November 2015
Available online 11 December 2015

Keywords:
Biopolymer
Fermentation
Medium-chain-length PHA
Palm kernel oil
Pseudomonas putida

ABSTRACT

Background: The paper reports on the utilization of palm kernel oil (PKO) as a low cost renewable substrate for medium-chain-length poly-3-hydroxyalkanoates (mcl-PHA) production by *Pseudomonas putida* BET001. Investigation on the effects of selected key variables on growth, mixed free fatty acids consumption and mcl-PHA production by the bacterial culture in the shaken flask system were carried out along with its kinetic modeling.
Results: The biomass production, fatty acids consumption and mcl-PHA production were found favorable when the strain was cultured in mineral medium at pH 6–7, 28°C, aeration surface-to-volume ratio of 0.4×10^6 m^{-1}, 250 rpm agitation rate for 48 h. Mcl-PHA production by this strain showed mixed growth and non-growth associated components as described by Luedeking–Piret kinetic model.
Conclusion: The findings of this study provided add to the literature on key variables in for achieving good microbial growth and mcl-PHA production in shake flasks culture. In addition, suitable kinetic model to describe cultivation in this system was also presented.

1. Introduction

Polyhydroxyalkanoates (PHA) are biodegradable and biocompatible polyesters. More than 150 monomers can be combined within this family to give materials with vastly different properties [1,2]. The resulting biopolymers can be either thermoplastic or elastomeric materials with melting points ranging from 40–180°C. The mechanical and biocompatibility properties of PHA can also be altered by blending, surface modification or combining the PHA with other polymers, enzymes and inorganic materials, expanding its range of applications [3].

Due to increasing environmental pollution caused by non-biodegradable materials, the potential usage of these biodegradable polymers is high on priority list [4]. The biodegradability, compostability, restorability and piezoelectricity of PHA incur their potential exploitation in applications such as biodegradable polymeric packaging materials, biofuels, paper sizing biomaterials and drug delivery devices [5]. Diversity in the monomeric composition of PHA resulted in potentially high-molecular weight polymeric materials with myriads of applications as compared to their chemically synthesized counterparts. In addition, the increasing demand of highly functionalized PHA for specialty applications warrants the enhancement of microbial bio-processing capable of efficiently accumulating these biodegradable polymers [6]. This polyester can be produced *via* microbial fermentation using fatty acids as a carbon source for growth [7,8,9]. To date, most of the researches on medium-chain-length poly-3-hydroxyalkanoates (mcl-PHA) have been focused on commercial free fatty acids as sole carbon source [10] and [11]. One of the potential renewable carbon feedstock for use as mcl-PHA fermentation substrate is palm kernel oil (PKO). PKO is derived from the kernel of oil palm [12]. This edible oil consists of highly saturated vegetable fats not unlike that of coconut oil and palm oil. The most abundant component in palm oil and coconut oil is 16-carbon atom length saturated fatty acid such as palmitic acid ($CH_3(CH_2)_{14}COOH$). However, in PKO, 12-carbon atom length saturated fatty acid *viz.* lauric acid is pre-dominant (Table 1).

In this study, the production of mcl-PHA from raw PKO by a wild-type *Pseudomonas putida* BET001 was investigated. The effects of pH, temperature, agitation rate, aeration surface-to-volume ratio and fermentation time were investigated in the shaken flask cultures. Kinetic studies were also carried out under investigated conditions to understand the relationships among cell growth, free fatty acid utilization and mcl-PHA production.

2. Materials and methods

2.1. Microorganisms

A bacterial strain previously isolated from palm oil mill effluent (POME) and identified as *P. putida* BET001 [11] was used in this study. It was grown and maintained on rich medium agar consisting

* Corresponding author.
E-mail addresses: hanom_ss@um.edu.my, hanomks@gmail.com (K. Simarani).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2015.07.005

Table 1
Fatty acid composition of PKO.

Fatty acids	Percentage (%)
C6:0 (caproic acid)	0.2
C8:0 (caprylic acid)	3.0
C10:0 (capric acid)	4.0
C12:0 (lauric acid)	48.0
C14:0 (myristic acid)	16.0
C16:0 (palmitic acid)	8.0
C18:0 (stearic acid)	3.0
C18:1 (oleic acid)	15.4
C18:2 (linoleic acid)	2.4
C20:0 (arachinoic acid)	0.1

Source: Ang et al. [20].

of (g L^{-1}): 10 yeast extract, 15 nutrient broths, 5 ammonium sulfate and 18 agar at 32°C for 24 h. This strain is known to produce medium-chain-length poly-3-hydroxyalkanoates with monomer composition ranging from C_4–C_{14} [11].

2.2. Saponification process

Saponification of the PKO was carried out according to Annuar et al. [6]. 2.8 g of sodium hydroxide was dissolved in 100 mL absolute ethanol; 8.0 g of PKO was added to this mixture which was stirred well inside a round-bottom flask. The mixture was refluxed gently for 1 h during which it was swirled from time to time. After this, flask was cooled under running water and excess ethanol evaporated by rotary vacuum evaporation; leaving behind the sodium salt of the fatty acids. These were dried and stored at room temperature.

2.3. Growth medium and shake flask cultivation

One loopful of a single colony grown on the nutrient agar medium was inoculated into 100 mL of sterile rich medium and incubated on an orbital shaker under agitation rate of 250 rpm at 32°C for 24 h. From this culture, 0.29 ± 0.02 g L^{-1} cell dry weight was inoculated into 100 mL of E2 medium in 250 mL Erlenmeyer flask. E2 medium consisted of (g L^{-1}): 0.24 ammonium, 10 saponified PKO, 5.73 dipotassium phosphate, and 3.7 potassium orthophosphate. To avoid precipitation during autoclaving, 10 mL 0.1 M $MgSO_4$ solution and 1.0 mL trace mineral solution were added aseptically after the medium has cooled down to room temperature. The trace element solution was made of (g 100 mL^{-1}): 2.7 $FeCl_3 \times 6H_2O$, 0.2 $ZnCl_2 \times 4H_2O$, 0.2 $CoCl_2 \times 6H_2O$, 0.2 $Na_2MoO_4 \times 2H_2O$, 0.1 $CaCl_2 \times 2H_2O$, 0.13 $CuCl_2 \times 6H_2O$, 0.05 H_3BO_3 and 10 mL concentrated HCl. The fatty acid composition of PKO was listed in Table 1.

2.4. Effects of selected fermentation variables on mcl-PHA production in shaken flask system

Studies on total biomass growth, mcl-PHA production and free fatty acid consumption in shake flask cultivation were undertaken as a function selected key variables namely the initial pH (5, 6, 7, 8 and 9), cultivation temperature of (25, 28, 32, 37 and 40°C), agitation speed (90, 170 and 250 rpm), aeration surface-to-volume ratio (1×10^6 m^{-1}, 0.4×10^6 m^{-1}, 0.2×10^6 m^{-1} and 0.1×10^6 m^{-1}) and incubation time (24, 48, 72 h). The shake flasks were prepared in triplicate and chosen randomly from a population of shaken flasks for analyses at regular intervals. Broth samples were centrifuged and cell mass was collected to determine the total biomass and mcl-PHA production while the supernatant was collected to determine residual free fatty acid concentration.

2.5. Kinetic studies

Kinetic analyses were performed on data obtained from the shake flask culture under investigated fermentation variables *viz.* initial pH 7, cultivation temperature of 28°C, aeration surface-to-volume of (1×10^6 m^{-1}), agitation rate of 250 rpm and cultivation time of 72 h.

2.6. Analytical methods

2.6.1. Determination of biomass production

The total biomass *i.e.* the residual biomass plus mcl-PHA was determined *via* gravimetry method. 1.0 mL of the culture was pipetted into a tared microfuge tube. The cells were spun down at 8000 rpm for 5 min. The supernatant was discarded and the pellet was washed twice with phosphate buffer solution. Then, the samples were oven dried at 90°C until constant weight. Measurements were carried out in triplicate.

2.6.2. Determination of residual free fatty acid concentration

Fatty acids were extracted using 50 mL isopropanol in a ratio of 1:10 of sample-to-solvent. The extracted fatty acids were titrated with 0.05 N NaOH in isopropanol until the mixture solution reached pH 7. The concentration of fatty acids titrated was determined using the following equation:

$$[\mathrm{FFA}]\left(\frac{\mathrm{mol}}{\mathrm{m^3}}\right) = \frac{V_{\mathrm{NaOH}}(\mathrm{mL}) \times \mathrm{Normality}_{\mathrm{NaOH}}\left(\frac{\mathrm{mol}}{\mathrm{L}}\right) \times 1000\left(\frac{\mathrm{L}}{\mathrm{mL}}\right)}{\mathrm{Volume\ of\ sample\ withdrawn}(\mathrm{m^3})} \quad \text{[Equation 1]}$$

where [FFA] is the concentration of residual fatty acid (mol/m^3) and V_{NaOH} is the total volume of alkali added (mL).

2.6.3. Determination of mcl-PHA

The mcl-PHA content was analyzed using gas chromatography (GC). Direct cell methanolysis method was used for the determination of intracellular mcl-PHA amount [6]. The machine used was Thermo Scientific Trace GC Ultra employing TG-5MS column (30 m × 0.32 mm × 0.25 μm). The column temperature program which was used to separate the different 3-hydroxyalkanoic acid methyl esters was as follows: program rate = 10°C min^{-1}; initial temperature = 50°C; final temperature = 280°C; hold time = 2. Helium was used as a carrier gas at 35 mL min^{-1}. A splitless injection of 1 μL was performed.

2.7. Numerical calculation

The kinetic of bacterial growth was described using a logistic model:

$$\frac{dX}{dt} = \mu \max\left(1 - \frac{X}{X \max}\right)X \quad \text{[Equation 2]}$$

where dX/dt is the rate of biomass production (g L^{-1} h^{-1}), X is the biomass concentration (g L^{-1}), μ_{max} is maximum specific growth rate (h^{-1}) and X_{max} is the maximum attainable biomass concentration (g L^{-1}).

The mcl-PHA production was calculated using the Luedeking–Piret (LP) model [13] as expressed in [Equation 3]:

$$\frac{dP}{dt} = \alpha\left(\frac{dX}{dt}\right) + \beta X \quad \text{[Equation 3]}$$

where dP/dt is the rate of mcl-PHA production (g L^{-1} h^{-1}), dX/dt is the rate of biomass production (g L^{-1} h^{-1}), α is the coefficients for growth associated product, β is the coefficient for non-growth associated product (h^{-1}) and X is the concentration of biomass (g L^{-1}).

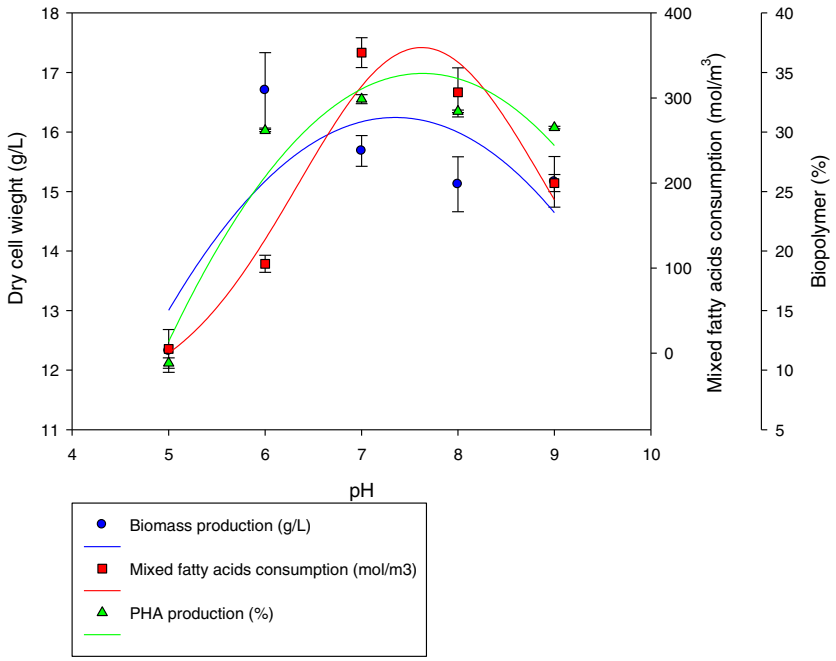

Fig. 1. Biomass growth, mixed fatty acid consumption and mcl-PHA production of *P. putida* BET001 as a function of different initial pHs (5, 6, 7, 8, and 9) with constant of other variables such as temperature (25°C), agitation (170 rpm), volume of media (100 mL) and incubation time (48 h) with 10% inoculum.

The rate of free fatty acid consumption as a growth substrate was described as shown in [Equation 4] below:

$$\frac{dS}{dt} = \frac{1}{YG}\left(\frac{dX}{dt}\right) + \frac{1}{Yps}\left(\frac{dP}{dt}\right) + ms(X) \qquad \text{[Equation 4]}$$

where dS/dt is the rate of free fatty acid utilization (g L^{-1} h^{-1}), dX/dt is the rate of biomass production (g L^{-1} d^{-1}), dP/dt is the rate of mcl-PHA formation (g L^{-1} h^{-1}), Y_G is coefficient biomass yield over substrate (g g^{-1}), Y_{ps} is coefficient product yield over substrate (g g^{-1}), m_s is cell maintenance coefficient (g g^{-1} h^{-1}) and X is biomass concentration (g L^{-1}).

2.8. Statistical analysis

All experiments were performed in triplicate. The numerical results are expressed as mean ± standard deviation. Statistical analyses were performed using Sigma Stat 12.0. Non-linear regression using Runge–Kutta–Fehlberg algorithm to fit the model with experimental data was carried out using Polymath 6 software.

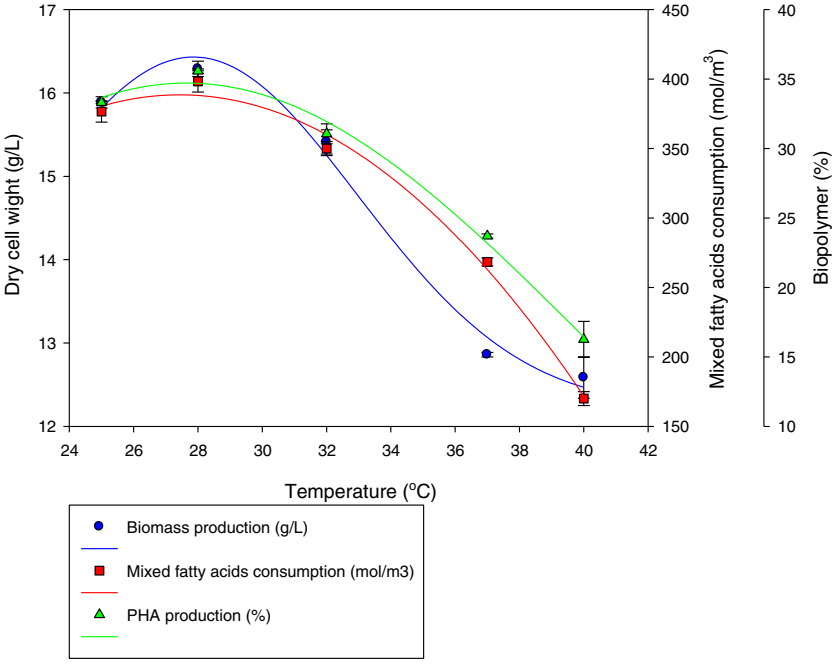

Fig. 2. Biomass growth, fatty acid consumption and mcl-PHA production of *P. putida* BET001 as a function of different temperatures (25, 28, 32, 37 and 40°C) with constant of other variables such as pH (7), agitation (170 rpm), volume of media (100 mL) and incubation time (48 h) with 10% inoculum.

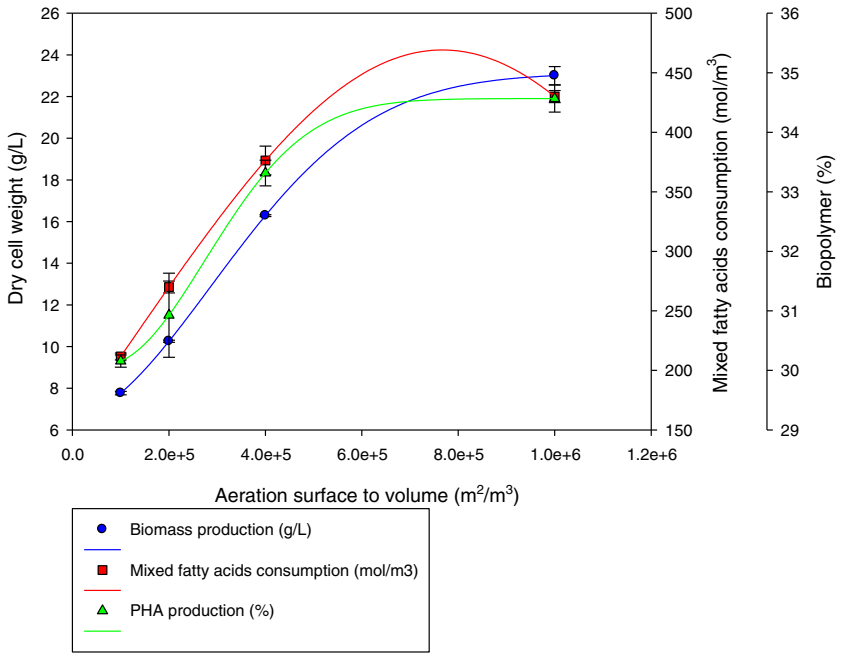

Fig. 3. Biomass growth, fatty acid consumption and mcl-PHA production of *P. putida* BET001 as a function of different aeration surface-to-volume ratio (1×10^6, 0.4×10^6, 0.2×10^6, 0.1×10^6) with constant of other variables such as pH (7), temperature (28°C), agitation (170 rpm), and incubation time (48 h) with 10% inoculum.

3. Results and discussion

3.1. Effects of fermentation variables on growth, mcl-PHA production and free fatty acid consumption

Investigations on the effects of selected key variables on total biomass, mcl-PHA production and free fatty acid consumption in shake flask cultivation were performed based on the adjustment of initial pH, temperature and an aeration surface-to-volume ratio. The total biomass, mcl-PHA production and free fatty acids by *P. putida* BET001 were clearly affected by the initial pH of culture medium. Fig. 1 showed the highest biomass concentration of 16.7 ± 0.63 g L^{-1} was obtained at pH 6. However, at pH 7 the highest free fatty acid consumption of 353 ± 17.56 mol m^{-3} was observed along with 33 ± 0.38% of total dried biomass weight mcl-PHA production. These favorable pH range for growth, biopolymer production and free fatty acid consumption in *P. putida* BET001 were similar with the studies of Lopez-Thomas et al. [14] that found that the optimum pH for growth of *P. putida* is between pH 6.5 and 7.

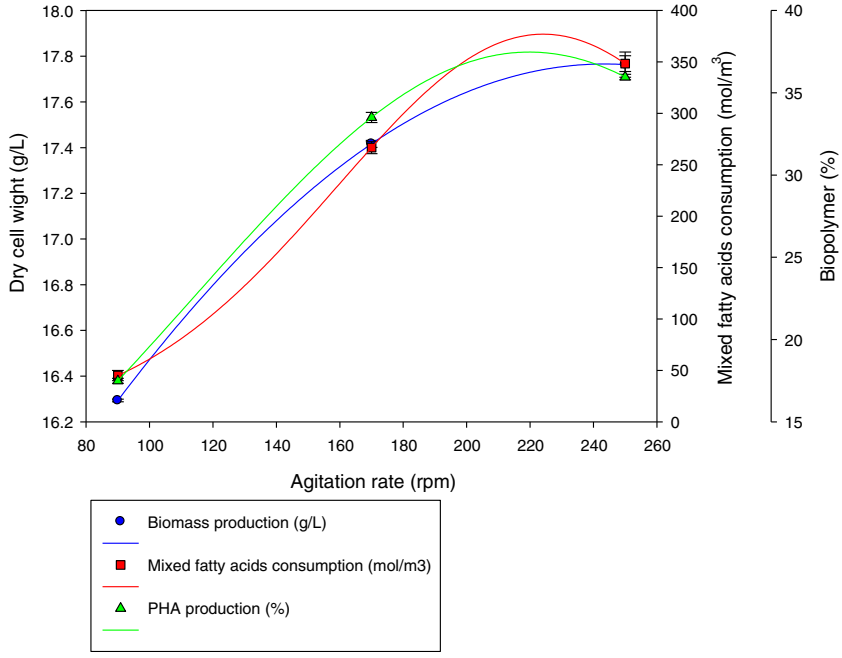

Fig. 4. Biomass growth, fatty acid consumption and mcl-PHA production of *P. putida* BET001 as a function of different agitation speeds (90, 170 and 250 rpm) with constant of other variables such as pH (7), temperature (28°C), volume of media (100 mL) and incubation time (48 h) with 10% inoculum.

Table 2
Biomass and mcl-PHA production by different types of bacteria and fatty acids as carbon sources.

Carbon source	Bacterium	Biomass (g)	PHA %	PHA mole fraction (mol%)										Reference
				C4	C5	C6	C7	C8	C10	C12	C14	C16	C18	
C6:0 (caprylic acid)		9.8	49.7	–	–	8.1	–	76.2	11.0	4.7	–	–	–	
C12:0 (lauric acid)		10.6	54.5	–	–	3.5	–	38.2	38.9	19.4	–	–	–	
	P. putida Bet001													[11]
C16:0 (palmitic acid)		14.2	65.3	–	–	4.1	–	36.9	34.8	18.0	6.3	–	–	
C18:1 (oleic acid)		15.5	68.9	1.5	–	5.0	0.7	31.8	24.1	22.9	14.1	–	–	
C12:0 dodecanoic acid	*P. entomophila* L48	NA	NA	–	–	–	–	44.5	38.9	–	–	–	–	[10]
PKO	*P. putida* Bet001	17.3	34.9	ND	ND	ND	ND	34.9	22.4	20.7	15.7	6.3	–	This study

Note: PHA (% of total dried biomass weight).

The temperature for cultivation was investigated at 25, 28, 32, 37 and 40°C. Comparable biomass concentrations of 16.7 ± 0.09, 15.9 ± 0.07 and 15.4 ± 0.15 g L^{-1} were obtained at 28, 25 and 32°C respectively (Fig. 2). As a mesophile microorganism from soil environment, the increase in cultivation temperature up to 37 and 40°C could lead to significant lower production of biomass (12.9 ± 0.03–12.6 ± 0.25 g L^{-1}). Similar reported by Mordocco et al. [15] and Harwood et al. [16] showed that optimum temperature range for *P. putida* growth is between 25 and 30°C. The free fatty acid consumptions were also significantly reduced to 170 ± 5.00 mol m^{-3} at 40°C from 268.33 ± 2.89 mol m^{-3} at 37°C. At the appropriate cultivation temperature of 28°C, *P. putida* BET001 could consume the free fatty acids at 398 ± 7.64 mol m^{-3} as a sole carbon and energy source with 36 ± 0.15% w/w production of mcl-PHA.

The aeration surface-to-volume ratio is another important variable influencing the growth of an aerobic *P. putida* BET001 in shake flask cultivation. The aeration surface-to-volume ratio of 1 × 10^6 m^{-1} gave the highest biomass concentration of 23 ± 0.44 g L^{-1} with mcl-PHA content of 35 ± 0.23% total cell dry weight with the free fatty acid consumption at 430 ± 5.00 mol m^{-3} under agitation rate of 170 rpm (Fig. 3). However, the growth (17.76 ± 0.06 g L^{-1}), consumption of free fatty acids (348.33 ± 7.64 mol m^{-3}) and biopolymer production (35.96 ± 0.17%) appeared to be favored at 250 rpm and aeration surface-to-volume of 0.4 × 10^6 m^{-1} (Fig. 4). This indicated that the appropriate combination of agitation rate and aeration surface-to-volume ratio played a crucial role in a highly aerobic fermentation such as mcl-PHA production from a reduced substrate such as fatty acids.

Cultivation of *P. putida* BET001 using saponified PKO as mixed free fatty acid substrate under investigated conditions for mcl-PHA production was compared to different strains and types of fatty acids as a major carbon source (Table 2). A variation of fatty acid identities in the PKO provide greater amount of reduced carbon substrate that could be oxidized for both cellular growth and mcl-PHA accumulation at a substantially advantageous cost compared to pure fatty acids. It was found that biomass yield was similar to when pure fatty acids were used as substrate as shown by the work of Razaif-Mazinah et al. [17] where the application of a mixture of heptanoic acid (C7) and oleic acid (C18:1) as a carbon substrate in *P. putida* BET001 cultivation also yielded approximately 37% of mcl-PHA content.

Gumel et al. [11] postulated that medium chain length fatty acids are more favorable for the bacterial strain cultivation as higher PHA production was obtained on oleic acid (C18:1). PKO consists of relatively high fraction of medium chain length fatty acids *i.e.* lauric acid (C12) at 48% (Table 1). The biomass fraction of mcl-PHA obtained in this study was comparable to Tan et al. [18] where they obtained approximately 37% of *P. putida* PGA 1 cellular content with PKO as a major carbon substrate.

3.2. Kinetic studies of growth, mcl-PHA production and mixed free fatty acid consumption in shaken flask cultivation

For a successful process optimization, acquisition of process kinetics information describing cell growth, product accumulation and substrate consumption is required [19]. Kinetic analyses were performed on data collected from the shake flask cultivation under favorable culture conditions of initial pH 7, cultivation temperature of 28°C, aeration surface-to-volume of (3.8 × 10^5 m^2/m^3), agitation rate of 250 rpm and cultivation time of 72 h to understand the relationship between cell growth and biosynthesis of mcl-PHA on PKO free fatty acids. Based from [Equation 2]–[Equation 4], the kinetic parameters associated with the rate of biomass formation (dX/dt), the rate of fatty acid utilization (dS/dt) and the rate of PHA production (dP/dt) were shown in Table 3. The average volumetric growth (dX/dt) was at 1.47 g L^{-1} h^{-1} with maximum specific growth rate (μ_{max}) of 0.04 h^{-1}. The microbial growth was increased until 60 h fermentation with the maximum biomass concentration of 17.5 g L^{-1} before declining afterwards (Fig. 5). The free fatty acid consumption with the respect of growth yield (Y_G) was determined at 1.7 g g^{-1} (biomass/fatty acids). However, the mcl-PHA content increased steadily (up to 37%) with growth until 48 h and it started to reach a plateau when 528 mol m^{-3} of fatty acids was utilized. Product yield (Y_{PS}) was calculated at 0.6 g g^{-1} (mcl-PHA/ fatty acids) with maintenance coefficient (m_s) at 0.03 h^{-1}. The average volumetric consumption of fatty acids was 2.16 g L^{-1} h^{-1}. For mcl-PHA production, the Luedeking–Piret parameters, α and β were determined at 0.49 and 0.01 h^{-1}, respectively (Table 3).

Model prediction under investigated conditions in the shake flask cultivations showed a good agreement with experimental data (Fig. 5). Hence, it provides a basis to determine beforehand key parameters such as cell harvesting time, time of maximum mcl-PHA accumulation and substrate depletion. This information is useful, for example, if the shake flask culture is to be used as subsequent starting culture for the next level fermentation.

4. Conclusions

The data presented in this study highlighted the key fermentation variables in shake flask cultivation when highly reduced substrates

Table 3
Kinetic parameters and coefficient of correlation of bacterial growth, free fatty acid utilization and PHA production.

Kinetic parameters	Cultivation of bacterium	R^2
Bacterial growth		
X_{max} (g L^{-1})	17.45 ± 0.11	0.9652
μ_{max} (g L^{-1})	0.043 ± 0.0001	
dX/dt(g L^{-1} h^{-1})	1.47	
Free fatty acid utilization		
Y_G (g g^{-1})	1.745 ± 0.011	0.9932
Y_{ps} (g g^{-1})	0.643 ± 0.001	
m_s (g g^{-1} h^{-1})	0.029 ± 0.000	
$-dS/dt$(g L^{-1} h^{-1})	2.16	
PHA production		
α	0.49 ± 0.003	0.9982
β (h^{-1})	0.0082 ± 0.000	
dP/dt(g L^{-1} h^{-1})	0.76	

R^2: coefficient of correlation.

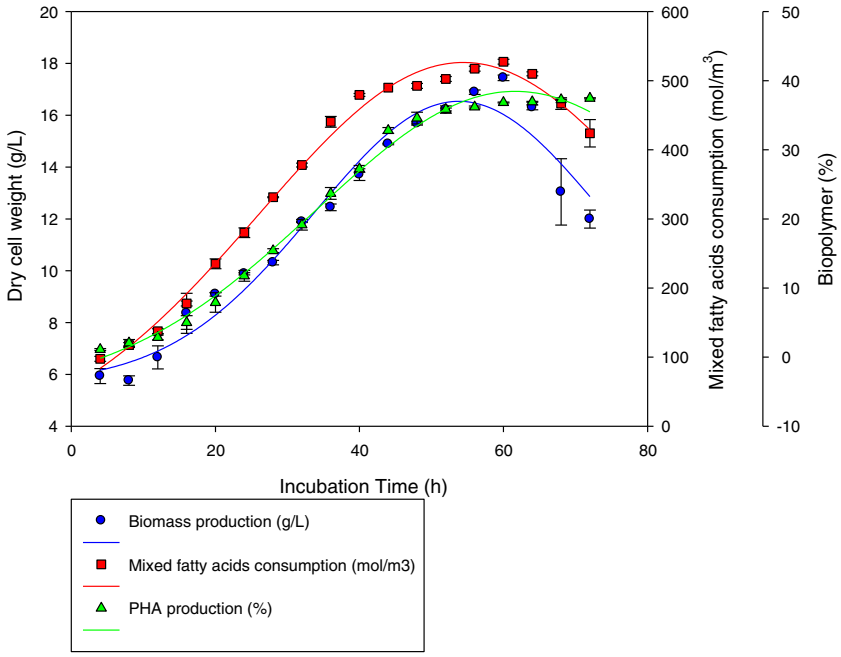

Fig. 5. Profiles of growth, fatty acid consumption and mcl-PHA production of *P. putida* BET001 in shake flask fermentation (0–72 h) with constant of other variables such as pH (7), temperature (28°C), agitation (250 rpm), volume of media (100 mL) with 10% inoculum.

such as fatty acids are used as a major carbon and energy source. The kinetic analyses and modeling performed also helped to predict important fermentation behavior in a highly dynamic culture system as the shaken flasks.

Financial support

The authors thanked the University of Malaya for research grants IPPP (PG055-2012B), UMRG program (RP024-2012D) and UMRG project (RG048-11B10).

References

[1] Doi Y, Steinbuchel A. Biopolymers. Weinhem: Wiler-VCH; 2002.
[2] Sharma R, Christi Y, Banerjee UC. Production, purification, characterization, and applications of lipases. Biotechnol Adv 2001;19:627–62. http://dx.doi.org/10.1016/S0734-9750(01)00086-6.
[3] Jacquel N, Lo CW, Wei YH, Wu HS, Wang SS. Isolation and purification of bacterial poly(3-hydroxyalkanoates). Biochem Eng 2008;39:15–27. http://dx.doi.org/10.1016/j.bej.2007.11.029.
[4] Treichel H, De Oliveira D, Mazutti MA, Di Luccio M, Oliveira JV. A review on microbial lipases production. Food Bioprocess Technol 2010;3:182–96. http://dx.doi.org/10.1007/s11947-009-0202-2.
[5] Jaeger KE, Ransac S, Dijkstra BW, Colson C, Hauvel MV, Misset O. Bacterial lipases. FEMS Microbiol Rev 1994;15:29–63. http://dx.doi.org/10.1111/j.1574-6976.1994.tb00121.x.
[6] Annuar MSM, Tan IKP, Ibrahim S, Ramachandran KB. Production of medium-chain-length poly(3-hydroxyalkanoates) from crude fatty acids mixture by *Pseudomonas putida*. Food Bioprod Process 2007;85:104–19. http://dx.doi.org/10.1205/fbp06017.
[7] Sylvia DM, Fuhrmann JF, Hartel PG, Zuberer DA. Principles and applications of soil microbiology. New Jersey: Pearson Education Inc.; 2005.
[8] Joseph B, Ramteke PW, Kumar PA. Studies on the enhanced production of extracellular lipase by *Staphylococcus epidermidis*. J Gen Appl Microbiol 2006;52: 315–20. http://dx.doi.org/10.2323/jgam.52.315.
[9] Mahler GF, Kok RG, Cordenons A, Hellingwerf KJ, Nudel BC. Effects of carbon sources on extracellular lipase production and *lip* A transcription in *Acinetobacter calcoaceticus*. J Ind Microbiol Biotechnol 2000;24:25–30. http://dx.doi.org/10.1038/sj.jim.2900764.
[10] Chung AL, Jin HL, Huang LJ, Ye HM, Chen JC, Wu Q, et al. Biosynthesis and characterization of poly(3-hydroxydodecanoate) by β-oxidation inhibited mutant of *Pseudomonas entomophila* L48. Biomacromolecules 2011;12:3559–66. http://dx.doi.org/10.1021/bm200770m.
[11] Gumel AM, Annuar MSM, Heidelberg T. Biosynthesis and characterization of polyhydroxyalkanoates copolymers produced by *Pseudomonas putida* BET001 isolated from palm oil mill effluent. PLoS One 2012: e45214. http://dx.doi.org/10.1371/journal.pone.0045214.
[12] Poku K. Origin of oil palm: Small-scale palm oil processing in Africa. Food and Agriculture Organization of the United Nations; 2002 148.
[13] Luedeking R, Piret EL. A kinetic study of the lactic acid fermentation. Batch process at controlled pH. J Biochem Microbiol Technol Eng 1959;1:393–412. http://dx.doi.org/10.1002/jbmte.390010406.
[14] Lopez-Thomas LA, Ordoñez JA, Mediavilla C, Rodríguez-Marin JL, Sarmiento P, Zamora A, et al. Effect of growth temperature and pH on the aminopeptidase activity of *Pseudomonas putida*, *P. flourescens* and *Flavobacterium odoratum*; the 4-nitroaniline test is reliable. Folia Microbiol 2008;53:423–6. http://dx.doi.org/10.1007/s12223-008-0064-4.
[15] Mordocco A, Kuek C, Jenkins R. Continuous degradation of phenol at low concentration using immobilized *Pseudomonas putida*. Enzyme Microb Technol 1999;25:530–6. http://dx.doi.org/10.1016/S0141-0229(99)00078-2.
[16] Harwood CS, Fosnaugh K, Dispensa M. Flagellation of *Pseudomonas putida* and analysis of its motile behavior. J Bacteriol 1989;171:4063–6.
[17] Razaif-Mazinah MRM, Annuar MSM, Sharifuddin Y. Effects of even and odd number fatty acids cofeeding on PHA production and composition in *Pseudomonas putida* Bet001 isolated from palm oil mill effluent. Biotechnol Appl Biochem 2015. http://dx.doi.org/10.1002/bab.1354.
[18] Tan IKP, Kumar KS, Theanmalar M, Gan SN, Gordon III B. Saponified palm kernel oil and its major free fatty acids as carbon substrates for the production of polyhydroxyalkanoates in *Pseudomonas putida* PGA1. Appl Microbiol Biotechnol 1997;47:207–11. http://dx.doi.org/10.1007/s002530050914.
[19] Baei Sharifzadeh M, Najafpour GD, Younesi H, Tabandeh F, Issazadeh H, Khodabandeh M. Growth kinetic parameters and biosynthesis of polyhydroxybutyrate in *Cupriavidus necator* DSMZ 545 on selected substrates. Chem Ind Chem Eng Q 2011; 17:1–8. http://dx.doi.org/10.2298/CICEQ100216043B.
[20] Ang CYW, Liu KS, Huang YW. Asian foods: Science and technology. Boca Raton, FL: CRC Press; 1999.