

Production of Medium-Chain-Length Poly(3-Hydroxyalkanoates) from Saponified Palm Kernel Oil by *Pseudomonas putida*: Kinetics of Batch and Fed-Batch Fermentations

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ABSTRACT

The kinetics of medium-chain-length poly(3-hydroxyalkanoates), PHA_{MCL} production by *Pseudomonas putida* PGA1 in batch and fed-batch fermentations were studied. With saponified palm kernel oil (SPKO) supplying the free fatty acids mixture as the sole carbon and energy source, PHA_{MCL} accumulation is encouraged under ammonium-limited condition, which is a nitrogen stress environment. The amount of PHA_{MCL} accumulated and its specific production rate, q_{PHA} were influenced by the residual ammonium concentration level in the culture medium. It was observed that in both fermentation modes, when the residual ammonium was exhausted ($< 0.05 \text{ gL}^{-1}$), the PHA_{MCL} accumulation (11.9%) and q_{PHA} (0.0062 h^{-1}) were significantly reduced. However, this effect can be reversed by feeding low amount of ammonium to the culture, resulting in significantly improved PHA_{MCL} yield (71.4%) and specific productivity (0.6 h^{-1}). It is concluded that the feeding of low ammonium concentration to the culture medium during the PHA_{MCL} accumulation has a positive effect on sustaining the PHA_{MCL} biosynthetic capability of the organism. It was also found that increasing SPKO concentration in the medium significantly reduced (up to 50%) the volumetric oxygen transfer coefficient ($K_L a$) of the fermentation system.

Keywords: poly-(3-hydroxyalkanoates), medium-chain-length, kinetics, ammonium, palm kernel oil.

INTRODUCTION

Poly(3-hydroxyalkanoates) (PHA) is a polymer of biological origin produced by a wide variety of microorganisms. The biopolymer has similar characteristics as the petrochemical derived plastics (Hocking and Marchessault, 1994). It has a huge potential of being an alternative to the synthetic plastics in numerous applications due to the fact that PHA is readily biodegradable. There are two major groups of PHAs: the short-chain-length PHA (PHA_{SCL}) with five or less carbon atoms, and medium-chain-length PHA (PHA_{MCL}) with six or more carbon atoms. Only the PHA_{SCL} have been commercially produced up to 500 tons per year, which was manufactured by Monsanto (Kellerhals *et al.*, 2000). The PHA_{MCL} is yet to make a significant impact as a viable choice due to the fact that it is very expensive to produce this polymer in bulk amount even for material testing purposes. To date, the final PHA_{MCL} yield and content obtained is relatively lower compared to those of PHA_{SCL}, which hampered development of its applications (Lee *et al.*, 2000).

PHA_{MCL} copolymers can be produced using a variety of substrates including plant oils. Due to their long carbon number, these substrates have high energy content which is excellent for good cell growth and energy metabolism. Furthermore, the structural similarity of various fatty acids with the linear PHA_{MCL} made it an even more attractive

choice. Preliminary studies on the production of PHA_{MCL} from renewable and cost-effective substrates like palm oil, palm kernel oil and their major fatty acids fractions by *Pseudomonas putida* have been reported (Tan *et al.*, 1997).

PHA accumulation by bacteria is a response to the imbalance in growth environment, with excess carbon source and simultaneous limitation of nutrients such as nitrogen, phosphorus, oxygen, magnesium etc; this physiological condition can be exploited in the fermentation process to achieve high yields and productivity. Ammonium ion is usually chosen as the limiting nutrient as it is relatively easier to make a bacterial culture ammonium-limited than other mineral ions (Suzuki *et al.*, 1986a).

Several PHA_{MCL} production strategies in the bioreactor such as batch and continuous (Durner *et al.*, 2001; Jung *et al.*, 2001), fed-batch (Beom, 2002) and high-cell-density process (Lee *et al.*, 2000) under various cultivation conditions have been described.

The aim of this study is to examine and compare the kinetics of PHA_{MCL} production from heterogeneous fatty acids mixture (supplied as saponified palm kernel oil, SPKO) by *Pseudomonas putida* in an ammonium-limited batch and fed-batch fermentations.

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MATERIALS AND METHODS

Microorganism

Pseudomonas putida PGA1 strain was a gift from Professor G.Eggink from the Agrotechnological Research Institute, Wageningen, The Netherlands.

Medium composition

Medium for both inoculum cultivation and bioreactor studies was the mineral salt solution containing (in gL⁻¹): 5.74, K₂HPO₄; 3.7, KH₂PO₄; 1.0 ml trace elements (MT) solution (Lageveen *et al.*, 1988), 10.0 ml 0.1M MgSO₄.7H₂O. Saponified palm kernel oil (SPKO) is supplied as the sole carbon and energy source with NaNH₄HPO₄.H₂O as the limiting nutrient. Saponification of palm kernel oil (PKO) was carried out according to Tan *et al.* (1997). PKO is the extract from the nut of the oil palm (*Elaeis guineensis* Jacq.) fruit. The oil consists of a mixture of C6-C18:2 fatty acids with approximately 82% saturated fatty acids and 18% unsaturated fractions (Table 1). The same medium composition was used for inoculum and fermenter cultivations to reduce or eliminate lag period.

To avoid precipitation during autoclaving, solutions of magnesium salt, trace elements and SPKO (pH 7.0) were sterilized separately before adding to the rest of the medium components.

Table 1: Fatty acids composition of palm kernel oil (Elson 1992)

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 (arachidonic acid)	0.1
SFA ^a	82.2
(SCFA) ^b	7.2
(MCFA) ^c	64.0
(LCFA) ^d	11.1
MUFA ^e	15.4
PUFA ^f	2.5

^aSaturated fatty acids

^bShort chain SFA

^cMedium chain SFA

^dLong chain SFA

^eMonounsaturated fatty acids

^fPolyunsaturated fatty acids

Inoculum

Inoculum in 120 ml culture volume was grown in 500 ml shake-flasks at 30°C and 250 rpm rotation using Hotech Model 718 orbital shaker incubator (Hotech Instruments Corp., Taiwan). During active growth at 18 h, the whole content of the flask was used to seed the bioreactor.

Bioreactor

Fermenter experiments were performed in a Biostat®B 3-liter fermenter (B.Braun Biotech International). A round bottom culture vessel (Type B2) made of borosilicate glass and stainless steel equipped with an outer thermostat jacket was used. The main geometric characteristics of the fermenter are shown in Table 2. Temperature, pH and oxygen partial pressure (pO₂) were monitored and controlled by a computer system.

Table 2: Dimensions of the fermenter and its components

Design parameters	Specifications
Total volume	3 L
Diameter of inner tank	130 mm
Height of tank	240 mm
Number of baffles	4
Baffle width	10.5 mm
Type of impellers	Rushton disc turbine
Number of impellers	2
Distance between impellers	79.5 mm
Distance of lower impeller from bottom plate	25 mm
Impeller diameter of disc	53 mm
Number of blades	6
Impeller blade width	10.5 mm
Impeller blade length	14.5 mm
Diameter of single ring sparger	48 mm
Number of holes	14
Distance of ring sparger from bottom plate	20 mm

Estimation of volumetric oxygen transfer coefficient (K_La)

The estimation of volumetric oxygen transfer coefficient, K_La was carried out in a batch bioreactor using static gassing-out method. The aqueous phase consisted of actual composition of the fermentation medium in 1.0 liter distilled water. The liquid medium was deoxygenated by sparging gaseous nitrogen until all traces of oxygen were stripped away. Then air was sparged into the bioreactor at 0.5 vvm and 600 rpm agitation rate. The increase in the oxygen partial pressure (pO₂) was recorded at regular intervals until readings became constant which indicated the saturation of the liquid medium with oxygen.

The K_La was determined using the relationship:

$$dC_L/dt = K_L a (C^*_L - C_L) \tag{1}$$

where C^*_L is the pO_2 saturation value and C_L is the pO_2 in the aqueous medium. Upon integration, equation (1) transformed into

$$\ln(C^*_L - C_L) = -K_L a \cdot t + \ln C^*_L \quad (2)$$

The plotting of $\ln(C^*_L - C_L)$ versus t yields a straight line with the $K_L a$ value determined directly from the slope.

Batch fermentation

Inoculum was added at 10% (v/v) and the fermenter working volume after inoculation was 1.2 L. The cells concentration at the start of fermenter cultivation was approximately 0.13 gL^{-1} . The temperature and the pH were maintained at $30 (\pm 0.5)^\circ\text{C}$ and $7.0 (\pm 0.05)$, respectively; with the agitation rate of 600 rpm and the aeration rate of 0.5 vvm of filtered air. Silicone anti-foam (BDH) was included in the fermenter at 1.0 gL^{-1} . Samples were withdrawn at regular intervals.

Fed-batch fermentation

The fermentation was carried out using fermenter setup as in the batch with the addition of the feeding routine. The fermentation was performed as batch start-up and at 9 h the feeding commenced. Feeding medium (0.4 gL^{-1} ammonium) consisted of fixed ratio of SPKO-to-ammonium (6.9mole:1mole) in the mineral salt solution. Feeding was carried out using Masterflex 7521-35 peristaltic pump at a constant flow rate of 0.14 L h^{-1} ($0.056 \text{ g ammonium h}^{-1}$) to avoid total depletion of ammonium in the fermenter. The contents of the feed reservoir were continuously stirred using magnetic stirrer to obtain a homogenous feed into the fermenter. The approximate working volume for all fermentations should not exceed 1.5 L after taking into account the total volume of the feeding solution and pH adjustment.

Analytical methods

All measurements were performed in triplicates. Total biomass concentration was estimated by first spinning down the cells in pre-weighed micro-centrifuge tubes. The cells were then washed twice with saline solution and dried at 90°C till constant weight.

The residual ammonium in the cell-free culture medium was determined using spectrophotometric method (Solorzano, 1969). The residual free fatty acids from SPKO in the culture medium supernatant were estimated by solvent titration (Cocks and van Rede, 1966).

To obtain PHA production profile, 8.0 mg of dried cells were subjected to acid-catalyzed methanolysis and the PHA amount and composition were determined by gas chromatography (GC) using benzoic acid methyl ester as the internal standard (Brandl *et al.*, 1988). The gas chromatograph used was Varian Star 3400CX equipped with a fused silica capillary column (30m x 0.53mm ID) (Supelco SPB™-608) and a flame ionization detector.

Data analysis and calculations

The calculations for kinetics data were performed using MATLAB 6.1 software (The MathWorks Inc.).

Calculation for volumetric productivity of PHA_{MCL} (Φ) was according to Yamane (1992), as shown in equation (3).

$$\Phi = [P] / (t_d + t_f) \quad (\text{g L}^{-1} \text{ h}^{-1}) \quad (3)$$

where $[P]$ = PHA_{MCL} concentration (gL^{-1}); t_d = down time (h) and t_f = fermentation time (h).

PHA_{MCL} yield from the SPKO supplied ($Y_{P/C}$) for the batch fermentation was calculated as in equation (4):

$$Y_{P/C} = \Delta(PHA_{maximum} - PHA_{initial}) / SPKO_{supplied} \quad (\text{g PHA g}^{-1} \text{ SPKO}) \quad (4)$$

where $PHA_{maximum}$ = maximum mass of PHA (g) attained; $PHA_{initial}$ = mass of PHA (g) at the start of cultivation; $SPKO_{supplied}$ = mass of SPKO (g) at the start of cultivation. Residual biomass yield from SPKO supplied ($Y_{R/C}$) for batch fermentation was calculated as in equation (5):

$$Y_{R/C} = \Delta(R_{maximum} - R_{initial}) / SPKO_{supplied} \quad (\text{g residual biomass g}^{-1} \text{ SPKO}) \quad (5)$$

where residual biomass = total biomass minus PHA mass (g); $R_{maximum}$ = maximum mass of residual biomass (g) attained; $R_{initial}$ = mass of residual biomass (g) at the start of cultivation.

In the case of fed-batch cultivation, the PHA_{MCL} yield from the SPKO ($Y_{P/C}$) was calculated as in equation (6):

$$Y_{P/C} = \Delta(PHA_{maximum} - PHA_{initial}) / (SPKO_{supplied} + SPKO_{fed}) \quad (\text{g PHA g}^{-1} \text{ SPKO}) \quad (6)$$

where $SPKO_{supplied} + SPKO_{fed}$ = mass of SPKO (g) at the start of cultivation plus mass of SPKO added during feeding (g).

Residual biomass yield from SPKO supplied ($Y_{R/C}$) was calculated as in equation (7):

$$Y_{R/C} = \Delta(R_{maximum} - R_{initial}) / (SPKO_{supplied} + SPKO_{fed}) \quad (\text{g residual biomass g}^{-1} \text{ SPKO}) \quad (7)$$

RESULTS

Effect of SPKO concentrations on the volumetric oxygen transfer coefficient ($K_L a$)

Since SPKO is a highly reduced substrate metabolized through an aerobic β -oxidation pathway, adequate oxygen supply to the cells in the PHA_{MCL} fermentation is important to maximize yield and productivity. Due to the soap-like properties of the SPKO, it would be reasonable to expect that its presence in the liquid medium might affect the efficiency of oxygen transfer to the fermentation system. Experiments to quantify this were carried out by determining the volumetric oxygen transfer coefficient, $K_L a$ (s^{-1}) in batch fermentation at increasing SPKO concentrations i.e. (gL^{-1}): 1.0, 5.0, 10.0, 15.0 and 20.0.

Similar conditions as in the actual fermentation were implemented during the procedure, with the following exceptions; (a) the determination was done under non-sterile conditions; (b) without the presence of the *P.putida* PGA1 biomass and; (c) SPKO concentrations in the bioreactor were increased by manual addition.

Only experimental results from SPKO concentrations of 1.0, 5.0 and 10.0 gL⁻¹ were obtained due to the problem of heavy foaming that occurred for the SPKO concentrations above 10.0 gL⁻¹. The foaming was extremely heavy during the K_La determination at SPKO concentration more than 10.0 g L⁻¹ and hence the whole procedure had to be terminated. The estimated K_La values for the three SPKO concentrations tested are shown in Table 3.

Table 3: Estimated K_La values at different SPKO concentrations.

K _L a (s ⁻¹)	SPKO concentration (g L ⁻¹)		
	1.0	5.0	10.0
	0.025 (±4%)	0.028 (±4%)	0.014 (±7%)

The K_La values for SPKO concentrations of 1.0 and 5.0 gL⁻¹ are very close to each other i.e. 0.025 (±4%) s⁻¹ and 0.028 (±4%) s⁻¹, respectively. However, an increase in SPKO concentration from 5.0 to 10.0 gL⁻¹ in the liquid medium caused a reduction in K_La value by 50%. It is suggested that further increase in the SPKO concentration would have caused the K_La to decline even further. This showed that the range of SPKO concentrations to be supplied batch wise should be between 5.0 to 10.0 gL⁻¹, and in fed-batch fermentation it should be controlled within this range by proper feeding of SPKO.

Batch fermentation

The initial amount of SPKO and ammonium provided were 5.0 gL⁻¹ and 0.4 gL⁻¹, respectively. SPKO was the sole carbon and energy source which was in excess for both growth and PHA_{MCL} production, and ammonium was the limiting nutrient in the medium. Growth immediately commenced after inoculation (Figure 1A).

Cells proliferation continued until 12h and a final 0.9 gL⁻¹ concentration of total biomass was obtained. The increase in total biomass is due to both cellular growth and PHA_{MCL} accumulation inside the cells. The profile of ammonium utilization reflected the growth trend (Figure 1B).

The increase in PHA_{MCL} concentration followed closely the trend of growth, indicating that under the present conditions PHA_{MCL} accumulation was growth associated (Figure 1C). In addition, the dynamics of intracellular PHA_{MCL} content is shown in Figure 1D, which may not necessarily, follows closely the trend of concentration profiles of biomass and PHA_{MCL}. Total biomass and PHA_{MCL} concentration reached their maxima at 12 h. Beyond 12 h, a decrease of total biomass was observed until the end of fermentation. This was due to

both the cells death and degradation of internal PHA_{MCL} via depolymerase action.

The time period where a decline in total biomass and active degradation of stored PHA coincided with the time period of ammonium exhaustion (from approximately 15-48 h) where its concentration in the culture broth was lower than 0.05 gL⁻¹, which indicated severe exhaustion and remained so until the end. The concentration of free fatty acids in the medium within the cultivation period remained above 2.5 gL⁻¹, which was still in excess. No oxygen limitation was observed as its partial pressure was never less than 50% air saturation.

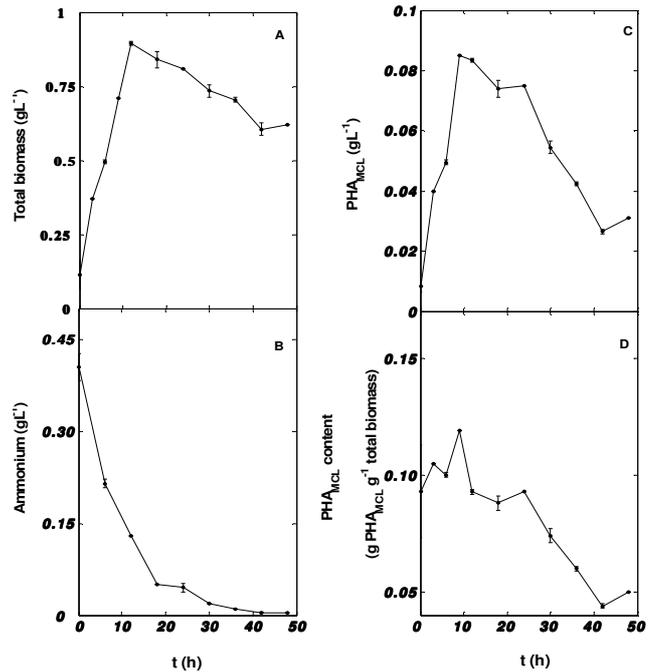


Figure 1: Total biomass (A), ammonium (B), PHA_{MCL} concentration (C) and intracellular content (D) profiles during batch fermentation of *P.putida* PGA1.

Kinetics of PHA_{MCL} biosynthesis in batch fermentation

In this batch culture, ammonium-limitation condition was characterized by active PHA_{MCL} accumulation whereas ammonium-exhaustion condition was represented by active internal degradation of stored PHA_{MCL}.

The basis of specific PHA production rate, q_{PHA} (g PHA produced g⁻¹ residual biomass h⁻¹) calculation was according to Suzuki *et al.* (1986b) and Yamane (1992) which is as follows: when the microorganism produces an intracellular storage material such as PHA, the total biomass [X] (gL⁻¹) consists of two main parts, namely PHA [P] (gL⁻¹) and residual biomass [R] (gL⁻¹), where [R] is calculated as the difference between the total biomass and the mass of PHA i.e. [R]=[X]-[P]. Then the total biomass in the culture is given by equation (8):

$$[X].V_{broth} = [P].V_{broth} + [R].V_{broth} \tag{8}$$

where V_{broth} is the working volume of the medium. $[R].V_{broth}$ can be considered as the anabolically active biomass including proteins and nucleic acids. Therefore the q_{PHA} based on the unit of residual biomass is described as follows:

$$q_{PHA} = \frac{1}{([R]_t.V_{broth})} \times \frac{d([P].V_{broth})}{dt} = \frac{1}{([X]_t.[P]_t).V_{broth}} \times \frac{d([P].V_{broth})}{dt} \quad (9)$$

The PHA accumulation data from batch fermenter (Figure 1C) was fitted using 4th degree polynomial (Figure 2A). The polynomial form was as follows:

$$[P](t) = -3.5(10^{-8})t^4 + 8.5(10^{-6})t^3 - 0.00056t^2 + 0.012t + 0.0081 \quad (10)$$

where $[P](t)$ represents PHA_{MCL} concentration data as a function of time t .

The polynomial function was differentiated and subsequently evaluated with respect to the residual biomass concentration at a particular time $[R]_t$. This gave the q_{PHA} (g PHA_{MCL} produced g^{-1} R h^{-1}) in the batch fermenter.

It was clear that ammonium exhaustion in the liquid medium (Figure 2B) was not a conducive condition for effective PHA_{MCL} production, because the PHA_{MCL} biosynthetic activities, as shown by the rapid decline of the organism's specific PHA_{MCL} biosynthesis rate, q_{PHA} (Figure 2C), were considerably reduced under this condition. The q_{PHA} calculated at the time when maximum PHA_{MCL} has been accumulated intracellularly (11.9% from the total dry biomass) was approximately 0.0062 g PHA_{MCL} produced g^{-1} R h^{-1} . The volumetric productivity of PHA_{MCL} was calculated to be 0.0028 g L^{-1} h^{-1} . Suzuki *et al.* (1986b) calculated the specific PHA_{SCL} formation rate, q_{PHA} (g PHA_{SCL} formed g^{-1} residual biomass h^{-1}) of *Protomonas extorquens* to be below 0.01 when the ammonium concentration in the liquid medium decreased to almost 0 ppm. Methanol was continuously supplied as a sole carbon and energy source in their cultivations.

Similar effect was observed by Bitar and Underhill (1990) in their batch culture of *Alcaligenes eutrophus*. They noted that without the ammonium supplementation at the beginning of PHA_{SCL} accumulation phase, the PHA_{SCL} accumulation rate (g PHA L^{-1} h^{-1}) was below 0.1 g L^{-1} h^{-1} . This is nearly 90% less than the maximum PHA_{SCL} accumulation rate attainable in their batch culture studies.

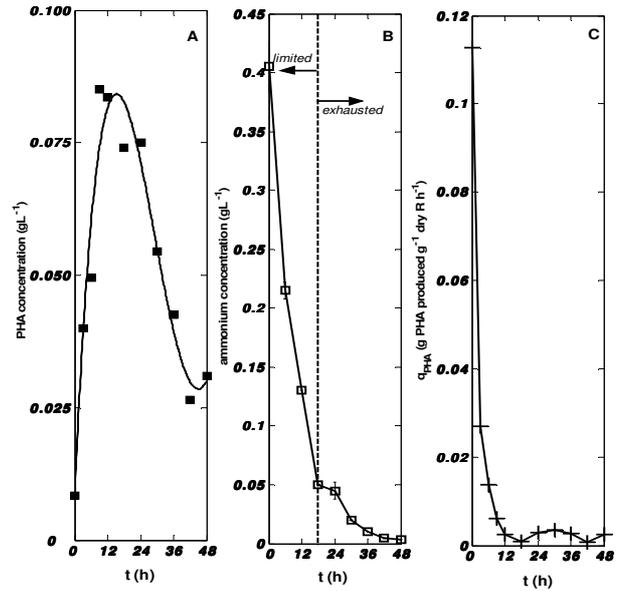


Figure 2: Fitting of PHA_{MCL} concentration time profile with 4th-degree polynomial. (A), the residual ammonium concentration with indicated limiting- and exhausted region (B), and the specific PHA_{MCL} production rate (C) in batch fermentation.

Fed-batch fermentation

The cultivation was started in a batch mode (1.0 L) and fed-batch was initiated *via* feeding. The initial amount of SPKO and ammonium in the fermenter were 5.0 $g L^{-1}$ and 0.16 $g L^{-1}$, respectively.

In the batch start-up phase, the cellular growth and intracellular PHA_{MCL} accumulation contributed to the increase in the concentration of total biomass (Figure 3A). Final total biomass obtained was 2.1 (± 0.1) $g L^{-1}$. The consumption of ammonium followed the growth trend until the commencement of feeding (Figure 3B).

The mass of R remained essentially constant from the start of feeding ($\sim 0.83 \pm 0.04$ g) until the end of the experiment ($\sim 0.84 \pm 0.02$ g). This shows that the increase in the total biomass was largely due to the increase in the PHA_{MCL} amount being accumulated intracellularly by the cells. Between 0-4 h, an increase in PHA_{MCL} concentrations were observed (Figure 3C). However, a slight decline occurred after 4h until the beginning of feeding, where the PHA_{MCL} concentrations started to increase again. The PHA_{MCL} content of the cells also decreased from approximately 0.5 g $PHA_{MCL} g^{-1}$ total biomass at $t=0$ h to 0.3 g $PHA_{MCL} g^{-1}$ total biomass at $t=8$ h, prior to the start of feeding (Figure 3D). When the feeding commenced, PHA_{MCL} content of the cells increased dramatically up to 0.7 g $PHA_{MCL} g^{-1}$ total biomass and remained so until the end of the fermentation. The ammonium concentration in the culture medium during the feeding period increased temporarily, and approaching 0.02 $g L^{-1}$ at 10h. After that, it decreased to near zero at

the end of fermentation. The decline of PHA_{MCL} concentration and its content coincided with the exhaustion of ammonium; with the concentration in the culture broth < 0.05 gL⁻¹ (Figure 3B). Similarly, this was observed in the batch fermentation i.e. when ammonium concentration < 0.05 gL⁻¹ in culture broth. This is regardless of the initial ammonium concentrations used in this study, which was 0.4 gL⁻¹ and 0.16 gL⁻¹ for the batch and fed-batch fermentations, respectively. The decline of PHA_{MCL} concentrations and content is ascribed to the action of intracellular PHA_{MCL} depolymerase.

The free fatty acids concentrations were estimated to be no less than 4.0 gL⁻¹ throughout the fermentation, and the oxygen partial pressure was never less than 50%.

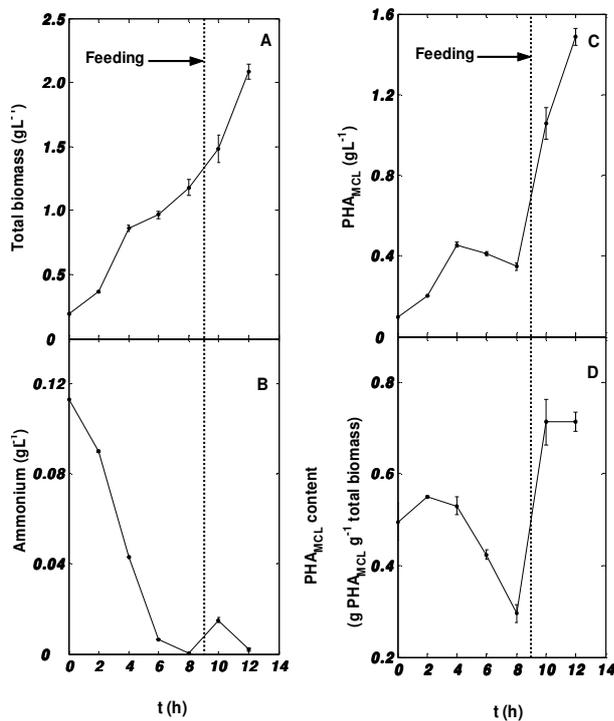


Figure 3: Total biomass (A), ammonium (B), PHA_{MCL} concentration (C) and intracellular content (D) profiles during fed-batch fermentation of *P. putida* PGA1.

Kinetics of PHA_{MCL} biosynthesis in fed-batch fermentation

The nitrogen source feeding (0.056 g ammonium h⁻¹) together with SPKO was to supply low concentration of ammonium and excess carbon source in the form of free fatty acids during the PHA_{MCL} biosynthesis stage.

The calculation for the q_{PHA} was the same as described for the batch cultivation with the exception that the culture volume (V_{broth}) is increasing with time. The PHA_{MCL} accumulation data from fed-batch fermenter was fitted using 4th degree polynomial (Figure 4A). The polynomial form was as follows:

$$[P](t) = -0.00018t^4 + 0.0067t^3 + 0.062t^2 + 0.22t + 0.068 \quad (11)$$

From Figure 4B, it can be seen that during the batch start-up phase of the cultivation, ammonium concentrations in the culture medium gradually declined to become almost zero. This resulted in a rapid reduction in the specific PHA_{MCL} biosynthesis rate, q_{PHA} (Figure 4C). However, when the feeding begins, certain level of ammonium remained was present in the culture liquid rather than completely exhausted, and the q_{PHA} started to recover and reached the maximum rate of approximately 0.6 g PHA_{MCL} produced g⁻¹ R h⁻¹; with only a slight decrease until the termination of the experiment at 12 h. The volumetric productivity of PHA_{MCL} for the fed-batch fermentation is 0.07 g L⁻¹ h⁻¹. Jung *et al.* (2001) obtained similar q_{PHA} i.e. 0.67 g PHA_{MCL} g⁻¹ residual biomass h⁻¹ in the chemostat cultivation of *P. oleovorans* using *n*-octane as carbon source.

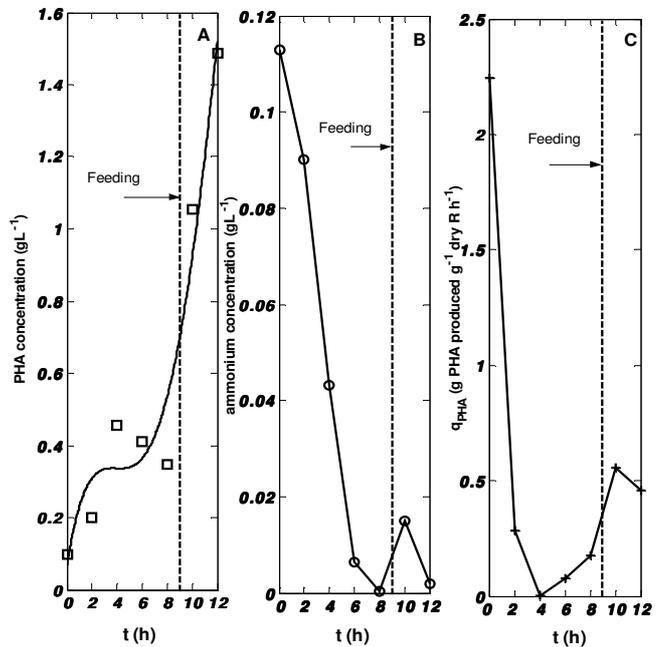


Figure 4: Fitting of PHA_{MCL} concentration time profile with 4th degree polynomial (A), the residual ammonium concentration profile (B), and the specific PHA_{MCL} production rate (C) in fed-batch fermentation.

In fed-batch cultures using fixed-rate ammonia feeding, 60% PHA_{SCL} content of the *Protomonas extorquens* was obtained faster at 58 hours with constant ammonia feeding rate at 0.08 g h⁻¹, as compared to nearly 100 h for the same PHA_{SCL} content in the case without ammonia feeding (Suzuki *et al.*, 1986b). Improved volumetric PHA_{SCL} synthesis rate from 0.1 to 0.99 g PHA_{SCL} produced L⁻¹ h⁻¹ was recorded by Bitar and Underhill (1990) during the period of ammonium supplementation to the batch culture of *Alcaligenes eutrophus*. A similar effect was seen with regards to the maximal specific rate of monomer production of PHA_{SCL}.

(0.125 g 3-hydroxyalkanoates monomer produced g⁻¹ residual biomass h⁻¹), where the rate was maintained for longer periods in nitrogen fed cultures than in the nitrogen exhausted culture (Aragao *et al.*, 1996).

The results obtained here are also comparable to that of pH-stat fed-batch fermentation using carbon-to-nitrogen (C:N) ratio of 20 g octanoic acid g⁻¹ ammonium nitrate (Beom, 2002). Using *P.oleovorans*, the highest PHA_{MCL} content of 75% was obtained at this C:N ratio with 0.63 g L⁻¹ h⁻¹ productivity.

In this study, the deterioration in the specific PHA_{MCL} production rate by *P.putida* PGA1 was successfully reversed by feeding mineral salt solution which contains fixed ratio of SPKO-to-ammonium. Furthermore, the feeding enhances the biosynthesis of PHA as seen by the concomitant increase in both the PHA_{MCL} concentration and content. The feeding of low ammonium concentration in the medium has a positive effect on the culture viability and in boosting the PHA accumulation.

Comparison between batch and fed-batch cultivations

The performance between the two cultivation modes was evaluated in terms of their volumetric and specific productivities, yield, biomass concentration, product concentration and PHA content (Table 4). It is clear that the fed-batch cultivation mode is superior to batch cultivation in every respect. In terms of volumetric productivity, Φ , the fed-batch is at least 25 times higher than the batch.

Table 4: Comparison between the batch and fed-batch fermentation modes.

Cultivation mode	X (g L ⁻¹)	P (g L ⁻¹)	P/X (%)	Φ (g L ⁻¹ h ⁻¹)	Y _{P/C} (gg ⁻¹)	q _{PHA} ^{a)} (g P g ⁻¹ R h ⁻¹)
Batch	0.90 ±0.01	0.09 ±0.0	11.9 ±0.0	0.0028	0.02	0.0062
Fed-batch	2.1 ±0.1	1.49 ±0.04	71.4 ±2.0	0.07	0.11	0.6

Keys: Maximum total biomass concentration, **X**; maximum PHA concentration, **P**; maximum PHA content, **P/X**; PHA_{MCL} volumetric productivity, Φ ; PHA_{MCL} yield from SPKO supplied, **Y_{P/C}**; specific PHA_{MCL} production rate, **q_{PHA}**.

^{a)}The maximum values calculated during the active PHA_{MCL} accumulation phase under ammonium-limitation.

Bitar and Underhill (1990) reported higher PHA_{SCL} content ca. 70% was achieved by ammonium supplementation to the batch culture of *Alcaligenes eutrophus* as compared to 59% without ammonium supply. In addition, PHA_{SCL} yield from glucose was 0.2 g PHA_{SCL} g⁻¹ glucose in normal batch culture compared with 0.447 g PHB g⁻¹ glucose in the ammonium supplemented culture.

It is clear that feeding with a small quantity of ammonium during the PHA accumulation phase improved the q_{PHA} of the *P.putida* PGA1 under ammonium-limited

condition, resulting in higher PHA concentration and content. This was opposite of the cultivation without ammonium feeding with poor q_{PHA} resulted in low PHA concentration and content.

DISCUSSION AND CONCLUSION

The presence of small amount of ammonium in the fermentation culture is important to maintain the growth capacity of the organism, as only viable residual biomass has the biochemical capability to synthesize and store the PHA. Thus, even though nitrogen source limitation stimulated PHA synthesis, complete nitrogen deficiency is detrimental to microbial PHA_{MCL} biosynthetic activity as was observed in the batch fermentation. Exhaustion of ammonium is detrimental to culture physiology resulting in biomass death and biodegradation of stored PHA_{MCL}. It is hypothesized that certain amount of residual nitrogen is needed by the metabolizing cells during active PHA_{MCL} production phase. The cultivation condition should be maintained under "ammonium limited" rather than "ammonium exhausted" in order to sustain culture viability and PHA_{MCL} biosynthesis capability. Other published works showed that biomass and PHA concentrations continue to increase, or at least remained stable after ammonium concentration dropped to extremely low values in batch fermentation (Sonnleitner *et al.*, 1979; Heinzle and Lafferty, 1980; Mulchandani *et al.*, 1989; Belfares *et al.*, 1995; Durner *et al.*, 2001). Bacterial strains used in these studies were *Alcaligenes eutrophus* and *Pseudomonas oleovorans*. Similar results however, were not observed in our experiments.

Kim *et al.* (1997) suggested that there would exist some optimal concentrations of ammonium and dissolved oxygen to maintain cell viability high enough to accumulate PHA_{MCL} since its concentration depends on both the cell concentration and its cellular content; therefore, a minimal supply of these are necessary to achieve a high PHA productivity in a jar fermenter operation. This was based on their results from the two-step fed-batch fermentation of *P.putida* BM01 using combined glucose and octanoate as carbon sources. Ammonium was used as the limiting nutrient and supplied in a fixed ratio to octanoate in feeding solution during the PHA_{MCL} accumulation phase.

Hence, it is concluded that PHA_{MCL} production can be improved by feeding low concentration of ammonium to the culture medium. This is supported by the comparison of the PHA_{MCL} production kinetics from the batch and fed-batch fermentations in this study.

NOMENCLATURE

Abbreviations

PHA _{MCL}	medium-chain-length poly-(3-hydroxyalkanoates)
PHA _{SCL}	short-chain-length poly(3-hydroxyalkanoates)
PKO	palm kernel oil
SPKO	saponified palm kernel oil

Symbols

Φ	volumetric productivity of PHA _{MCL} , g L ⁻¹ h ⁻¹
PHA _{initial}	mass of PHA at the start of cultivation, g
PHA _{maximum}	maximum mass of PHA, g
Q _{PHA}	specific PHA production rate, g PHA g ⁻¹ residual biomass h ⁻¹
R	residual biomass
R _{initial}	mass of residual biomass at the start of cultivation, g
R _{maximum}	maximum mass of residual biomass attained, g
SPKO _{supplied}	mass of SPKO supplied at the start of cultivation, g
SPKO _{fed}	mass of SPKO added during feeding, g
t _d	fermenter downtime, h
t _f	fermentation time, h
V _{broth}	working volume of the fermenter, L
Y _{P/C}	PHA yield from SPKO, g PHA g ⁻¹ SPKO
Y _{R/C}	residual biomass yield from SPKO, g R g ⁻¹ SPKO
[P]	PHA concentration, gL ⁻¹
[R]	residual biomass concentration, gL ⁻¹
[X]	total biomass concentration, gL ⁻¹

Subscript

t	time, hour
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