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Development of Bio-PORec® system for polyhydroxyalkanoates (PHA) production and its storage in mixed cultures of palm oil mill effluent (POME)

Mohd Fadhil Md. Din [a], Ponraj Mohanadoss [a,*], Zaini Ujang [b], Mark van Loosdrecht [c], Salmiati Muhd Yunus [a], Shreeshivadasan Chelliapan [a], Vasudeo Zambare [d], Gustaf Olsson [e]

[a] Institute of Environmental and Water Resource Management (IPASA), Faculty of Civil Engineering, University Technology of Malaysia, 81310 Skudai, Johor, Malaysia
[b] Faculty of Natural and Chemical Engineering, University Technology of Malaysia, 81310 Skudai, Johor, Malaysia
[c] Delft University Of Technology, Kluyver Laboratory for Biotechnology, Department of Biochemical Engineering, Julianalaan 67, NL-2628 BC Delft, The Netherlands
[d] Sequence Biotech Pvt. Ltd., Wadivarhe, Tal-Igatpuri, Dist-Nashik 422403, India
[e] Department of Industrial Electrical Engineering and Automation (IEA), Lund University, Box 118, SE-22100 Lund, Sweden

HIGHLIGHTS

- PHA accumulation could be achieved in mixed cultures.
- Feast–famine regime study gave the measure of actual accumulation phase for PHA.
- A successful lab scale, batch study fabrication system was demonstrated.

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ABSTRACT

High PHA production and storage using palm oil mill effluent (POME) was investigated using a laboratory batch Bio-PORec® system under aerobic-feeding conditions. Results showed that maximum PHA was obtained at a specific rate (q_p) of 0.343 C-mol/C-mol h when air was supplied at 20 ml/min. The PHA yield was found to be 0.80 C-mol/C-mol acetic acid (HAc) at microaerophilic condition and the mass balance calculation showed that PHA production increased up to 15.68 ± 2.15 C-mmol/cycle. The experiments showed that short feeding rate, limited requirements for electron acceptors (e.g. O_2, NO_3) and nutrients (N and P) showed lower tendency of glycogen accumulation and contributed more to PHA productivity.

1. Introduction

Polyhydroxyalkanoates (PHAs) accumulation in mixed culture has been widely reported by many researchers. PHA consists of various carbon contents and organic wastes (Salehizadeh and Van Loosdrecht, 2004) and both show properties similar to conventional plastic. PHA is recognized as a major component raw material in fabricating biodegradable plastics. In addition, biodegradable plastic is demanded now-a-days to reduce the unfavorable decomposition processes. On the other hand, the decomposition of biodegradable plastic can be achieved in a short period of time either by natural degradation or landfill disposal. Typically, the PHAs are known as polyesters of various hydroxyalkanoates and predominantly synthesized by numerous microorganisms as energy reserve materials. It could be synthesized under unbalanced growth conditions, i.e. by limitation of some essential nutrient and excess carbon source (Serafim et al., 2004). Many reports are available in the literature on microbial PHA production (Zafar et al., 2012). Moreover, PHA production using mixed culture from organic acid has been carried out (Salmiati et al., 2007). It has the potential to enhance the PHAs contents at low cost operations, i.e. simple equipment, friendliness environment, and cost effective substrates including industrial, municipal and agricultural wastes at massive scale (Rodríguez et al., 2011). Selection of a suitable carbon substrate is an important factor for optimizing the PHA production, as it could affect the PHA content, composition and the polymer properties. Over 40% of total operating expense for PHA production is related to the raw materials (Lee et al., 1999). Thus, the use of a low-cost carbon source is required in order to reduce the high production cost of PHA. The approach of mixed culture conditions could enhance the fast substrate utilization and thus more

* Corresponding author. Tel.: +60 143149291; fax: +60 75531575.
E-mail address: goldking1977@gmail.com (P. Mohanadoss).

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economical for higher PHA production where Salehizadeh and Van Loosdrecht (2004), have summarized various required environmental conditions for robust PHA production using organic waste substrates, including C:N ratio, oxygen and detention time.

An agricultural waste is recommended as a suitable carbon source for PHA production, as it improves the requirement of mixed culture development. The nature of mixed culture production from agricultural waste will produce both degradable and non-degradable polymers with other value-adding waste treatment process such as biogas production. The selection of organic waste from POME is predetermined since its composition is reported to have high organic acids and it is free from toxic substances. POME has been evaluated by various researchers in terms of its characteristics, treatment and recovery processes (Lam and Lee, 2011). Those studies from POME cultivation have been reported for two-stage cultivation or pure culture systems in a simple and direct way. Both organic removal and PHA-producing microbial organisms are cultivated in the same system which is called a hybrid fed-batch system. As described by many published works, a feast (availability of carbon source) and famine (exhausted of carbon source) regime have been reported as appropriate condition for 'quick' PHA production (Van Loosdrecht and Heijnen, 2002). Most of the feast and famine regimes could be achieved under hybrid batch or a batch reactor. The batch system used in this study is commercially known as Bio-PORec® system, and it is similar to the sequencing batch reactor (SBR). The Bio-PORec® system was operated for mixed culture development during growth phase and PHA production during accumulation phase. PHA content of the harvested solid biomass at initial stage of mixed culture development was typically obtained at range 10–30% of cell dried weight (CDW). However, after the steady-state condition had been achieved the PHA concentration in the bacteria cells improved up to 80% of CDW. Since the PHA development occurred in a single fed-batch culture, the cycle length of each operation is cultivated between 8 and 12 h.

The main objective of this study was to select appropriate experimental operating conditions (such as dynamic aerobic feast–famine condition) in a mixed culture for high PHA production. Although there are number of research on anaerobic and two-stage PHA production, there is less reported study on the aerobic-dynamic substrate feeding in a SBR system using POME. Accordingly, the current study focuses on the kinetic analysis, specific rates and nutrient behavior on PHA production.

2. Methods

2.1. Substrate

The raw POME was collected from Bukit Besar Palm Oil Mill, Kulai Malaysia and the activated sludge from a waste stabilization pond was obtained from the Universiti Teknologi Malaysia, Skudai campus. The composition of raw POME was determined using Gas Chromatography (GC, Chrompack, Germany).

2.2. Development of the reactor system and the inoculums

The SBR system, here called as BioPORec, with a working volume of six liters (with 50% discharge level) has been developed. The configuration of the overall reactor system is shown in Table 1. The mixing has been controlled by two standard geometry six-bladed impeller (stainless steel material) to improve the thermodynamic effects in the reactor. A heterotrophic aerobic bacterium was cultured from a facultative anaerobic pond of POME, located at one of the existing wastewater treatment plants in the factory territory Bukit Besar, Kulai, Johor, Malaysia and the activated sludge from a waste stabilization pond was collected in order to enhance the capability of mixed culture development. The heterotrophic aerobic bacterium and activated sludge cultured wastewater were prepared at 1:2 ratio, followed by premixing with raw POME and distilled water. Within three days, the reactor system was continuously operated by supplying nutrient (Vishniac solution) into the reactor for several weeks, in order to reach a steady-state condition.

2.3. Experimental set-up

The experiments were conducted in series of serial and parallel (also known as growth and accumulation phase) configurations for at least eight hours operation, similar to previous operations reported (Serafim et al., 2004; Dionisi et al., 2004). The microorganisms were grown in serial configuration under non-limiting nutrient conditions with the aim of biomass growth whereas in the parallel configuration the nutrient existence was controlled during a certain period to minimize biomass growth and to increase the PHA production intracellularly. The temperature of the reactor was maintained at 30 °C using a water-bath, while the pH was maintained at 7.00 ± 0.1 using an automated-buffer peristaltic pump with 2 N HCl or 2 N NaOH. The operating principles of the system are characterized by the three discrete periods: fill, react and draw (discharge). The purpose of this operating principle is to maximize the growth rate and fast storage polymer formation (PHA production).

The system was also operated in continuous reaction period, which means no settling or allowing the idle phase. The length of each phase can be varied independently of the treatment task. The influent is pumped into the tank and mixed with the biomass that settled during the previous cycle until the time for filling is reached. The filling phase can be mixing in aerated (oxygen as electron donor), anoxic (nitrate or nitrite as electron donor) or micro-aerophilic-aerobic (controlling the oxygen level) conditions.

A two consecutive stage bioprocess was developed to obtain high rate of PHA accumulation. This was necessary to promote the bacterial growth that contains minimal total phosphorus before starting the subsequent PHA accumulation phase.

2.4. Analytical procedures

Samples were taken from the reactor system with a 60 ml syringe. The DO concentration in the reactor was monitored online with a DO electrode as percentage of air saturation using data acquisition (ISTEK®, Korea). The samples were centrifuged at 10,000 rpm for 10 min at 4 °C using (Sorval RC-5B) and then filtered by using PVDF-syringe filter and analyzed for TOC, COD, PHA, VFA, VSS, CDW, NH_4, NO_3 and PO_4 in accordance with Standard Methods (APHA, 1995). The carbon concentration in the supernatant was measured by gas chromatography (GC), while NH_4^+, NO_3^- and PO_4^{2-} concentrations in the supernatant were measured at 630, 450 and 520 nm, respectively with auto analyzers (HACH Spectrophotometer DR-4000U, USA). The supernatant consisting of VFAs were measured with GC and a flame ionization detector (FID) by injecting into a supelco fused-silica capillary column (diameter 0.25 mm × 25 m). The quantification of CDW was performed using the VSS and ash technique according to the Dutch Standard (NNI. NEN: 1982).

The PHA was determined from dried biomass obtained from the reactor by extraction, hydrolyzation, and esterification in a mixture of hydrochloric acid, 1-propanol and dichloroethane, respectively at 100 °C. Benzoic acid (2 ml) was used as an internal standard throughout the procedure. Then 2 ml of chloroform was added to the mixture. The whole was taken in a screw-cap bottle and digested at 100 °C for 2 h using digester reflux (HACH digester,

Table 1
Operating phase with POME as substrate.

Experiment(s)	Operating time (min)				
	Aerobic mineral feeding	Aerobic feeding	Aerobic reactor	Anoxic reactor	Draw/discharge
Growth	355–360	0–60	60–345	–	345–355
CNP_{pome} [a]	no fill	0–60	60–350	–	350–360
Air_{pome} [b]	no fill	0–60	60–350	–	350–360
HRT_{pome} [c]	no fill	0–60	up to 770	–	up to 780
FR_{pome} [d]	no fill	up to 150	up to 200	–	up to 360
$ANae_{pome}$ [e]	no fill	0–60	up to 232	up to 203	up to 360
$MICae_{pome}$ [f]	no fill	0–60	60–350	–	350–360

[a] Experiment of fully aerobic with different ratio of C:N:P, fixed $Q_{airflow}$, HRT = 12 h, Q_{feed} = 20 ml/min.
[b] Experiment of fully aerobic with different air flow rate, fixed C:N:P ratio, HRT = 12 h, Q_{feed} = 25 ml/min.
[c] Experiment of fully aerobic with different cycle length, fixed C:N:P ratio, Q_{feed} = 20 ml/min and $Q_{airflow}$ = 0.5 l/min.
[d] Experiment of fully aerobic with different Q_{feed}, fixed C:N:P ratio, HRT = 12 h, $Q_{airflow}$ = 0.5 l/min.
[e] Experiment of intermittent aerobic-anoxic condition with fixed C:N:P ratio, HRT = 12 h, $Q_{airflow}$ = 0.5 l/min, Q_{feed} = 20 ml/min.
[f] Experiment of intermittent low aeration condition with fixed C:N:P ratio, HRT = 12 h, $Q_{airflow}$ = 0.5 l/min, Q_{feed} = 20 ml/min.

USA). The screw-cap bottles were allowed to cool and 3 ml of distilled water was added into each bottle. Then, it was shaken for 10 min to allow the organic and inorganic layer to mix properly. Upon the completion of digestion, 1 μl of the chloroform phase (bottom layer) was injected into the GC and the PHA quantification can be obtained by percentage (%) or concentration (mg/L) of CDW. The calculation for PHA content, production and rates in biomass cell was adapted from (Cavalheiro et al., 2009).

2.5. Experimental analysis

The specific mass balance was determined as proposed by (Van Aalst-van Leeuwen et al., 1997). Elemental mass balances on the measured conversions of substrate, biomass, PHA, CO_2, O_2 and NH_4^+ were performed in order to check the consistency of the data. The Macrobal software (Beun et al., 2000a,b) was used in the experiment for balancing all the converted amounts and calculating errors, to define the feast and famine period separately in terms of converted compounds.

The observed yield, Y_{obs} (C-mmol/C-mmol VFAs), corresponding to the amount of VFAs converted into active biomass and hyroxy butyrate (HB), was determined according to:

$$Y_{obs} = 1 - \frac{\int OURv(t)dt}{\Delta VFAs} \quad (1)$$

where, OUR_v denotes the volumetric oxygen uptake rate (OUR) converted into carbon considering that 1 mmol of O_2 corresponds to 1 mmol of carbon, ΔVFAs is the substrate consumed during the "feast" phase. This parameter, in terms of carbon material balance, can be expressed by:

$$Y_{obs} = Y_{p/s} + Y_{x/s} \quad (2)$$

where $Y_{p/s\ (storage\ over\ production)}$, $Y_{x/s\ (storage\ over\ biomass)}$...The material balance for VFAs can be represented by Eq. (3)

$$\Delta Y = Y_{p/s} + Y_{x/s} + \frac{\int OURv(t)dt}{\Delta VFAs} \quad (3)$$

3. Results and discussion

The composition of raw POME is shown in Table 2, with lactic and acetic acid depicted as the major fatty acid concentration. In addition, the characteristics of POME at the end of the growth and accumulation phase showed the performance of utilization and production rate of microbial activities. The acetic acid was found to be the most depicted fatty acid utilized by microorganisms, since the value was recorded at the highest concentration during growth phase. The lactic acid was also discovered as a similar trend, which may have contributed from the growth of lactic acid bacteria (Zakaria et al., 2010).

3.1. Respirometric kinetics in Bio-PORec® system

The study was carried out with mixed cultures for comparing dynamic responses of sludge by continuous, intermittent and without feeding and showed the effect of various operational conditions which includes both significant changes of microbial population and different physiological state of microorganisms.

As shown in Table 3, intermittently fed sludge typically exhibit faster substrate uptake and higher yield was obtained than continuously fed ones. A distinction between storage and accumulation was first proposed by Cech and Chudoba (1983) based on the form of substrate profiles versus time and the current study can been explained for substrate utilization in the presence of microorganisms

Table 2
POME characteristics using Bio-PORec® cultivation.

Parameters	Nomenclature	Raw POME (gL^{-1})	End of growth phase (gL^{-1})	End of accumulation phase (gL^{-1})
Lactic acid	$C_3H_5O_3$	3.90 ± 1.22	4.60 ± 0.44	1.20 ± 0.05
Formic acid	CH_2O_2	0.23 ± 0.66	5.22 ± 0.20	0.03 ± 0.02
Acetic acid	$C_2H_4O_2$	3.62 ± 0.31	7.20 ± 0.12	0.01 ± 0.02
Propionic acid	$C_3H_6O_2$	0.12 ± 1.05	2.10 ± 0.05	0.12 ± 0.15
Butyric acid	$C_4H_8O_2$	0.08 ± 0.14	0.50 ± 0.01	NA
Orthophosphate	PO_4^{3-}–P	0.194 ± 1.06	0.550 ± 0.01	0.440 ± 0.08
Ammoniacal-nitrogen	NH_4^+–N	0.108 ± 2.23	0.09 ± 0.03	0.01 ± 0.05
Nitrate-nitrogen	NO_3^-–N	0.016 ± 1.26	0.440 ± 0.10	0.220 ± 0.11
Total COD	COD_{tot}	87.30 ± 0.54	42.10 ± 0.30	12.50 ± 0.30
Soluble COD	COD_{sol}	41.52 ± 0.61	19.30 ± 0.50	5.50 ± 0.23
Total solids	TS	31.05 ± 0.49	–	–
Total volatile solids	TVS	27.60 ± 0.94	32.10 ± 0.12	44.10 ± 0.50

Table 3
Comparison of respirometric analysis on continuous and batch cultures.

Experiments	Batch culture/continous culture					Refs.
	ΔY (C-mol/C-mol HAc)	Y_{obs} (COD/COD)	SUR (COD mg/gSS. h	$Y_{storage}$ (COD/COD)	Storage cap. (g/g SS)	
$CODNPpome_{ave}$	0.66–0.69	0.32*	342–450	0.46	0.40	This study
$Airpome_{ave}$	0.57–0.78	0.44*	300–520	0.52	0.33	
$HRTpome_{ave}$	0.42–0.65	0.52*	420–610	0.33	0.21	
$FRpome_{ave}$	0.59–0.72	0.39*	350–540	0.34	0.20	
$ANaepome_{ave}$	0.32–0.47	0.60*	890–1200	0.55	0.53	
$MICaepome_{ave}$	0.53–0.80	0.65*	800–1420	0.57	0.64	
Continuous-fed bulking	n.a.+	n.a.+	200(b)	n.a.	n.a.	Cech and Chudoba, 1983
Intermittent–fed-well settling	n.a.+	0.56(°)+	1730(a)	n.a.	0.65(a)	
Continuous-fed bulking	n.a.+	0.46+	200–260	0.35	-	Majone et al. (1999)
Intermittent-fed well settling	n.a.+	0.33+	800–1000	0.75	-	
Intermittent-fed well settling	n.a.+	0.52+	740–920	0.7	0.47	Beccari et al. (1998)
Continuous-fed batch	n.a.+	0.60	191–500	0.40	0.62	Dionisi et al. (2001)
Continuous-fed bulking	0.23–0.33	0.49+	690–1050	0.50	0.33	Beccari et al. (2002)

Note: Original units of most data have converted to suit the table. Y_{obs} from respirometry studies apart from values indicated with (°). (a) accumulation phase, (b) storage phase, (*) Cmmol/Cmmol VFAs, (+) Continous culture.

that are most able to store substrates quickly during the imposed dynamic conditions. Due to the lower capacity for accumulation with respect to storage, the observed substrate uptake rate is first quickly decreasing (saturation of the accumulation) and then remains constant (storage still far from saturation). Therefore, it has been suggested that both mechanisms (storage and accumulation) are acting when the sludge is intermittently fed, while only storage is most favorable for the continuously fed sludge. The importance of storage response under dynamic conditions for mixed cultures has been confirmed by direct determination of stored polymers in sludge Majone et al. (1999) have shown that for both intermittently and continuously fed sludge, the storage response is the main mechanism of the dynamic response and the growth response occurred to a slight increase or no change in settled sludge.

With reference to previous works, as shown in Table 3, the result was compared with several mixed culture experiments. The microaerophilic–aerobic condition showed highest storage activity compared with other studies the PHA production was (ΔY = 0.53 to 0.80 C-mol/C-molHAc) and the conversion rate of HAc to active biomass and PHA production was (Y_{obs} = 0.65 COD/COD). During batch culture, the diluted POME was tested using respirometric vessel and high storage capacity (g/g SS) occurred again under microaerophilic–aerobic condition. This was explained via the behavior of sludge utilization rate (SUR) at rate 800–1420 mg COD/g SS h.

In anoxic condition, heterotrophs showed the capability of switching their mode of metabolic activity and readily adapting themselves to consume nitrate as the terminal electron acceptor instead of dissolved oxygen. Regularly during cyclic operation, the significant observation is that the rate of electron acceptor utilization under anoxic conditions is always lower than what may be achieved under aerobic conditions. Therefore, the study was conducted under cycling of anoxic and aerobic ($ANaepome_{ave}$) to provide an adequate electron acceptor (O_2 and NO_3) in the reactor. As a result, the storage capacity and $Y_{storage}$ reached a higher value for anoxic compared to only aerobic conditions (e.g. $Airpome_{ave}$ and $FRpome_{ave}$) Table 3.

3.2. Mass balance of substrates during feast–famine period

The mass balance values during feast, famine and total cycle of selected experiments during a pulse cycle of POME as carbon source is shown in Table 4 and the measurements were carried out in triplicates and the results in the table are shown as the average of three. The consistency of the mass balance was checked using Macrobal software analysis by performing the C-balance over the total cycle. The total amount of substrate added in one cycle was consumed during the feast period. An important fraction of substrate was converted and stored as polymer storage compounds.

For comparison, an overview of all converted amounts in C-mmol/cycle and their standard deviations as balanced with Macrobal are shown in Table 4. Values for cultures fed with acetate or glucose as single substrate at different SRTs obtained by Beun et al. (2000a) is summarized in this Table 4 as well. However, data from Carta et al. (2001) stated that both acetate and glucose was calculated and identified as mixed substrates. In order to compare those findings, the example of microaerophilic–aerobic result has been analyzed in the last column of the Table 4.

It can be seen that a large fraction of external substrate is stored as PHA or glycogen. In the system with a mixed substrate (except this study), the conversion of acetate and glucose in PHA and glycogen, respectively, is the same. Since this study was conducted using high substrate concentration (four to eight times higher than other studies), the PHA production had increased up to 15.68 ± 2.15 C-mmol/cycle at feast period. Consequently, this contributed a higher production compared to the study of Reddy and Mohan (2012) who showed that the time taken for high PHA accumulation increased with increase in organic load [OLR3 at 60th h (40.3%)] and then showed a decrement thereafter (72th h, 26.3%) due to lower availability of substrate.

3.3. Development of PHA productivity (Δf_{PHA})

In Fig. 1, the fraction of net polymer produced per unit of active biomass (Δf_{PHA}) during the "feast" phase is presented for all experimental data. The experiments also considered the optimum yield and kinetic rates. The Δf_{PHA} had been used to confirm the productivity of PHA during "standard feast" with minor modification on specific rates (q_p and $-q_s$). The result also indicated that nitrogen substances are important parameters to be controlled in the reactor. Nitrogen limitation caused a decrease in the cell growth rate and led to an increase in the polymer storage yield and productivity. The rate of polymer production varied directly with the substrate concentration in the range 150–300 C-mmol/l, but

Table 4
Comparison of converted amounts for measured compounds in aerobic pulse-fed SBR processes.

	SRT Substrate Compound	Carta et al. (2001); 6.1 days; acetate/ glucose ((C) mmol/cycle)	Beun et al. (2000a); 3.8 days; acetate ((C) mmol/cycle)	Beun et al. (2000a); 9.5 days; acetate ((C) mmol/cycle)	Dircks et al. (2001); 3.6 days; glucose ((C) mmol/cycle)	This study; limited oxygen; HRT = SRT; POME ((C) mmol/cycle)
Feast	Substrate[a]	−6.21 ± 0.25	−13.54 ± 0.55	−11.44 ± 0.27	−12.46	−48.79 ± 7.45
	Biomass	1.2 ± 0.3	1.36 ± 1.34	0.23 ± 0.77	0.68	24.39 ± 3.33
	Glycogen	−6.2 ± 0.8				–
	PHA	3.66 ± 0.48	5.57 ± 0.75	7.16 ± 0.64	–	15.68 ± 2.15
	CO_2	**3.41 ± 1.02**	**6.61 ± 1.19**	**4.05 ± 0.27**	**0.84**	**8.72 ± 6.44**
	O_2	−3.02 ± 1.04	–	−3.15 ± 0.26	–	−6.70 ± 2.30
	NH_4^+	−0.22 ± 0.06	−0.23 ± 0.23	−0.04 ± 0.13	−0.13	−12.77 ± 2.03
	PO_4^{2-}	–	–	–	–	−5.13 ± 0.44
Famine(∗)	Biomass	3.77 ± 0.47	2.61 ± 0.85	2.85 ± 0.73	5.75	22.44 ± 6.32
	Glycogen	−4.16 ± 0.14			−10.96	–
	PHA	−3.66 ± 0.48	−5.57 ± 0.75	−7.16 ± 0.64	–	−15.68 ± 4.88
	CO_2	**4.06 ± 0.49**	**2.96 ± 0.17**	**4.31 ± 0.20**	**6.21**	**6.76 ± 2.55**
	O_2	−4.76 ± 0.51	−3.63 ± 0.15	−5.18 ± 0.19	–	−4.88 ± 1.41
	NO_3^-	–	–	–	–	−1.71 ± 2.75
	PO_4^{2-}	–	–	–	–	−1.70 ± 0.95
Total	Substrate	–	–	–	–	−48.79 ± 7.02
	Biomass	4.93 ± 0.49	3.97 ± 1.04	3.08 ± 0.22	–	46.83 ± 4.23
	CO_2	**7.46 ± 0.10**	**9.57 ± 1.18**	**−8.36 ± 0.18**	–	**15.48 ± 5.55**
	O_2	–	−9.53 ± 1.19	−8.33 ± 0.18	–	−10.58 ± 0.58

Note: A minus sign indicates consumption of the compound. Standard deviations after plus/minus signs, while bold values are calculated values. (a) single substrate or readily biodegradable COD, Xs and Ss (∗)overall famine period.

decreased significantly for more than 450 C-mmol/l (data not shown). At the same time, a high increment in oxygen saturation will also lead to the polymer storage. As depicted in air flow rate experiments, the Δf_{PHA} will slow down because of the limitation of air supply into the reactor. The limited concentration of oxygen is significant for storage capacity of the cells. Therefore, all experiments were conducted in a period of less than 2.5 l/min.

Based on the previous study, a high substrate concentration (more than 450 C-mmol/l) favored PHA accumulation, even though the specific storage rate decreased due to substrate inhibition. In order to overcome inhibition, the same volume of carbon substrate was added to the reactor applying five different feeding rates with the duration of the flow rates between 30 and 120 min. The Δf_{PHA} was found to be higher under slow feeding rate (e.g. 20 ml/min) compared to the fast feeding (e.g. 75 ml/min). The maximum amount of PHA depicted in this aerobic dynamic feeding was around 50%, as reported by (Chelme et al., 2008; Serafim et al., 2004). It was postulated that during the initial substrate pulse addition, substrate will be converted mainly for growth of the organism then later will result in accumulation of polymer when the biomass becomes saturated.

In order to understand the behavior of polymer storage, the anoxic and microaerophilic conditions were also conducted in a single fed-batch system. The PHA storage capacity was discovered to be higher in an "aerobic" condition than in "anoxic". Even though production of polymer occurred for each condition, the Δf_{PHA} decreased from 0.1 to 0.4 C-mmol/l. Therefore, the study only focussed on aerobic condition in order to obtain a specific finding of PHA production rate. In addition, the storage of PHA is an important mechanism of substrate removal, and even a mixture of carbon sources is simultaneously utilized by different biomass (Dionisi et al., 2004).

3.4. Effect of C:N:P ratio on PHA production

The effect of C:N:P ratio on the PHA production was evaluated and the results showed that at limited N and P (feed), the biomass has the ability to store PHA at faster mode before the biomass uses it for cell growth and anabolic metabolism. Therefore, the optimum storage of PHA content was obtained at a COD/N ratio of approximately 400 and a COD/P ratio of about 200. The enrichment of PHA producing bacteria by operating under alternating periods of growth and nutrient limitation conditions was an effective way to achieve high PHA production when the substrate was a mixture of VFAs (Chinwetkitvanich et al., 2004; Santhanam and Sasidharan, 2010). PHA accumulated under P limitation is higher than under limitations of N or other essential nutrients. Under adequate N and P limitations, the biomass loss (approximately 17% of total cycle) was obtained after 4–5 h. This was also indicated by the peak of PHA accumulation occurring in a much shorter time period (fast uptake rate). Several researchers (Du et al., 2001; Mumtaz et al., 2009) have explained that the PHA accumulated under P limitation is higher than under limitations of N or other essential nutrients. It was also observed that O_2 will act as electron acceptor to the biomass but low concentration of NO_3 was favoring the PHA production and by manipulating DO instead of limiting nitrogen or phosphate availability showed higher PHA production which utilized nutrient rich feed stocks (Pratt et al., 2012).

3.5. Effect of feeding regime

In the presence of external substrate (S_s), the organisms have a choice to use the substrate for growth or storage purposes. Traditionally, it is assumed that competing microorganisms will maximize their growth rate, and storage capacity will only occur when some growth related compound becomes limited such as N and P. As depicted in Fig. 2, the growth rate consists of two parts, one resulting from growth on S_s and limited by the amount of protein synthesizing system in the biomass and a second part when the S_s is depleted resulting from growth on PHA. It is notable that μ increases in the short period of S_s presence. The turn-over of PHA was observed clearly, when the feed rate over cycle length (FR/CL) increased, the PHA content under feast and famine will be reduced. The PHA content during the feast period will be decreased, while it will start to increase during the famine period. This pattern was also shown by Van Loosdrecht and Heijnen (2002), which indicates that the bacteria always compete on substrate uptake rate and not for growth rate. Therefore, the appropriate feeding is required to maximize the PHA production rate under short cycle length study. As a conclusion, the growth and PHA production rate can be controlled by varying the ratio of FR/CL. The preferred ratio was projected at 0.5 (feeding rate at 20–25 ml/min).

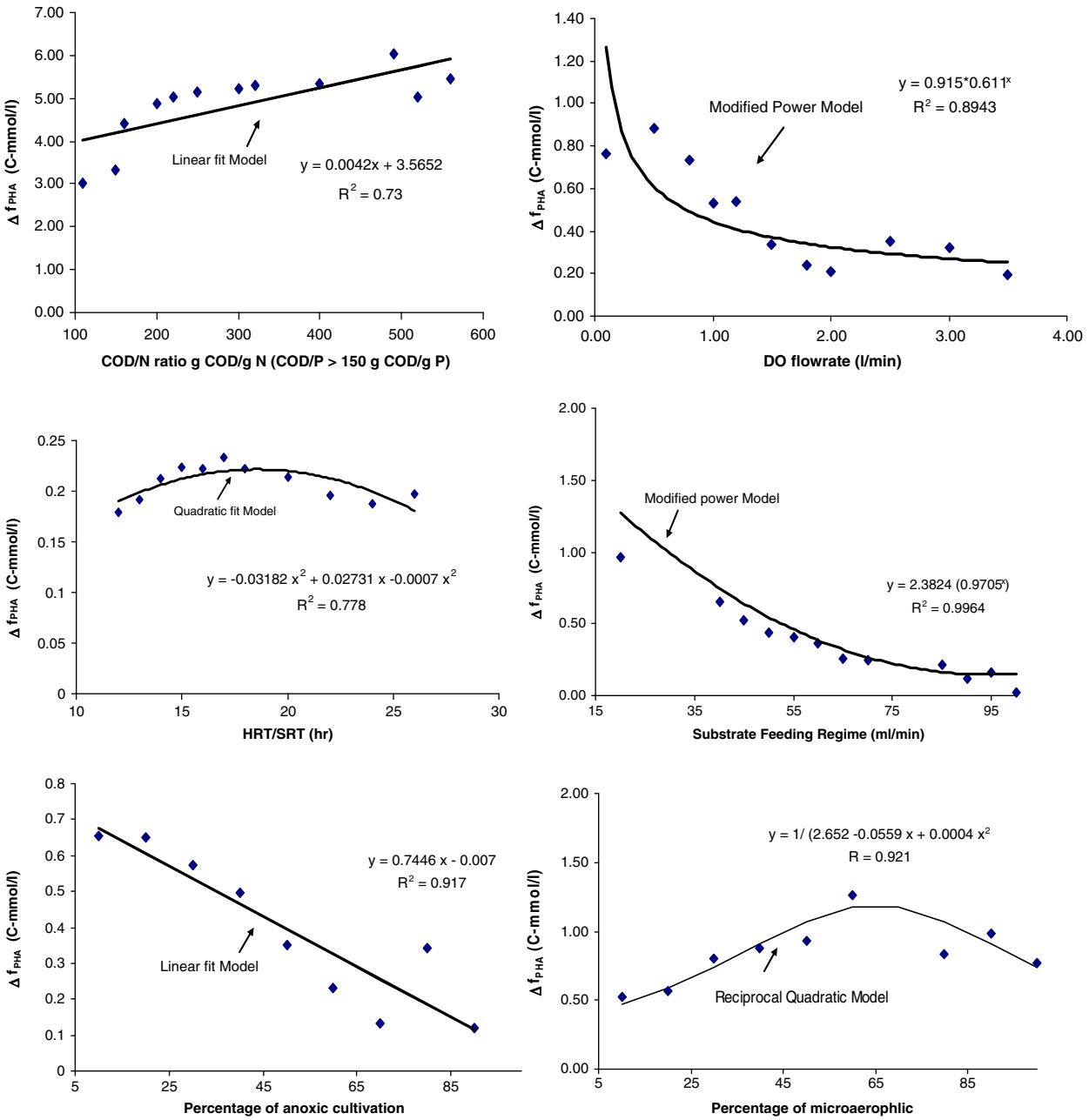

Fig. 1. PHA produced from biomass concentration (Δf_{PHA}) on C/N ratio, DO flow rates, HRT = SRT and temperature conditions, feeding rates, anoxic and microaerophilic conditions.

3.6. Effect of intermittent anoxic conditions

The difference in specific growth rate (μ) between the feast and the famine period was smaller under anoxic compared to aerobic condition (Table 5). The lower growth rate in feast period under anoxic conditions can be explained by the lower $-q_s$ (0.493C-mole/C-mol h). In addition, it also produces a high rate of PHA (q_p = 0.343 C-mol/C-mol h) which is comparable with other research findings (Beun et al., 2000a,b; Lishman et al., 2000). The transient response to a substrate spike was investigated for mixed cultures under anoxic/aerobic environment at different operating conditions (COD:N:P ratios and feed length) (Reddy and Mohan 2012a) and the results showed that PHA accumulation was high at higher substrate load [OLR3, 40.3% of dry cell weight (DCW)], low nitrogen (N_1, 45.1% DCW) and low phosphorous (P_1, 54.2% DCW) and 75% COD removal for the period of 72 h.

Dionisi et al. (2001a) have reported that the specific yields and rates on PHA, substrate and biomass were not strongly affected by the external electron acceptor (such as O_2 or NO_3). In the present study, a remarkable observation was that the anoxic specific substrate uptake rate was 3–4 times lower than the aerobic condition due to nitrate uptake has a rate limiting factor (Beun et al., 2000b).

In contrast with the results obtained by Dionisi et al. (2001a,b), it was found that certain microorganisms could perform the aerobic-denitrification as well as anoxic condition. This circumstance has already been reported from a single culture of *T. pantotropha* which can simultaneously utilize oxygen and nitrate during acetate removal. This can be achieved under aerobic condition at high growth rates and without nitrate concentration (Dionisi et al., 2001a). It is proven that aerobic denitrification can always occur with the mixtures of substrates concentration (Beccari et al., 2002). The behavior of the microorganisms appeared to be very

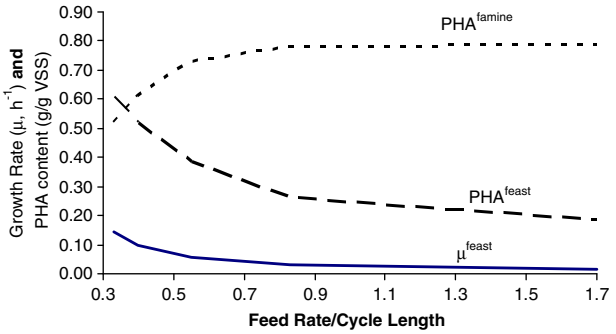

Fig. 2. Relative length of feeding period (FRpome experiment) in fed-batch SBR. *Note:* Growth rate of bacteria in feast period (bottom line), cellular content of PHA at the end of the feast (dashed line) and famine periods (dotted line).

similar to that reported by Beun et al. (2000b). The reason for the reduction of PHA yield under anoxic condition can be explained by the fact that dissolved oxygen limits the availability of adenosine triphosphate and restricts the capacity for high energy-demanding process required for biomass growth. This is because the bulk of adenosine triphosphate available is used only for substrate transport and leads to the low-energy-demanding process of PHA production (Pratt et al., 2012). The proposed mixed culture in this study is believed to limit their specific growth rate. The results show that substrate uptake, PHA degradation and electron transport were the limiting factors. The main difference between completely anoxic and the anoxic/aerobic SBR was the accumulation and subsequent degradation of nitrite in the completely anoxic SBR (Beun et al., 2000b). Under completely anoxic conditions the nitrite reduction rate falls behind the nitrate reduction rate. The degradation of PHA during the famine period resulted in an increase of growth rate under anoxic conditions. The similar specific growth rate (μ) in the famine period under both anoxic and aerobic conditions can be explained by the same level of PHA consumption rate. On the other hand, changes from aerobic to anoxic conditions could be adapted fast with exhibiting full denitrifying activity and no lag phases (Kornaros et al., 1996). In general, both anoxic and aerobic conditions are appropriate for the accumulation of PHA inside the cells. The microorganisms present in the medium have very slow adaptability to aerobic environment especially under long lag phases during the anoxic–aerobic conversion period. This also implies that the DO (dissolved oxygen) seemed to act as an inhibitor to the activity of the denitrifying reductase rather than as a repressor of their synthesis (Kornaros et al., 1996). Therefore, it can be concluded that the maintenance mechanism was the same under aerobic and anoxic conditions. The PHA degradation and/or production are influenced by the type of electron acceptor whereas the substrate uptake rate is independent of substrate composition (Saito et al., 2004).

3.7. Effect of microaerophilic ($MICae_{pome}$) conditions

Fig. 3 shows the profiles of PHA and CDW of the microaerophilic reactor with N and P limitations and the PHA production increased rapidly during the first 30 h after N and P were eliminated from the feed. The production of PHA was observed during steady-state of microaerophilic phase. It was shown that the PHA accumulation under 70% Microaerophilic continued to increase and reached the maximum content of 70%/CDW, 50 h after N and P were eliminated. At the same cultivation period, the PHA production declined from 40 to only 30% under 20% Microaerophilic. As mentioned previously, the reduction of PHA content will significantly reduce the efficiency of COD removal from solution. This observation is different from what is observed in typical biological nutrient removal (BNR) systems. PHA production in a BNR system is observed during the anaerobic phase where most of the COD is removed from the solution. On the other hand, PHA consumption occurs in the aerobic phase, when it is used as a source of carbon and energy for biomass growth and polyphosphate storage. After PHA content reached the maximum percentage value of 48%/CDW, it then decreased simultaneously with the biomass concentration. Reddy and Mohan (2012b) studied anoxic microenvironment documented higher PHA production, while aerobic microenvironment showed higher substrate degradation from food waste and acidogenic effluents using aerobic consortia.

In contrast to typical BNR systems, when PHA production was maximized using microaerophilic/aerobic cycling with N and P limitation, most of the COD was consumed during the aerobic period, for example, COD consumption during the microaerophilic period was negligible (Punrattanasin et al., 2001). Reduction of soluble COD was not observed after 30 h without N and P addition, and COD during the aerobic period was higher than the influent concentration. This is expected since N and P are essential nutrients required for cellular growth by all living organisms. The results also suggest that the commercially developed PHA production strategy of first developing a culture without nutritional limitations, and then subjecting it to one or more limitations for a short period before harvesting, is most likely to be a successful strategy for PHA production utilizing activated sludge.

Table 5
Comparative study on intermittent anoxic/aerobic experiments.

Parameters	Dimension	Beun et al. (2000a)	Beun et al. (2000b)	Current study
SRT	days	3.8	6.3	<1[a]
C_x	C-mmol/l	45	49.5	660
$-q_s$	C-mol/C-mol.h	0.640	0.170	0.493
q_p	C-mol/C-mol.h	0.270	0.064	0.343
$q_p/-q_s$	C-mol/C-mol	0.410	0.370	0.695
$\mu^{overall}$	h^{-1}	0.011	0.007	0.110
μ^{feast}	h^{-1}	0.065	0.019	0.165
μ^{famine}	h^{-1}	0.008	0.004	0.052
$\mu^{famine,anoxic}$	h^{-1}	–	0.006	0.024
$\mu^{famine,aerobic}$	h^{-1}	–	0.003	0.003
$\mu^{feast}/\mu^{overall}$	–	5.900	2.900	1.499
$\mu^{famine}/\mu^{overall}$	–	0.700	0.600	0.472
$\mu^{famine,anoxic}/\mu^{overall}$	–	–	0.900	0.218
$\mu^{famine,aerobic}/\mu^{overall}$	–	–	0.400	0.031
μ^{feast}/μ^{famine}		8.400	4.600	3.172

[a] Based on HRT (the study used HRT = SRT).

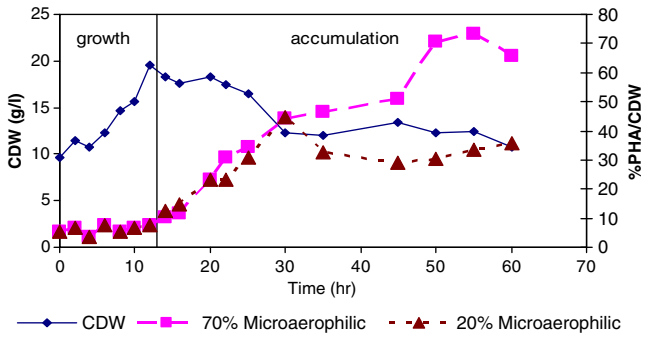

Fig. 3. Changes of PHA production and CDW at 70% and 20% of microaerophilic conditions. Growth rate of bacteria in feast period (bottom line), cellular content of PHA at the end of the feast (dashed line) and famine periods (dotted line).

As a conclusion, the study from Du et al. (2000) found that the limitation of oxygen could also result in the accumulation of acetyl-CoA and a low intracellular concentration of free CoASH (co-enzymes that are important to build the hydroxybutyric acid polymer). The increase of the acetyl-CoA/CoASH ratio partially relieved the inhibition of β-ketothiolase, which favors the formation of PHA. PHA concentration and content was increased rapidly at the early stage of oxygen limitation, then slowly reduced its rate at a later stage (as shown at 20% of microaerophilic experiment). Compared to previous works, the PHA production could reach 80% (Du et al., 2001; Mumtaz et al., 2009). However, in this study, the PHA production is only reached 74% (Fig. 3). This indicated that the oxygen limitation is more advantageous in accumulation PHA because under aerobic process the bacteria utilized the substrate only for its growth and showed a minor response for the storage of PHA especially due to very slow adaptability during long lag phases and the final PHA production could not reach more than 80% of dried biomass.

4. Conclusions

The control of the N and P levels in the reactor system can increase the storage of PHA in the biomass cells. A process of PHA production using mixed cultures and dynamic conditions (cycle length, feeding rate, anoxic and microaerophilic conditions) could be implemented by supplying the carbon source with pulses, controlled by the oxygen flow rate and mixed fatty acid components. The high specific PHA storage rates as well the high sludge PHA contents achieved by mixed cultures make this process competitive with those based on pure cultures.

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