Intracellular biopolymer productions using mixed microbial cultures from fermented POME

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Abstract This study aimed to produce polyhydroxyalkanoates (PHAs) from organic wastes by mixed bacterial cultures using anaerobic-aerobic fermentation systems. Palm oil mill effluent (POME) was used as an organic source, which was cultivated in a two-step-process of acidogenesis and acid polymerization. POME was operated in a continuous flow anaerobic reactor to access volatile fatty acids (VFAs) for PHAs production. During fermentation, VFA concentration was produced in the range of 5 to 8 g/L and the COD concentration reduced up to 80% from 65 g/L. The VFA from anaerobic fermentation was then utilised for PHA production using a mixed culture in availability of aerobic bioreactor. Production of PHAs was recorded high when using a high volume of substrates because of the higher VFA concentration. Even though the maximum PHA content was observed at only 40% of the cell dried weight (CDW), their production and performance are significant in mixed microbial culture.

Keywords Biopolymers; mixed culture; oil palm waste; PHAs; recovery

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Introduction

Bioplastics are alternatives to conventional plastics because of their high rate of biodegradability and their origination from renewable resources (Dionisi *et al.*, 2005). Polyhydroxyalkanoates (PHAs) are an example of bioplastic which consists of polyesters of various hydroxyalkanoates and is synthesised by a wide variety of microorganisms. Selection of a suitable substrate is an important factor for PHA optimisation, inducing their content, composition and polymer properties. Since the availability of substrate is a major cost factor of PHA production, thus the usage of waste organic carbon from mixed culture could significantly reduce the total production cost of PHA (Serafim *et al.*, 2004; Lemos *et al.*, 2006). Therefore, the used of a cheaper carbon source is required in order to reduce the high production cost of PHA.

Palm oil mill effluent (POME) has been reported for its suitability for PHA production (Hassan *et al.*, 1997, 2002; Md Din *et al.*, 2004, 2006). POME consists of high organic acids, therefore it is suitable to be used as a carbon source. However, POME is usually present in a complex form that cannot be directly utilised by PHA-producing bacterial species for PHA synthesis. Therefore, anaerobic treatment has been proposed to reduce their characterised compound. Hydrolysis and acidogenesis are the first step to convert the wastes to short-chain VFAs (i.e. acetic, butyric and propionic acids). After that, the VFAs will be utilised by PHA-producer for PHA production (Lee and Yu, 1997). Hassan *et al.* (1997 and 2002) found that 15 g/L of organic acids could be obtained from POME and utilised for PHA production with pure cultures.

Many studies found that most microbial cultures could consume VFA and store it as intracellular biopolymer (Reis *et al.*, 2003; Serafim *et al.*, 2004; Dionisi *et al.*, 2005; Lemos *et al.*, 2006). Some studies also found the maximum PHA cell concentration to be as high as a 0.54 g PHA/gVSS (Du and Yu, 2002). Since it could produce up to 72% of PHA, hence it is beneficial for cost reduction.

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Considering the interest of using fermented substrate as a carbon source for the production of PHA, this study aimed at understanding how the VFA concentration influences the yield of polymer production. In addition, it was to observe the optimum sludge retention time (SRT) with respect to acidification for the anaerobic treatment. This knowledge is important for the mixed culture, since it allows the setting of the final polymer production as a function of the PHA reactor feed production. Therefore, the objective of this study was to optimise the PHAs production using fermented POME as carbon sources using mixed cultures.

Materials and methods

Anaerobic digestion of POME

POME was collected from local resources, the wastewater treatment plant Felda Bukit Besar Palm Oil Mill, Kulai. Initially, the anaerobic digestion reactor of this organic waste was conducted at room temperature (28–30 °C) with a working volume of 19 L, the dimensions are shown in Figure 1. The substrates were fed into the reactor at the bottom and the culture medium contained the supernatant discharge circulated at the rate of approximately 40 mL min^{-1} through granulated sludge bed. The support material used in the fixed bed was oil palm fibres; in the form of sphere shapes approximately 1 cm diameter (total specific surface area was 0.15 cm^2 m^{-3}). The acidogenesis process was conducted in 45 days. Typically, before it can be used as a substrate to aerobic reactor, the reactor was operated without pH control and aeration nor stirring to avoid acidogenesis process. Every 7 days, acidic slurry was pumped to the aerobic reactor for the PHA production.

PHA production coupled with POME digestion

An aerobic reactor with a working volume of 6 L with 25 and 50% discharge levels were used to produce PHA. The initial mixed microbial culture was developed using 10% of activated sludge from sewage treatment and 90% from POME. The acclimatisation studies

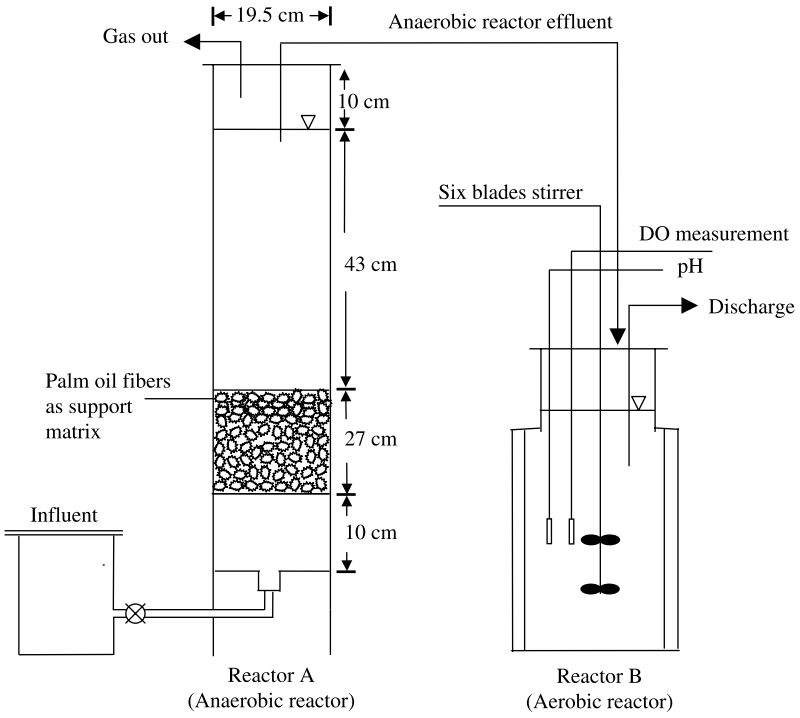

Figure 1 Anaerobic and aerobic reactors used for the PHA production POME

were conducted for at least 40 days to reach steady-state conditions. The steady-state condition was determined either by cell dried weight (CDW) or O_2 profiles. The supernatant was stored in a refrigerator for COD and PHA analysis and in the freezer for VFA, CDW and ash constituents. Figure 1 shows the set of anaerobic digestion (Reactor A) for VFA production and aerobic reactor (Reactor B) for PHA production. The fermentative acids in the acidic slurry were transferred into the reactor B, and were utilised as carbon source for PHA synthesis. The aerobic reactor was operated at room temperature (28–30 °C) and pH of 7.00 ± 0.1 using 2 M HCl or 2 M NaOH solutions. The reactor was well aerated with airflow of less than 2.0 L min^{-1} (controlled by a mass-flow controller) and stirred with two standard geometry six-blade stirrer. The process was conducted in a turbulence regime to ensure completely mixed conditions using a mixing speed of 400 rpm, approximately.

Fed-batch tests were performed in two consecutive phases: growth and accumulation periods. The reactor was operated in a famine phase of 2–6 h, a feast phase of 2–3 h, 5–10 min of withdrawal and 5 min of additional mineral medium. In this case, the acid slurry volume of 1,500 mL and 3,000 mL was added in pulses. The first pulse of VFA was added 5 min after the mineral solution was added. The second pulse was added when the VFA in the reactor was exhausted (due to the increment of dissolved oxygen). Since the duration of the cycle was always inconsistent, the hydraulic retention time (HRT) changed along the operation periods.

Analytical techniques

The dissolved oxygen (DO) concentration in the reactor was measured online with a DO-electrode as percentage of air saturation. COD, VFA, CDW analyses were conducted in accordance with *Standard Methods* (APHA, 1995). PHA was determined by GC and the PHA content (%) was defined as the percentage of the ratio of PHA concentration to CDW.

Results and discussion

COD removal and VFA production from POME

The COD removal achieved was as high as 80% in approximately SRT of 6 days of fermentation, as shown in Figure 2. The COD removal actually occurred during the fermentation process where a high amount of COD removal was used in the generating and synthesising of new bacterial cells for the anabolism route (De la Rubia *et al.*, 2006). This process begins with the hydrolysis of complex organic compounds in the initial POME to more soluble intermediates. Through the process of acidogenesis, these intermediates are primarily broken down into VFAs and other monomer species. However, the differences in SRT influenced the VFA results; the concentration of the VFA was increased significantly for the SRT of 6 days and reduced for 7 days. As shown in Figure. 2, at SRT of 7 days acidogenic conditions prevail, while at SRT $\geq$ 7 days methanogenic condition prevail. It shows that the SRT strongly affects the type and rate of bioconversion process under anaerobic conditions (Miron *et al.*, 2000).

Respirometric analysis and substrates concentration

The organisms have a choice to consume external substrate either for growth or storage purposes. It is widely known that the competing microorganisms maximise their growth rate and storage capacity only occurs when the nutrients (N and P) are limited (Serafim *et al.*, 2004). Many organisms subjected to feast–famine conditions maximise their substrate uptake rate, regardless of their growing factors, whether it will be reduce or increase their yield. Storing substrate and subsequent growth on it leads to a slightly reduced net growth yield (Beun *et al.*, 2002).

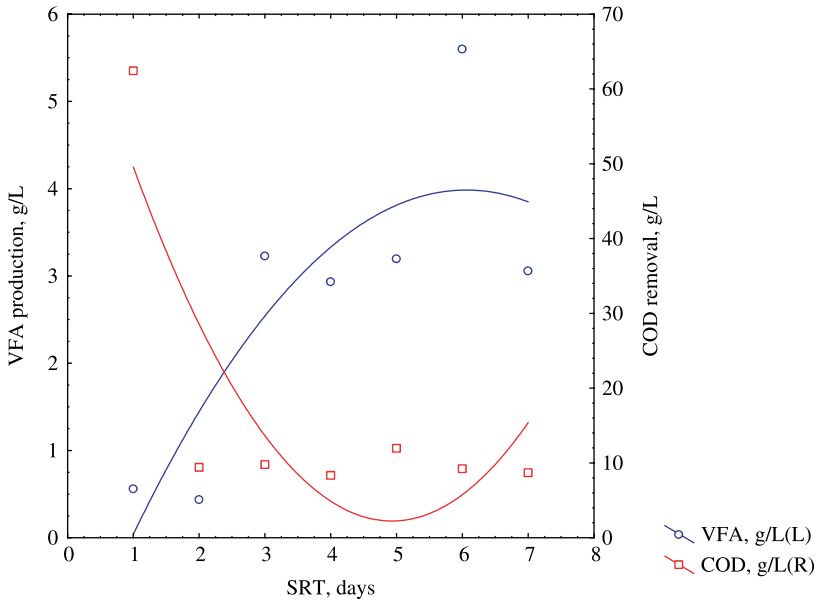

Figure 2 Profile of VFA and COD concentration using anaerobic process at room temperature (28–30 °C)

The oxygen uptake rate (OUR) is usually determined to monitor the dynamic response of the culture, and in general this study found that the OUR increased drastically for the first 50 min, as shown in Figure 3(a). During the limiting condition (known as PHA accumulation), the OUR curve reached a peak at 42 mg O_2/L.min and then decreased to only 15 mg O_2/L.min after 100 min. The plateau trend within 50 to 140 min indicates that the biomass utilised readily biodegradable substrate (S_S) in a short period of time. This resulted in a fast uptake rate since the S_S, such as acetic acid, can be removed directly by the microorganisms, similar results were obtained by Majone *et al.* (2001) and Dionisi *et al.* (2001). The substrate can also be removed quickly at early accumulation stage for storage and slow response to respiration analysis because lack of nutrients will cause a slow down of the metabolic process. This was hindering the present biomass to duplicate their cells. These mechanisms clearly indicate that the substrate is transported into the cell and maintained in an almost unchanged form or transformed into low-molecular weight metabolic intermediates. The type and extent of biomass response depends on its microbial composition and on the physiological state of the different microorganisms, which in turn are defined by the previous history of the biomass. Therefore, the PHA production rate was proven higher under limiting nutrient conditions rather than with sufficient nutrients (Serafim *et al.*, 2004).

In contrast, as shown in Figure 3(b), the OUR measurement gave a gradual increment to indicate that the S_s is insufficient for microorganisms at the beginning of cultivations. In this growth phase, the second increase of the OUR curve occurred after 200 min of cultivation. During this period, the increment of OUR curve can be explained by the adaptation to the organic substrate or it could be caused by growth of the biomass. Then, the curve exhibits a sudden drop (after 300 min) indicating that the added substrate has been consumed from the medium. The sudden drop of the OUR curve is then followed by continuously decreasing respiration. The respiration in this phase is due to the transformation of a secondary substrate (storage polymer) or it could be due to the accumulation of the substrate in the flocs, which caused different access to the remaining primary substrate.

The batch tests with the fermented POME showed a different OUR pattern. Figure 3(a) shows a consistent increase until it reached a plateau after 100 min when the OUR suddenly

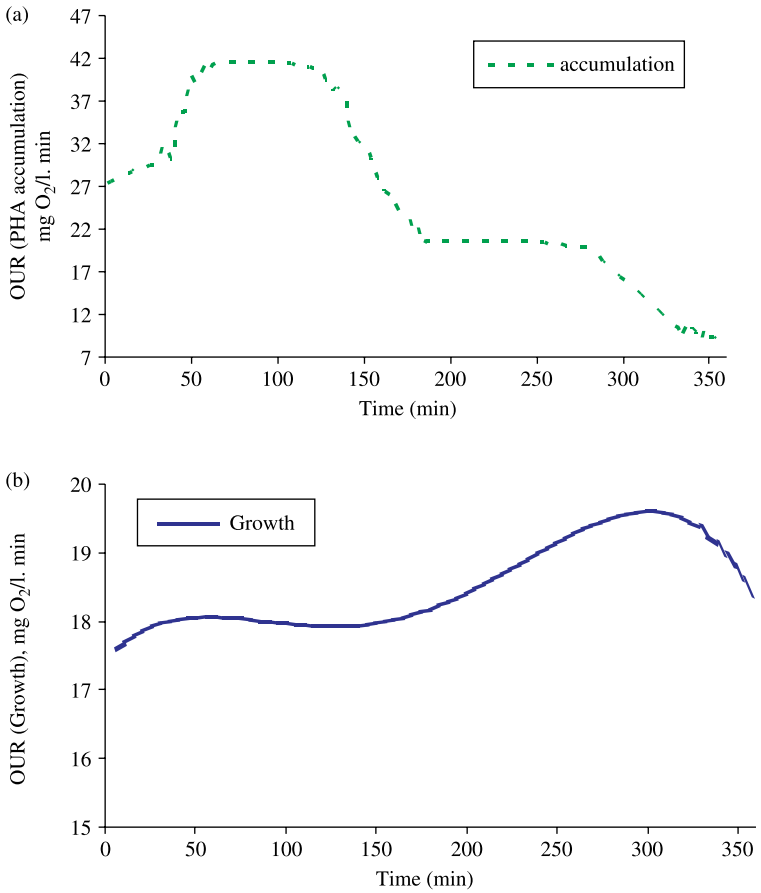

Figure 3 (a) Comparison of OUR analysis during PHA accumulation (no nutrient) and, (b) growth phase (nutrient available). Both used diluted POME

decreased. However, in Figure 3(b), the trend of OUR shows two phases before the micro-organism respiration turned back to the endogenous stage. The main differences in the OUR profiles could be explained by the availability of high carbon sources in the reactor and the low nutrient present during the feast period, which influenced the sudden decreasing of OUR. The presence of a second OUR phase, as shown in Figure 3(a), seems to indicate that a significant fraction of the analytical soluble COD is actually slowly biodegradable, as already reported in previous studies (Beccari *et al.*, 2002).

Based on observation in terms of the biomass content, for 50% feeding (second phase of feeding), the biomass content is obviously higher than for 25% feeding (first phase) as seen Table 1. The concentration of biomass produced increased directly with substrate volumes. In addition, the amount of polymer produced per substrate consumed increased concurrently with VFA production rate. The concentration of a substrate supplied affects the amount of polymer produced (Beun *et al.*, 2002; Serafim *et al.*, 2004). In other words, the amount of PHA in the biomass will be accumulated and increased directly proportional to the amount of the initial carbon source for approximately 40% from cell dried weight (see Table 1). Besides, the volume of substrates influenced the time of accumulation. The length of the feast–famine period depends on the amount/volume of substrate. If the substrate fed was in a smaller volume then the usage of air for oxidising and for substrate storage into micro-organism, it becomes shorter, as shown in Table 1 and Figure 4.

Table 1 The concentration of parameters for the two step process

Parameters	Anaerobic (Stage A)		Aerobic (Stage B)	
	Influent	Effluent	25% substrate	50% substrate
COD(g/L)	65	8	25	32
COD removal (%)	–	80	45	60
VFA (gCOD/L)	NA	10.1	4.2	12.2
Cell dried weight (CDW) (%)	–	–	13	30
PHA (% CDW)	–	–	12	40
Length of feast periods (min)	–	–	170	280

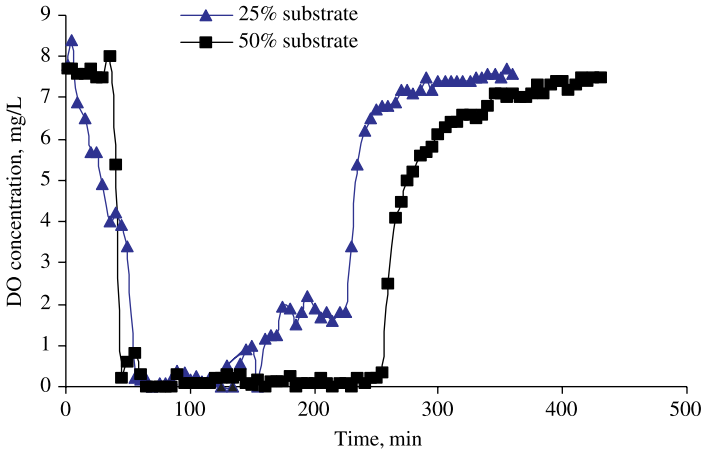

Figure 4 Trend of the concentration of substrate

The identification of feast and famine periods were clearly recorded from DO trends, as shown in Figure 4. The end of the feast period and the beginning of the famine period was easily identified by the sudden increase in DO concentration. Upon addition of the feeding substrate (respectively at 1 h), the percentage of DO decreased significantly. The minimum DO achieved was 0.1–2.0 mg/L within the period ranging from 1.5 to 6 h for both cycles (feast–famine). DO concentration decreased immediately after the substrate feed to the reactor, it will remain constant during the feast period and rises again after carbon source exhaustion. At this point, biomass begins to consume intracellular PHA slowly, thus reducing the PHA concentration in the famine period. These results clearly indicate that the amount of substrate consumed during the feast period was significantly used for polymer storage (PHA), cell growth and maintenance processes (Serafim *et al.*, 2004). In summary, PHAs were stored in the feast period and degraded in the famine period as internal sources of carbon and energy. Therefore, PHA concentration was maximum in correspondence to substrate depletion and minimum at the end of the cycle (Dionisi *et al.*, 2005).

Conclusions

The possibility of PHA production from organic wastes in a two-stage-process consisting of anaerobic acidogenic fermentation and step aerobic processes has been studied. The experiment showed that POME represents a potential feedstock substrate for the production of PHA from high storage capacity mixed cultures under feast–famine conditions. POME fermentation was effective in producing VFAs, in addition, it consistently removed over 80% of COD and VFAs production was in the range of

5–8 g/L for 6 days of the SRT. The percentage of CDW and PHA production increased simultaneously with an increase of substrate where the maximum value is 40% of CDW.

Acknowledgements

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References

American Public Health Association (APHA) (1995). *Standard Methods for Examination of Water and Wastewater*, 18th edn, American Public Health Association, Washington DC, USA.

Beccari, M., Dionisi, D., Giuliani, A., Majone, M. and Ramadori, R. (2002). Effect of different carbon sources on aerobic storage by activated sludge. *Water Sci Technol.*, **45**(6), 157–168.

Beun, J.J., Dirks, K., Van Loosdrecht, M.C.M. and Heijnen, J.J. (2002). Poly-(hydroxybutyrate) metabolism in dynamically fed mixed microbial cultures. *Water Res.*, **36**, 1167–1180.

De la Rubia, M.A., Perez, M., Romero, L.I. and Sales, D. (2006). Effect of solid retention time (SRT) on pilot scale anaerobic thermophilic sludge digestion. *Proc. Biochem.*, **14**, 79–86.

Dionisi, D., Caruccia, G., Petrangeli Papinia, M., Riccardi, C., Majone, M. and Carrasco, F. (2005). Olive oil mill effluents as a feedstock for production of biodegradable polymers. *Water Res.*, **39**, 2076–2084.

Dionisi, D., Majone, M., Tandoi, V. and Beccari, M. (2001). Sequencing batch reactor: influence of periodic operation on performance of activated sludge in biological a astewater treatment. *Ind. Eng. Chem. Res.*, **40**, 5110–5119.

Du, G. and Yu, J. (2002). Green technology for conversion of food scraps to biodegradable thermoplastic polyhydroxyalkanoates. *Environ. Sci. Technol.*, **36**, 5511–5516.

Hassan, M.A., Shirai, N., Umeki, H., Abdul Karim, M.I., Nakanishi, K. and Hashimoto, K. (1997). Acetic acid separation from anaerobically treated palm oil mill effluent for the production of polyhydroxyalkanoate by *Alcaligenes eutrophus*. *Biosci. Biotechnol. Biochem.*, **61**, 1465–1468.

Hassan, M.A., Nawata, O., Shirai, Y., Nor Aini, A.R., Yee, P.L., Arif, A. and Abdul Karim, M.I. (2002). A proposal for zero emission from palm oil industry incorporating the production of polyhydroxyalkanoates from palm oil mill effluent. *J. Chem. Eng. of Japan.*, **35**(1), 9–14.

Lee, S.Y. and Yu, J. (1997). Production of biodegradable thermoplastics from municipal sludge by a two-stage bioprocess. *Res. Conserv. Recycl.*, **19**, 151–164.

Lemos, P.C., Serafim, L.S. and Reis, M.A.M. (2006). Synthesis of Polyhydroxyalkanoates from Different Short-chain fatty acids by mixed cultures submitted to aerobic dynamic feeding. *J. Biotechnol.*, **122**, 226–238.

Majone, M., Beccari, M., Dionisi, D., Levantesi, C. and Renzi, V. (2001). Role of storage phenomena on removal of different substrates during pre-denitrification. *Water Sci. Technol.*, **42**(3), 151–158.

Md Din, M.F., Ujang, Z. and Van Loosdrecht, M.C.M. (2004). Polyhydroxybutyrate (PHB) production from mixed cultures of sewage sludge and palm oil mill effluent (POME): the influence of C/N ratio and slow accumulation factor. *Wat. Env. Manag. Series*, **7**, 112–115, IWA Publishing, London.

Md Din, M.F., Ujang, Z., Muhd Yunus, S. and Van Loosdrecht, M.C.M. (2006). Storage of polyhydroxyalkanoates (PHA) in fed-batch mixed cultures. Fourth Seminar on Water Management (JSPS-VCC), July 11–13, Johor, Malaysia.

Miron, Y., Zeeman, G., van Lier, J. and Lettinga, G. (2000). The role of sludge retention time in the hydrolysis and acidification of lipids, carbohydrates and proteins during digestion of primary sludge in CSTR systems. *Water Res.*, **34**(5), 1705–1713.

Reis, M.A.M., Serafim, L.S., Lemos, P.C., Ramos, A.M., Aguiar, F.R. and van Loodrecht, M.C.M. (2003). Production of polyhydroxyalkanoates by mixed microbial cultures. *Bioprocess Biosystem Eng.*, **25**(6), 377–385.

Serafim, L.S., Lemos, P.C., Oliveira, R. and Reis, M.A. (2004). Optimization of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions. *Biotechnol.Bioeng*, **87**(2), 145–160.