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Polyhydroxyalkanoates (PHAs) Production from Complex Polymer Organic Waste Using Anaerobic and Aerobic Sequence Batch Reactor

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ABSTRACT

A novel method was developed to convert complex polymer organic waste into polyhydroxyalkanoates (PHAs). Palm oil mill effluent (POME) was used as carbon source within this study. The designed laboratory scale system includes two coupled reactors; an anaerobic fermenter for volatile fatty acids (VFAs) production followed by an aerobic sequencing batch reactor (SBR) to obtain PHA. During fermentation, VFAs was produced in the range of 5 to 7 g/L and the COD reduction was up to 80% from 65 g/L at a sludge retention time (SRT) of 6 days. Acetic acid was the predominant component of the fermentative VFAs. In the succeeding aerobic SBR with a mixed culture, microaerophilic-aerobic conditions increase the PHA production. PHA, analyzed as PHB, reached up to 4.0×10^{-2} g/gVSS. The maximum PHA content observed was only 40% of cell dried weight (CDW). The indication is that the PHA production and process performance will benefit from a mixed microbial culture.

INTRODUCTION

With the increasing environmental pressure to replace conventional plastics with biodegradable polymers, the reduction of production costs of the latter materials became critical [1]. Polyhydroxyalkanoates (PHAs) are considered to be good candidates for biodegradable plastics due to their large range application and capability of being produced from renewable resources [2-4]. These materials have attracted interest because of their potential use as biodegradable alternatives to petroleum-based synthetic plastics such as polypropylene and polyethylene [5]. However, the production cost of PHAs has limited their use in wide range applications [6]. Efforts have been made to produce PHAs by microbial fermentation organic waste (kitchen waste, olive oil and POME). The production of biodegradable polymers from oil palm industry can be seen as beneficial to the environment as well as contributing to sustainable development. Until recently, the remaining 90% (empty fruit bunches, fibers, fronds, trunks, kernels, POME) was discharge as waste, and either burned in the open air or left settle in waste ponds. By utilizing the POME and empty palm oil fiber bunch (EPFB) as carbon source and

support matrix, the disposal of POME that needed further treatment could be reduced. In addition, the production of biodegradable polymers from POME can provide multiple benefits to the environment, and contribute to sustainable development [7,8].

POME has been reported for its suitability for PHA production [4, 9]. POME consists of high organic acids, which makes it suitable as a carbon source. However, POME is usually present in complex form that contains significant amount of triacylglycerides, di- and monoacyl glycerides and monoglycerides and some derivatives of fatty acids [10]. These forms cannot be directly utilized by PHA-producing species bacterium for PHA synthesis. Therefore, anaerobic treatment has been proposed to reduce the complex POME polymers. Hydrolysis and acidogenesis are the first steps to convert the wastes to short-chain VFAs i.e: acetic, butyric and propionic acids. After that, the VFAs will be utilized by PHA-producer for PHA production [8,11]. At present, there are technological difficulties in coupling the waste acidogenesis and PHA synthesis steps. In essence, both acid-producing and PHA-producing cells can be cultivated in a mixed culture, and the

acid released by the former one is directly utilized by the latter one. However, PHA content of the solid mass is not enough for effective polymer recovery, due to the considerable amount of non-biodegradable matter in the POME, as well as the biomass of non-PHA producing microbes. Furthermore, a high acid concentration due to the high microbial activity of acidogenic microbes would inhibit the microbial activity of PHA-producing cells [7].

This study is aimed at understanding the mechanism of VFA influences on the yield of polymer production. In addition, optimize the PHAs production using fermented POME as carbon sources using mixed cultures. The improved system contain two separate bioreactors, in order to satisfy the different physiologies and metabolic activities of the two types of microbes; one for acidogenesis of POME and a second for mixed microbial culture of PHA-producing strains.

MATERIAL AND METHODS

Reactor Design

In order to investigate the PHA production in POME and mixed culture, two types of bioreactor was designed and used to meet metabolic activities in one cycle. The anaerobic part was designed in a cylindrical shape with a working volume of 19 litres, interior diameter 19.5 cm and height 90 cm. The sampling ports were designed along 10 cm height intervals from the bottom. A perforated piping system was used at the bottom of the reactor to ensure homogenous distribution of flow into the reactor and no recirculation of effluent was practiced.

A support material used in the fixed bed, which is: empty palm oil fiber bunch (EPFB); in sphere form shape with approximately 1cm diameter (total specific surface area was $0.15 \text{ cm}^2\text{m}^{-3}$). The acidogenesis process was conducted for 45 days for the inoculation. The reactor was operated at room temperature ($28 \pm 2 \text{ }^\circ\text{C}$) and at varied SRT/HRT. At each HRT, the reactor was operated for six weeks to reach steady state conditions. Steady state conditions were established when the variation in the product concentration were constant (effluent VFAs and COD concentrations).

Typically, the reactor was operated without pH control and aeration nor stirring to avoid the acetogenesis process that transforms VFA to other forms. Acidic slurry produced in anaerobic reactor was then pumped to the aerobic reactor as substrate feeding for the microorganisms that produce PHA. This supply can be varied depending on each SRT/HRT.

The aerobic part was fabricated in two double-jacketed laboratory-scale reactor with six litres effective volume. The experiment has demonstrated under microaerophilic-aerobic (controlling the oxygen level) conditions. The operation of aerobic reactor was based on the sequencing batch reactor (SBR) system under feast-famine regime which was conducted on samples taken from fermented POME from anaerobic reactor.

Seeding sludge detailed study procedures

Raw POME and waste sludge from a third sludge pond was collected from a local palm oil mill wastewater treatment plant. A fresh activated sludge taken from an aeration tank of a local municipal wastewater treatment was used as seed sludge for both of reactor. Waste sludge, POME and sewage sludge were introduced as inoculate to acclimatize the autotrophic and/or heterotrophic bacteria in each reactor. The ratio of the inoculums is 1:2:1. For the anaerobic reactor, the substrates

were fed into the reactor at the bottom and the culture medium contained supernatant discharge circulated at the rate of about 40 mL/min through a granulated sludge bed. A perforated piping system was used at the bottom of the reactor to ensure homogenous distribution of flow into the reactor and no recirculation of effluent was practiced. For the aerobic reactor, at least more than 50% of the working volume must be designed to be discharged as supernatant.

The operating principles of a batch activated sludge system are characterized in just three discrete periods: fill, react and draw (discharging). After two to three days, the aerobic system was continuously operated by supplying nutrient adaptation for several weeks, in order to reach a steady-state condition. At steady-state conditions, some parameters has been extensively monitored (eg. pH, DO, and samples was collected every hours for analysis COD, TOC, $\text{NH}_4^+\text{-N}$, P, VFAs CDW and ash constituent).

In order to maximize the growth rate and fast substrate uptake rate and storage polymer formation, the system was operated in continuous reaction period, which means no settling or allowing the idle phase ($\text{HRT}=\text{SRT}$). The length of HRT and SRT in each treatment depends on the microorganisms' reaction after feeding. The cycle of operation system depends on substrate concentrations. The detailed features of the two reactors are described in Table 1.

Table 1. Reactor description and condition of each experiments

Reactor	Anaerobic reactor	Aerobic reactor
Working volume	19-L (laboratory scale)	6-L (laboratory scale)
Influent feed	Raw POME	VFA (fermented POME)
Temperature	$28 \pm 2 \text{ }^\circ\text{C}$	$28 \pm 2 \text{ }^\circ\text{C}$
pH	4.0 – 10.0	7 ± 0.1
Aeration and DO	No aeration	$1 - 1.7 \text{ Lmin}^{-1}$
HRT /SRT	7-8 hours/ 5-10 days	1 hour/ 4-8 hours
Stirrer	-	300 - 400 rpm

The acidic supernatant is pumped into the reactor and mixed with biomass that settled during the previous cycle until the time for filling is reached. A mineral solution with composition was added for the growth phase only [12] is shown in Table 2. In general, the overall operation period of fermented POME cultivation is shown in Table 3. The filling phase can be mixed in either aerated (oxygen as electron donor) or microaerophilic-aerobic conditions.

In order to control the oxygen concentration properly, the gas concentrations was controlled using gas flow meter. The reaction phase can also be mixed in similar condition with the common environment needed by bacteria to live. This reactor was equipped with peristaltic pumps for influent feeding and effluent withdrawal, and air compressor was employed for aeration. The procedures of reactor operation, such as feeding, aerating and withdrawing were controlled automatically by timers and temperature was maintained at $28 \pm 2 \text{ }^\circ\text{C}$ in a temperature controlled-room. The dissolved oxygen (DO) concentration was measured as percentage of the saturation concentration ($100\% = 9.1 \text{ mgL}^{-1}$). DO concentration and pH were measured continuously. Volatile fatty acid, cell dried weight (CDW) and PHA determination was conducted according to the Comeou *et al* [13].

Table 2. The composition of the mineral solution.

Compound	Concentration in the medium (g/L)
NH ₄ Cl	15.5
KH ₂ PO ₄	7.59
MgSO ₄ .7H ₂ O	0.2
FeCl ₃ .5H ₂ O	1.50
H ₃ BO ₃	0.15
CuSO ₄ .5H ₂ O	0.03
MnCl ₂ .4H ₂ O	0.12
Na ₂ MoO ₄ .2H ₂ O	0.06
ZnSO ₄ .7H ₂ O	0.12
CoCl ₂ .6H ₂ O	0.15
EDTA	10.0
KI	0.18

Source: [12,14]

Table 3. Operating phase with POME as substrate.

Experiment (s)	Operating time (min)			
	Aerobic mineral feeding	Aerobic feeding	Aerobic reactor	Draw/ discharge
Growth	0 - 60	0 - 60	60 - 330	330 - 340
DO (pretreated POME)	No fill	-	0 - 600	-
Mic ae (pretreated POME)	No fill	-	0 - 500	-

Analytical procedures

Samples were taken from the reactor with a 60 mL syringe (Syphon, United Kingdom). The syringe was always rinsed with the content of the reactor before sampling. Part of the sample was stored in the refrigerator for analysis. The remaining supernatant was centrifuged at 10,000 rpm for 10 minutes. The centrifugation for separating the debris and supernatant was performed using Sorval RC-5B (Hermmicks, Germany) for 15 minutes at 2000 rpm and 4 °C. The supernatant was then filtered by using PVDF-syringe filter. Samples for analysis of NH₄-N, PO₄-P, TOC and COD and VFA were immediately centrifuged and filtered using 0.45 µm filters to separate the bacterial cells from the liquid, 0.2 µm cone-syringe filters were used for soluble analysis.

The supernatant was stored in the refrigerator at -4 °C (for PHA analysis) and at -5°C (for VFA, MLSS, MLVSS, CDW, NH₄⁺, NO₃⁻, PO₄²⁻ and COD). All analytical measurements performed in this study were conducted according to *Standard Methods for the Examination of Water and Wastewater* ([15]). PHA determination was conducted according to the Comeou *et al.* [13].

RESULTS AND DISCUSSION

COD removal and VFA production from fermented POME

Organic wastes are usually complex in nature, and cannot be directly utilized by PHA-producing microbes for PHA synthesis. POME consists of high organic acids; therefore it is suitable to be used as a carbon source. Typically, raw POME is difficult to degrade because it contains significant amounts of oil (tryacylglycerols) and degradative products such as diacylglycerols and monoacylglycerols and fatty acids [10]. The fatty acids composition (C₁₂-C₂₀) of each of this fraction are different from one another and contribute to a high value of pollution load in POME. The typical characteristics of raw POME are given in Tables 4 and 5 showing the composition of lipids in POME as used in this study.

POME is usually present in a complex form that cannot directly be utilized by PHA-producing bacterial species for PHA synthesis. Therefore, anaerobic treatment has been proposed to reduce their POME characteristic. It is one of the naturally occurring processes involving decomposition and decay, in which complex organic matter is broken down into its chemical constituents [16]. 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 utilized by PHA-producers for PHA production [7,17].

Table 4. Characteristics of raw POME obtained from a local palm oil mill factory.

Parameters	Units	Results
pH	-	4.8 ± 0.21
Total suspended solids	mg/L	35,000 ± 200
Turbidity	NTU	21,000 ± 300
COD	mg/L	65,000 ± 800
BOD ₅	mg/L	27,000 ± 800
O&G	mg/L	8,000 ± 300
TOC	mg/L	12,300 ± 570
Phosphorus	mg/L	142 ± 19
Ammonia Nitrogen	mg/L	62 ± 10

Note: values represent means of triplicate determination

Table 5. The composition and concentration of lipids in POME used in this study.

Lipids	Concentrations (%)
Tryglycerides	81.5
Diglycerides	7.0
Monoglycerides	0.5
Free fatty acids	11.0

The fermentation reactor started to produce acids immediately after inoculation. The fermenter biomass consisted of a large number of small free-living bacteria and some small aggregates. To optimize the yield of VFAs and COD removal in the sludge fermentation, it was conducted in batch anaerobic reactor at varies of SRT (1 to 12 days). The COD removal achieved was as high as 80% in approximate SRT of 6 days, as shown in **Fig 2**. The COD removal actually occurred during the fermentation process where a high amount of COD removal was used in the generating and synthesizing new bacterial cells for the anabolism route [18]. 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 broken down primarily 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 the plateau was reached after about 10 days of the fermentation. Then the production rate decreased after 11 days.

As shown in **Fig. 2** where at SRT of 11 days, acidogenic conditions prevail while at SRT ≥ 11 days, methanogenic conditions prevail. It shows that the SRT strongly affects the type and rate of bioconversion process under anaerobic conditions [19]. The initial slurry had a solids content of 35 g/L and 70% or 10 g/L was digested in six days as shown in **Fig. 3**. The solid mass declined quickly in three days and almost 50% of the digestible POME was consumed during this period of time. At the same time, the pH value dropped rapidly from 7 to below 5. After that, acid release rate only resulted in slower pH

reduction and about 10% of digestible POME was digested in the last 8 days.

The slowdown of solid digestion was not only attributed to the low pH level, but also to the residual solids that became difficult to digest. This indicated that most of the easily digestible components in POME were quickly consumed in the early stage [20]. Obviously, during POME digestion, the pH dropped due to the accumulation of the large amounts of organic acids, and the digestion rate (VFAs concentration) was influenced by the change of pH. These situations well exceeded the threshold limits that inhibit methanogenetic bacteria [7].

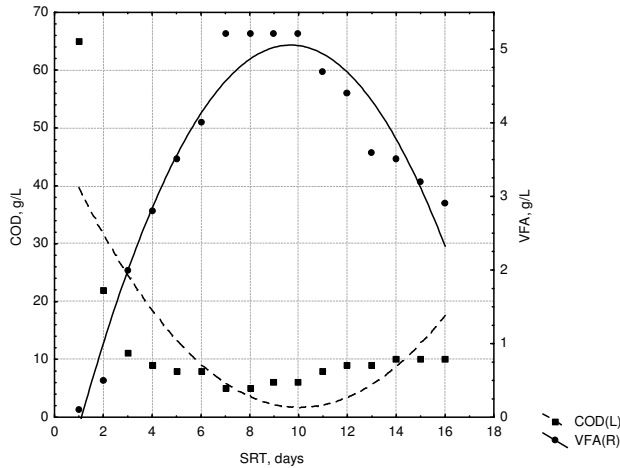


Fig 2. Profile of VFA and COD concentration using anaerobic process at room temperature.

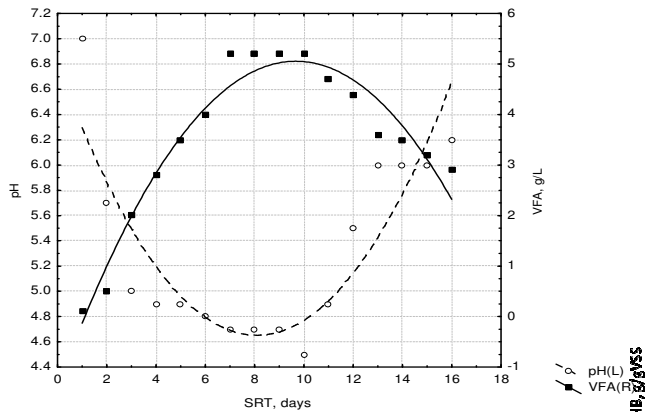


Fig 3. Profile of pH values and VFA concentrations using the anaerobic process at room temperature (28 ± 2°C).

The different VFA distribution can have a great influence on the composition of the polymer produced in the subsequent steps, namely the percentage of HV monomers within the copolymer P(HB-HV). Indeed, VFAs containing an even number of carbon atoms (i.e. acetic and butyric acids) mostly lead to formation of HB monomers, whereas VFAs containing an odd number of carbon atoms (i.e. propionic and valeric acids) mostly lead to the formation of HV monomers [7,21]. As an increase of HV content within the copolymer generally

improves its thermal and mechanical properties, the negative effect of centrifugation on propionic and valeric acids production has to be considered along with the important beneficial effects discussed above [21].

VFA characterization has been conducted using fermented POME in anaerobic reactor. Table 6 shows the composition of VFAs found in this study. Fermented POME composition constitute up to 50% of acetic acid, 30% of butyric acid, and 10% of propionic and lactic acids. Earlier studies have shown that the VFAs (acetic, butyric, propionic, etc) have been used as carbon source by bacteria, as individual or mixed or of the VFAs [3,7,21].

Table 6. VFAs composition (%) of fermented POME in Anaerobic reactor.

Systematic name	Trivial name	Compound	Values
Ethanoic Acid	Acetic acid	CH ₃ COOH	57
Butanoic Acid	Butyric acid	C ₃ H ₇ COOH	33
Propanoic Acid	Propionic acid	C ₂ H ₅ COOH	6
Pentanoic Acid	Valeric acid	C ₄ H ₉ COOH	4

For the cultivated fermented POME composition, an overall effect on growth and accumulation factor has been proposed, as shown in Fig. 4. The concentration of PHA biomass component was determined as PHB and the value obtained (gPHB/gVSS) from the system could reach up to 4.0 x 10⁻² g/gVSS. Under this situation, the cell growth (active biomass) increased with the initial feed of VFAs and reached steady after five hours, ranging from 2 to 5 g/L. Immediately, the PHB content increased to the maximum levels up to 4.20 x 10⁻² g/g VSS, before sharply decreasing to 2.0 x 10⁻² g/g VSS. The low concentration of PHB during this preliminary experiment may be affected by low concentration of VFAs in the medium. However, the type of polymer was homopolymer (PHB). This suggests that the mixture of acetic acid and butyric acid is a good carbon source for PHA synthesis, but unlike propionic acid, valeric acid cannot be used for synthesis of hydroxyvalerate (HV) monomer, even though both acids have three carbons.

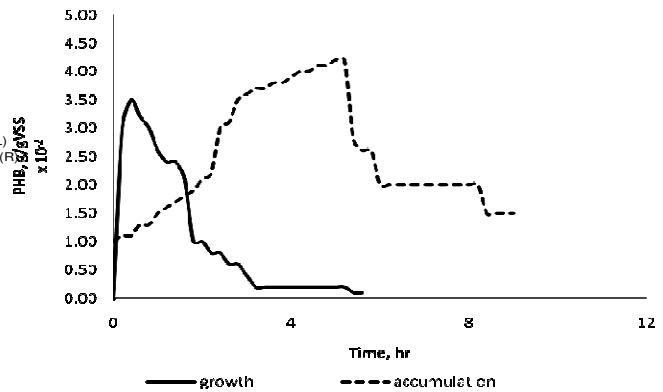


Fig. 4. PHB production during pre-determined growth and accumulation conditions.

Substrates concentration

With current biological PHA production practice, the feedstock is estimated at 30 to 50% of the total production costs [22]. These estimates vary somewhat with the microbial species utilized, carbon source, PHA yield and PHA production capacity [23]. The quantity and form of the carbon substrate dictates the polymeric structure and yield of the PHA [24].

In this study, 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 shown in Table 6. 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 [1]. In other words, the amount of PHA in the biomass will be accumulated and increased directly in proportion to the amount of the initial carbon source for about 40% from cell dried weight (see Table 7). 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 smaller volume, then the usage of air for oxidizing and for substrate storage into microorganism becomes shorter as shown in Table 7.

Table 7. 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 (g COD/L)	NA	10.1	4.2	12.2
Cell dried weight (CDW) (%)	-	-	13	30
PHA (% CDW)	-	-	12	40

CONCLUSIONS

This study has successfully shown the possibility of PHA production from POME in a two-stage-process consisting of anaerobic acidogenic fermentation and step aerobics processes. The experiment has demonstrated that POME is a potential feedstock substrate for the production of PHA from high storage capacity mixed cultures under microaerophilic-aerobic conditions. The cycle of microaerophilic-aerobic condition influences the PHA production. POME fermentation was effective in producing VFAs, and consistently removed over 80% of COD, and VFAs production was in the range of 5–7 g/L between 6-8 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.

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