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Full Length Research Paper

Comamonas sp. EB172 isolated from digester treating palm oil mill effluent as potential polyhydroxyalkanoate (PHA) producer

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Eleven potential polyhydroxybutyrates (PHB) producers were successfully screened from treated palm oil wastewater using acetate as sole carbon and energy sources and on nitrogen limiting media with Nile Blue A dye to screen for PHB accumulation. Orange fluorescence color, produced upon binding of the dye to polymer granules in the cell, was detected when directly exposed under ultra violet light and it was further confirmed using fluorescence microscopes. Positive isolate designated as PHB5 gave the highest PHB content of 44% (g PHB/g CDW) in shake flask experiment. The metabolic properties of the bacteria was characterized using BIOLOG program and this bacterium was identified belongs to the genus *Comamonas* sp. Based on the peaks detected from ^{1}H-NMR analysis, it is clearly indicated that the polyhydroxyalkanoates (PHA) produced from the bacteria was poly-(hydrobutyrate-co-hydroxyvalarate) (PHBV) when the bacterium was supplied with 10 g/L acids mixture (acetic 5 : propionic 3 : butyric 2) derived from palm oil mill effluent (POME) treatment.

Key word: Screening, polyhydroxyalkanoates, palm oil mill effluent, *Comamonas* sp.

INTRODUCTION

Polymeric materials occurring naturally or produced from renewable resources were recognized as extremely useful many years ago. These materials have become increasingly more interesting and attractive in recent years as alternatives to petrochemical-based polymers and plastics. Meanwhile, consumers and the plastics industry have shown a growing concern about the disposal of plastics and their environmental impact. The industry is actively looking for ways to minimize the unnecessary use of plastics to complement recycling and reuse programs. Others are working on new materials or modifications to old ones to reduce the environmental impact of plastics.

Palm oil mill effluent (POME) is the most polluted organic residues generated from palm oil processing and composes of high nutrient content mainly oil and fatty acids is able to support bacterial growth with the degradation of the waste to reduce its pollution strength. Most of the mills in Malaysia adopted open lagoons and open digester tanks for POME treatments. Under anaerobic conditions, biogas will be generated and consist mainly methane gas. Other potential by-products recovered from the anaerobic process of POME is volatile fatty acids mainly consists of acetic, propionic and butyric acids (Hassan et al., 1997). These acids then will be used for polyhydroxyalkanoate (PHA) production (Hassan et al., 2002). A few studies reported on the isolation of PHA accumulating bacteria from the environments (Redzwan et al., 1997; Alias and Tan, 2005; Berlanga et al., 2006)

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Abbreviations: **PHA**, Polyhydroxyalkanoates; **PHB**, polyhydroxybutyrates; **POME**, palm oil mill effluent.

utilizing oils and glucose as carbon and energy source. Since organic acids are abundantly available from the anaerobic process of POME, and due to existing intellectual property or licensing in commercialize it in future, an attempt to screen and isolate the wild type strains from the anaerobic digester treating POME was carried out using conventional Nile Blue A staining method. Thus the aim of this study is to screen and isolate PHA producer from local source with the ability to consume organic acids as carbon source and accumulate high PHA yield. This paper also describes the isolation process, characterization of the polymer produced and bacterial strains for PHA production. The significance of this study is that potential PHA producers was successfully isolated from oil palm wastewaters and showed significant poly-(hydrobutyrate-co-hydroxyvalarate) (PHBV) accumulation and those acids derived from anaerobically treated POME was utilized by this bacterium.

MATERIALS AND METHODS

In this study, sludge was obtained from open digester tank (ODT) treating POME at the Serting Hilir mill located in the Negeri Sembilan, Malaysia. ODT capacity was 3600 m^3 which was able to treat POME at HRT 20 days daily. The treated effluents in the ODT was withdrawn and collected in the Schott bottles and kept on ice prior use.

Screening of PHA-producing bacteria from ODT was done by direct plating and enrichment techniques. In direct plating technique, 50 ml of activated POME sludge from ODT was homogenized by vortex at moderate speed and a dilution series up to $10x^{-9}$ was made to obtain a desired c.f.u. Aliquots of 0.1 ml were plated onto nutrient agar (Merck, Germany) and the plates were incubated for 24 – 48 h at 30 or 37^{0}C. Colonies developed on the agar were differentiated by color, elevation, form, and edge appearances (Dawes and Senior, 1973). For enrichment technique, the sludge was centrifuged at 3,500 rpm for 10 min at 4^{0}C to remove residual oils. The supernatant was decanted and the pellet was transferred into enrich media containing 2 g/L of sodium acetate. The flask was agitated at 250 rpm speed for 2 weeks at 30 or 37^{0}C. Concentration of the sodium acetate was maintained at 2 g/L by weekly addition of sodium acetate solutions into the enriched media. Loopfuls of the culture media was withdrawn and streak onto the nutrient agar plates using spread plating technique. Selected isolates were purified by dilution-streaking to obtain single colonies.

The isolation for PHA producing bacteria was done by randomly picking out and culturing the colonies onto solid mineral salt medium (MSM) containing as proposed by Berlanga et al. (2006). All MSM compositions were same except the concentration of NaCl. MSM was supplemented with 2.5 g sodium acetate/L. 0.5 mg Nile blue A (Sigma, St. Louis, MO, USA) (DMSO)/ml was added into the MSM media. The cultures were incubated for 4 – 5 days at 30 - 37^{0}C. The grown colonies from solid MSM were exposed under UV illuminator at the wavelength 280 - 360 nm. The orange fluorescence color observed from PHA positive colonies were different in brightness and it depends on the types of microorganisms able to store polymeric compounds.

The cultures from agar slants were then transferred in the 250 ml shake flask containing 50 ml NB. The cultures were incubated at 30^{0}C for 18 h. The cells were centrifuged at 6,000 rpm for 5 min at 4^{0}C. Then the cultures were transferred into 250 ml shake flask containing 50 ml nitrogen limiting media with 2.5 g /L sodium acetate. The compositions of the nitrogen limiting media were as mentioned above excluding agar. The cultures were incubated for 4– 5 days at 30 - 37^{0}C. As for PHBV productions, the MSM medium was supplied with 10 g/L of acids mixture from anaerobically treated POME with ratio (acetic 5: propionic 3: butyric 2).

Biochemical properties and screening for carbon substrates utilized of the test strains was performed with the BIOLOG GN2 microplate system release 4.0 by following manufacturer's instruction. Cell concentration was determined by measuring the cell dry weight (CDW) of culture broth. The relationship between turbidity and the CDW of the organism was established by measuring the turbidity of a series of dilutions of the bacterial suspension. For the PHA accumulation detection, 1 ml culture broth was transferred into an eppendorf tubes and centrifuged at 10,000 rpm for 5 min. The PHA detection using HPLC was performed according to Hassan et al. (2002). Nile blue A staining was carried to all cultures to confirm the cell inclusion of PHA (Ostle and Holt, 1982). PHA was extracted from the cells according to the method proposed by Brandl et al. (1988). In order to determine the PHA components, proton (^{1}H) NMR analysis was done and the spectrum was recorded on a 500-MHz JEOL JNM-ECP500 FT NMR system. Chloroform-*d* was used as solvent. Chemical shifts were reported as δ values (ppm) relative to internal tetramethylsilane (TMS) in $CDCl_3$. Expected ^{1}H NMR chemical shifts were predicted using a ChemNMR program in a CS ChemDraw Ultra version 6.0.

RESULTS AND DISCUSSION

In this study, approximately 200 colonies were randomly screened for their ability to accumulate polyhydroxybutyrates (PHB). All colonies were transferred onto the selective medium containing the Nile Blue A dye with limiting nitrogen source. After several days of incubation, the viable colonies that showed a bright orange colour under UV light were selected for further processed indicated the isolates were able to accumulate PHA. All the colonies that have native colours (orange or yellow) were discarded during the screening process because this colour will influence the orange fluorescence exhibited and all the colour strains tested previously did not promoted the PHB accumulation. Only the white colonies were selected for further analysis. Eleven colonies were successfully isolated from the palm oil wastewaters using acetate as sole carbon and energy sources (Table 1). Further studies have shown that only two colonies observed shown significant of high PHB accumulation in their cells. Both strains namely PHB1 and PHB5 were rod shapes and gram negative bacteria. The highest PHB accumulated was detected in PHB1 and PHB5 in the shake flask experiment using acetate were 29% (w/w) and 44% (w/w), respectively. In this regards, PHB5 was selected for further study for growth and PHB accumulation. Results obtained from BIOLOG identification revealed that culture PHB5 was related to *Comamonas testosteroni* with 65% similarity. However, a comparative study was performed between these two strains by referring to existing other *Comamonas* sp. (Willems and De Vos, 2006) and resulted *Comamonas* sp. EB172 was proposed for the PHB5 culture. Growth studies of *Comamonas* sp. EB172 was performed using various organic acids intended to obtain suitable acids for PHB

Table 1. Isolated PHA producer and PHB accumulation using acetate as a carbon source

Isolates	PHB (g/L)	CDW (g/L)	PHA % (w/w)	Gram	Morphology
PHB1	0.06	0.21	29	-ve	Rod
PHB2	0.01	0.18	6	-ve	Coccus
PHB3	0.02	0.25	8	-ve	Roccus
PHB4	0.06	0.32	19	+ve	Rod
PHB5	0.20	0.45	44	-ve	Rod
PHB6	0.006	0.12	5	-ve	Rod
PHB7	0.002	0.21	1	-ve	Coccus
PHB8	0.005	0.14	4	-ve	Rod
PHB9	0.004	0.13	3	+ve	Coccus
PHB10	0.004	0.11	4	+ve	Rod
PHB11	0.008	0.16	5	+ve	Rod

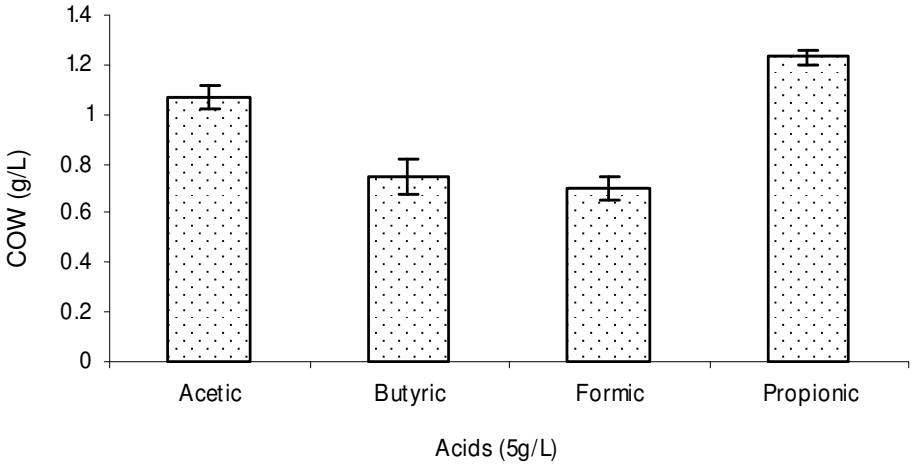

Figure 1. Growth profiles of *Comamonas* sp. EB172 using various acids (synthetic).

and poly-(hydrobutyrate-co-hydroxyvalarate) (PHBV) production. As showed in Figure 1, propionic acid is the best carbon source for growth of *Comamonas* sp EB172 as determined by CDW, followed by acetic and butyric acids. The results obtained showed that this bacterium was able to consume propionic acids and possibly could produce higher hydroxyvalerate (HV) monomer units during PHA accumulation with mixture acids. Other studies was conducted in order to improve the growth of the culture by additional of nitrogen sources and supplements. Further experiments shows that the addition of 1 g/L of yeast extract will improve the growth of *Comamonas* sp. EB172 and the best in comparison with other supplements. The addition of sodium chloride (NaCl) and ammonium sulfate $(NH_4)2SO_4$ did not show any growth improvement. It was expected that the PHA produced from the cells is a PHBV due to the feedstock used during fermentation. In order to confirm this speculation, the extracted PHA sample was analyzed by ^{1}H NMR and the spectrum is shown in Figure 2. The assignments of PHBV signals have been reported previously (Doi et al., 1986; Kamiya et al., 1990). Based on the reports and by the estimation of ^{1}H NMR chemical shifts using a ChemNMR program in a CS ChemDraw Ultra version 6.0, the peaks in Figure 1 were assigned. The assignments of the ^{1}H NMR signals revealed that the PHA produced by *Comamonas* sp. EB172 was a PHBV and hence, confirmed the speculation.

It is necessary to note that PHA accumulation experiment was carried out in this study was under uncontrolled pH and dissolved oxygen using shake flasks. It is certain that with the control pH, dissolved oxygen (DO) concentration and optimum C/N ratio, the PHAs content will further substantially increase. Thus, the effect of nitrogen limitation on cell growth and PHA synthesis needs further investigations by batch as well as fedbatch culture in a fermenter where pH and DO are well controlled. In future, genotype identification of the PHA producer *Comamonas*

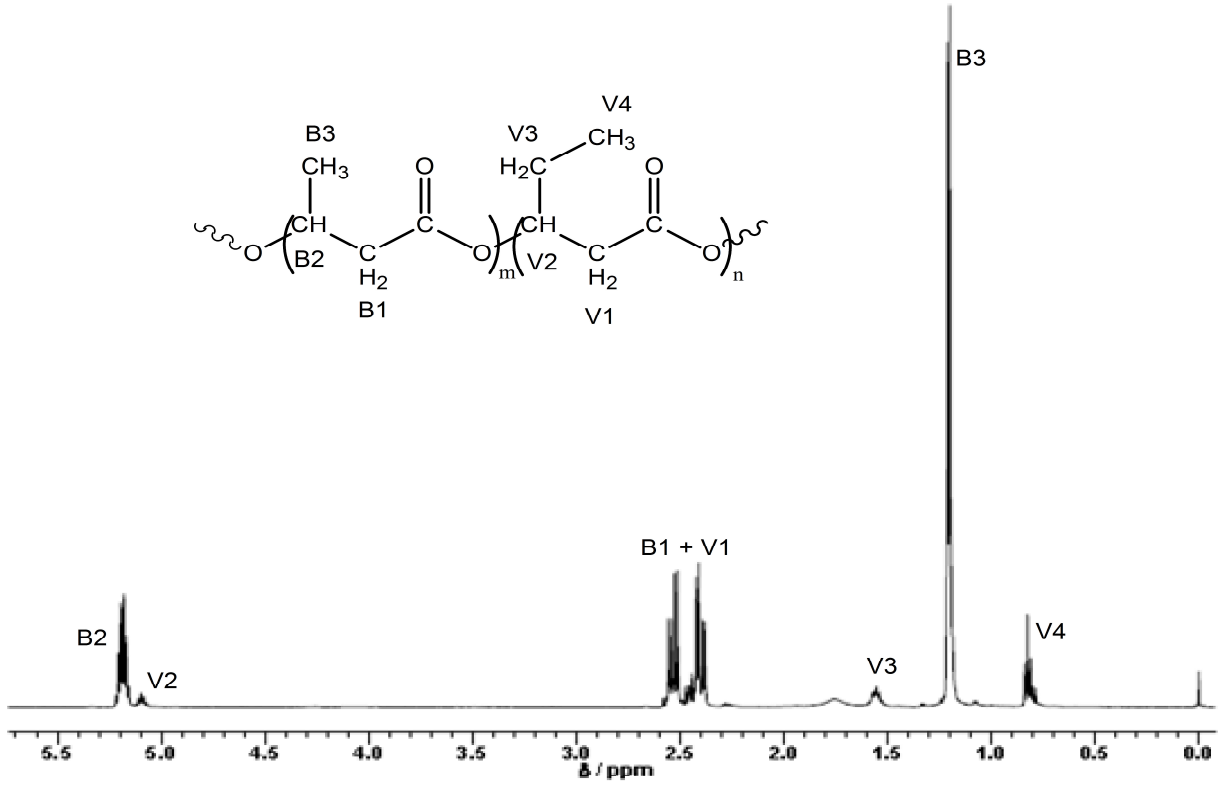

Figure 2. 500 MHz ^{1}H-NMR spectrum of PHBV produced by *Comamonas* sp. EB172 when supplied with 10 g/L organic acids derived from POME treatment.

sp. EB172 will be carried out using 16S rDNA gene sequences.

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