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Biosynthesis and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer from wild-type *Comamonas* sp. EB172

Mohd Rafein Zakaria [a,*], Hidayah Ariffin [a], Noor Azman Mohd Johar [a], Suraini Abd-Aziz [a], Haruo Nishida [b], Yoshihito Shirai [b], Mohd Ali Hassan [a]

[a] Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[b] Department of Biological Functions and Engineering, Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu-ku, Kitakyushu, Fukuoka 808-0196, Japan

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ABSTRACT

Poly(3-hydroxybutyrate) [P(3HB)] homopolymer and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] copolymer was produced by *Comamonas* sp. EB172 using single and mixture of carbon sources. Poly(3-hydroxyvalerate) P(3HV) incorporation in the copolymer was obtained when propionic and valeric acid was used as precursors. Incorporation of 3HV fractions in the copolymer varied from 45 to 86 mol% when initial pH of the medium was regulated. In fed-batch cultivation, organic acids derived from anaerobically treated palm oil mill effluent (POME) were shown to be suitable carbon sources for polyhydroxyalkanoate (PHA) production by *Comamonas* sp. EB172. Number average molecular weight (M_n) produced by the strain was in the range of 153–412 kDa with polydispersity index (M_w/M_n) in the range of 2.2–2.6, respectively. Incorporation of higher 3HV units improved the thermal stability of P(3HB-*co*-3HV) copolymer. Thus the newly isolated bacterium *Comamonas* sp. EB172 is a suitable candidate for PHA production using POME as renewable and alternative cheap raw materials.

1. Introduction

Polyhydroxyalkanoate (PHA) is a biopolymer and biodegradable thermoplastics that have been produced by various types of bacteria as carbon and energy reserve materials in their cytoplasm [1,2]. It has been reported that bacteria could accumulate up to 90% of their cell dry weight (CDW) without disruption of their osmotic pressure [1,3,4]. Poly(3-hydroxybutyrate) [P(3HB)] and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] are the most studied polyesters in the PHA family. These polymers share the physical and mechanical properties similar to petroleum derived thermoplastics polypropylene (PP) and polyethylene (PET) [5]. However, higher price of PHAs in comparison to conventional plastics has limited the wide application of these biodegradable plastics for the time being. Much attention has been spent on optimizing the PHA production process, recovery and blending with other polymers to reduce the total PHA production cost. Approximately 40% of total PHA production was derived from carbon substrates [6]. Incorporation of other substitute hydroxyalkanoic acids (HA) in the P(3HB) homopolymer resulted in improvement in their thermal stability and physical properties from stiff and brittle to elastomeric rubber-like materials [7]. Renewable and cheaper raw materials, such as palm oil mill effluent (POME), have been used as nutrient supplements for bacterial PHA production as substitutes to synthetic carbon sources [8,9]. Attempts to obtain high poly(3-hydroxyvalerate) P(3HV) units in the P(3HB-*co*-3HV) copolymers have been carried out using mixed organic acids from anaerobically treated POME by wild-type *Comamonas* sp. EB172 as an option for reducing PHA production cost [10–12]. The ability of this bacterium to adapt, grow and produce the P(3HB-*co*-3HV) under high acids concentration has encouraged us to expand our study on this bacterium.

The present study aimed to explore the ability of the strain to produce PHA from various carbon sources in one-step cultivation process. The composition of 3HV units produced can be varied by regulating the initial medium pH and propionic acid concentration. Biosynthesis of P(3HB-*co*-3HV) copolymer using mixed organic acids derived from anaerobically treated POME by *Comamonas* sp. EB172 was performed by fed-batch cultivation and the polyesters produced were further characterized.

* Corresponding author. Tel.: +60 3 89467590; fax: +60 3 89467593.
E-mail address: moraza81@yahoo.com (M.R. Zakaria).

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2. Materials and methods

2.1. Bacterial strain

Comamonas sp. EB172 used in this study was isolated from open digester tanks treating POME collected in Serting Hilir palm oil mill located in the Negeri Sembilan, Malaysia [10]. For preparation of inoculum, the culture was grown at 30 °C under aerobic condition in nutrient rich medium (10 g of peptone, 3 g of meat extract, 5 g of yeast extract, and 2 g of sodium acetate in 1 l distilled water) for 12–15 h. The cultures were stored at −80° in 25% (v/v) glycerol for maintenance purposes.

2.2. PHA biosynthesis by one-step cultivation

The pre-grown cells (10% v/v) were transferred into 50 ml mineral salts medium (MSM) in 250 ml shaken flasks containing (per liter of distilled water) KH_2PO_4, 6.7 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4$, 0.2 g, 0.02 $CaCl_2$ and 1 ml microelements solution. The preparation of the microelements solution was as reported previously [8]. Technical grade organic acids and their mixtures (total acids 5 g/l) were added into the medium and were extrapolated to the desired ratios. The initial pH of the medium was adjusted to 7.0 with 2 M NaOH and 2 M H_2SO_4. The cultures were incubated at 30 °C for 48 h under aerobic condition with agitation speed 200 rpm. Cell growth was monitored by measuring the optical density (OD) of the broth at 600 nm.

2.3. Fed-batch cultivation of Comamonas sp. EB172 using organic acids from POME

100 ml of pre-grown cells from growing stage were transferred into 900 ml MSM in 2 l bioreactors (Sartorious, Germany). The substrate used were organic acids derived from POME comprises acetic, propionic and butyric acids at the ratio and total concentration of 5:3:2 and 50 g/l, respectively [11]. Detailed medium composition and fermentation conditions were discussed elsewhere [12].

2.4. Analysis of polymer properties

2.4.1. Gas chromatography

A total of 25 mg of lyophilized cells were subjected to methanolysis in the presence of methanol and sulfuric acid [85%: 15% (v/v)]. The resulting hydroxyacyl methyl esters were then analyzed according to standard method [13]. PHA content and composition of the lyophilized cell was determined using gas chromatography (GC) according to Zakaria et al. [12].

2.4.2. PHA extraction film preparation

As for PHA extraction, the PHA was extracted from freeze-dried cells using solvent extraction method [14]. About 3.0 g freeze-dried cells were stirred in 600 ml chloroform for 24 h at 30 °C. The extracts were filtered to remove cell debris, and the chloroform was concentrated to a volume of about 15 ml using a rotary evaporator (Rotavapor 220, Buchi, Switzerland). The concentrated solution was then added drop-wise to 150 ml of rapidly stirred cold methanol to precipitate the PHA. The precipitated PHA was recovered by filtration using a Whatman filter paper no. 1 and dried overnight in vacuum to completely eliminate the solvent. All PHA samples (standard and biosynthesized from fermentation) were solution cast as reported previously [15].

2.4.3. Gel permeation chromatography (GPC)

The molecular weight of the polyester samples were measured by size exclusion chromatography (SEC) on a TOSOH HLC-8120 GPC system with a refractive index (RI) detector at 40 °C using TOSOH TSKgel Super HM-M column and chloroform eluent (0.6 ml/min). The sample (12 mg) was dissolved in chloroform (2 ml) and the solution was filtered through a membrane filter with 0.45 mm pore size.

2.4.4. Thermogravimetry analysis (TGA)

Thermal analysis of the extracted PHAs were conducted on Seiko Instruments Inc. EXSTAR 6200 TG system in aluminum pans (5 mm ø). A blank aluminum pan was used as reference. PHA film sample (4–6 mg) in the aluminum pan was set in TG and heated at heating rates (φ) 1 and 10 °C/min in the range of 60–400 °C under a steady flow of nitrogen (100 ml/min).

2.4.5. Differential scanning calorimetry (DSC)

DSC measurements were carried out under a nitrogen flow of 20 ml/min using a Seiko Instruments Inc. EXSTAR6000-DSC6200 calibrated with indium. The film samples (5 mg) were encapsulated in aluminum pans and heated from 30 to 250 °C at a heating rate of 10 °C/min. The first, second, and third scans were recorded in all cases. The melting temperature (T_m) was taken at the peak point.

3. Results and discussion

3.1. Biosynthesis of PHA during one-step cultivation

The ability of *Comamonas* sp. EB172 to accumulate PHA during one-step cultivation process was investigated in shake flasks experiments using different types of carbon sources. Carbon sources such as acetic acid, propionic acid, n-butyric acid, DL-lactic acid, valeric acid, and glucose were used solely or in combination (1:1) to observe the cells growth and accumulation of PHAs. It was found that *Comamonas* sp. EB172 was able to produce P(3HB) homopolymer and P(3HB-*co*-3HV) copolymer when fatty acid and/or its mixture were used as carbon and energy sources. Table 1 shows PHA content, CDW and monomers composition for fermentation at various carbon sources. P(3HB) homopolymer was produced when acetic, DL-lactic and n-butyric acid were used as carbon sources. On the other hand, propionic and valeric acids led to the production of P(3HB-*co*-3HV) copolymers. The highest CDW (3.8 g/l) was achieved when n-butyric acid was used as the carbon source with P(3HB) accumulation of 50 wt.%. DL-Lactic acid showed the highest P(3HB) accumulation at 66 wt.% but the CDW obtained was slightly lower (3.0 g/l). Fermentations with other carbon sources such as glucose and fructose were also performed but no significant growth and PHA accumulation were observed. It was supported by the

Table 1
Cell dry weight, PHA content and PHA composition produced from various carbon sources by *Comamonas* sp. EB172.[a]

Carbon sources	Cell dry weight (g/l)	PHA content (wt.%)[b]	PHA composition (mol%)[b]	
			3HB	3HV
Acetic acid	2.1	16	100	0
n-Butyric acid	3.8	50	100	0
Propionic acid	1.1	15	71	29
DL-Lactic acid	3.0	66	100	0
Valeric acid	1.5	10	42	58
Acetic + Valeric	3.4	27	56	44
Acetic + Propionic	3.0	20	91	9
Fructose	0.3	0	0	0
Glucose	0.4	0	0	0

*Values are means of two replicates.
[a] Fermentation was conducted by single or in combination (1:1) of carbon sources with total 5 g/l of substrates, 30 °C, 200 rpm for 48 h.
[b] Determined by GC from lyophilized cells.