Biosynthesis and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) copolymers using jatropha oil as the main carbon source

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ABSTRACT

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] copolymers were produced using jatropha oil as the carbon source. P(3HB-*co*-3HV) with a 3HV monomer as high as 42 mol% was produced by wild-type *Cupriavidus necator* H16 from a mixture of jatropha oil and sodium valerate, and P(3HB-*co*-3HHx) with a 3HHx monomer of 3 mol% was produced by transformant *C. necator* PHB⁻4/pBBREE32d13 harboring *Aeromonas caviae* PHA synthase, using jatropha oil as the sole carbon source. The results of differential scanning calorimetry, thermogravimetric analysis and gel permeation chromatography revealed that the copolymers produced from jatropha oil were essentially the same as those produced from other, more established carbon sources, such as sugars and other plant oils. This study demonstrates that jatropha oil is a potential renewable carbon source for the large-scale production of copolymers by *C. necator* and its transformant.

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1. Introduction

Polyhydroxyalkanoate (PHA) is a polymer that is accumulated by bacteria as a carbon and energy storage material in the presence of an excess amount of carbon and a limited supply of another nutrient, which can be in the form of nitrogen, sulfur, phosphorus, magnesium or oxygen [1]. Among various known biodegradable polymeric materials, PHA is known to be a fully biodegradable alternative to conventional plastics [2]. In terms of its applications, PHA is a good material for bioplastics and implant biomaterials due to its biodegradability, biocompatibility, thermoprocessibility and sustainability [3].

Although PHA is a good substitute for petroleum-based plastics, the high cost of production makes PHA more expensive than conventional plastics [4]. In an effort to reduce the production cost of PHAs, various carbon sources, such as plant oils and sugars, have been explored for use in PHA production. To date, the use of various plant oils, including soybean oil [5], palm oil [6,7], coconut oil [6] and corn oil [8], has been reported to result in high yields of PHA.

However, the use of these edible oils as primary carbon sources for bioplastic production may not be sustainable [9]. Continuous conversion of edible oils to bioplastics may lead to a shortage in food supplies and cause inflation in certain countries, especially in third world countries. Therefore, our study aims to evaluate the use of jatropha oil, which is a non-edible plant oil, for the production of PHA copolymers. In a previous study, we showed that jatropha oil can be used by *Cupriavidus necator* H16 to grow and synthesize poly(3-hydroxybutyrate) [P(3HB)] homopolymer [10]. However, it is not known if jatropha oil affects the incorporation of comonomer and the properties of PHA copolymers. *Jatropha curcas*, also known as physic nut, is a multipurpose shrub that can reach a height of 20 ft and has glabrous branchlets [11]. The life expectancy of *J. curcas* is almost 50 years, and this shrub is distributed natively in Central and South America, including in Mexico, Brazil and Argentina and can now also be found in many parts of Africa and Asia [12]. *J. curcas* is characterized as a hardy, highly adaptable, drought-resistant crop and consequently has high ecological adaptability. Furthermore, *J. curcas* is a disease-resistant plant because only a few insects or fungi can transmit their diseases to the plants [12]. Jatropha oil can be considered to be a biodiesel fuel because it has a low oil viscosity when compared to soybean, cottonseed and sunflower oils [13]. The blackish *J. curcas* seeds contain five main toxins, which are phorbol

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esters, phytates, trypsin inhibitors, lectins and curcin [14]. Besides, a variety of terpene alcohols and sterols were detected in jatropha oil [15,16]; thus, to some extent, jatropha oil is toxic and classified as a non-edible oil. Regarding its use as a carbon source for PHA biosynthesis, it is not known whether the toxins and/or any other unknown components in the jatropha oil have any effects on the various monomer supplying pathways involved in the synthesis of the PHA copolymers.

In Malaysia, *Jatropha* has been put into consideration as a supplementary renewable resource in addition to palm oil to balance the renewable energy supply and exportation of biodiesel [17], and jatropha oil is a potential renewable feedstock for bioplastics. Recognizing the potential of *Jatropha* as a renewable energy source, many industrial companies are now investing in its research and cultivation. By the end of 2010, the cultivation area for *Jatropha* is expected to reach 1 million ha [17]. For the above reasons, this study extended the evaluation of jatropha oil for the production and characterization of PHA copolymers. The ability to biosynthesize PHA copolymers from the co-feeding of jatropha oil and precursors (sodium valerate and sodium propionate) was investigated using wild-type *C. necator* H16. In addition, copolymer biosynthesis from jatropha oil using transformant *C. necator* PHB⁻4/pBBREE32d13 was evaluated.

2. Materials and methods

2.1. Bacterial strain and maintenance

C. necator H16 (formerly known as *Alcaligenes eutrophus*, *Ralstonia eutropha* and *Wautersia eutropha*) and transformant *C. necator* PHB⁻4/pBBREE32d13 harboring the PHA synthase gene of *Aeromonas caviae* [18] were used throughout this study. For short-term maintenance, the bacterial strains were routinely streaked onto nutrient-rich (NR) agar plates with the following composition (per liter): 10 g peptone, 10 g meat extract and 2 g yeast extract [19]. Kanamycin at a concentration of 50 mg/L was added into the agar for maintenance of the transformant strain plasmid. For long-term storage, the bacteria were maintained in a 25% (v/v) glycerol stock solution. The glycerol stock was prepared by the addition of 12.5 mL of pure glycerol to an overnight culture of the bacterial cells in 50 mL of NR. The tubes were stored in aliquots of 1 mL at −20 °C.

2.2. Carbon source

Jatropha oil (Sarawak, Malaysia) was used solely and in conjunction with 3HV precursors to produce P(3HB-*co*-3HHx) and P(3HB-*co*-3HV), respectively. The fatty acid composition of jatropha oil was described in the previous paper [10]. Jatropha oil was filtered with a hydrophobic PTFE membrane filter (0.2 μm pore size) and subsequently autoclaved at 121 °C for 15 min before the oil was added into mineral medium (MM) broth. Sodium valerate and sodium propionate were used as 3HV precursors. Stock solutions of both precursors at 20% (w/v) were prepared and autoclaved separately.

2.3. Cultivation and PHA synthesis

One-stage batch cultivation in shake flasks was conducted for PHA biosynthesis. The bacterial cells were first grown in NR medium to enrich the cells. Two loops of bacteria, cultured for 16–18 h, from the NR plate were grown for 6 h in 50 mL of NR medium at 30 °C and 200 rpm. Approximately 3% (v/v) of the inoculum (OD$_{600nm}$ = 4.5–5) was transferred into 100 mL of MM broth and incubated for 48 h at 30 °C and 200 rpm for PHA accumulation. The MM was prepared according to the following composition (per liter): 3.32 g Na$_2$HPO$_4$, 2.80 g KH$_2$PO$_4$, 0.54 g (NH$_2$)$_2$CO, 0.25 g MgSO$_4$·7H$_2$O and 1 mL trace element solution [19]. The trace element solution consisted of 0.22 g CoCl$_2$·6H$_2$O, 9.7 g FeCl$_3$, 7.8 g CaCl$_2$, 0.12 g NiCl$_2$·6H$_2$O, 0.11 g CrCl$_3$·6H$_2$O and 0.16 g CuSO$_4$·5H$_2$O in 1 L of 0.1 N HCl [5]. In addition, 50 mg/L of kanamycin was added for cultures of the transformant strain. To induce the biosynthesis of P(3HB-*co*-3HV), 3HV precursors were added after 12 h of cultivation. The cells were harvested at the end of the 48-h cultivation period. Centrifugation at 8000 rpm and 4 °C for 5 min using a KUBOTA 6500 was conducted to pellet the cells. Approximately 20 mL of hexane was added to the cell pellet followed by vortexing and centrifugation at 8000 rpm and 4 °C for 3 min to remove the residual oil. The final centrifugation (8000 rpm, 4 °C for 5 min) was performed after adding 50 mL of distilled water to the pellet to remove the remaining hexane. The harvested cells were frozen at −20 °C for about 24 h before freeze drying.

2.4. Residual oil measurement

Approximately 2 mL of culture broth was collected at 48 h and centrifuged. The supernatant was mixed with 5 mL of hexane and vortexed for 1 min to dissolve the residual oil. Subsequently, 1 mL of the upper layer (hexane layer) was transferred to a pre-weighed plastic plate and left to dry in the fume hood until a constant weight was obtained.

2.5. Analytical procedures

PHA content and composition were determined by gas chromatography (GC) analysis. Approximately 20 mg of lyophilized cells were subjected to methanolysis in the presence of 15% (v/v) sulfuric acid and 85% (v/v) methanol for 140 min at 100 °C. The resulting hydroxyacyl methyl esters were then analyzed by GC [20]. To extract PHA from the lyophilized cells, approximately 3 g of freeze dried cells were refluxed in 300 mL of chloroform in a ratio of 1:100 for 4 h at 60 °C. The refluxed solution was cooled to room temperature and filtered to remove the cell debris. The filtrate was then concentrated using a rotary evaporator before it was added, dropwise, into vigorously stirred, cool methanol. The precipitated and purified polymer was then collected and air dried in the fume hood.

2.6. Copolymer characterization

The purified and dried extracted polymer was used for molecular weight determination. The molecular weight was determined at 40 °C using a gel permeation chromatography (Agilent 1200 GPC) system equipped with a refractive index detector and SHODEX K-802 and K-806M columns. The samples were prepared by dissolving the extracted PHA in chloroform at a concentration of 1 mg/mL. Chloroform was used as the eluent at a flow rate of 0.8 mL/min. The weight-average molecular weight (M_w), number-average molecular weight (M_n), and polydispersity index (M_w/M_n) were determined from the curve that was obtained. Calorimetric measurements (DSC) of the PHA were conducted using a Perkin Elmer Pyris 1 differential scanning calorimetry (DSC) thermal analysis system in the range of −30 °C to 200 °C at a heating rate of 20 °C/min. The glass transition temperature (T_g), crystalline melting point (T_m) and enthalpy of fusion (ΔH_m) were determined from the DSC thermogram of the second scan. Thermogravimetric analysis (TGA) was performed using a Mettler-Toledo TGA/SDTA 851 thermobalance with STAR^e thermal analysis software. TGA heating of 10 mg of PHA under a nitrogen atmosphere started at 30–900 °C at a heating rate of 20 °C/min. The decomposition temperature (T_d) at 5% weight loss was determined.

3. Results

3.1. Biosynthesis of P(3HB-co-3HV) copolymer from mixtures of jatropha oil and sodium valerate or sodium propionate

In a previous study [10], jatropha oil was evaluated and found to be a suitable, sole carbon source for the biosynthesis of poly(3-hydroxybutyrate) [P(3HB)] homopolymer. However, it is not yet known how jatropha oil will perform when added together with precursor carbon sources for the biosynthesis of PHA copolymers. In this study, the biosynthesis of P(3HB-*co*-3HV) copolymer was investigated for mixtures of jatropha oil and sodium valerate or sodium propionate (Table 1). The total carbon from jatropha oil and precursor for cell growth and PHA biosynthesis was fixed at 9.51 g/L, which was equivalent to the carbon content of jatropha oil at the optimal concentration (12.5 g/L) used for the biosynthesis of P(3HB) in previous experiments. When the concentrations of the precursors were increased, the concentration of jatropha oil was decreased to result in a total carbon concentration of 9.51 g/L. The concentration of precursors mentioned in the text always refers to the carbon concentration. Here, sodium valerate or sodium propionate was co-fed with jatropha oil to produce P(3HB-*co*-3HV) copolymers with different 3HV monomer compositions. The 3HV precursors, such as propionic and valeric acids, have certain level of toxicity to the cells [21,22] and have to be fed in a timely and controlled manner. P(3HB-*co*-3HV) copolymers biosynthesized from late feeding of the precursors resulted in the formation of copolymer blends having different 3HV molar fractions [7]. In addition, the high content of readily accumulated P(3HB) in cells could reduce the utilization of precursors and incorporation of 3HV [22]. However, the feeding of precursors at an early stage would have stronger