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Biological recovery and properties of poly(3-hydroxybutyrate) from *Cupriavidus necator* H16

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ABSTRACT

In the present study, we observed that laboratory rats readily consumed lyophilized cells of *Cupriavidus necator* H16 cultivated using palm oil containing 39 wt% poly(3-hydroxybutyrate) [P(3HB)] as sole diet source. The test animals excreted whitish fecal pellets containing 82–97 wt% P(3HB). The remaining impurities could be easily removed by washing the pellets with water and/or low concentrations of detergent, which resulted in P(3HB) granules of a high purity. The molecular masses and thermal properties of P(3HB) obtained by this method were almost similar to P(3HB) extracted from bacterial cells using chloroform. The method reported here is simple and eliminates the need for solvents and strong chemicals, thus resulting in P(3HB) production that is more ecofriendly.

1. Introduction

Biodegradable polymers are attractive as these materials can be returned to the environment in the form of their basic building blocks either hydrolytically or enzymatically [1].

Being biodegradable and structurally diverse, polyhydroxyalkanoates (PHAs) have been extensively researched as a potential substitute for some conventional plastics [2,3]. PHAs are naturally occurring lipid inclusions synthesized by various microorganisms as carbon and energy storage compounds. Gram-positive and Gram-negative bacteria from at least 75 different genera are known to accumulate PHAs [4]. Among these PHA producers, *Cupriavidus necator* H16 is one of the most investigated strains due to its well understood physiology and ability to grow to high cell densities with enormous PHA content [5,6]. The publication of the whole genome sequence of *C. necator* H16, the "Knallgas" bacterium, is anticipated to permit wider genetic manipulations in PHA biosynthetic pathways [7].

For the past 50 years, the biotechnological interest on this non-pathogenic bacterium has focused mainly on PHA production [7]. Prior to this (in the 1960s), however, *C. necator* H16 which fixes hydrogen as a source of reducing power and energy was studied as a potential candidate to regulate ambient air composition while serving as a source of regenerated food during prolonged space flights. Striking a parallel with human consumption of probiotic cultures in fermented dairy products, a search for bacterial strains that could utilize chemical by-products of atmospheric regeneration systems was initiated [8]. As a feed for animals, *C. necator* H16 cells were found to be composed of protein of high biological value comparable to casein, and it was well-tolerated by rats [9,10]. Nevertheless, accumulation of poly(3-hydroxybutyrate) [P(3HB)] in these cells due to limited availability of oxygen was undesirable since it was poorly digested (below 10%) by rats [5,11]. In recent years, several studies have been dedicated to the conversion of these energy-rich substances to increase the metabolizable energy of the animal feed [12–16].

The present study was initiated following the observation that PHA was excreted in the fecal pellets of rats fed with this bacterium. This was due to the absence of PHA depolymerizing enzymes in the gastrointestinal tract, and/or to insufficient time for such enzymes to act [12–16]. The well-being and tolerability of the rat model (Sprague Dawley) to lyophilized *C. necator* H16 cells containing P(3HB) cultivated using crude palm kernel oil (CPKO) as the sole diet source have been reported elsewhere [17]. The present study demonstrates that *C. necator* H16 cells is

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a suitable protein source for rats that concurrently produce recoverable P(3HB) granules.

In contrast to earlier studies that focused on the utilization of PHAs as a component of animal feed to increase the metabolizable energy content [12–16], the present study directed the excretion of undigested PHA towards development of a new biological recovery method of PHA. The animals on a bacterial cell diet excreted whitish fecal pellets while animals fed on commercial food produced normal dark-colored fecal pellets. Gas chromatography (GC) analysis confirmed that these white fecal pellets were comprised of 82–97 wt% P(3HB). Size exclusion chromatography (SEC) measurements of P(3HB) granules recovered biologically showed negligible reduction in weight average molecular weight (M_w) when compared with chloroform-extracted P(3HB) [17]. This paper describes the characterization of P(3HB) from the fecal pellets by subjecting them to various standard analyses for PHA. In addition, further purification of these granules using water or low concentrations of detergent was also attempted.

2. Materials and methods

2.1. Bacterial strain and P(3HB) biosynthesis in 2000 L fermenter

Cupriavidus necator H16 (ATCC 17699) was used throughout this study. The bacterium was maintained on Nutrient rich (NR) agar containing 14 g/L of bacteriological agar powder. The cells were grown using modified culture method developed by Budde and co-workers in which the use of gum arabic was avoided [18]. The detailed fermentation conditions had been described elsewhere [17]. The lyophilized cells used for feeding experiments contained a moderate amount of P(3HB) content, 39 ± 1 wt% and the crude protein content was determined to be 43 ± 3 wt%.

2.2. Feeding studies: animals and experimental procedures

Sprague Dawley (SD) rats of both sexes between 8 and 12 weeks weighing initially 150–200 g were used in this study. The experimental details on the evaluations of the tolerability and safety of rats given lyophilized cells of *C. necator* H16 as sole diet source had been described [17]. The whitish fecal pellets excreted by test animals given bacterial cell diet were collected and dried overnight in an oven at 60 °C to reduce water content prior to characterization studies.

2.3. Purification of the fecal pellets

The collected fecal pellets were subjected to further purification processes using water, sodium dodecyl sulfate (SDS) and sodium dodecylbenzenesulfonate (SDBS). The fecal pellets were ground to powder prior to treatment. For each of the treatment, the respective solution (water, 2 wt% SDS and 2 wt% SDBS) containing 20 wt% of biologically extracted P(3HB) granules (volume of water: fecal pellet was 4:1) was stirred at 250 rpm at room temperature for 24 h. After treatment, the solution was centrifuged at 8000*g* for 15 min. The supernatant was discarded and the pellet was then re-suspended in distilled water followed by centrifugation. This step was repeated twice. At the end, the pellet was transferred to a clean test tube. The resulting granules were then dried overnight in an oven at 60 °C.

2.4. Polymer extraction using chloroform (solvent extraction)

P(3HB) accumulated in the cells was extracted by refluxing 1 g of lyophilized cells in 100 mL chloroform for 4 h at 60 °C. The resulting chloroform extract was cooled to room temperature and residual biomass was removed by filtration. The filtrate was concentrated to 10 mL using a rotary evaporator (Eyela, Japan) and then precipitated by adding the concentrated extract drop-wise into 100 mL of vigorously stirred chilled methanol. The resulting white polymer material was recovered by centrifugation at 10,000*g* for 10 min and air dried overnight.

2.5. Analytical procedures

2.5.1. Gas chromatography (GC)

The P(3HB) content of the untreated and purified fecal pellets were determined according to standard methods [19] using Shimadzu GC-2010 (Shimadzu, Japan) equipped with AOC-20i Auto-Injector. Approximately 15 mg of samples were subjected to methanolysis in the presence of 2 mL of 85% (v/v) methanol acidified with 15% (v/v) sulfuric acid and 2 mL of chloroform for 140 min at 100 °C. The resulting methyl esters were then quantified with caprylic methyl ester as an internal standard. The column temperature was initiated at 70 °C and then increased to 280 °C in a continuous step of 14 °C/min.

2.5.2. Size exclusion chromatography (SEC)

Molecular mass data were obtained by size exclusion chromatography (SEC) analysis at 40 °C using the Agilent 1200 GPC (Agilent Technologies, USA) system fitted with a refractive index detector (RID) and Shodex K-806M and K-802 columns. Chloroform was used as the eluent at a flow rate of 0.8 mL/min. Sample concentration of 1.0 mg/mL was applied after being filtered using 0.45 μm PTFE filter (Sartorius, Germany). Polystyrene standards with a low polydispersity were used to construct a calibration curve.

2.5.3. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) data were recorded in the temperature range of −120 to 200 °C on a Perkin-Elmer Pyris 1 (PerkinElmer Inc., USA) instrument equipped with a cooling accessory under a nitrogen flow rate of 20 mL/min. Samples were encapsulated in aluminum pans and heated from 25 to 200 °C at a heating rate of 20 °C/min (first heating scan). The melt samples were then maintained at 200 °C for 1 min and followed by rapid quenching to −120 °C. They were heated again from −120 to 200 °C at a heating rate of 20 °C/min (second heating scan). The glass transition temperature (T_g) was taken as the midpoint of the heat capacity change. The melting temperature (T_m) and the heat of fusion (ΔH_m) were determined from the DSC endotherm.

2.5.4. Thermogravimetric analysis (TGA)

The TGA analysis was carried out using TA Instruments Q500 (TA Instruments, USA). Sample (<5 mg) was loaded in aluminum pan and heated from room temperature to 500 °C at a heating rate of 10 °C/min under nitrogen atmosphere.

2.5.5. Rheological characterization

Dynamic mechanical measurements were performed using a TA Instruments AR 2000 Rheometer (TA Instruments, USA) equipped with a convection oven purged with nitrogen gas. The samples were compression molded before the measurements for 2 min at 180 °C into disks with a diameter of 15 mm. Time sweep measurements were performed in the linear viscoelastic region at 180 °C at a frequency of 10 Hz and constant strain amplitude of 2%.

2.5.6. Scanning electron microscope (SEM)

The lyophilized cells and powdered fecal pellets were subjected to SEM observation. The samples were mounted on aluminum stubs, sputter-coated with gold for 15 s prior to observation under