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A new biological recovery approach for PHA using mealworm, *Tenebrio molitor*

Paramasivam Murugan[a], Lizhu Han[a], Chee-Yuen Gan[b], Frans H.J. Maurer[c], Kumar Sudesh[a,*]

[a] *Ecobiomaterial Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia*
[b] *Analytical Biochemistry Research Centre, Universiti Sains Malaysia, 11800 Penang, Malaysia*
[c] *Department of Chemistry, Polymer & Materials Chemistry, Lund University, Box 124, Lund SE-221 00, Sweden*

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ABSTRACT

Bacterial polyhydroxyalkanoates (PHA) are expensive partly due to the recovery and purification processes. Thus, many studies have been carried out in order to minimize the cost. Here we report on the use of mealworm, which is the larva of mealworm beetle (*Tenebrio molitor*) to recover PHA granules from *Cupriavidus necator*. Mealworms were shown to readily consume the freeze-dried *C. necator* cells and excrete the PHA granules in the form of whitish feces. Further purification using water, detergent and heat resulted in almost 100% pure PHA granules. Comparison with chloroform extraction showed no signs of reduction in the molecular weight and dispersion of the PHA molecules. Scanning electron microscopy and dynamic light scattering measurements revealed that the biologically recovered PHA granules retained their native spherical morphology. The PHA granules were subjected to a battery of tests to determine their purity and properties in comparison to the chloroform extracted PHA. This study has demonstrated the possibility of using mealworms as a biological agent to partially purify the PHA granules.

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1. Introduction

In this modern age, plastics are ubiquitous and human life without plastic materials is unimaginable. Although plastics are beneficial for human's day to day life, dumping of plastic waste products into the natural environment has led to plastic pollution. This has negatively affected the land and marine wildlife (Eriksen et al., 2014; Nelms et al., 2015). Recent revelations regarding microplastics have aggravated the problem and revealed the greater dangers of plastic materials (Law and Thompson, 2014; Lönnstedt and Eklöv, 2016). A possible solution for these problems is the production and use of biodegradable plastics. Polyhydroxyalkanoates (PHAs) are biological polyesters produced by certain bacteria as cell inclusion bodies when there is a limitation of essential nutrients but in the presence of excessive carbon source (Anderson and Dawes, 1990; Pohlmann et al., 2006). PHAs are known to be degradable by soil bacteria and fungi (Boyandin et al., 2013) thus making them environmentally friendly plastics. Therefore, one can expect the PHA-based microplastics to be completely mineralized in the environment. Among the various types of PHAs that can be synthesized by bacteria, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] is a thermoplastic that has similar mechanical and physical properties comparable to some commodity plastics of petrochemical origin (Doi et al., 1995). However, the cost of PHAs is more than petrochemical plastics. The production and use of PHAs at industrial level is still at its infancy due to the high cost (Li et al., 2016). One of the reasons for the high cost of PHAs is the recovery process. Since PHAs are accumulated in the bacterial cell cytoplasm, it is necessary to lyse the cells in order to recover the PHA granules. In addition, the PHA granules are closely associated with several types of proteins which are located on the surface of the granules (Bresan et al., 2016; Sudesh et al., 2004). Most of the PHA extraction processes are developed using organic solvents such as chloroform and dichloromethane (Choi

Abbreviations: PHA, polyhydroxyalkanoate; C.necator, Cupriavidus necator; P(3HB-*co*-3HHx), Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)-; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; GC, gas chromatography; CME, caprylic methyl ester; M_w, weight average molecular weight; M_n, number average molecular weight; TGA, thermo gravimetric analysis; SEM, scanning electron micrograpy; ATR-FTIR, attenuated total reflectance- fourier transform infrared spectroscopy; CDW, cell dry weight; NMR, nuclear magnetic resonance spectroscopy; DSC, differential scanning calorimeter; T_m, melting temperature.

* Corresponding author.
E-mail address: ksudesh@usm.my (K. Sudesh).

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and Lee, 1999). The limitation of solvent based recovery method is such that it requires large amount of solvents which needs a recycling process. Reducing the amount of solvent for recovery leads to high viscosity of the polymer which makes the process difficult and affects the purity of the polymer. Many alternative methods were developed for solvent based recovery method such as chemical disruption by sodium hydroxide (NaOH) and sodium dodecyl sulphate (SDS), enzymatic digestion with the help of thermal pretreatment and digestion using sodium hypochlorite (Choi and Lee, 1999; Thakor et al., 2005). The detergent based recovery method is very efficient in disrupting the cell components and there is no negative effect to the PHA, which is very important for the recovery process (Choi and Lee, 1999). The drawback of detergent based recovery method is low purity of the resulting PHA and high detergent cost. Moreover it needs large amount of detergent to recover a small quantity of PHA and a large amount of water to remove the cell debris and detergent from the PHA (Jiang et al., 2006). Proteolytic enzymes such as alcalase can digest the proteins completely (De Koning et al., 1997). Dissolution of protein, lipids and other cell components can be achieved by micelles formation using anionic detergents such as SDS (Womack et al., 1983). The combination of alcalase and SDS is effective at the optimum pH and temperature. The reaction time can be reduced with a combination of enzymes and detergents (De Koning et al., 1997). In addition to these methods, many innovative methods are being investigated such as killer bug (Martínez et al., 2016) and secretion of the PHA (Rahman et al., 2013). The present study presents an alternative approach whereby the use of solvents and chemicals can be minimized. More importantly, in the process of recovering the PHA from the bacterial cells, the method described here makes use of the non-PHA cellular materials. As a proof of concept, we have recently demonstrated the use of bacterial cells with PHA as animal feed and subsequent recovery of the PHA from the animal's feces (Kunasundari et al., 2013). Here, we report a further improvement of the biological recovery process by using mealworms, the larvae of mealworm beetle, *Tenebrio molitor*. Mealworm can be grown in high densities, require less water and space, breed prolifically and can consume up to 10 wt% feed of their body weight per day. We have found that mealworm can be used to recover the PHA granules while the non-PHA cellular materials are digested by the mealworm. In contrast to a recent report on the degradation of polystyrene by mealworm (Yang et al., 2015), we observed no reduction in the molecular weights of the recovered PHA granules. The PHA granules can be further purified using water and SDS. The purified PHA granules were characterized by gas chromatography, gel permeation chromatography, differential scanning calorimetry, thermogravimetric analysis and rheology. This study builds on and contributes to the concept of biological recovery process that we proposed recently (Kunasundari et al., 2013).

2. Materials and methods

2.1. Biosynthesis of PHA

Cupriavidus necator Re2058/pCB113 kindly provided by Prof. Anthony Sinskey (MIT) was used to synthesize and accumulate PHA by growing them in mineral medium with palm olein as the sole carbon source as described previously (Budde et al., 2011). The cells containing 54 wt% poly(3-hydroxybutyrate-*co*-25 mol% 3-hydroxyhexanoate) [P(3HB-*co*-25 mol% 3HHx)] were chosen for this study. The cells were harvested and freeze-dried according to standard procedures.

2.2. Maintenance of mealworm

Mealworms (*Tenebrio molitor*) obtained from local pet shop were used in this study. The feeding experiment was started with 50 g of mealworms (30–35 days old) which were fed 5% of their body weight per day for 16 days with the freeze-dried *C. necator* cells. The mealworms were kept in plastic containers at ambient conditions. The whitish fecal pellets excreted by the mealworms were collected, sieved using a 0.5 mm mesh (No. 35, DER SHUENN) and dried overnight in an oven at 60 °C prior to characterization studies. In control experiments, mealworms were fed with oats (Captain, Malaysia) which produced darker feces.

2.3. Purification of the fecal pellets

The collected fecal pellets containing PHA granules were subjected to further purification using water and 1% (w/v) SDS (with and without heating at 50 °C). The respective solutions (volume of solution: fecal pellet was 5:1) were stirred using a magnetic stirrer at 250 rpm at room temperature up to 10 h. Finally, the fecal pellets were washed with 0.001N HCl. Then, the resulting pellet was transferred to a clean petri dish and dried overnight in an oven at 60 °C.

2.4. Sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE)

The presence of protein from the biologically recovered and purified PHA granules was checked using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970). The purified PHA granules were mixed at a ratio of 8:2 (w/v) with 5 × loading buffer consisting of 0.5 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol. The mixture was heated to 95 °C for 10 min to denature the protein. Ten microliter per well was loaded onto 12% separating and 5% stacking gel. The gel was run at 80 V for 2 h using Mini PROTEAN II electrophoresis chamber (BioRad, USA). Pre-stained protein marker weight standards (10–170 kDa, Thermo scientific, Rockford, USA) were used for molar mass estimation. The gels were stained with Coomassie Brilliant Blue for 30 min and destained overnight with destaining buffer (distilled water, methanol, and acetic acid in the ratio of 8:2:1, respectively).

2.5. PHA extraction using chloroform

The freeze-dried cells (1 g) were stirred in 100 mL chloroform for 5 days at room temperature. The residual biomass was removed by filtration using Whatman No.1 filter paper. The filtrate was concentrated to 10 mL using rotary evaporator (Eyela, Japan) and then precipitated by adding the concentrated polymer drop-wise into 100 mL of vigorously stirred chilled methanol. The resulting white precipitate was recovered by vacuum filtration with 0.2 μm PTFE filter and air dried overnight.

2.6. Isolation of native PHA granules

PHA granules were isolated by sucrose density gradient centrifugation according to Wieczorek et al. (1995). The freshly cultivated cells were washed and re-suspended with Tris-HCl (pH 7.2). The suspension was subjected to three passages through a French press (1000 psi) (Thermo IEC, FRENCH, Pressure cell press, FA-003 cell). Sucrose gradient was prepared by gently adding 1 mL of each 2.0 M, 1.6 M, 1.3 M, and 1.0 M sucrose concentration. Approximately, 1 mL of cell lysate was loaded on the sucrose density gradient. After ultracentrifugation (Beckman Coulter, USA) (210,000 × *g* for 2 h at 4 °C), a whitish band containing native

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