Disassembly and Reassembly of Polyhydroxylkanoates: Recycling through Abiotic Depolymerization and Biotic Repolymerization

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Abstract

An abiotic-biotic strategy for recycling of polyhydroxyalkanoates (PHAs) is evaluated. Base-catalyzed PHA depolymerization yields hydroxyacids, such as 3-hydroxybutyrate (3HB), and alkenoates, such as crotonate; catalytic thermal depolymerization yields alkenoates. Cyclic pulse addition of 3HB to triplicate bioreactors selected for an enrichment of *Comamonas*, *Brachymonas* and *Acinetobacter*. After each pulse, poly(3-hydroxybutyrate) (P3HB) transiently appeared: accumulation of P3HB correlated with hydrolysis of polyphosphate; consumption of P3HB correlated with polyphosphate synthesis. Cells removed from the cyclic regime and incubated with 3HB under nitrogen-limited conditions produced P3HB (molecular weight > 1,000,000 Da) at 50 % of the cell dry weight (< 8 h). P3HB also resulted from incubation with acetate, crotonate, or a mixture of hydrolytic depolymerization products. Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) resulted from incubation with valerate or 2-pentenoate. A recycling strategy where abiotic depolymerization of waste PHAs yields feedstock for customized PHA re-synthesis appears feasible, without the need for energy-intensive feedstock purification.

Keywords

Polyhydroxyalkanoate, PHB, PHBV, polyphosphate, recycling

1. Introduction

Polyhydroxyalkanoates (PHAs) are a diverse class of biopolymers that can potentially replace petroleum-based plastic products (Houmiel et al., 1999). Many species of bacteria synthesize PHA granules when they are supplied with carbon and electron equivalents but lack another nutrient needed for cell replication, such as nitrogen or phosphorus (Anderson and Dawes, 1990; Lee, 1996). Microorganisms use the granules as reservoirs of carbon and reducing equivalents for later use when conditions become favorable for cell replication (Doi, 1990; Pieja et al., 2011). While PHAs are currently more expensive than their fossil carbon-derived counterparts (Arun et al., 2006), they are of great interest due to their diversity of function, biocompatibility, and lack of persistence in the environment (Johnson et al., 2009; Steinbuchel, 2001). As shown in Figure 1-b, PHAs are renewable both aerobically and anaerobically. Under aerobic conditions, they degrade to CO₂ (Akmal et al., 2003), and the CO₂ can be recycled by photosynthesis back into PHAs. Under anaerobic conditions, they degrade to methane-rich biogas that can be captured and used to create virgin poly(3-hydroxybutyrate) (P3HB) through the activity of type II methanotrophic (methane-utilizing) bacteria (Pieja et al., 2011). Molecular recycling of PHAs through such lengthy ecological pathways leads to energy inefficiencies and longer time scales for polymer recycling. Shorter recycling pathways are needed. In principle, PHAs can be recycled through conventional sorting and re-melting (Figure 1-a), but downcycling results, as desirable properties are lost with successive reuse and shortening of the polymer chains (Chan Sin et al., 2010). Strategies are needed for regeneration of PHAs without downcycling.

In this study, we propose and test a “short-circuit” PHA recycling strategy that combines abiotic depolymerization (via base hydrolysis or pyrolysis) with biochemical repolymerization, enabling rapid regeneration of PHAs without downcycling (Figure 2). Specifically, we demonstrate that microbial enrichments can repolymerize abiotic depolymerization products to high quality PHAs (high molecular weight, low polydispersity), as illustrated in Figure 2. Abiotic PHA depolymerization has been previously investigated using both chemical and thermal methods (Ariffin et al., 2010; Yu et al., 2005), but these studies had limited scope in terms of materials tested and the methods used. No study to date has coupled abiotic depolymerization to biotic repolymerization or evaluated the feasibility of repolymerization without energy-intensive purification steps.

2. Materials and methods

2.1. Base-catalyzed hydrolysis of poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV)

Hydrolysis of PHBV was carried out by methods similar to that reported for P3HB (Yu et al., 2005). Twelve mg of microbially-generated PHBV with 5 mol% 3HV content (Sigma-Aldrich, St Louis, MO, USA) was added to 10 mL of 0.1 M sodium hydroxide solution and charged into sealed cylindrical glass vials (pH 13). The vials were placed in a water bath, shaken continuously at 200 rpm, and incubated at 60 °C. Vials were periodically removed from the water bath and analyzed for soluble degradation products.

Two-milliliter samples were extracted and centrifuged for 15 min at 14,000 rpm. After centrifuging, 1.25 mL of supernatant was added to a 2 mL centrifuge tube containing 25 μ L of 1.44 M HEPES Buffer and 200 μ L of 800 mg/L sodium benzoate. The solution was frozen at -80 °C, lyophilized until dry, then amended with 250 μ L of methanol. Twenty microliters of the dissolved sample was transferred to a glass GC vial and amended with 120 μ L of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The solution was microwaved in a conventional microwave oven (Emerson Model # MW9332BFC) at a power level of 6 for 3 min. Samples of this solution were analyzed by GC/MS using an Agilent 6890N gas chromatograph (GC) coupled to an Agilent 5973 mass selective detector (MSD). The GC was equipped with a HP-5MS column (Agilent Model Number 19091s-433 30.0 m long x 250 μ m i.d. x 0.25 μ m film thickness). The oven temperature program was as follows: 50 °C for 3 min, ramp increase to 60 °C over 9 min, 60 °C for 3 min, ramp increase to 260 °C over 9 min, ramp increase to 300 °C over 6 min, and held at 300 °C for 6 min. Helium (1.0 mL $\cdot$ min⁻¹) was the carrier with inlet pressure maintained at 56.7 kPa (8.22 psi). The Mass Selective Detector (MSD) was operated in scanning ion mode at mass to charge ratios (m/z) from 33 to 250. The ions used for quantification of 3HB, crotonate, 3HV, 2-pentenoate, 3-pentenoate, and benzoate were m/z = 147, 191; 143, 69; 147, 131; 83, 143, 157; 157, 117, 41, 83; and 77, 105, 135, 179 respectively. The ion peak areas for a particular product were summed and divided by the sum of the integrated benzoate peaks. For each sample, the result was compared to standard curves.

2.2. Base-catalyzed pyrolysis of P3HB

The pyrolysis of P3HB was carried by the method of Ariffin and coworkers (Ariffin et al., 2010). Unpurified P3HB (natural origin, Sigma-Aldrich, St Louis, MO, USA) was ground with magnesium hydroxide (Fisher Scientific, Pittsburgh, PA) (9 wt% relative to the P3HB) using a mortar and pestle. This mixture (370 - 400 mg) was placed on a metal scoopula, and the scoopula inserted into a 2.54 cm diameter quartz tube. The tube was inserted into a Thermo Scientific Lindberg Mini-Mite Tube Furnace, and subjected to a vacuum. Two furnace programs were investigated (Temperature Profile 1 and Temperature Profile 2). Temperature Profile 1 was as follows: ramp increase from 25 °C to 160 °C over 15 min, 160 °C for 30 min, ramp increase to 240 °C over 15 min, and 240 °C for 30 min. Temperature Profile 2 was as follows: ramp increase from 25 °C to 200 °C over 5 min, 200 ± 30 °C for 30 min, ramp increase to 240 °C over 5 min, and 240 ± 30 °C for 30 min. After cooling, the vacuum was released. Pyrolyzed products were collected by rinsing the tube with acetone and the solvent was evaporated *in vacuo*. The resulting products were weighed and analyzed by ¹H-NMR and ¹³C-NMR in deuterated chloroform (CDCl₃). The percentage of *trans*-crotonic acid in the products was determined by comparing the integration of *trans*-crotonic acid peaks with those from oligomers in the NMR spectra.

2.3. Microbial enrichment adapted to 3HB

Fresh activated sludge was obtained from the aeration basin at the Palo Alto Regional Water Quality Control Plant in Palo Alto, CA. Large material was removed by filtering through a 100-µm cell strainer (BD Falcon Biosciences, Lexington, TN, USA). The dispersed cells were centrifuged to create a pellet. The pellet was resuspended in medium W1 and shaken to obtain a dispersed cell suspension. Medium W1 contained the following

chemicals per liter of solution: 0.8 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.13 mM CaCl_2 , 1.2 mM NaHCO_3 , 2.4 mM KH_2PO_4 , 3.4 mM K_2HPO_4 , 2.1 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 1 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 10 μM Fe-EDTA, 1 mL trace metal solution, and 10 mL vitamin solution. The trace stock solution contained the following chemicals per liter of solution: 500 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 400 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg $\text{MnCl}_2 \cdot 7\text{H}_2\text{O}$, 50 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 15 mg H_3BO_3 , 250 mg EDTA. The vitamin stock solution contained the following chemicals per liter of solution: 2.0 mg biotin, 2.0 mg folic acid, 5.0 mg thiamine $\cdot \text{HCl}$, 5.0 mg calcium pantothenate, 0.1 mg vitamin B12, 5.0 mg riboflavin, and 5.0 mg nicotinamide. Aliquots (15 mL) of the dispersed cell suspension were added to a 250-mL flask (Corning CellBIND 75 cm², Corning Inc., NY, USA) containing 40 mL of Medium W1. A 3HB-degrading enrichment was prepared by adding 3HB stock solution (1 mL) and ammonium stock solution (1 mL) every 12 h for two weeks. After addition of 1 mL of 3HB stock (2.4 M sodium 3-hydroxybutyrate; > 99.0 % purity; Sigma-Aldrich, St Louis, MO, USA), the concentration of 3HB in the flask was 60 mM. After addition of 1 mL ammonium stock solution (1.35 M NH_4Cl ; > 99.8 % purity; Mallinckrodt Inc., St. Louis, MO), the ammonium concentration in the flask was 33.75 mM. The 3HB enrichment was grown for two weeks at 30 °C on orbital shakers (200 rpm) to a final optical density OD_{600} of 2.2, then centrifuged (3,000 X g). The pellet was resuspended in 15 mL of W1 medium. The suspension was divided into 5-mL aliquots for inoculation of three pulse-fed flask reactors (flask volume 500 mL; Corning Inc., NY, USA). Each pulse-fed reactor initially contained 5 mL of inoculum; 33 mL of medium W1, 1 mL of 3HB stock, and 1 mL of ammonium stock (total volume 40 mL).

2.4. Long-term operation of cycling pulse-fed flask reactors

After a 12-h incubation, cyclic operation of the three batch flask reactors commenced with establishment of an alternating carbon-nitrogen feed for all three reactors. This was a long-term continuous “repeating cycle”. To initiate cycling (Day 0), 20 mL of culture was removed from each flask, and 20 mL of feed solution was added (18 mL medium W1, 1 mL 3HB stock, 1 mL ammonium stock). A 12-h cycle was established for all three flask reactors: during the fill phase (0-5 min), each reactor received 20 mL feed; during the react phase (5-715 min), 3HB degraded; and during the decant phase, 20 mL of liquid culture was removed (715-720 min). The 20-mL samples removed from each pulse-fed reactor were centrifuged and suspended in fresh medium, amended with 3HB (no nitrogen), incubated for 12 h, harvested by centrifugation (11,000 rpm), and freeze-dried. We refer to this removal of cells and their subsequent incubation as the PHA production step. This repeating cycle with a PHA production step was repeated for >300 days, with reproducible patterns of Chemical Oxygen Demand (COD) consumption and PHA production (typical results in Figure 5). Preserved samples were assayed for PHA content. The liquid culture that remained in the pulsed-fed reactors (20 mL) was used as the inoculum for the next cycle. In some cycles, the PHA production step was modified to evaluate possible production of different types of PHA: 3HB was replaced by addition of crotonate (60 mM), acetate (120 mM), valerate (48 mM), 2-pentenoate (48 mM), or a mixture of P3HB hydrolysis products (50 mL mixture/L media, COD of the mixture = 68,000 mg COD/L) generated by complete dissolution of 2.51 grams of P3HB in a 1 M NaOH solution.

2.5. Analytical methods

To determine soluble (filtered) COD, 0.2-mL samples were removed from each flask reactor, diluted 1:10 with Milli-Q water, then assayed using the standard protocol for COD analysis with Hach High-Range COD Digestion Vials (Hach Co., Loveland, CO, USA). 3HB concentrations were estimated assuming 1.38 mg COD per mg 3HB.

P3HB was assayed by GC per the protocol of Braunegg et al. (Braunegg et al., 1978). Sub-samples (5-10 mg) of freeze-dried samples were weighed then transferred to a 12-mL glass vial. Each vial was amended with 2 mL of methanol containing sulfuric acid (3 %, vol/vol) and benzoic acid (0.25 mg/mL methanol), supplemented with 2 mL of chloroform, and sealed with a Teflon-lined plastic cap. All vials were shaken then heated at 95-100 °C for 3.5 h. After cooling to room temperature, 1 mL of Milli-Q water was added. The reaction cocktail was mixed on a vortex mixer for 30 s then allowed to partition until phase separation was complete. The organic phase was sampled by syringe and analyzed using a GC (Agilent 6890N) equipped with an HP-5 column (containing 5 % phenyl-methylpolysiloxane; Agilent Technologies, Palo Alto, CA, USA) and a flame ionization detector. DL-hydroxybutyric acid sodium salt (Sigma-Aldrich, St Louis, MO, USA) was used to prepare external calibration curves. The P3HB content of the samples (% by mass) was calculated by normalizing to initial dry mass.

The 3HV content of PHBV synthesized by cells incubated with sodium valerate, sodium 2-pentenoate was analyzed with the same GC protocols described above. Standards were PHBV powers (Sigma-Aldrich, St Louis, MO, USA) with varying 3HV molar ratios (5, 8 and 12 mol%).

For total suspended solids (TSS) analysis, 0.1 to 0.5 mL of cell suspension was filtered through pre-washed, dried and pre-weighted 0.2 µm membrane filters (Pall, Port Washington, NY, USA). These filters were then weighed on an AD-6 autobalance (Perkin Elmer, Norwalk, CT, USA) after drying at 80 °C for 24 h.

Ammonium was quantified according to EPA Method 353.2 (U.S. Environmental Protection Agency, 1993). Samples were centrifuged at 12,000 X g for 2 min and the supernatants diluted 1:500 in Milli-Q water before colorimetric analysis using a Westco Smartchem 200 discrete analyzer (Brookfield, CT, USA).

Polyphosphates were assayed according to EPA Method 365.1 (U.S. Environmental Protection Agency, 1993). For analysis of total phosphorus, samples were digested in an autoclave for 30 min at 121 °C and 103-138 kPa (15-20 psi) with ammonium persulfate and sulfuric acid to convert all phosphorus to orthophosphate. The digested samples were analyzed on Westco Smartchem 200 discrete analyzer (Brookfield, CT, USA). For analysis of polyphosphate, samples were digested as before, but without persulfate. This step promoted hydrolysis of polyphosphate to orthophosphate, but prevented hydrolysis of organic phosphorus. The digested samples were analyzed on the Westco Smartchem 200 discrete analyzer to obtain the concentration of hydrolysable phosphorus, including both polyphosphates and orthophosphates. The polyphosphate concentration was estimated as the difference between the orthophosphate concentration and the concentration of hydrolysable phosphorus.

PHA granules were extracted from the cells by suspending 500 mg of freeze-dried cell material in 50 mL Milli-Q water, adding 400 mg sodium dodecyl sulfate (> 99.0 % purity; Sigma-Aldrich, St Louis, MO, USA) and 360 mg of EDTA, followed by heating to 60 °C for 60 min to induce cell lysis. The solution was then centrifuged at 4,700 rpm for 15 min, and the pellet washed three times. To purify the PHA, pellets were washed with a 50-mL sodium hypochlorite (bleach) solution (chlorox 6.15 %), incubated at 30 °C with continuous stirring for 60 min, then centrifuged at 3,000 X g for 15 min. This process was repeated three times.

Extracted PHA samples were characterized using gel permeation chromatography (GPC). Samples were dissolved in chloroform at a concentration of 5mg/mL for 90 min at (60 °C), filtered through a 0.2 µm PTFE filter, and analyzed with a Shimadzu UFLC system (Shimadzu Scientific Instruments, MD, USA) equipped with a Shimadzu RID-10A refraction index detector. The GPC was equipped with a Jordi Gel DVB guard column (500Å) and three Jordi Gel DVB analytical columns (500Å, 104 Å, and 105 Å). The temperature of the columns was maintained at 40 °C, and the flow rate of the mobile phase (chloroform) was 1 mL • min⁻¹. Molecular weights were calibrated with polystyrene standards from Varian (Calibration Kit S-M2-10, USA).

Peak melting temperature of extracted PHA samples were analyzed using TA Q2000 differential scanning calorimetry (DSC, TA Instruments, New Castle, DE, USA). Thermal data were collected under a nitrogen flow of 10 mL • min⁻¹. Three to five milligrams of melt-quenched PHA samples encapsulated in aluminum pans were heated from -40 °C to

200 °C at a rate of 10 °C • min⁻¹. The peak melting temperatures were determined from the position of the endothermic peaks.

2.6. Bacterial community analysis

After establishing a repeating cycle of operation (Section 2.4), 100-μL samples were removed from reactor, and the genomic DNA (gDNA) was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA), as per the manufacture's protocol. Bacterial 16S rRNA was amplified using the bacterial primers BAC-8F (5'-AGAGTTTGATCCTGGCTCAG-3') and BAC-1492R (5'-CGGCTACCTTGTTACGACTT-3') (Stackebrandt and Goodfellow, 1991). A Polymerase chain reaction (PCR) was performed using Accuprime Taq DNA Polymerase System (Invitrogen, Carlsbad, CA, USA) and the following thermocycling steps: (i) 94 °C for 5 min; (ii) 30 cycles consisting of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 80 s; and (iii) an extension at 68 °C for 10 min. Amplicon presence and quality of PCR reaction were verified via 1.5 % agarose gel electrophoresis.

PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Chatsworth, CA, USA), then cloned using pGEM-T Easy Vector System with JM109 competent *E. coli* cells (Promega, Madison, WI, USA) per the manufacture's protocol. Randomly selected clones were sequenced by Elim Biopharmaceuticals Inc. (Hayward, CA, USA), generating 120 near-full length 16S rRNA gene sequences. Retrieved DNA sequences were compared with sequences obtained using the Basic Local Alignment Search Tool (BLAST) and EzTaxon (Kim et al., 2012).

2.7. Electron microscopy and energy dispersive X-ray spectroscopy

Samples from different time points were fixed with 2 % glutaraldehyde and 4 % paraformaldehyde in 0.1 M sodium cacodylate buffer ($\text{Na}(\text{CH}_3)_2\text{AsO}_2 \cdot 3\text{H}_2\text{O}$), pH 7.4 for 48 h at 4°C. To coat cells in gelatin, cells were washed in the buffer and resuspended in 10 % warm gelatin for 5 min, placed on ice for 5 min, then cut into blocks and post-fixed using cold osmium tetroxide (OsO_4). Post-fixed samples were dehydrated using ethanol and acetonitrile, embedded in an epoxy resin mixture, then cut into ultra-thin sections, which were then mounted on copper grids. The grids were observed with a JEOL TEM1230 microscope equipped with a Gatan 967 slow-scan, cooled CCD camera. Images were processed using Digital Micrograph, Digital Montage, and TEM Auto tune.

To verify the presence of granules and to differentiate PHA and polyphosphate, the grids were analyzed using energy dispersive X-ray spectroscopy (EDS) to determine the elemental composition of a variety of components in the samples. Inclusion bodies were examined by FEI Titan 80–300 (80 kV) electron microscope equipped with energy-dispersive X-ray detector.

3. Results and discussion

3.1. Abiotic depolymerization

Two means of abiotic PHA depolymerization were evaluated: base-catalyzed hydrolysis and catalytic thermal depolymerization (low temperature pyrolysis). Hydrolysis of PHBV was carried out in a manner analogous to that reported for P3HB (Yu et al., 2005) by

suspending a sample of PHBV (5 mol% 3HV) in aqueous base (pH 13) at 60 °C. This reaction could theoretically yield five possible products: 3-hydroxybutyrate (3HB), crotonate, 3-hydroxyvalerate (3HV), 2-pentenoate, and 3-pentenoate. As shown in Table 1, 3HB and crotonate were major products; 3HV and 2-pentenoate were detected, but 3-pentenoate was obtained in only trace amounts.

The thermal decomposition of commercial P3HB (natural origin, Sigma-Aldrich, St Louis, MO, USA) and 9 wt% Mg(OH)₂ in a vacuum tube furnace was studied under two temperature profiles. In a typical experiment, 337 - 364 mg of unpurified P3HB was ground up with solid Mg(OH)₂ (9 wt%) and placed in a quartz vacuum tube furnace. The use of Temperature Profile 1 resulted in 55 % recovery (based on the original mass of P3HB) of a white crystalline material, of which 98 % was revealed by ¹H-NMR and ¹³C-NMR to be *trans*-crotonic acid, with the remainder likely to be higher oligomers of the nature. No *cis*-crotonic acid was detected. The use of Temperature Profile 2 resulted in 69 % recovery (based on the original mass of P3HB) of a white crystalline material, 98 % of which was revealed by ¹H-NMR and ¹³C-NMR to be *trans*-crotonic acid. The ¹H-NMR spectra and ¹³C-NMR spectra from both pyrolysis experiments were virtually identical. The results confirm observations made by Ariffin and coworkers (Ariffin et al., 2010) that the use of Mg(OH)₂ results in almost exclusive degradation of P3HB to *trans*-crotonic acid, although we report a lower overall percentage yield. Our lower yield may be due to the fact that the P3HB was used as received, without any purification, and simply mechanically ground with the Mg(OH)₂ prior to pyrolysis. This is in contrast to the previous study, in which the

P3HB was thoroughly purified, before a film of the polymer with $\text{Mg}(\text{OH})_2$ was cast prior to pyrolysis (Ariffin et al., 2010).

3.2. Pulse-fed enrichment cultures: community composition

Microbial enrichment cultures fed 3HB were initially obtained by seeding three batch reactors with activated sludge from a local wastewater treatment plant. A repeating cycle was imposed in which 3HB was added as a pulse every 12 h. This cycle selected for a community dominated by three genera: *Comamonas*, *Brachymonas* and *Acinetobacter* (Figure 3). *Pseudomonas* and other minor genera accounted for the remaining bacteria.

3.3. Pulse-fed enrichment cultures: inclusion granules

Harvested cells isolated from the enrichment cultures contained inclusion granules visible by light microscopy. Samples were analyzed by TEM to evaluate the morphology and composition of the granules. TEM images confirmed the presence of light and dark granules inside harvested cells from the pulse-fed enrichments. The composition of dark and light granules was compared to cytoplasmic background by energy dispersive X-ray spectroscopy (EDS) analysis. Light granules contained mostly carbon and oxygen; dark granules contained mostly phosphorus and oxygen; and background cytoplasm contained carbon, oxygen, and phosphorus. The ratio of phosphorus to the sum of carbon, oxygen and phosphorus, $\text{P}/(\text{C}+\text{O}+\text{P})$ was computed for light and dark granules (where element signals reflect X-ray counts). This value was divided by the same ratio for the background cytoplasm to obtain a “normalized P abundance” (Figure 4). GC analyses of the lysate extract confirmed the presence of 3HB indicating that the light granules with no

phosphorus were likely P3HB. Phosphorus analysis of the filtered and unfiltered culture indicated luxury uptake of phosphorus, indicating that the dark granules were likely composed of polyphosphate. These granules had phosphorus levels twelve times that of the background cytoplasm.

3.4. Pulse-fed enrichment cultures: repeating cycle

Patterns of substrate consumption and product formation were evaluated for the batch reactors fed 3HB on a repeating cycle. These patterns were reproducible for > 300 days of operation. Figure 5 illustrates the behavior for a typical 12-h repeating cycle (Day 90). Also shown is a 12-h period for P3HB accumulation using biomass harvested from the fed-batch reactors and incubated separately with 3HB. The errors bars are standard deviations, and indicate a high degree of reproducibility.

As shown in Figure 5-A, the concentration of 3HB decreased sharply at the beginning of each cycle (Figure 5-A), indicating its rapid use as a carbon and energy source. Within 0.5 h, the level of phosphate increased in solution, indicating hydrolysis of polyphosphate (Figure 5-D). After 2 h, polyphosphate level stabilized, and P3HB increased from 8 to 22 % by weight (Figure 5-C). During this period, total suspended solids (TSS) increased rapidly (Figure 5-A). From 4 h to 12 h, phosphate levels decreased as polyphosphate accumulated (Figure 5-D), and TSS increased more slowly (Figure 5-A). At the same time, P3HB levels declined to the baseline value of 8 % (Figure 5-C), suggesting oxidation of P3HB coupled to formation of polyphosphate.

3.5. Pulse-fed enrichment cultures: PHA production step

In addition to illustrating a typical repeating cycle, Figure 5 also illustrates a typical 12-h PHA production step. During this period, half of the biomass removed from the repeating cycle at 12 h was incubated with 3HB in the absence of added nitrogen. Nitrogen levels were negligible throughout the PHA production period (Figure 5-B). 3HB level decreased at the beginning of each cycle (Figure 5-A), but at a slower rate compared to the rate observed the beginning of the repeating cycle. Within 4 h, aqueous phosphate levels increased, indicating hydrolysis of polyphosphate (Figure 5-D). Within 8 h, P3HB levels increased from 8 to 50 % by weight (Figure 5-C), and TSS levels increased (Figure 5-A). From 8 h to 12 h, polyphosphate levels increased (Figure 5-D), and P3HB levels decreased to 44 % by weight at the end of the PHA production step (Figure 5-C).

A significant result was the observation that these enrichments were able to generate PHAs from a wide variety of monomers derived from abiotic depolymerization. This is likely due to the fact that alkenoates and hydroxyacids are readily interconverted through known biochemical mechanisms (Eggers and Steinbüchel, 2013; Janssen and Schink, 1993).

In some cycles, we replaced 3HB by other substrates to evaluate the potential for production of different types of PHA. Incubation with acetate, crotonate, or a mixture of depolymerization products resulted in the formation of up to 45 wt% P3HB. Incubation with valerate or 2-pentenoate resulted in the formation of up to 38 wt% PHBV containing up to 24 mol% 3HV.

The number of carbon atoms in monomer side chains depends upon the number of carbon atoms in the substrates added during the polymer production phase: when this number is

even, the side chains contain an odd number of carbon atoms; when it is odd, the side chains contain an even number of carbon atoms. For acetate, beta oxidation can promote incorporation of 3HB units via successive formation of acetyl CoA, acetoacetyl-CoA, 3-hydroxybutyryl-CoA, and ultimately 3HB monomers, with methyl side chains. We anticipate that longer side chains may result through addition of longer alkanates containing an even number of carbon atoms (Loo and Sudesh, 2007). For added alkanates containing an odd number of carbon atoms, such as valerate, hydroxy acyl units may be added via beta oxidation, with formation of acyl-CoA, 2-enoyl-CoA, 3-hydroxyacyl-CoA, and incorporation of 3-hydroxyacyl units (resulting in side chains with an even number of carbon atoms, such as ethyl groups) (Loo and Sudesh, 2007). For alkenates, such as crotonate and 2-pentenoate, a possible pathway is thiolase-mediated formation of enoyl-CoA, hydratase-mediated formation of 3-hydroxyacyl-CoA, and PHA-synthase incorporation of 3-hydroxyacyl units (Loo and Sudesh, 2007). All of these pathways lack substrate specificity (Antonio et al., 2000; Rehm and Steinbüchel, 1999), suggesting that other copolymers such as block-poly-3-hydroxyhexanoate (PHB-b-PHHx) or poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (P3HB4HB) might be produced. Such modifications can confer many useful properties, such as impact resistance, toughness, and flexibility (Haddouche et al., 2011; Sheu et al., 2012).

3.6. PHA accumulation and utilization by polyphosphate accumulating organisms (PAOs)

The strong selection pressure exerted by cyclic addition of 3HB resulted in a community dominated by a few major species of *Comamonas*, *Brachymonas* and *Acinetobacter* (section 3.2). At the species level (> 97 % sequence similarity), *Comamonas aquatica*,

Brachymonas chironomi and *Acinetobacter junii* were the major species. *Comamonas aquatica* and *Acinetobacter junii* are known polyphosphate accumulating organisms (PAOs). They also are known to produce intracellular P3HB (Ciesielski et al., 2013; Liu et al., 2005; Mehlig et al., 2013; Momba and Cloete, 1996). Although there is no direct evidence for the species *Brachymonas chironomi* yet, *Brachymonas* genus is also believed to be responsible for biological phosphorus removal (Halet et al., 2007; Shi and Lee, 2007; Shi et al., 2007). After each 3HB pulse, P3HB transiently appeared: accumulation of P3HB correlated with hydrolysis of polyphosphate; consumption of P3HB correlated with synthesis of polyphosphates. Polyphosphate serves many functions including energy storage, ATP substitution, source of phosphorus for synthesis of key macromolecules, buffer against alkali, and stress response regulator (Kornberg, 1995). The inverse correlation between polyphosphate consumption and PHA production is evidence that the function of PHA in these enrichments is energy storage (Comeau et al., 1986; Wentzel et al., 1986). According to the Comeau-Wentzel model, polyphosphate kinase cleaves polyphosphate, producing ATP from ADP. This ATP is used to produce acetyl-CoA (one mole of acetyl-CoA per mole of ATP), a precursor to P3HB. Some acetyl-CoA is also used to produce NADH (four moles of NADH produced by oxidation of one mole of acetyl-CoA), a source of reducing equivalents also needed for P3HB synthesis. In the absence of 3HB, P3HB disappeared (Figure 5). This pattern is also observed in PAOs that use the TCA cycle to generate ATP for growth and replacement of intracellular polyphosphate granules storage (Comeau et al., 1986; Wentzel et al., 1986).

3.7. Pulse-fed enrichment cultures: kinetic modeling parameters

For both the repeating cycle and the PHA production step, the specific 3HB utilization rate (q_{3HB}) correlated linearly with the concentration of 3HB ($3HB$):

$$q_{3HB} = -(d(3HB)/dt)/X = k_{3HB}(3HB) \quad (1)$$

where X is concentration (mg/L) of total suspended solids (TSS).

A regression analysis of q_{3HB} against 3HB concentration gave a pseudo-second order rate coefficient k_{3HB} of $2.4 \pm 0.8 \text{ L} \cdot \text{mg TSS}^{-1} \cdot \text{h}^{-1}$ for the repeating cycle ($r^2 = 0.93$) and $1.8 \pm 0.6 \text{ L} \cdot \text{mg TSS}^{-1} \cdot \text{h}^{-1}$ for the PHA production step ($r^2 = 0.73$).

Specific 3HB utilization rate (q_{3HB}) also correlated linearly with the specific growth rate (μ_X) for the repeating cycle and with the specific P3HB accumulation rate (q_{P3HB}) for the PHA production step:

$$\mu_X = (dX/dt)/X = q_{3HB}Y_{X,3HB} - b_X \quad (2)$$

$$q_{P3HB} = (d(3HB)/dt)/X = q_{3HB}Y_{P3HB,3HB} - b_{P3HB} \quad (3)$$

For the repeating cycle, a linear regression of μ_X versus q_{3HB} gave a conversion yield $Y_{X,3HB}$ of $0.76 \pm 0.11 \text{ mg TSS} \cdot \text{mg 3HB}^{-1}$ and a specific biomass decay rate b_X of $0.06 \pm 0.01 \text{ mg TSS} \cdot \text{mg TSS}^{-1} \cdot \text{h}^{-1}$ ($r^2 = 0.88$). For the PHA production step, regression of q_{P3HB} versus q_{3HB} gave a $Y_{P3HB,3HB}$ of $0.73 \pm 0.11 \text{ mg P3HB} \cdot \text{mg 3HB}^{-1}$ and b_{P3HB} of $0.05 \pm 0.01 \text{ mg P3HB} \cdot \text{mg TSS}^{-1} \cdot \text{h}^{-1}$ ($r^2 = 0.81$).

An overall conversion yield ($Y_{NET, P3HB,3HB}$) can be derived from $Y_{X,3HB}$ for the repeating cycle, and $Y_{P3HB,3HB}$ and the fraction of P3HB (f_{P3HB}) for the PHA production step, where:

$$Y_{Net,P3HB,3HB} = [(1 - f_{P3HB})/f_{P3HB}Y_{X,3HB} + 1/Y_{P3HB,3HB}]^{-1} \quad (4)$$

The overall conversion yield ($Y_{NET, P3HB,3HB}$) calculated using the values from the above kinetic models is $0.37 \text{ g P3HB} \cdot \text{g 3HB}^{-1}$. This conversion yield is typical for heterotrophic PHA production, and can be increased in optimized systems (Johnson et al., 2009).

Overall, the kinetic modeling indicated that the specific PHA utilization rate is highly correlated with 3HB concentration whereas the specific PHA decay rate is independent of 3HB concentration and stable throughout the cycle. This suggests possibilities for future process optimization, especially during the PHA production step, when the non-PHA biomass is stable. For these conditions, pulse addition of high levels of PHA precursors would be expected to increase PHA production relative to decay.

3.8. Molecular weight characterization of the PHA generated

Table 2 illustrates molecular weight and molecular weight distributions ($PDI = M_w/M_n$) of P3HB and PHBV generated in the pulse fed reactors (Day 183). These values were compared to those of commercially available P3HB and PHBV powders (Sigma-Aldrich, St Louis, MO, USA). The results indicate that the produced P3HB has higher molecular weight and lower PDI. Both traits are desirable: high molecular weight polymers can offer improved tensile strength and elongation to break, and a lower PDI value more uniform polymer chain lengths (Atkins and Paula, 2009).

3.9. Melting temperatures of the PHA generated

DSC analysis on PHA polymers produced from the 3HB-pulse fed bioreactors had a peak melting temperature (T_m) of 176 °C and an onset glass transition temperature (T_g^0) of 10 °C, values typical of P3HB (Loo and Sudesh, 2007). After incubation with valerate or 2-pentenoate, the PHA polymers (PHBV with 5 mol% 3HV) had a lower peak melting temperature (T_m) of 167 °C and an onset glass transition temperature (T_g^0) of 6 °C, values typical of PHBV (Loo and Sudesh, 2007).

4. Conclusions

Our results demonstrate that the abiotic depolymerization of waste PHAs collected at end-of-life can be used to generate low molecular weight products that can be subsequently used as feedstock for the production of high quality PHAs. Cyclic pulse-feeding of a key depolymerization product (3HB, in this case) enabled development of a microbial community capable of reproducible PHA production. The proposed strategy requires fewer steps for recycling than the natural carbon cycle via CO₂ or methane and permits production of customized PHA molecules through feedstock control during the PHA production step.

The following files are available as supplementary data in an electronic annex: NMR data, Nile-red fluorescence method, hydrolysis products of PHBV, TEM images of PHA granules, GPC profiles, GC/FID profiles, and GC/MS profiles.

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Figure Captions

Figure 1. Recycling paths for PHAs. The thick lines highlight chemical routes for PHA recycling based upon 1) abiotic depolymerization of PHAs into precursor molecules, including chemical precursors, such as hydroxyalkanoate monomers and alkenoic acids, and biochemical precursors, such as acyl-CoA and acyl-ACP (Chen, 2010), and 2) biotic polymerization of precursors into PHA granules.

Figure 2. Overall scheme of direct recovery of PHA from waste PHA (two dotted lines represent the key steps proposed in this paper)

Figure 3. Genus-level bacterial community structures based on one hundred and twenty 16S rRNA gene sequences retrieved from the 3HB-utilizing bioreactors (sample for Day 96). Triplicate reactors had similar proportional composition.

Figure 4. EDS analysis of inclusion granules in cell biomass (Day 65) after the PHA production step. Normalized P abundance for the light granules, dark granules, and background cytoplasm.

Figure 5. Reactor performance during a repeating cycle (0-12 h), in which the harvested cells are used in a PHA production step (12-24 h). (A) Concentrations of 3HB (×) and biomass (●); (B) concentration of ammonium (□); (C) percent P3HB in biomass (△); (D) concentration of soluble phosphate (○) and percent polyphosphates in biomass (◆).

Figures

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Tables

Table 1. Hydrolysis and elimination products from PHBV degradation under basic conditions at 60 °C. 3-hydroxybutyrate (3HB), crotonate (CA), 3-hydroxyvalerate (3HV), 2-pentenoate (2P), and 3-pentenoate (3P)

		Percentage of total carbon					
		3HB	CA	3HV	2P	3P	Mass recovered
Time (h)	6	7.4	5.6	0.8	0.4	0.2	14.4
	12	23.4	15.8	1.3	1.4	0.1	42.0
	18	51.7	37.8	4.9	3.6	0.1	98.1

Table 2. Peak molecular weight and polydispersity index (PDI) of extracted polymers (Day 183) compared to commercially available PHAs.

	Peak molecular weight	PDI
Commercially available P3HB	7.38 E+05	2.02
Commercially available PHBV	4.48 E+05	2.18
Produced P3HB	1.14 E+06	1.65
Produced PHBV	9.87 E+05	1.81