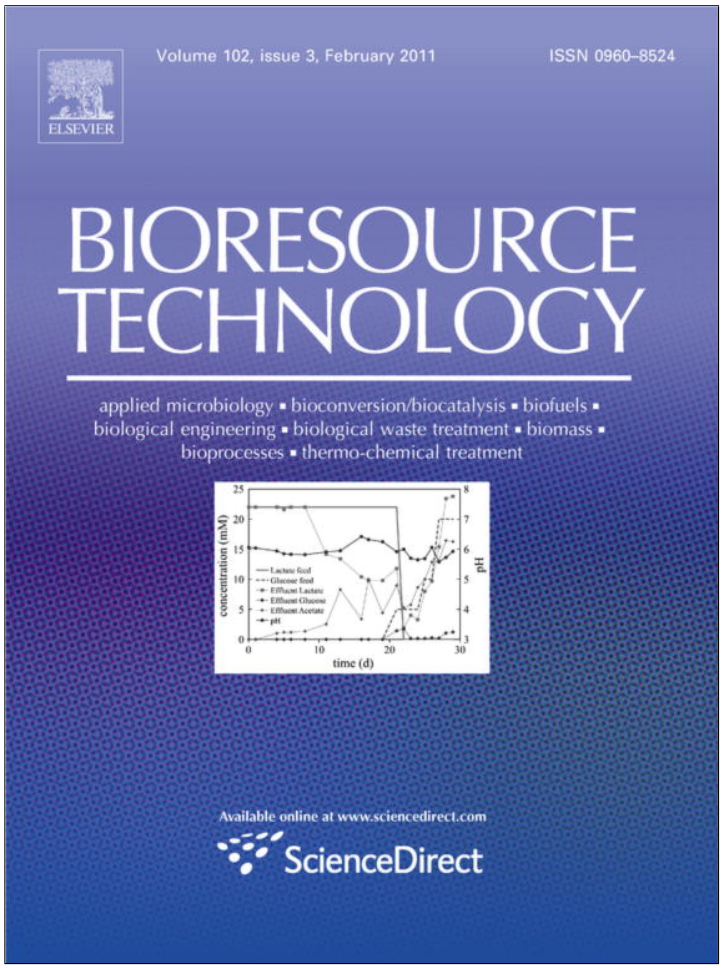

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Polyhydroxybutyrate synthesis on biodiesel wastewater using mixed microbial consortia

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ABSTRACT

Crude glycerol (CG), a by-product of biodiesel production, is an organic carbon-rich substrate with potential as feedstock for polyhydroxyalkanoate (PHA) production. PHA is a biodegradable thermoplastic synthesized by microorganisms as an intracellular granule. In this study we investigated PHA production on CG using mixed microbial consortia (MMC) and determined that the enriched MMC produced exclusively polyhydroxybutyrate (PHB) utilizing the methanol fraction. PHB synthesis appeared to be stimulated by a macronutrient deficiency. Intracellular concentrations remained relatively constant over an operational cycle, with microbial growth occurring concurrent with polymer synthesis. PHB average molecular weights ranged from 200–380 kDa, while thermal properties compared well with commercial PHB. The resulting PHB material properties and characteristics would be suitable for many commercial uses. Considering full-scale process application, it was estimated that a 38 million L (10 million gallon) per year biodiesel operation could potentially produce up to 19 metric ton (20.9 ton) of PHB per year.

1. Introduction

Biodiesel represents a potentially sustainable supplement to petroleum-based diesel fuels that can readily be generated from renewable agricultural resources. However, sustainability of this commodity is in part compromised due to the high strength crude glycerol (CG) co-product waste stream generated. Crude glycerol is produced at a rate of approximately 1 kg per 12.6 L of biodiesel created, and although purification and refining processes to reduce CG volumes may be practiced at larger production sites, smaller scale producers often find the associated costs to be prohibitive (Thompson and He, 2006). While some downstream CG recycling opportunities do exist and have been leveraged (e.g., crude glycerol does retain some inherent caloric value and thus has been used to augment livestock feed (Mach et al., 2009)), it nonetheless remains broadly perceived as a waste (*i.e.*, contains minimal-to-no value; managing it is simply a cost of doing business). Considering that the production of biodiesel is only expected to grow in coming years, there is a prescient need to develop new opportunities for using the CG waste stream.

Crude glycerol principally consists of residual ethanol or methanol, glycerol, fatty acid ethyl (or methyl) esters, and residual fatty acids (Ashby et al., 2004). In regard to converting this resource to a commodity, these carbon sources are direct precursors for the bacterial synthesis of polyhydroxyalkanoates (PHAs) (Yamane, 1993). PHAs are biologically produced, biodegradable thermoplastics with many potential commercial applications. Over 300 bacterial species are known to synthesize PHAs, which are carbon and energy storage reserves, as cytoplasmic granules (Lee, 1996b). Synthesis is stimulated by excess soluble carbon with a concurrent macronutrient limitation (typically nitrogen or phosphorus), a limitation in a terminal electron acceptor (typically oxygen), or a feast–famine environment wherein microorganisms realize a transient excess of soluble carbon without any nutrient limitations (Dionisi et al., 2004). The form of carbon substrate dictates the PHA structure (Madison and Huisman, 1999), with the most common forms being poly-3-hydroxybutyrate (PHB), poly-hydroxyvalerate (PHV), and poly-4-hydroxybutyrate (P4HB). PHB exhibits similar properties to polypropylene (Madison and Huisman, 1999).

While PHA is attractive as a renewable substitute to petroleum-based thermoplastics, current commercial production practices exhibit higher fossil fuel demands and greenhouse gas emissions than conventional plastics; these demands are largely associated with substrate production and bioreactor operations (Gerngross, 1999). Research to minimize these impacts has focused on the use of pure cultures with waste streams rich in carbon precursors (Ashby et al., 2004; Kahar et al., 2004), pure cultures with waste streams derived from refined waste feedstocks (Du and Yu, 2002), or mixed microbial consortia (MMC) grown on synthetic feedstocks (Beun et al., 2000; Dionisi et al., 2004; Serafim et al.,

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2004). PHA synthesis using CG has also been previously explored, although only with pure microbial cultures. In these studies, PHB has been the predominant form of PHA produced utilizing glycerol (Cavalheiro et al., 2009; Ibrahim and Steinbuchel, 2010; Mothes et al., 2007), methanol (Yezza et al., 2006; Zhao et al., 1993), or both (Braunegg et al., 1999) as carbon substrate. PHB synthesized using CG has been shown to exhibit properties similar to those produced from other common substrates, like acetic acid and glucose, with molecular weights ranging from approximately 620 to 960 kDa (Cavalheiro et al., 2009; Mothes et al., 2007).

In contrast to pure cultures, the use of MMC to synthesize PHA represents an opportunity to further reduce the process' environmental footprint, principally due to reduced energy usage associated with eliminating the requirement to maintain axenic conditions. While the concept of using MMC with organic waste streams as feedstock for PHA synthesis has been investigated (Coats et al., 2007; Dionisi et al., 2005), CG has not previously been considered as a candidate substrate. The objective of the research presented herein was to assess the potential to produce PHA on CG using MMC derived from a full-scale wastewater treatment plant (WWTP). Specific goals were to (i) determine the forms of PHA produced by MMC on CG, and identify the CG carbon forms utilized; (ii) optimize reactor operations to maximize intracellular PHA and overall reactor yields; (iii) identify potential initiator(s) of PHA production on CG; and (iv) characterize the material properties of the PHA produced.

2. Methods

2.1. Material sources

The MMC inoculum for the investigations was obtained from the Moscow, ID, WWTP aerobic basin. Crude glycerol was acquired from two sources: the Biological and Agricultural Engineering (BAE) Department pilot-scale biodiesel manufacturing facility at the University of Idaho (Moscow, ID, USA) and GEN-X Energy Group, Incorporated (Burbank, WA, USA). Pure glycerol was obtained from Acros Organics (Morris Plains, NJ, USA).

2.2. Crude glycerol characterization

The CG from the University of Idaho BAE Department was generated during biodiesel production using multiple vegetable oil sources, primarily canola, rapeseed, and mustard. Sodium methoxide (CH_3ONa) was utilized as the process catalyst. The unrefined CG contained approximately 355 g L^{-1} glycerol and 239 g L^{-1} methanol. The unrefined GEN-X CG contained a maximum of 221 g L^{-1} methanol.

2.3. Bioreactor operations

A total of 14 laboratory-scale PHA bioreactors were operated for these investigations (Table 1). For tracking purposes, each reactor was labeled with the initials "BD" (biodiesel) followed by a number. The array of reactors covered three solids residence times (SRTs) (10, 20, and 30 days), four hydraulic residence times (HRTs) (6, 8, 20, and 30 days), and three cycle lengths (4, 5, and 10 days). Each reactor was operated as a continuously mixed, fully aerobic batch reactor over the entirety of the cycle, with sludge and effluent withdrawal performed manually at the end of the cycle. Reactor substrate addition, introduced at the beginning of the cycle, consisted of 10% glycerol (v/v) and 90% tap water (v/v), with feed volumes adjusted based on SRT, HRT, and cycle length. Ammonium chloride solution (150 g L^{-1}) was added as a nitrogen source. Fully mixed aerobic conditions were maintained in reactors BD1-BD3 using stone diffusers and stir plates. In the remaining BD reactors, aerobic conditions were maintained by dispersing air through Sanitaire membrane disc diffusers (ITT Water and Wastewater, Brown Deer, WI, USA). Residual dissolved oxygen (DO) concentrations were maintained at a minimum of 2 mg L^{-1}. Foaming was controlled with an infrequent addition of Antifoam A Concentrate (Sigma Aldrich, St. Louis, MO, USA).

2.4. PHA-rich biomass recovery

Recovered biomass was suspended in 6.25% sodium hypochlorite to lyse the cells (Berger et al., 1989) and to arrest bacterial metabolic activity during the recovery process. Biomass was centrifuged at approximately 10,000 rpm for 10 min, and the pellets were rinsed with deionized water and frozen at −20 °C. Samples were lyophilized for at least 2 days (Labconco FreeZone 4.5 lyophilizer; Labconco Corporation, Kansas City, MO, USA) at −47 °C using a Welch 1402 DuoSeal vacuum pump (Gardner Denver Thomas Inc., Niles, IL, USA) at a vacuum pressure of 2.0 Pa. Following lyophilization, the samples were stored at room temperature in a dry location.

2.5. Polymer recovery for determining material properties

Known quantities of lyophilized PHA-rich biomass were prewashed with acetone (20 volumes, boiling, 15 min) and transferred into round bottom flasks (250 mL), to which chloroform was added (50 volumes). The biomass slurry was then refluxed in boiling chloroform under constant stirring overnight (approximately 16 h); the obtained chloroform extract was separated from the biomass slurry by filtration and concentrated under vacuum. PHA was precipitated by adding five volumes of cold petroleum ether (boiling point range 35–60 °C) into the concentrated chloroform solution (containing crude PHA) under constant stirring. Purified PHA was recovered by centrifugation (3000 rpm, 5 min) and evaporation of solvent. Purified PHA was dried to constant weight at ambient temperature in a vacuum oven and sealed in plastic bags before characterization.

2.6. Glycerol and methanol quantification

Glycerol and methanol concentrations were quantified through high performance liquid chromatography (HPLC) with a refractive index (RI) detector (Hewlett–Packard 1047A (Palo Alto, CA, USA)). Samples diluted with deionized water were filtered with 0.22 μm filters and injected using a SpectraSYSTEM AS300 autosampler (Thermo Electron Corporation, Waltham, MA, USA) with a 20 μL injection loop. A 0.01 N sulfuric acid solution was utilized as the HPLC eluant (0.5 mL min^{-1}, Waters Associates Inc. model 6000 (Milford, MA, USA)) and separation performed on a Rezex ROA organic acid column (Phenomenex, Torrance, CA, USA; 300 mm, 7.8 mm internal diameter) at 65 °C. Data was recorded using a Hewlett–Packard (Palo Alto, CA, USA) 3393A integrator. Glycerol and methanol concentrations were quantified by comparing retention times and areas with pure glycerol and methanol standard curves ($R^2 > 0.98$).

2.7. PHA content analysis

Biomass PHA content was determined by gas chromatography/mass spectrometry (GC–MS), following the method of Braunegg et al. (1978). Briefly, lyophilized PHA-rich biomass samples were digested at 100 °C in 2 mL each of acidified methanol (3% v/v sulfuric acid) and chloroform. Benzoic acid was added to the chloroform as an internal standard. Following vigorous vortexing of the mixture with 1 mL deionized water, the organic (chloroform) phase

Table 1
Summary of reactor operating parameters, feedstock, and range of intracellular PHB content (end of cycle).

Reactor	SRT (days)	HRT (days)	Vol. (L)	Cycle (days)	Feed[a]	Feed per cycle (L)	NH4Cl (mg N L^{-1})	PHB (% d.w.)
BD1	20	8	0.5	4	BAE crude glycerol	0.25	393	30–59
BD2	10	6	0.5	4	BAE crude glycerol	0.33	786	45–62
BD3	20	6	0.5	4	BAE crude glycerol	0.33	393	42–61
BD4	20	20	12	5	BAE crude glycerol	3.0	393	20–55
BD5	20	20	4	10	BAE crude glycerol	2.0	393	17–26
BD6	20	20	4	10	BAE crude glycerol	2.0	197	11–30
BD7	20	20	4	10	GEN-X crude glycerol	2.0	393	8–24
BD8	20	20	2	5	BAE crude glycerol	0.5	0	0
BD9	30	30	4	5	BAE crude glycerol	0.67	393	6–13
BD10	20	20	4	5	GEN-X crude glycerol	1.0	393	14–37
BD11	20	20	4	5	BAE crude glycerol	1.0	197	10–31
BD12	20	20	2	5	Pure glycerol	0.5	393	5–8
BD13	20	20	4	5	Distilled GEN-X crude glycerol	1.0	393	5–15
BD14	20	20	4	5	BAE crude glycerol	1.0	786	11–18

[a] Feed consisted of 10% glycerol source (v/v) and 90% tap water (v/v), supplied at beginning of each cycle.

was recovered and dried with anhydrous sodium sulfate prior to analysis. GC–MS was performed on a PolarisQ iontrap GC–MS instrument (Thermo Electron Corporation, Waltham, MA, USA) in positive ei mode. The sample was introduced using split injection. Separation was achieved on a ZB1 capillary column (30 m × 0.25 mm Ø, Phenomenex, Torrance, CA, USA) with helium as the carrier gas (1.2 mL min^{-1}) using a temperature program 40 °C (2 min) ramped to 200 °C at 5 °C min^{-1}. Data was analyzed using the software program Xcalibur v2 (Thermo Electron Corporation, Waltham, MA, USA). The compounds were confirmed by retention time and mass spectral matching with known standards (Lancaster Synthesis, Ward Hill, MA, USA) as methyl ester derivatives, and quantified based on the internal standard. Total intracellular PHA content was determined on a percent dry weight cell basis (mass PHA/mass of biomass, w/w).

2.8. Molecular weight determination

The weight average molecular weight (M_w) of recovered PHA was determined using a gel permeation chromatography (GPC) system equipped with a Waters 717 plus Autosampler, a Waters 2478 RI detector, and a Viscotek 270 dual detector (low angle laser light scattering (LALLS), right angle laser light scattering (RALLS), and differential viscometer; Viscotek Corporation, Houston, TX, USA). PHA (approximately 10 mg) was dissolved in chloroform (5 mL) in a Teflon-capped vial and stirred constantly at 35 °C (2 h). A sample volume of 100 μL was injected onto the GPC column (Viscotek ViscoGEL, I-MBHMW-3078) at 40 °C using stabilized tetrahydrofuran (THF) as eluant (1 mL min^{-1}). A narrow width polystyrene standard (Viscotek, M_w = 98.946 g mol^{-1}) was used for constructing a calibration curve. Chromatography integration and molecular weight calculations were conducted using OmniSEC 4.1 (Viscotek) software.

2.9. Thermal analysis

Melting temperature (T_m), glass transition temperature (T_g), and degree of crystallinity were determined by differential scanning calorimetry (DSC) on a TA Instruments (New Castle, DE, USA) Q200 device equipped with refrigerated cooling. Indium was used to calibrate the system before testing. Approximately 5 mg of dry PHA was enclosed in an aluminum T-zero pan. Test procedures included first heating to 180 °C to destroy any pre-thermal history, then cooling to −50 °C, and finally, re-heating to 180 °C for the determination of thermal transitions. An equilibration time of 3 min was applied after each heating and cooling cycle. All temperature ramping rates used in the testing were 10 °C min^{-1}. T_m was taken as the peak point of the crystallite melting peak. The degree of crystallinity was calculated by dividing fusion enthalpy by 146 J g^{-1} – fusion enthalpy for a 100% crystalline PHA (Barham et al., 1984). T_g was recorded as the inflection point in the thermogram.

2.10. Tensile analysis

PHB films prepared by chloroform solvent casting were subjected to tensile testing on a dynamic mechanical analysis (DMA) test instrument (TA Instruments Q800 DMA (New Castle, DE, USA)). PHB films were dried under vacuum for 24 h and were then conditioned (25 °C, 50% relative humidity) for one week before testing. Sample dimensions were approximately 15 × 4 × 0.06 mm. Tensile testing was conducted at a constant force of 1 N min^{-1} until break. Tensile strength, Young's modulus, and elongation at break were determined from the constructed stress–strain curve using Universal Analysis software (TA instruments).

3. Results and discussion

3.1. PHA synthesis on CG: toward process optimization

Initial investigations focused on determining optimal bioreactor operating conditions to maintain a stable MMC capable of PHA synthesis. Considering that CG is carbon-rich, but poor in readily bio-available macronutrients (Thompson and He, 2006), our hypothesis was that PHA synthesis would be driven by macronutrient limitation or a feast/famine mechanism. Performance of the first three reactors tested (BD1-BD3; Table 1) confirmed the potential to generate biomass with high PHA content – in excess of 50% (w/w) (note that only PHB was synthesized). The bioreactors were operated under fully aerobic conditions, thus PHB synthesis was not driven by electron acceptor deficiency. Furthermore, the prototypical feast/famine PHA response (rapid PHA synthesis concurrent with organic carbon utilization (Dionisi et al., 2004)) was not observed. Therefore, it would appear that PHB synthesis was driven by a macronutrient deficiency. Regarding longer-term operational stability, these reactors maintained high PHB content but were prone to foaming, most likely due to surfactant contaminants in the CG. In particular, reactors BD2 and BD3 experienced loss of volume due to excess foaming, even with Antifoam A Concentrate added. Reactor BD2, with its shorter SRT and corresponding larger volume of feed each cycle, experienced the most frequent process upsets, while BD1 (longer SRT, less feed volume per cycle) experienced fewer upsets. As would be expected, longer SRTs also yielded higher mixed liquor suspended solids (MLSS)

concentrations (5 g L^{-1} in BD1; 1.5 g L^{-1} in BD2; 3 g L^{-1} in BD3). Combined with PHB yield, the longer SRT reactors would produce more total polymer per cycle.

Due principally to enhanced process stability, the BD1 operational scenario was scaled up as the "control" and "production" reactor BD4 (*i.e.*, BD1 was used as the inoculum microbial population for BD4). Other larger scale reactors (Table 1) were established with fresh inoculum from the Moscow WWTP, with operational parameters modified based on the preliminary results. Specifically, the HRT was increased, because longer HRTs were considered to be favorable for maximizing microbial growth and PHB production on the bio-available nutrient-poor CG. Additional variables included longer cycle times (BD5–7; more time for microbes to convert CG to PHA), variable ammonia–nitrogen (BD6, BD11, and BD14; evaluating nutrient limitation), and a longer SRT (BD9; assessing effects of a larger consortium). A longer cycle length (BD4 vs. BD5, 6) appeared to reduce PHB production, as did longer SRT (BD4 vs. BD9). Reduced ammonia-N in the feed led to a similar outcome (BD4 vs. BD8, 11), as did higher nitrogen in the feed (BD4 vs. BD14). Notably, the reactor with no ammonium chloride added (BD8) produced no PHB, which suggests that a minimum amount of readily bio-available nitrogen must be added to stimulate PHB synthesis.

The CG predominantly used in this research was obtained from the UI biodiesel production facility, which operates at a production capacity of 95,000 L $year^{-1}$ (25,000 gal $year^{-1}$). To add further breadth to our study, investigations were performed using CG from a commercial biodiesel facility (GEN-X Energy Group, Burbank, WA, USA). Using this waste stream, reactors BD7, 10, and 13 were operated and tested as replicates to reactors fed UI CG (Table 1). As shown, PHB production was generally comparable to that observed in the UI CG-fed reactors. The GEN-X CG, which was derived from biodiesel production using waste food-service oils vs. the pure seed oil used in the UI biodiesel program, did contain less methanol than the UI CG, although the concentration was relatively high. Removing methanol from the GEN-X CG (BD13 vs. BD10) reduced PHB synthesis; comparable results were observed for a reactor fed pure glycerol (BD12). Both glycerol and methanol can be used by MMC to synthesize PHA, although from a bio-energetic perspective microbes will preferentially use methanol first (higher oxidation state). Collectively, these results indicate that the enriched MMC was using methanol as the principal precursor for PHA synthesis. Further, these results indicate that our bioreactor operational criteria and conditions were effectively optimized to enrich for microbes that would preferentially synthesize PHA on methanol. These results reinforce that the proposed PHA production process may exhibit some variability depending on CG feedstock, and that the process would need to be optimized to the CG waste stream.

3.2. Assessing PHB production potential

Based on the preliminary investigations, reactor BD4 was selected for further performance analyses. Of the reactor configurations tested, BD4 had the largest volume (12 L), operated for the longest time period (more than a year), and consistently produced high PHB levels. Parameters monitored and/or assessed over several BD4 cycles included (i) substrate utilization (methanol and glycerol), (ii) PHB production and consumption, and (iii) microbial population trends.

Several operational cycles for BD4 were analyzed to observe substrate utilization and PHB synthesis (Fig. 1). As shown, glycerol concentrations remained relatively constant, while methanol concentrations were significantly reduced (to near complete depletion). Considering the favorable microbial energetics for methanol use over glycerol (*i.e.*, methanol exhibits a higher oxidation state), this preferable use of carbon by the MMC under aerobic

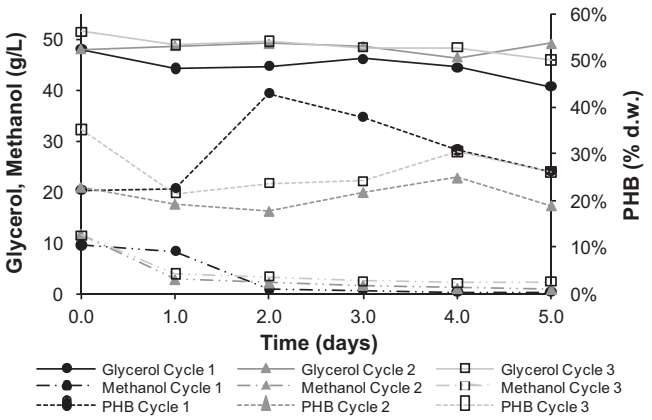

Fig. 1. BD4 profile of glycerol and methanol concentrations, along with intracellular PHB content, over three cycles within approximately 1 month.

conditions was not a surprise; these results and conclusion align with the observations from reactors fed glycerol only (Table 1). Regarding PHB synthesis, these results suggest an enriched methylotrophic population was metabolizing methanol to form PHB. Previous research has shown that methylotrophs prefer C1 sources (Kim et al., 1999; Yezza et al., 2006). Braunegg et al. (1999) reported similar results, as the facultative *Methylomonas extorquens* utilized methanol before glycerol when fed a CG mixture containing 20% methanol. Finally, the relative consistency of intracellular PHB concentration over all three cycles sampled suggests a relatively stable population of microbes capable of PHB synthesis on the methanol fraction in the CG.

While data presented in Fig. 1 on intracellular PHB concentrations might suggest minimal net polymer production in reactor BD4 (*i.e.*, relatively constant intracellular concentration over a cycle), a true measure of polymer production must consider biomass growth. Biomass concentrations were monitored through total solids (TS) testing, as the CG residual in the liquid phase made suspended solids testing (in accordance with Standard Methods (Clesceri et al., 1998)) impossible. Glycerol contamination in the TS testing was avoided by gently rinsing samples with deionized water following centrifugation. PHB production was assessed over two contiguous operational cycles (Figs. 2A and 2B). Glycerol concentrations (data not shown) remained effectively constant over the cycles, similar to that shown in Fig. 1, and again methanol appeared to be the primary carbon source used by the microbes. In total, reactor PHB quantities increased by 20–44% over each cycle (end minus beginning) (Fig. 2B), attributed primarily to biomass

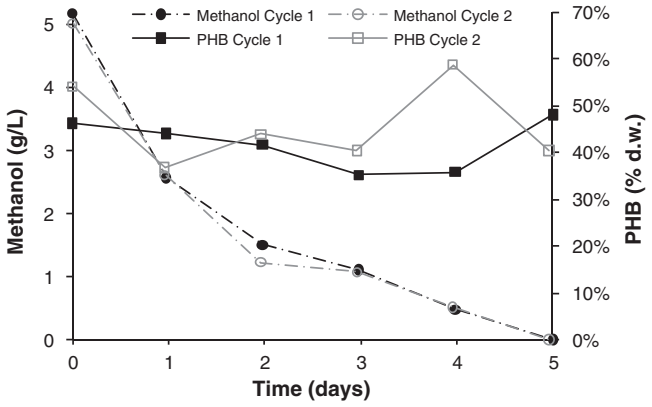

Fig. 2A. Methanol concentration and intracellular PHB content in BD4 over two cycles.

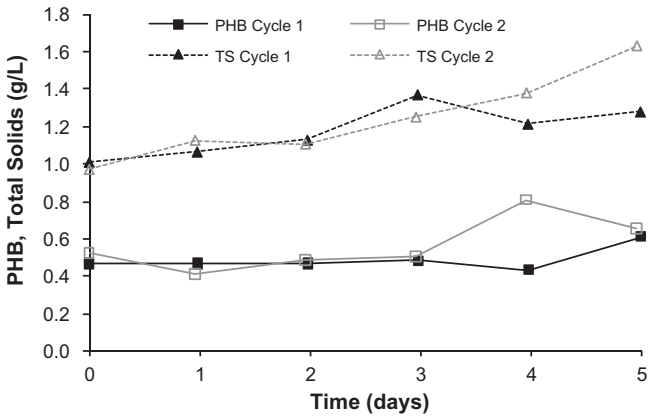

Fig. 2B. Concentration of PHB and total solids in BD4 over two cycles.

growth; within the cycle, intracellular PHB content fluctuated relatively significantly (37–59%, w/w; Fig. 2A). These results suggest that the microbial consortium was storing PHB at a relatively constant rate while also exhibiting slow growth. Growth-associated PHB storage has been observed previously with pure cultures using either glycerol (Ibrahim and Steinbuchel, 2010) or methanol (Yezza et al., 2006). The consortium in this study exhibited similar tendencies – maintaining relatively constant bulk solution PHB concentrations over a cycle without peaks that would be associated with feast/famine or nutrient-limited PHA synthesis.

Intracellular PHB concentrations measured in reactor BD4 biomass were comparable to that observed by others using pure cultures and glycerol or methanol substrates (Braunegg et al., 1999; Cavalheiro et al., 2009; Mothes et al., 2007). Regarding PHB yield, at a maximum of 0.10 g PHB produced per g methanol utilized, results from this research compares similarly to those of de Almeida et al. (2007) (0.10 g PHB per g glycerol) and Zhao et al. (1993) (0.12 g PHB per g methanol). However, PHB yields observed by Braunegg et al. (1999) (0.23 g PHB per g methanol + glycerol), Ibrahim and Steinbuchel (2010) (0.29 g PHB per g glycerol), and Cavalheiro et al. (2009) (0.36 g PHB per g glycerol) suggest that higher PHB yields on CG could be achieved. Contrasting our work with that showing higher polymer yields, principal differences in experimental setup include the use of isolated PHB-producing pure cultures, the regulation of pH and/or temperature, and the addition of more macronutrients to culture media. In this research, the mixed consortium survived and produced PHB with only the addition of ammonium chloride, with other nutrient value assumed intrinsic to the CG feed. While this less-than-ideal nutrient scenario resulted in lower microbial populations, considering long term environmental and economic sustainability, additional costs were not incurred by using excess quantities of synthetically-derived nutrients. Beyond potential macronutrient limitations, other research using CG has also indicated that residual sodium remaining from the biodiesel transesterification process can adversely impact PHB production and yield due to cellular osmoregulation (Cavalheiro et al., 2009; Mothes et al., 2007). Mothes et al. (2007) reported a yield of 0.14 g PHB per g methanol when using glycerol polluted with NaCl, a yield similar to that of this research. Sodium methoxide was used as a catalyst in the process from which our CG was derived.

The carbon-to-nitrogen (C:N) ratio may also have impacted the consortium's PHB synthesis potential. The experimental design was based on a C:N ratio of approximately 100 Cmol $Nmol^{-1}$ based on research by Wang et al. (2007), with the original assumption that glycerol would be used as the primary carbon source. However, considering that the consortium instead used methanol, the resultant C:N ratio was less than 10. Hydrolysis of organic nitrogen in the CG may have reduced the C:N ratio even further. Although the methanol concentration (near 0.5–1% of the reactor, v/v) should have been adequate for enhanced PHB production (Yezza et al., 2006), higher C:N ratios typically result in greater PHB accumulation (Cavalheiro et al., 2009). The lower C:N ratio, with implicitly more nitrogen, suggests that to a certain degree the environmental conditions may have favored growth/maintenance over PHB synthesis (*i.e.*, no nitrogen limitation relative to carbon).

3.3. Toward enhancing PHB production

The parent PHB "production" reactor (BD4) yielded moderate quantities of polymer and appeared to be semi-optimized in terms of operational criteria. However, it was not clear if PHB synthesis was maximized for this consortium. To better understand PHB production potential and also the PHB synthesis "trigger" mechanism, a set of sidestream reactors were evaluated, with each reactor inoculated using biomass obtained from reactor BD4. The initial sidestream investigation (identified as SS1) was conducted to evaluate how the consortium would respond to receiving new substrate at the end of a 5-days operational cycle (a continued operational cycle, but with only new CG and without additional nutrient supplement (reactor SS1, operated for 5 days)). The consortium was fed in an identical manner to BD4, but without ammonium chloride. Under these altered substrate conditions, the consortium exhibited a gradual increase in intracellular PHB concentration (49–60% d.w.; Table 2 and Fig. 3A) but experienced limited growth (total solids increased from 1067 to 1287 mg L^{-1}; Fig. 3B) over the cycle. The intracellular PHB concentration increased most rapidly in the first 24 h, then, similar to the parent reactor, the PHB concentration remained essentially constant thereafter (Fig. 3A). Compared to the parent reactor, the consortium produced a larger increase in intracellular PHB over the operational cycle (an increase of approximately 22%). Again PHB synthesis appeared driven by methanol utilization, and the PHB yield on methanol remained low (Table 2). Results from this sidestream reactor suggest that (i) additional production could potentially be realized beyond that produced in the parent reactor, and

Table 2
Summary of sidestream operating parameters, change in total solids over a cycle, maximum intracellular PHB contents, and maximum PHB yields (g PHB produced per g methanol utilized).

Sidestream reactor	Operations differing from BD4	Initial vol. (L)	TS change[a] (mg/L)	Max. PHB (% d.w.)	Max. yield (g PHB/MeOH)
SS1	Not provided NH_4Cl dose at beginning of cycle	2	220	61	0.07
SS2	BD4 feed/NH_4Cl dose split into daily increments for pulse feed effect	1.6	−52	67	0.17
SS3	None	1	111	17	0.09
SS4	Phosphorus sources at feed: 250 mg of both KH_2PO_4 and $Na_2HPO_4{\cdot}2H_2O$	1	−190	6	0.02
SS5	SS4 nutrients plus 450 mg $MgSO_4$, 52 mg $CaCl_2$, and 75 mg EDTA at feed	1	746	7	0
SS6	SS4 and SS5 nutrients plus 2 mL of trace micronutrient solution at feed	1	860	4	0

[a] Change in total solids concentration over the cycle.

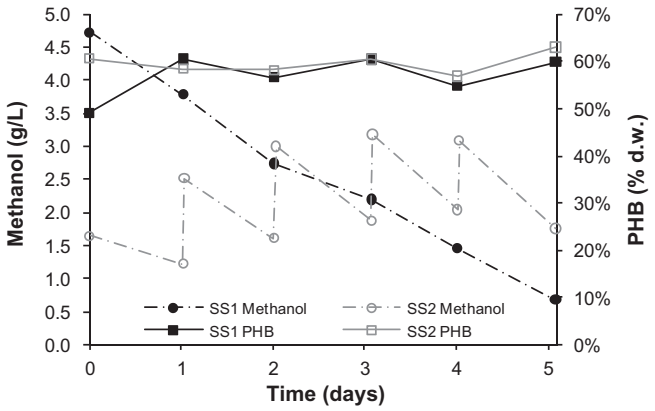

Fig. 3A. Profile of methanol concentration and intracellular PHB content in SS1 (fed BD4 regimen without ammonium chloride) and SS2 (step-fed BD4 regimen).

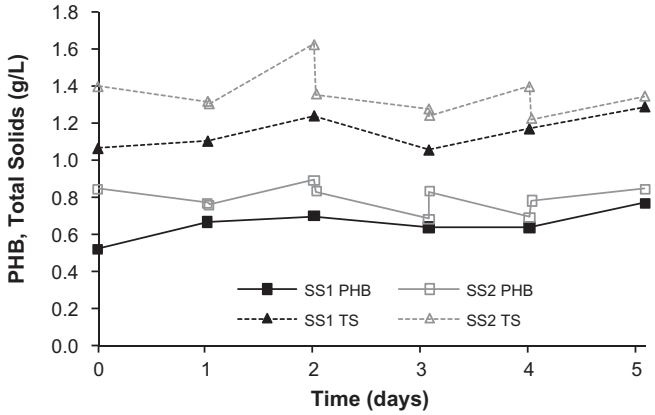

Fig. 3B. Concentration of PHB and total solids in SS1 and SS2.

(ii) enhanced nutrient limitation (*i.e.*, higher C:N ratio) stimulated more PHB synthesis over growth.

In contrast to batch feeding, pulse feeding substrate to mixed microbial consortia has been shown by others to induce excess PHA synthesis (Serafim et al., 2004, 2008), although the phenomenon is typically associated with a better quality substrate than CG and also with feast–famine PHA synthesis. Employing this approach, a second sidestream reactor (SS2) was operated in an attempt to increase PHB storage over a cycle. Using an inoculum from BD4, at the beginning of each day (and each day thereafter, for 5 days) an equal fraction of the total BD4 crude glycerol and ammonium chloride feed was provided to the consortium. As shown (Figs. 3A and B) and summarized (Table 2), the consortium produced minimally more PHB than realized in the parent reactor (2% increase in intracellular concentration over the cycle), although polymer yield on methanol increased. The microbes readily used the new methanol provided each day, but the resulting effluent methanol concentration was higher than observed in parent reactor BD4 or reactor SS1. The TS concentration also remained effectively constant over the cycle. Overall, it appears that pulsing substrate maintained the consortium in somewhat of a stationary growth phase which was not conducive to maximizing PHA production.

Finally, a concurrent set of sidestream reactors (SS3-6) were operated to better understand the potential effect of macronutrient availability on PHB synthesis. These four sidestream reactors were operated concurrently for a 5 days cycle, and all were inoculated from reactor BD4. Reactor SS3 was operated as per BD4 (as a control), while reactors SS4–SS6 sequentially were augmented with additional macronutrients (for details see Table 2). In effect, these sidestream investigations supported the hypothesis that PHB synthesis was stimulated and/or enhanced by a macronutrient limitation in the substrate. As the consortium received increasing quantities of macronutrients, PHB synthesis decreased significantly (Table 2). Even further, with the additional macronutrients, the consortium used its PHB reserves for growth and cell maintenance. In fact, the total solids concentrations for reactors SS5 and SS6, which received significant quantities of nutrients, approximately tripled over the cycle.

3.4. PHB on crude glycerol: material properties

Little data has been published regarding the material characteristics and thermal properties of PHA produced by mixed microbial consortia on waste organic streams (Dias et al., 2006). To begin to address this critical deficiency, the scale of our primary PHB reactor (BD4) was leveraged to collect sufficient quantities of PHB-rich biomass for materials characterization. Specifically, recovered PHB was characterized for weight average molecular weight (M_w), thermal properties (including melting temperature (T_m), glass transition temperature (T_g), and crystallinity), and tensile properties.

Polymer chain size greatly governs material properties; therefore, the determination of M_w provides vital information regarding these properties and thus the practical uses of PHAs. For example, PHB for industrial processing such as extrusion and injection molding has been documented to require a M_w over 500 kDa (Mothes et al., 2007). It has also been shown that the M_w of PHA greatly varies with the microorganisms and carbon feed stocks used for PHB synthesis (van der Walle et al., 2001). In this study, PHB M_w were monitored daily over 1 month together with intracellular PHB content. Fig. 4A presents the dynamic changes of the content and M_w of PHB recovered from lyophilized biomass, averaged over four cycles. As shown, PHB content decreased immediately after feeding at the beginning of the cycle, followed by an increase later in the cycle. The average M_w of PHB remained relatively constant around 300 kDa within each cycle. In contrast, microbes producing PHA on organic acids under feast/famine conditions exhibited a relatively significant variation in M_w over a synthesis cycle (Serafim et al., 2008).

Long-term monitoring can help evaluate the potential for operational stability of bioreactors, which is essential in industrial applications. In this regard, for a one month operational period (Fig. 4B), PHB content was observed to vary from 10–35% (w/w) while the M_w ranged from 200–380 kDa. The PHB M_w obtained in this study was lower than the values reported by Mothes et al. (2007) (620–750 kDa) and Cavalheiro et al. (2009) (790–960 kDa); however, both of these studies achieved PHB synthesis

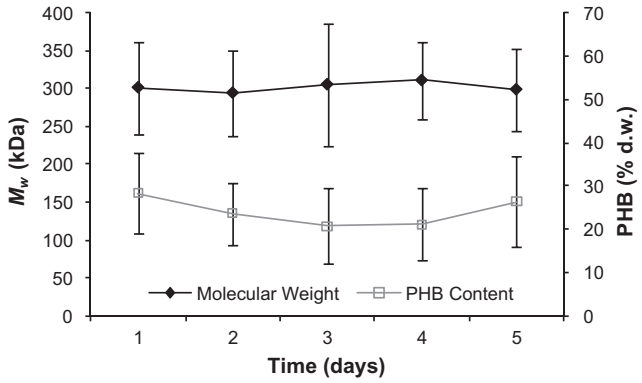

Fig. 4A. BD4 intracellular PHB content and molecular weight of PHB – average values over four cycles.

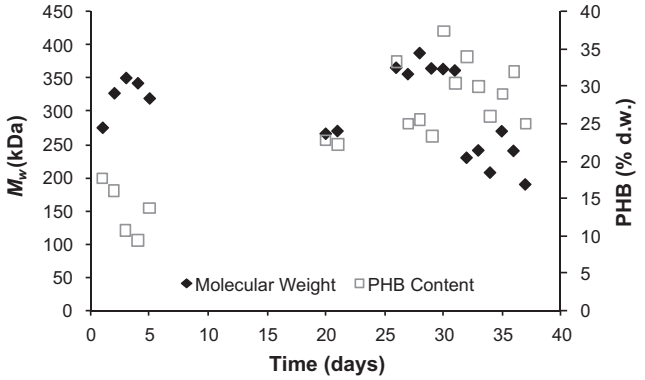

Fig. 4B. One-month profile of PHB content and PHB molecular weight in BD4.

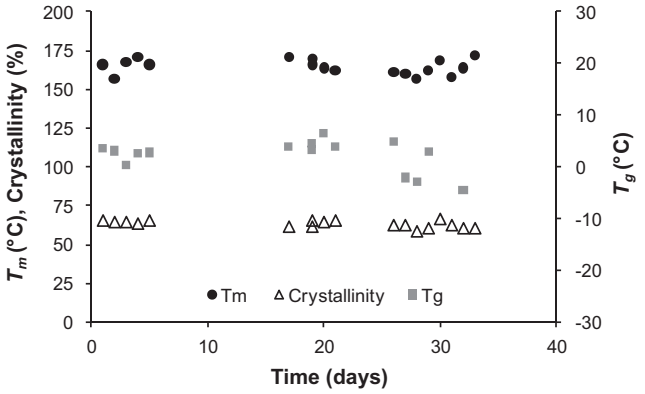

Fig. 4D. One-month profile of PHB thermal properties in BD4.

on crude glycerol using pure cultures. The use of a mixed culture and the inherent nutrient limitations of the feedstock could explain the lower M_w values from our study.

Thermal properties of semicrystalline polymeric materials are generally evaluated by parameters such as T_m, T_g, and degree of crystallinity – all of which greatly affect potential polymer applications. Similar to the above M_w investigations, the thermal properties of recovered PHB were monitored daily over the same one-month period. As shown (Fig. 4C), there was limited dynamic change of the average thermal parameters. Both T_m and T_g of the recovered PHB were relatively constant throughout each cycle. PHB crystallinity remained stable within the biosynthesis cycle at approximately 60%, close to values reported by van der Walle et al. (2001). Fig. 4D shows the one-month profile for the thermal properties of the recovered PHB. T_m, T_g and degree of crystallinity of PHB ranged respectively from 158 to 175 °C, −5 to 5 °C, and 58% to 65%, all of which reasonably compare with the thermal properties of PHB widely reported previously (Bengtsson et al., 2010; Lee, 1996a).

The tensile strength and Young's modulus of isolated PHB were 14 MPa and 1.8 GPa, respectively, and were lower than that of commercial (Aldrich) PHB (tensile strength of 20 MPa and Young's modulus of 2.3 GPa). This is most likely attributable to the lower molecular weight of isolated PHB (308 kDa) than the commercial PHB (437 kDa). However, the tensile strength obtained for the isolated PHB was comparable to those reported by Thellen et al. (2008) at 14–16 MPa. The Young's modulus and elongation at break (1.3%) values obtained were similar to other studies on PHB films (Dekoning, 1995; Thellen et al., 2008). Stress–strain curves of recovered and Aldrich PHB are shown in Fig. 4E.

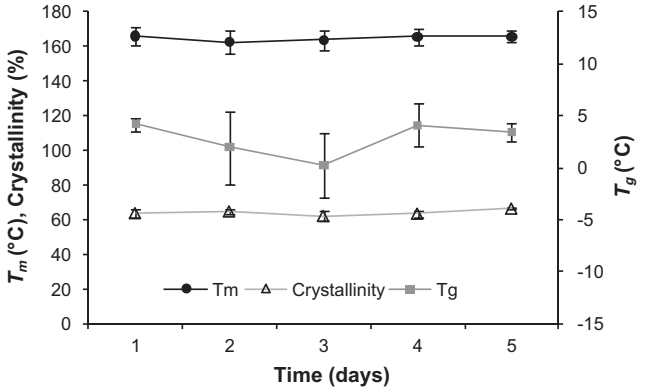

Fig. 4C. Thermal properties of BD4 PHB over one cycle (melting and glass transition temperatures).

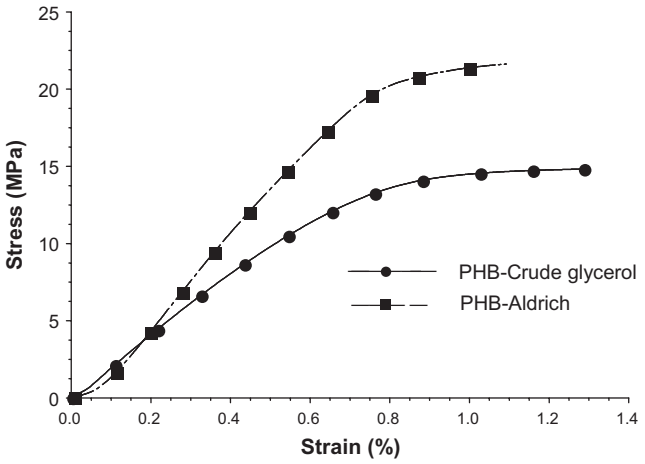

Fig. 4E. Stress–strain curves for commercial (Aldrich) and recovered PHB.

3.5. Estimating PHA production capacity for a full-scale system

PHB production capacity using a mixed microbial consortium fed CG was estimated based on the semi-optimized BD4 operational data (Figs. 2A and B) and assuming a 38 million L (10 million gallons) per year biodiesel facility that produced 2.4 million L (0.63 million gallons) of crude glycerol per year (Thompson and He, 2006). In this scenario, the PHB production was estimated at 266 kg (590 lb) per 5-days cycle or more than 19 metric ton (20.9 ton) per year. Regarding commodity value, the current estimated price for PHA pellets is nearly $5 per kg (DiGregorio, 2009). Thus, gross profit from PHB production would be approximately $96,000 per year. Polymer processing and purification costs would impact this potential revenue stream, although opportunities exist to produce commodities that significantly minimize PHA processing requirements (Coats et al., 2008). Further, with less crude glycerol requiring disposal or refinement, additional cost savings could be realized.

4. Conclusions

The research presented herein assessed the potential to produce PHA on crude glycerol (CG) using mixed microbial consortia (MMC). It was observed that MMC will synthesize PHB on methanol present in the CG, with PHB synthesis seemingly driven by a macronutrient deficiency. Over an operational cycle, microbes maintain a relatively constant intracellular PHB concentration while producing additional biomass. The produced PHB exhibited

material properties and characteristics suitable for many commercial uses. Finally, a scaled-up process could potentially produce up to 19 ton (20.9 ton) of PHB per year for a 38 million L (10 million gallon) per year biodiesel facility.

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