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ORIGINAL PAPER

Efficient Utilization of Crude Glycerol as Fermentation Substrate in the Synthesis of Poly(3-hydroxybutyrate) Biopolymers

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Abstract One refined and two crude glycerol (from biodiesel production) samples were utilized to produce poly(3-hydroxybutyrate) (PHB) by *Pseudomonas oleovorans* NRRL B-14682. A batch culture fermentation protocol including 1% glycerol and an aeration rate of 3 standard liters per minute proved best for PHB synthesis (av. yield = 1.0 ± 0.2 g/L at 48 h) and efficient glycerol utilization. PHB molecular weights decreased as MeOH concentration increased. Refined glycerol resulted in PHB polymers with number average molecular weights (M_n) of 314,000 g/mol which decreased by 17 and 90% as MeOH media concentrations increased to <0.005 and 0.85%, respectively. Proton (^{1}H) NMR demonstrated the presence of glycerol- and methoxy-based end-capping, which was confirmed by ^{1}H diffusion experiments (DOSY analyses). NMR diffusion analyses of the PHB polymers established their diffusivities, and confirmed that their relative molecular sizes were dependent on the impurities in the glycerol. In addition, DOSY analyses indicated that each end-capped PHB polymer and the glycerol or methoxy groups bound to it had the same diffusion constants, demonstrating that they migrated together as covalent complexes. Non-covalent complexation was eliminated by physically mixing free glycerol with PHB synthesized from oleic acid; their respective diffusivities were notably faster.

Keywords Poly(3-hydroxybutyrate) · Glycerol · Fermentation · *Pseudomonas oleovorans* · End-capping · Diffusion constants

Introduction

As the world moves towards a more petroleum-free existence, expectations are mounting to achieve a more bio-based dependence especially relating to renewable energy. In fact, in the United States alone, a number of goals have been established to implement bio-based research which will improve production of and reliance on renewable energy. Some of the more salient target dates include: (1) 2017, the target year under a proposal by President George W. Bush in his 2007 State of the Union Address for achieving the production and utilization of at least 35 billion gallons of alternative fuels within the United States, (2) 2022, the target year for the annual production of 36 billion gallons of renewable fuels in the United States under the Renewable Fuel Standard, as called for in the Energy Independence and Security Act of 2007, (3) 2025, the target year set by the "25 × ′25 coalition" for renewable energy to reach 25% of total energy use in the United States, and (4) 2030, the target year set by the Department of Energy (DOE) to displace 30% of the gasoline demand (2004 levels) in the United States with biofuels, primarily ethanol.

To reach these goals, provisions must be enacted to reduce the cost of production. Currently, technology exists that allows the production of renewable biofuels but the comparable production costs to petroleum-based equivalents make bio-based fuels less economically appealing. Whether the target product is bioethanol, biogas, biodiesel or other fuels, the utilization of low-cost renewable

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materials (preferably without ties to food) such as lignocellulosic biomass for the production of bioethanol and biogas [1, 2] and/or algal oils for the production of biodiesel [3, 4] is vital for the economic development of that particular fuel. Another possibility is the discovery of new outlets for the coproducts that are derived from biofuel production. By establishing new uses, coproduct value can be maintained giving biofuel producers an alternative means of offsetting the higher production costs of biofuels by selling the coproducts.

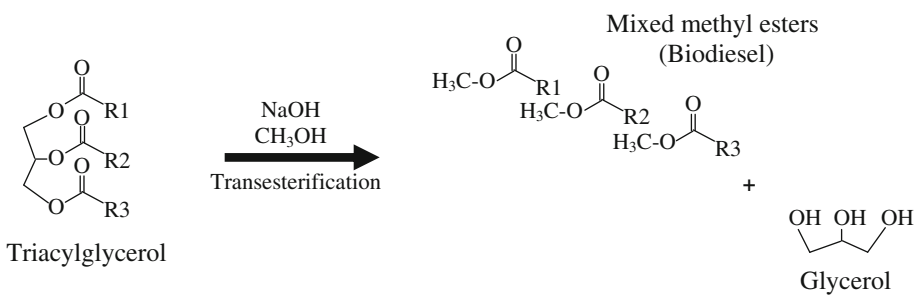
Biodiesel can be synthesized by chemical, enzymatic and microbial means [5], however; the most frequent approach generally involves base-catalyzed transesterification with short-chain alcohols (generally methanol) to produce the fatty acid alkyl esters (biodiesel) according to Scheme 1. One drawback to this method is the formation of a relatively large crude glycerol coproduct which, because of the amount produced, has very little value.

Historically, glycerol has occupied numerous applications in the drug, food, beverage, chemicals and synthetic material industries. It is used in cosmetics, toiletries, sweeteners, solvents, softening agents, cough syrups, surface coatings, paints and many other products. However, these applications generally require high quality glycerol as produced through chemical synthesis from petroleum feedstocks. Today, all glycerol is derived from biological sources. The transesterification reaction involved in the biodiesel production process results in a crude material whose composition differs based on the type of TAG used, the efficiency of the transesterification reaction and the effectiveness of biodiesel and methanol recovery. Generally, crude glycerol exits the biodiesel plant in formulations that are between 75 and 90% glycerol, less than 1% methanol with the remainder composed of water and small amounts of salts, ash, free fatty acids (FFAs), fatty acid esters, mono- (MAGs) and diacylglycerols (DAGs). Because of the rate of expansion of the biodiesel industry, crude glycerol is being produced at rates that have caused its price to drop to as low as a few cents per pound and while this price has recovered to a small degree, the expected rate of future biodiesel production necessitates new uses for crude glycerol to help it maintain value.

One possible fate of the crude glycerol coproduct is to refine it to a high purity and while there are certainly applications that require refined glycerol, only companies that produce biodiesel on a "large-scale" such as Cargill or Archer Daniels Midland (ADM) have the financial capacity to absorb the prohibitive costs of refining. Most of the smaller scale biodiesel producers (who are vital to achieve the country's bioenergy goals) do not have the capital required to refine the crude glycerol coproduct and in fact, to do so would be economically counterproductive. Therefore, it is important that new applications be found that can utilize the coproduct in its crude form and thus eliminate the need for refining.

One such possibility is using crude glycerol as a fermentation substrate. While refined glycerol has been known to be a good substrate for microbial growth for years, it has only been within the last decade that crude glycerol has been explored as a possible carbon source for the fermentative production of value-added products such as 1,3 propanediol [6–8], hydrogen [9], succinic acid [10], glycolipid biosurfactants (sophorolipids) [11], citric acid [12, 13], and single cell oils [14] among others.

Poly(hydroxyalkanoates) (PHA) are bacterial polyesters that are synthesized by numerous bacterial species from many carbon substrates including simple sugars [15], free fatty acids [16], simple alkanes and alkanols [17], and triacylglycerols [18, 19]. They are generally categorized into two groups based on the length of their monomeric side-chains. Short-chain-length PHA (*scl*-PHA) polymers consist of 3-hydroxyalkanoic acids with monomeric repeat units of 4–5 carbons, while medium-chain-length PHA (*mcl*-PHA) polymers are composed of monomeric repeat units that are ≥6 carbons in length. Because of their side-chain variability, PHA polymers exhibit a wide range of properties from semicrystalline thermoplastics (comparable to polyethylene and/or polypropylene) to amorphous elastomers. Within the past 5 years, focus has shifted to the use of less valuable, renewable materials as substrates for PHA production in an attempt to reduce production costs. Some



Scheme 1 The alkaline catalyzed transesterification reaction in the formation of biodiesel from triacylglycerols (TAGs) (R1, R2 and R3 correspond to unique fatty acids attached to the glycerol backbone of the TAG or alkyl ester)

of these substrates have included soy molasses [20], crude glycerol [21–26], and wheat-based coproducts [27, 28] and while some of these studies reported remarkable success in the production of large amounts of PHB polymers (especially from wheat-based coproducts; ~40 g/L under batch culture conditions and ~163 g/L under fed-batch conditions using a co-feed of wheat hydrolysate and pure glucose) [28], many of these studies, especially on crude glycerol, were either conducted on a small scale (<1-L) or were conducted in fed-batch culture where substrate was periodically added in an attempt to boost polymer production at the cost of unutilized carbon source. In this paper we discuss our efforts to improve the efficiency of (PHB) production from crude glycerol by altering fermentation conditions thereby improving substrate utilization and shortening fermentation time. In addition, we utilized ^{1}H-NMR diffusion techniques to verify glycerol- and methoxy-based end-capping, as well as discuss the ways in which viscosity and relative molecular size are affected in PHB polymers derived from three compositionally distinct glycerol substrates.

Materials and Methods

Materials

All simple salts and the tetramethylsilane (TMS) and tetrakis(trimethylsilyl)silane (TMSS) used as NMR reference materials were obtained from the Sigma Chemical Company (St. Louis, MO). Yeast extract and tryptone [components for Luria–Bertani (LB) broth] were purchased from Difco (Detroit, MI). Three separate glycerol samples, one reagent grade (≥99.0%) and two crude co-products were obtained from various sources and maintained compositions according to Table 1. All organic solvents were HPLC grade and purchased from Burdick and Jackson (Muskegon, MI). The silylation reagents for GC/MS analyses including *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and pyridine were purchased from Pierce (Rockford, IL).

Crude Glycerol Composition Determination

Reagent grade glycerol was used as received without compositional analysis. Two different crude glycerol substrates (Samples CG-G and CG-IS, Table 1), both derived from biodiesel production processes, were analyzed by HPLC for glycerol and methanol content. Samples were filtered through a 0.22-μm filter and placed in an auto sampler vial. An Agilent 1200 series HPLC equipped with an ion exclusion column (Aminex HPX-87H, Bio-Rad, Hercules, CA) at 60 °C and a refractive index detector was used to analyze the samples. A 5-μL injection was eluted at 0.6 mL/min with 5 mM sulfuric acid prepared in HPLC-grade water. Data were processed and analyzed using the Agilent Chemstation software. The HPLC was calibrated with standards containing glycerol and methanol. Standards were verified with a secondary standard after every 10 samples. Each sample was injected twice and the results for each sample were averaged. Material organics not glycerol (MONGs; consisting of unrecovered alkyl esters and unreacted FFA, TAG, DAG, and MAG) were determined gravimetrically after extraction in hexane. The crude glycerol samples (100 g) were extracted in triplicate with 50-mL of hexane. Hexane fractions were combined and the hexane was removed by evaporation under nitrogen, and dried in vacuo (25 Torr) for 24 h to constant weight. The flasks were reweighed and the MONGs concentrations were calculated by difference from the starting weights. The total concentration of alkyl esters, FFA, TAG, DAG and MAG was determined for each crude glycerol sample by dissolving a known amount of MONGs back into hexane and using an established HPLC procedure [29] (data not shown). Water content was determined by difference after glycerol, methanol and MONGs content was known.

Strain Information and Fermentation Parameters

Pseudomonas oleovorans NRRL B-14682 was obtained from the National Center for Agricultural Utilization Research (NCAUR), ARS, United States Department of Agriculture, Peoria IL. Stock cultures of the organism were

Table 1 Source and compositions of glycerol feedstocks used in PHB fermentations

Sample	Source	Glycerol (%)	MeOH (%)	MONGs (%)[a]	H_2O (%)
RG	Sigma Chemical Company (St. Louis, MO)	≥99	–	–	–
CG-G	Griffin Industries (Butler, KY)	77	<0.5	0.4	22
CG-IS	Dr. Jon van Gerpen Iowa State University (Ames IA)[b]	47	40	7	6

[a] MONGs = Material organic not glycerol and is composed of unrecovered alkyl esters and unreacted free fatty acids, tri-, di-, and monoacylglycerols

[b] Dr. Jon van Gerpen has since relocated to the Department of Biological and Agricultural Engineering, University of Idaho, Moscow, ID

prepared as described previously [30]. Bench-top fermentations were conducted in 10-L volumes (Bioflo 3000 with a 12-L working volume, New Brunswick Scientific, Edison, NJ) in Medium E* (starting pH 7.0) (for media composition see reference 31). Medium E* salts and glycerol were autoclaved separately to sterilize and then aseptically combined into the fermentation vessel resulting in 10-L of Media E* containing 1 or 2% (w/v) glycerol (for the crude glycerol samples mathematical calculations were made based on the glycerol content in each crude coproduct to result in the appropriate glycerol concentration for each fermentation). The inoculum for each 10-L fermentation was prepared by inoculating 50-mL of Luria–Bertani (LB) broth (1% tryptone, 0.5% NaCl, 0.5% yeast extract) with 1.5-mL of bacteria from a frozen stock culture and incubating the cultures at 30 °C, 200 rpm for 24 h. After 24 h, the entire 50-mL culture was used as inoculum and added to a 2-L Erlenmeyer flask containing 1-L of LB medium and the flask was again incubated as above. The 1-L LB culture was aseptically centrifuged (8,000×*g*, 20 min, 4 °C) to pellet the cells, the supernatant was discarded, and the cell pellets were resuspended in Media E* + glycerol from the 10-L fermentation vessel. The cell pellets were aseptically combined and added to the 10-L fermentation as the inoculum. The fermentations were conducted at 30 °C, impeller speed of 250 rpm and aeration at either 2 or 3 standard liters per minute (SLPM). In some instances, salts were replenished into the media at 48 h using a fed-batch concept (500 mL of 10× concentrated Media E* salts without glycerol) in order to stimulate more efficient glycerol utilization. In other instances, fermentations were carried out to completion under simple batch culture conditions. The duration of all fermentations was 96 h. At the appropriate time cells were harvested by centrifugation (conditions described above), washed twice in deionized water and lyophilized (24 h) to a constant weight.

Cell Growth and Glycerol Utilization

Cell growth was monitored gravimetrically based on 1-mL and 100-mL culture aliquots that were removed periodically from the fermentations. At predetermined times (0, 5, 22, 28, 48, 53, 72, 77 and 96 h post-inoculation) one 100-mL aliquot and three 1-mL aliquots were removed from the fermentations. The 1-mL aliquots were placed into separate tared Eppendorf tubes and spun at 10,000 rpm for 2 min at 4 °C to pellet the cells. The supernatants were combined, filtered through a 0.45-μm filter and used to determine glycerol and methanol utilization by HPLC as described previously. Each 1-mL cell pellet was then washed with 1-mL of deionized water and re-spun under the conditions previously noted. The water washes were discarded and the 1-mL cell pellets were frozen, lyophilized to dryness (24 h) and reweighed. The 100-mL aliquots were centrifuged at 8,000×*g*, 20 min, and 4 °C to pellet the cells. The cells were then washed once with deionized water, re-spun and the water fraction discarded. The cell pellets were frozen, lyophilized to dryness and weighed. These fractions were subsequently used to determine cell growth and polymer yields over time.

Polymer (PHB) Yield

Intracellular PHB polymers were isolated from the 100-mL lyophilized cells by chloroform extraction at 30 °C overnight with shaking at 250 rpm. Cellular debris was removed by filtration through Whatman #1 filter paper and the chloroform evaporated from the filtrate to give the crude polymers. Each crude polymer was redissolved in a small volume of chloroform and precipitated a total of three times by dropwise addition to cold methanol. The polymer was recovered, placed into a tared vial, dried in vacuo for 24 h and weighed to determine polymer yield.

Instrumental Procedures

Repeat unit compositions were determined by GC/MS of the trimethylsilyl derivatives of the 3-hydroxymethyl esters. Samples were prepared according to Brandl et al. [31] and were silylated by reacting 10 μL of each sample with 250 μL BSTFA and 200 μL pyridine. The mixtures were heated at 70 °C for 30 min and allowed to cool to room temperature. Finally, 150 μL of hexane was added to each sample and the samples were analyzed by GC/MS as previously described [32]. Percent composition was obtained by reconstructing the chromatograms selecting the 175 ion, indicative of silylated 3-hydroxymethyl esters, and identifying the molecular ion-15 amu (CH_3 group; M-15) ions as described by Lee and Choi [33].

Molecular weight averages were determined by gel permeation chromatography (GPC). Number average molecular weight (M_n) and weight average molecular weight (M_w) of the PHB polymers were determined using an LC-20AD liquid chromatograph equipped with a SIL-20A autosampler and RID-10A refractive index detector (Shimadzu, Japan) as described previously [24]. Briefly, the polymers were dissolved in chloroform at a final concentration of 0.7 mg/mL and the resulting polymer solution was filtered (PTFE membrane, 0.22 μm) before analysis. An SDV 8 × 300 mm column with 5-μm porosity was used (Polymer Standards Service, USA) with an oven temperature of 40 °C. The mobile phase was chloroform with a flow rate of 1 mL/min. Standard curves were created using polystyrene standards with a range from 682 to 1,670,000 g/mol and low polydispersities (Polystyrene High Mw Standards Kit, Polymer Standards Service, USA). The molecular weights of the polymers were calculated relative to these standards.

Nuclear Magnetic Resonance (NMR) Spectroscopy was performed to verify composition and determine the presence of glycerol- and methoxy-based end capping and the relative diffusion constants of the polymers in chloroform. Solution-state NMR spectra were recorded at 9.4 Tesla on a Varian INOVA NMR Spectrometer, using a 5-mm dual broadband probe, with *z*-axis pulsed field gradients, operating at 20 °C. All samples were dissolved in deuterated chloroform ($CDCl_3$) with TMS added as an internal ^1H reference. The ^1H (proton) spectra, at 400 MHz, had a sweep-width of 4,500 Hz, were acquired with a 90° pulse angle and a 1 s relaxation delay.

The polymers were also analyzed by a Doneshot ^1H diffusion experiment for "DOSY" analyses [34]. The PHB polymer samples were analyzed at very high concentrations to enable the observation of glycerol- and methoxy-based end-capping, and also at more dilute (less viscous) concentrations of ~0.1 mg/0.3 mL $CDCl_3$ to enable more accurate measurement of their diffusion constants. All samples contained TMS and TMSS as internal references for chemical shift and molecular size comparisons [35, 36]. These experiments were performed with a pulsed gradient driver capable of 18–20 G/cm along the *z*-axis. A series of 20–40 spectra of 16–32K data points and 1,024–2,048 scans were collected, with four steady-state scans per increment. The pulse gradient power was incremented in gradient-squared steps from 17 to 3 G/cm. The diffusion delay was optimized for each sample to ensure observation of the full exponential decay of the NMR signals of interest; its value was typically 150–300 ms. The gradient stabilization time was 2 μs, the relaxation delay was 2 s, and the temperature was maintained at 20 °C. In the concentrated samples, the parameters were optimized to observe the exponential decay of the glycerol resonances down to ~10% of their initial intensity, but an equivalent decay of the PHB polymer signal could not be observed due to viscosity and molecular self-association of the polymers. In order to estimate the diffusion coefficients of the polymers, the samples were serially diluted to ~0.1 mg/0.3 mL $CDCl_3$, which enabled the measurement of the exponential decay of the PHB polymers down to ~10% of their initial intensities. It was not practical to dilute the polymers any further, as it did not provide enough NMR signal for diffusion measurements. The diffusion spectra were processed and analyzed using Varian VNMR 6.1C software and the DOSY ToolBox [37].

Results and Discussion

With the ever-increasing amount of biodiesel being produced worldwide, glycerol is also being produced at historic levels making it essential that new outlets be found to aid the biodiesel industry and make production costs competitive with petroleum products. It is well-known that glycerol can be utilized by many different microorganisms as a carbon substrate for growth. However, until recently little work was done to utilize glycerol as a substrate for the biological production of value-added products with potential industrial uses.

In this report we have concentrated on the utilization of refined and two different crude glycerol samples with widely varying compositions to demonstrate the possibilities for using glycerol in PHB synthesis. Initial studies were performed using refined glycerol (RG, Table 1) in order to minimize potential effects from the impurities in the crude glycerol samples (CG-G and CG-IS, Table 1). A 2% RG concentration was arbitrarily chosen to supply the bacterium with ample carbon source for growth and PHB production. Under batch culture conditions, the cell dry weight (CDW) and PHB_{RG} yields (polymer composition was determined by GC/MS as described in "Materials and Methods") reached a maximum of 3.0 and 1.14 g/L, respectively (Fig. 1a) giving a maximum cell productivity [(polymer yield ÷ CDW) × 100] of 38% after 72 h. However, only 45% of the glycerol was utilized to achieve these values (Fig. 1b) leaving 55% of the glycerol as unused and ultimately wasted. Based on these results it was determined that glycerol media concentration was not the limiting factor in causing bacterial growth and PHB_{RG} synthesis to plateau at 72 h and begin to decline thereafter. In an attempt to stimulate additional PHB_{RG} synthesis and utilize more of the glycerol in the media, a fed-batch procedure was instituted that was designed to replenish the salts in the media at 48 h post-inoculation without adding additional glycerol to the fermentation. The results of the fed-batch procedure showed that although PHB_{RG} synthesis was slightly improved (mostly through inhibiting polymer decline after 72 h), most of the effect was directed to an increased CDW to between 3.6 and 3.8 g/L from 72 to 96 h (Fig. 1a). This improvement resulted in a slight boost in glycerol utilization (52.5% utilized after 72 h) but still resulted in 47.5% of the starting glycerol as unutilized. At least one factor that may contribute to incomplete glycerol utilization is the intracellular breakdown of PHB by the organism. PHB is known to serve as a carbon and energy storage material for the producing strain. Figures 1a and 2a show decreasing PHB yields after 72 h which indicates that later in the fermentation the organism may begin to preferentially utilize the PHB polymer itself rather than the extracellular glycerol which would ultimately retard glycerol utilization. This drawback must be alleviated in order to maximize PHB production and glycerol use by this bacterial strain to a point where this process could be considered economically profitable.

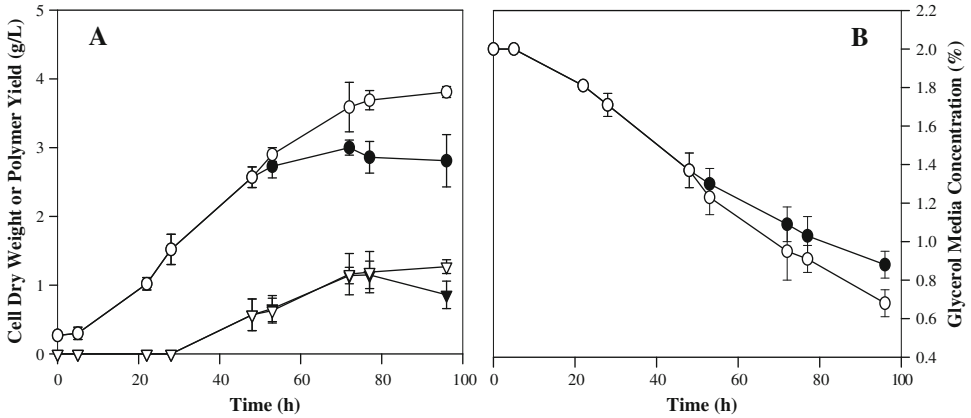


Fig. 1 Cell dry weight (CDW), PHB_{RG} yield (**a**) from *P. oleovorans* NRRL B-14682 grown on refined glycerol under batch and fed-batch culture conditions [CDW batch culture (*filled circles*), CDW fed-batch culture (*open circles*), PHB_{RG} yield batch culture (*inverted filled triangles*), PHB_{RG} yield fed-batch culture (*inverted triangles*)] and glycerol media concentration (**b**) as a function of time in the 10-L batch (*filled circles*) and fed-batch (*open circles*) cultures (starting glycerol concentration = 2%)

In the present study, since approximately half of the 2% (w/v) glycerol remained regardless of whether the fermentations were conducted under batch or fed-batch protocols, the RG media concentration was reduced to a starting concentration of 1% (w/v) in order to determine if PHB_{RG} yields could be maintained in batch culture when the initial glycerol concentration was halved. The results of these studies are seen in Fig. 2a. A comparison of the maximum CDW and PHB_{RG} yields from these batch cultures showed that the CDW and PHB_{RG} yields were comparable to those obtained with 2% glycerol and reached maximum values of 2.85 and 1.06 g/L (cell productivity = 37%), respectively at 72 h. However, glycerol utilization was more efficient starting at 1% (w/v). Figure 2b shows the comparative glycerol utilization under batch culture starting at 1 and 2% (w/v). It can be seen that the rate of utilization was approximately equal based on the parallel nature of the curves. However, in the 1% glycerol cultures at 72 h where maximum PHB_{RG} production occurred, 90% of the glycerol had been used leaving only 1 g/L unutilized (compared to 11 g/L after 72 h in the cultures with 2% initial glycerol concentration), a more efficient process resulting in less waste.

Transferring this procedure to crude glycerol (CG-G, Table 1) showed similar characteristics. The CDW and $PHB_{CG\text{-}G}$ yields reached a maximum of 2.54 and 0.80 g/L, respectively at 72 h (Fig. 3a) with a residual media glycerol concentration of 0.22% (Fig. 3b). In an attempt to further improve PHB polymer production efficiency, the aeration in the fermentation was increased from 2 to 3 SLPM and the CDW, $PHB_{CG\text{-}G}$ yield and glycerol utilization was monitored for the duration of the fermentations (96 h). It was determined that by increasing culture aeration $PHB_{CG\text{-}G}$ production efficiency was improved by reducing the time required to achieve maximum productivity by approximately 24 h yet maintaining comparable amounts of cell mass (2.68 g/L) and $PHB_{CG\text{-}G}$ yields (0.74 g/L; Fig. 3a). To do this, the bacterium utilized the glycerol at a comparatively faster rate when aeration was 3 SLPM but by reducing the time necessary to achieve maximal $PHB_{CG\text{-}G}$ synthesis, the total amount of glycerol utilized was also reduced and at 53 h 30% of the glycerol remained unutilized compared to 47% of the glycerol at 2 SLPM (Fig. 3b). Therefore, the tradeoff for a faster fermentation time is the utilization of slightly less glycerol, but on condition that the price of crude glycerol remains low (in the range of cents/lb), the energy and time saved outweighs the extra 8% [difference between residual glycerol for 3 SLPM at 53 h (30%) where maximum PHB yields occurred and residual glycerol for 2 SLPM at 72 h (22%) where maximum PHB yields occurred] of the glycerol that could be utilized in a fermentation with reduced aeration rates and longer durations.

The second crude glycerol sample (CG-IS, Table 1) contained an appreciable amount (40%) of MeOH. In fact, preparation of fermentation media containing 1% (w/v) glycerol from CG-IS resulted in a media MeOH concentration of 0.85% (w/v) as determined by HPLC (see "Materials and Methods"). This increase in MeOH resulted in a change in the PHB production characteristics by causing both the CDW and polymer production trends to plateau at 2.84 and 1.04 g/L, respectively at 48 h. While these values continued to improve slightly (0.006 g/L h for CDW and 0.004 g/L h for $PHB_{CG\text{-}IS}$ yield) up to the termination of the fermentation (96 h), there was no noticeable decrease in either of the aforementioned parameters as was evident with RG and CG-G (Fig. 4a). This plateau effect could be the result of a slower rate of utilization of glycerol in the presence of MeOH. In fact, at 48 h 61% (w/v) of the glycerol was unused in the fermentation

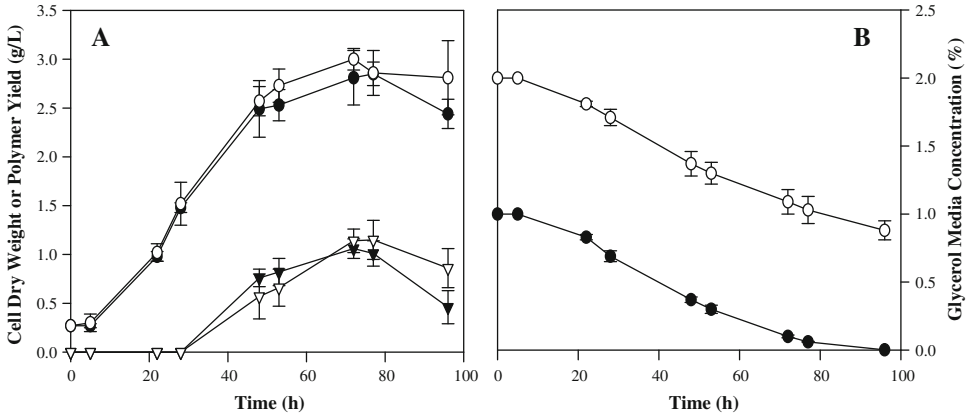


Fig. 2 Cell dry weight (CDW), PHB_{RG} yield (**a**) from *P. oleovorans* NRRL B-14682 grown in batch culture on 1 and 2% refined glycerol [CDW 1% glycerol (*filled circles*), CDW 2% glycerol (*open circles*), PHB_{RG} yield 1% glycerol (*inverted filled triangles*), PHB_{RG} yield 2% glycerol (*inverted triangles*)] and glycerol media concentration (**b**) as a function of time in the 10-L batch cultures containing 1% glycerol (*filled circle*) and 2% glycerol (*open circle*)

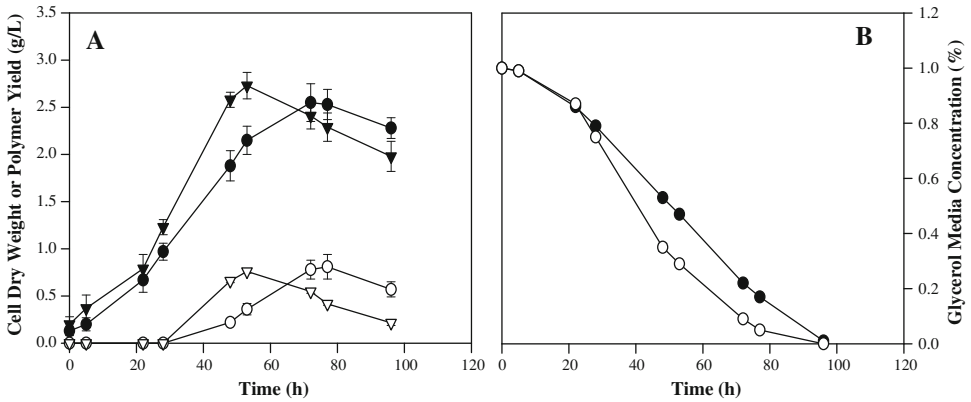


Fig. 3 Cell dry weight (CDW), $PHB_{CG\text{-}G}$ yield (**a**) from *P. oleovorans* NRRL B-14682 grown in batch culture on 1% CG-G (see Table 1) with aeration rates at 2 and 3 standard liters per minute (SLPM) [CDW; aeration = 2 SLPM (*filled circles*), CDW; aeration = 3 SLPM (*inverted filled triangles*), $PHB_{CG\text{-}G}$ yield; aeration = 2 SLPM (*open circles*), $PHB_{CG\text{-}G}$ yield; aeration = 3 SLPM (*inverted triangles*)] and glycerol media concentration (**b**) as a function of time in the 10-L batch cultures containing 1% CG-G with aeration rates at 2 SLPM (*filled circles*) and 3 SLPM (*open circles*)

(Fig. 4b). This was interesting considering that glycerol utilization in CG-G under identical fermentation conditions was utilized 38% faster at 48 h than the glycerol from CG-IS. Previous studies have shown that a transition from a carbon-excess to carbon-limited environment with yeasts, molds or bacteria can stimulate lipophilic compound degradation under appropriate growth conditions [38, 39] however; in the present case while increased MeOH concentrations in the fermentation media may play a role in decreasing the rate of glycerol utilization, CG-IS also provides a higher extracellular MONG (consisting of unrecovered alkyl esters and unreacted FFA, TAG, DAG, and MAG) media concentration whose free fatty acid and fatty acid ester components can also be metabolized by the bacterium. Because there is more carbon source (glycerol + MONGs) available for a longer period of time, $PHB_{CG\text{-}IS}$ production does not undergo the same decline as was seen with CG-G later in the fermentation. This could be a benefit for two reasons, (1) crude glycerol could be used to produce PHB without the need to remove MeOH, a benefit to polymer producers but perhaps not biodiesel producers, (2) the plateau effect would broaden the fermentation harvest time thus eliminating the need for stringent fermentation controls.

It was evident based on GC/MS analyses that all of the polymers produced from the three glycerol substrates were PHB. However, the molecular weights of the PHB produced from each substrate were noticeably different. Table 2 shows the molecular weights from the PHB derived from each of the glycerol substrates at 48, 72 and 96 h. It was seen that PHB_{RG} had the largest average molecular weights (~314,000 g/mol) followed by $PHB_{CG\text{-}G}$ (~261,000 g/mol) and then $PHB_{CG\text{-}IS}$ (~31,000 g/mol). Previous studies have shown that PHB produced in the

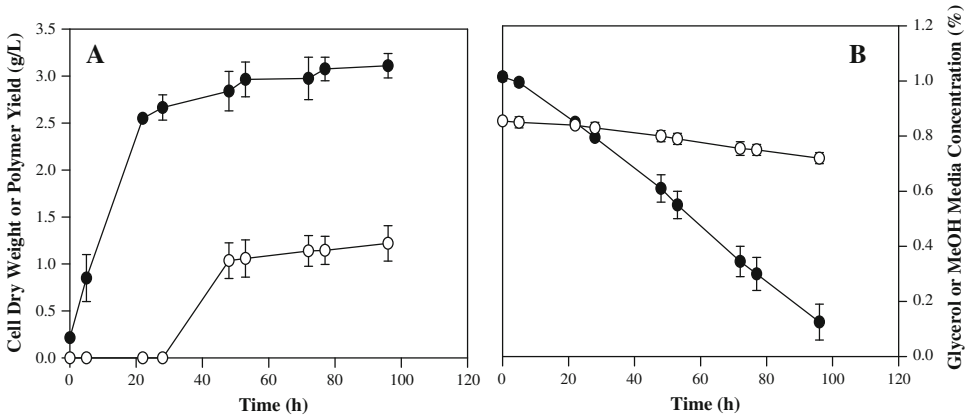


Fig. 4 Cell dry weight (*filled circles*), $PHB_{CG\text{-}IS}$ yield (*open circles*) (**a**) from *P. oleovorans* NRRL B-14682 grown in batch culture on 1% CG-IS (see Table 1), aeration at 3 SLPM and media concentration (**b**) of glycerol (*filled circles*) and methanol (*open circles*) as a function of time in the 10-L batch cultures containing 1% CG-IS with an aeration rate of 3 SLPM

Table 2 Molecular weight results for PHB polymers derived from *P. oleovorans* NRRL B-14682 grown on refined and crude glycerol

Sample[a]	M_n (g/mol × 1000)	M_w/M_n
Carbon source: refined glycerol		
48 h	308	1.62
72 h	299	1.77
96 h	336	1.59
	Average (x) = 314	Average (x) = 1.66
Carbon source: crude glycerol (CG-G)		
48 h	223	2.02
72 h	321	1.71
96 h	240	2.01
	Average (x) = 261	Average (x) = 1.91
Carbon source: crude glycerol (CG-IS)		
48 h	33	2.50
72 h	30	3.30
96 h	29	2.50
	Average (x) = 31	Average (x) = 2.77

[a] Samples are denoted by the fermentation duration post-inoculation. For example, the 48 h samples are PHB polymer samples obtained from the bacterial cell masses 48 h after inoculation etc.

presence of polyhydroxylated compounds such as glycerol and/or poly(ethylene glycol) (PEG) show decreased molecular weights due to premature chain termination via end-capping [24, 40–42]. It was therefore necessary to prove the existence of glycerol-based end-capping in the present study. This was accomplished by NMR using both one-dimensional ^1H spectra and two-dimensional DOSY (diffusion ordered spectroscopy), where the latter spectroscopically separated the solution components on the basis of their diffusion constants in $CDCl_3$. Figure 5a shows the 1D-^1H spectra and the 2D-DOSY spectra of the PHB_{RG} after 48 h, whereas Fig. 5b shows the corresponding spectra for PHB produced from oleic acid (PHB_{oleic}) mixed with refined glycerol at a nearly equal concentration and molar ratio as that found in PHB_{RG} prior to analysis. Whereas the 1D NMR spectrum of the PHB_{oleic} + glycerol mixed sample (Fig. 5b) had only a single peak arising from the free, refined glycerol (centered at ~3.6 ppm), the PHB_{RG} had multiple peaks in the range of ~3.5–4.6 ppm, which arise from the incorporation of the glycerol into the polymer. This same pattern was observed in the $PHB_{CG\text{-}G}$ and $PHB_{CG\text{-}IS}$ samples (data not shown).

Integration analysis of the 1D-^1H spectra revealed that ester linkages of alkyl groups (e.g., methoxy) were three times higher in the 48-h $PHB_{CG\text{-}G}$ sample, compared to the 48-h PHB_{RG} sample. The 48-h PHB_{RG}, on the other hand, had 40% more glycerol in the final product. The $PHB_{CG\text{-}IS}$ sample (for the 1D-^1H-NMR spectrum of $PHB_{CG\text{-}IS}$ see Fig. 6) had 3.8 times more glycerol-based end-capping than the PHB_{RG} sample and 100 times more methoxy-based end-capping than the $PHB_{CG\text{-}G}$ sample.

The diffusion NMR analysis (DOSY) of the highly concentrated PHB sample derived from oleic acid (PHB_{oleic}, produced in the absence of glycerol) clearly demonstrated that when free glycerol was mixed with PHB_{oleic}, its smaller size allowed it to diffuse at a much faster rate than that of the polymer with which it was mixed (diffusion constant, $D_{free\ glycerol}$ ~3×10^{-10} $m^2\ s^{-1}$ vs. $D_{PHBoleic}$ ~0.08×10^{-10} $m^2\ s^{-1}$; Fig. 5b). This is the expected behavior for a simple mixture in which there is neither covalent, nor non-covalent, association of the components. However, both the PHB polymer and the glycerol in the PHB_{RG} sample diffused at the same rate, indicating that they were moving as a complex ($D_{complex}$ ~0.3×10^{-10} $m^2\ s^{-1}$; Fig. 5a). Since the analysis of the simple mixed sample ruled out the possibility of non-covalent interactions, the concurrent diffusivities of PHB_{RG} and the glycerol in the PHB_{RG} sample can only arise from a covalent complex involving glycerol-based end-capping. The DOSY analysis of $PHB_{CG\text{-}G}$ showed similar results: both the glycerol moiety and the PHB polymer itself diffused at the same rates (D ~0.08×10^{-10} $m^2\ s^{-1}$). As noted in the "Materials and Methods", high concentrations were needed to observe the glycerol signal, but the resulting increase in viscosity and self-association of the polymers resulted in unreliable diffusion rates for the PHB polymers at high concentrations.

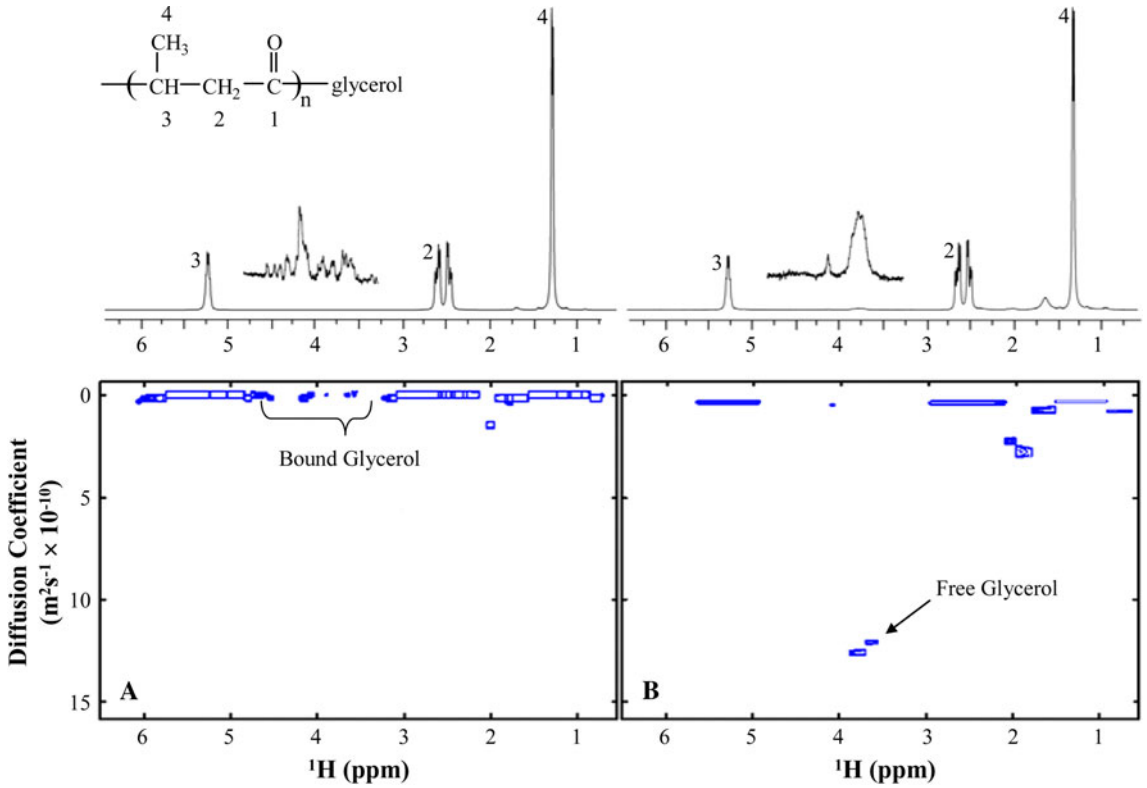


Fig. 5 One-dimensional ^{1}H NMR spectra and two-dimensional DOSY (diffusion ordered spectroscopy) for highly concentrated PHB_{CG-G} (**a**) and PHB_{oleic} + free glycerol (**b**)

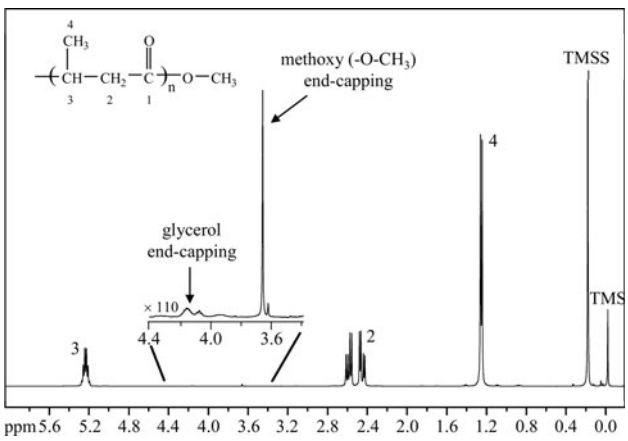


Fig. 6 One dimensional ^{1}H-NMR spectrum of PHB_{CG-IS}

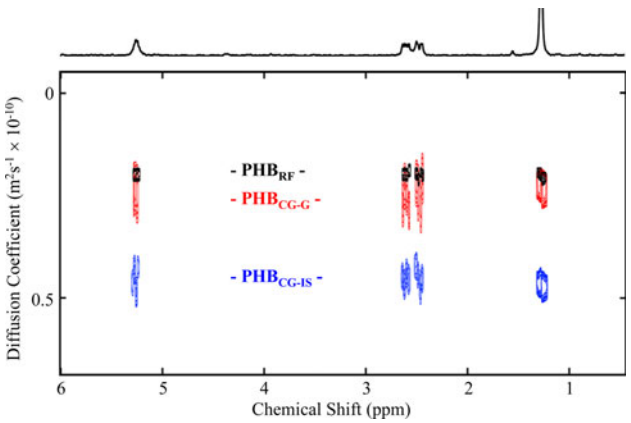


Fig. 7 Two-dimensional DOSY (diffusion ordered spectroscopy) spectra of dilute PHB_{RG}, PHB_{CG-G}, and PHB_{CG-IS}

The molecular diffusion coefficients of the polymers could be better estimated using much lower polymer concentrations (~0.1 mg/0.3 mL). The resulting diffusion constants were indicated such that PHB_{RG}, having the largest molecular weight, diffused the slowest (D_{PHB-RG} ~0.19 × 10^{-10} m^2 s^{-1}), followed closely by PHB_{CG-G} ($D_{PHB-CG-G}$ ~0.23 × 10^{-10} m^2 s^{-1}), while PHB_{CG-IS}, which had the smallest molecular weight, diffused appreciably faster ($D_{PHB-CG-IS}$ ~0.46 × 10^{-10} m^2 s^{-1}; Fig. 7). These observed trends in self-diffusion constants are consistent with the relative molecular weights, but experimental limitations prevent a more accurate analysis of the diffusion and molecular size (see "Materials and Methods").

In conclusion, it has been shown that glycerol; both in its refined and crude state can be utilized by *P. oleovorans* NRRL B-14682 to synthesize PHB polymers. The conditions of the fermentations that resulted in the most efficient production system in terms of PHB synthesis, glycerol utilization and fermentation time was a batch culture protocol with 1% (w/v) initial glycerol

concentration and aeration at 3 SLPM. These conditions resulted in maximum PHB yields of 1.0 ± 0.2 g/L after 48 h. Proton (^{1}H) NMR demonstrated the presence of glycerol- and methoxy-based end-capping, and was confirmed by ^{1}H diffusion experiments (DOSY analyses). The latter technique indicated that both end-capped PHB and the glycerol bound to it had the same diffusion constant indicating that they were moving together as a covalent complex. The possibility of non-covalent complexation was ruled out by physically mixing free glycerol with PHB_{oleic} and noting that their respective diffusivities were faster. The low molecular weight of the $PHB_{CG\text{-}IS}$ suggested that, although each glycerol sample caused premature chain termination through end-capping, the MeOH concentration in the crude glycerol may exacerbate chain termination by itself serving as a chain terminating agent. This was supported by the large concentration of methoxy linkages in the $PHB_{CG\text{-}IS}$ polymer. A smaller molecular weight will ultimately weaken the mechanical properties of the PHB polymers. Therefore, it is fortuitous that most biodiesel producers remove and recycle the methanol from the transesterification reaction prior to discarding the crude glycerol coproduct.

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References

1. Lin Y, Tanaka S (2006) Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 69:627–642
2. Taherzadeh M, Karimi K (2008) Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. Int J Mol Sci 9:1621–1651
3. Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25:294–306
4. Lardon L, Hélias A, Sialve B, Steyer JP, Bernard O (2009) Life-cycle assessment of biodiesel production from microalgae. Environ Sci Technol 43:6475–6481
5. Adamczak M, Bornscheuer UT, Bednarski W (2009) The application of biotechnological methods for the synthesis of biodiesel. Eur J Lipid Sci Technol 111:808–813
6. Papanikolaou S, Ruiz-Sanchez P, Pariset B, Blanchard F, Fick M (2000) High production of 1, 3 propanediol from industrial glycerol by a newly isolated *Clostridium butyricum* strain. J Biotechnol 77:191–208
7. Mu Y, Teng H, Zhang DJ, Wang W, Xiu ZL (2006) Microbial production of 1,3 propanediol by *Klebsiella pneumoniae* using crude glycerol from biodiesel preparations. Biotechnol Lett 28:1755–1759
8. Oh BR, Seo JW, Choi MH, Kim CH (2008) Optimization of culture conditions for 1,3-propanediol production from crude glycerol by *Klebsiella pneumoniae* using response surface methodology. Biotechnol Bioprocess Eng 13:666–670
9. Ito T, Nakashimada Y, Senba K, Matsui T, Nishio N (2005) Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. J Biosci Bioeng 100:260–265
10. Scholten E, Renz T, Thomas J (2009) Continuous cultivation approach for fermentative succinic acid production from crude glycerol by *Basfia succiniciproducens* DD1. Biotechnol Lett 31:1947–1951
11. Ashby RD, Nuñez A, Solaiman DKY, Foglia TA (2005) Sophorolipid biosynthesis from a biodiesel co-product stream. J Am Oil Chem Soc 82:625–630
12. Rywińska A, Rymowicz W (2009) Citric acid production from raw glycerol by *Yarrowia lipolytica* wratislavia 1.31. In: Aggelis G (ed) Microbial conversions of raw glycerol. Nova Science Publishers Inc, New York, pp 19–30
13. Papanikolaou S, Muniglia L, Chevalot I, Aggelis A, Marc I (2002) *Yarrowia lipolytica* as a potential producer of citric acid from raw glycerol. J Appl Microbiol 92:737–744
14. Papanikolaou S, Aggelis G (2009) Biotechnological valorization of biodiesel derived glycerol waste through production of single cell oil and citric acid by *Yarrowia lipolytica*. Lipid Technol 21:83–87
15. Huijberts GNM, Eggink G, de Waard P, Huisman GW, Witholt B (1992) *Pseudomonas putida* KT2442 cultivated on glucose accumulates poly(3-hydroxyalkanoates) consisting of saturated and unsaturated monomers. Appl Environ Microbiol 58:536–544
16. Eggink G, van der Wal H, Huijberts GNM, de Waard P (1993) Oleic acid as a substrate for poly-3-hydroxybutyrate formation in *Alcaligenes eutrophus* and *Pseudomonas putida*. Ind Crops Prod 1:157–163
17. de Smet MJ, Eggink G, Witholt B, Kingma J, Wynberg H (1983) Characterization of intracellular inclusions former by *Pseudomonas oleovorans* during growth on octane. J Bacteriol 154:870–878
18. Ashby RD, Foglia TA (1998) Poly(hydroxyalkanoate) biosynthesis from triglyceride substrates. Appl Microbiol Biotechnol 49:431–437
19. Solaiman DKY, Ashby RD, Foglia TA (2002) Physiological characterization and genetic engineering of *Pseudomonas corrugata* for medium-chain-length polyhydroxyalkanoates synthesis from triacylglycerols. Curr Microbiol 44:189–195
20. Solaiman DKY, Ashby RD, Hotchkiss AT, Foglia TA (2006) Biosynthesis of medium-chain-length poly(hydroxyalkanoates) from soy molasses. Biotechnol Lett 28:157–162
21. Ashby RD, Solaiman DKY, Foglia TA (2004) Bacterial poly(hydroxyalkanoate) polymer production from the biodiesel co-product stream. J Polym Environ 12:105–112
22. Cavalheiro JMBT, de Almeida MCMD, Grandfils C, da Fonseca MMR (2009) Poly(3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol. Proc Biochem 44:509–515
23. Shrivastav A, Mishra SK, Shethia B, Pancha I, Jain D, Mishra S (2010) Isolation of promising bacterial strains from soil and marine environment for polyhydroxyalkanoates (phas) production utilizing *Jatropha* biodiesel byproduct. Int J Biol Macromol 47:283–287
24. Zhu C, Nomura CT, Perrota J, Stipanovic AJ, Nakas JP (2010) Production and characterization of poly-3-hydroxybutyrate from biodiesel-glycerol by *Burkholderia cepacia* ATCC17759. Biotechnol Prog 26:424–430
25. Mothes G, Schnorpfeil C, Ackermann JU (2007) Production of PHB from crude glycerol. Eng Life Sci 7:475–479
26. Kawata Y, Aiba S (2010) Poly(3-hydroxybutyrate) production by isolated *Halomonas* sp. KM-1 using waste glycerol. Biosci Biotechnol Biochem 74:175–177
27. Koutinas AA, Xu YX, Wang R, Webb C (2007) Polyhydroxybutyrate production from a novel feedstock derived from

a wheat-based biorefinery. Enzyme Microb Technol 40: 1035–1044
28. Xu Y, Wang RH, Koutinas AA, Webb C (2010) Microbial biodegradable plastic production from a wheat-based biorefining strategy. Proc Biochem 45:153–163
29. Foglia TA, Jones KC (1997) Quantitation of neutral lipid mixtures using high performance liquid chromatography with light scattering detection. J Liq Chrom Relat Tech 20:1829–1838
30. Ashby RD, Solaiman DKY, Foglia TA, Liu CK (2001) Glucose/lipid mixed substrates as a means of controlling the properties of medium chain length poly(hydroxyalkanoates). Biomacromolecules 2:211–216
31. Brandl H, Gross RA, Lenz RW, Fuller RC (1988) *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl Environ Microbiol 54:1977–1982
32. Ashby RD, Foglia TA, Solaiman DKY, Liu CK, Nuñez A, Eggink G (2000) Viscoelastic properties of linseed oil-based medium chain length poly(hydroxyalkanoate) films: effects of epoxidation and curing. Int J Biol Macromol 27:355–361
33. Lee EY, Choi CY (1995) Gas chromatography–mass spectrometric analysis and its application to a screening procedure for novel bacterial polyhydroxyalkanoic acids containing long chain saturated and unsaturated monomers. J Ferm Bioeng 80:408–414
34. Pelta MD, Morris GA, Stchedroff MJ, Hammond S (2002) A one-shot sequence for high-resolution diffusion-ordered spectroscopy. Magn Reson Chem 40:S147–S152
35. Macchino A, Ciancaleoni G, Zuccaccia C, Zuccaccia D (2008) Determining accurate molecular sizes in solution through NMR diffusion spectroscopy. Chem Soc Rev 37:479–489
36. Price WS (1998) Pulsed-field gradient nuclear magnetic resonance as a tool for studying translational diffusion: part II. Experimental aspects. Concepts Magn Reson 10:197–237
37. Nilsson M (2009) The DOSY toolbox: a new tool for processing PFG NMR diffusion data. J Magn Reson 200:296–302
38. Alvarez HM, Kalscheuer R, Steinbuchel A (2000) Accumulation and mobilization of storage lipids by *Rhodococcus opacus* PD630 and *Rhodococcus ruber* NCIMB 40126. Appl Microbiol Biotechnol 54:218–223
39. Fakas S, Galiotou-Panayotou M, Papanikolaou S, Komaitis M, Aggelis G (2007) Compositional shifts in lipid fractions during lipid turnover in *Cunninghamella echinulata*. Enzyme Microb Technol 40:1321–1327
40. Ashby RD, Solaiman DKY, Foglia TA (2005) Synthesis of short-/medium-chain-length poly(hydroxyalkanoate) blends by mixed culture fermentation of glycerol. Biomacromolecules 6:2106–2112
41. Ashby RD, Solaiman DKY, Foglia TA (2002) Poly(ethylene glycol)-mediated molar mass control of short-chain and medium-chain-length poly(hydroxyalkanoates) from *Pseudomonas oleovorans*. Appl Microbiol Biotechnol 60:154–159
42. Ashby RD, Shi F, Gross RA (1997) Use of poly(ethylene glycol) to control the end group structure and molecular weight of poly(3-hydroxybutyrate) formed by *Alcaligenes latus* DSM 1122. Tetrahedron 53:15209–15223