

## Non-Edible *Vernonia galamensis* Oil and Mixed Bacterial Cultures for the Production of Polyhydroxyalkanoates

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### Abstract

Since the oil crisis of the 1970s much attempt has been made, albeit with varying degrees of success, to source the ideal substrate and bacteria for the production of PHA. The non-edible, naturally epoxidized seed oil from *Vernonia galamensis* and mixed cultures consisting of *Alcaligenes latus* (ATCC 29712), *Cupriavidus necator* (ATCC 17699), *Escherichia coli* (DH5 $\alpha$ ) and *Pseudomonas oleovorans* (ATCC 29347), were evaluated for PHA production under batch and fed-batch fermentations. PHA production, optimized by the mixed culture of *E. coli* and *C. necator*, was 0.4-19% (%wt/wt, cdw) for batch and fed-batch fermentations. Analyses of PHA by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) and Gas Chromatography Mass Spectrometry (GC/MS) identified the 3-hydroxybutyrate (3HB) monomeric unit. The PHA ester bond stretching vibration (C=O), was confirmed at absorption 1740.66 cm<sup>-1</sup>, using Fourier Transform Infrared Spectroscopy (FTIR). Gel Permeation Chromatography (GPC) indicated peak molecular weights between 3.8 $\times$ 10<sup>3</sup>-1.12 $\times$ 10<sup>6</sup> Da with melting points ( $T_m$ ), 60-90°C. The data further illustrates that inedible oils could be the ideal carbon source for the production of PHA.

**Keywords:** Polyhydroxyalkanoate; Mixed cultures; *Vernonia galamensis*; Batch/fed-batch fermentation; Mass spectrometry

### Introduction

The exponential growth of the human population compounded with increased consumption of non-renewable resources and pollution have reinvigorated the interest in polyhydroxyalkanoates (PHA), a class of bacterial biodegradable and biocompatible elastomers. PHA accumulates intracellularly as inclusion bodies with a diameter of 0.2 to 0.5  $\mu$ m, and may be utilized by bacteria from 75 genera as energy/carbon storage materials [1-4]. These materials may contain about 120 different (R)-3-hydroxy acid (3HA) monomeric units with the most common being poly(3-hydroxybutyrate), P(3HB). Blends of P(3HB) and other hydroxyacids are classified as copolymers, which may be short chain length (scl, 3 to 5 carbon atoms) or medium chain length (mcl, 6 to 16 carbon atoms). Scl-PHAs are often stiff and brittle, whereas, mcl-PHAs are elastomeric with lower crystallization. PHAs may have molecular weights ranging from 50 to 1000 kDa with a polydispersity (PD) of  $\approx$  2, and melting points ( $T_m$ ) of 60 to 180°C [5-7].

The current production of PHAs is primarily done with conventional substrates such as glucose and organic acids in conjunction with pure cultures. Although these substrates have generated PHA contents of 48 to 77% of cell dry weight (cdw), they are costly, edible and are therefore not competitive for industrial-scale production of PHA [8-11]. With high gross operating expenses and the resulting cost of PHA at ca. US\$ 16/kg versus polyethylene or polypropylene (US\$ 1/kg), there is an urgent need to develop and optimize strategies to economically produce the material [12].

The utilization of substrates such as plant oils may provide the solution since they contain more carbon atoms (compared to glucose), generate PHA with higher molecular masses and may produce a theoretical yield coefficient of 0.65 to 0.98 g PHA/g plant oil, versus 0.32-0.48 g PHA/g glucose [13-17]. Similarly, a high percentage of PHA has been generated using edible oils; therefore, non-edibles such as *Vernonia galamensis* oil may be a competitive alternative [14,18-20]. *V. galamensis* is a tropical hardy plant which requires low rainfall, marginal conditions, is widely available and can be cheaply produced [21]. The unique property of *V. galamensis* lies in the seed oil (35-42% of seed), which contain 72-80% low viscosity, vernolic acid (cis-12, 13

epoxy-cis-9-octadecenoic or 18:1 epoxy); and fatty acids: linoleic (12-14%), oleic (4 to 6%), steric (2 to 3%), palmitic (2 to 3%), and trace amount of arachidic acid [21]. Previously, 42.8 wt% P(3HB) and P(3HB-co-3HV) were produced from saponified *V. galamensis* under batch conditions using *Cupriavidus metallidurans* (formerly *Alcaligenes eutrophus*) [1].

The utilization of pure cultures for PHA production has been associated with several advantages and disadvantages. For example, *Cupriavidus necator* can utilize organic acids such as lactate, acetate and butyrate, but not glucose, fructose or xylose [22]. The bacterium produces PHA when grown on emulsified plant oil medium with gum arabic [23], and can generate a PHA concentration and content of 8.37 g/L and 39.52% respectively when grown on condensed corn substrates [24]. The isolate will accumulate PHB with mass range 6 $\times$ 10<sup>5</sup> to 1 $\times$ 10<sup>6</sup> Da [4]. Nevertheless, only a few isolates can utilize the unmodified triacylglycerides (TAG), and oils must therefore be saponified [25,26]. Unlike *C. necator*, *A. latus* is a growth associated producer of PHA [27], utilize sucrose and can produce a P(3HB) content and productivity of 98.7 g/L (83%) and 4.94 g P(3HB)/L/h respectively [28]. However, the performance of *A. latus* may be susceptible to extreme temperature, pH, carbon to nitrogen ratio in the feed, concentration of substrates, trace elements, ionic strength, agitation intensity and dissolved oxygen level [29,30]. Likewise, wild type *Pseudomonas oleovorans*, is the best characterized mcl-PHA producer; however, it does not utilize substrates such as fructose or glucose, but grows on substrates such as fatty acids

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or carbon sources which can undergo fatty acid *de novo* synthesis (n-alkanoic acids, n-alkanals and n-alkanes) [14,31-34]. *Pseudomonas* spp. could produce PHA with molecular weights in range  $5 \times 10^4$  to  $6 \times 10^4$  Da [35]. PHA can only be produced by recombinant *Escherichia coli* (transformed with the PHA operon). The isolate affords a rapid turnover and the production of 90% (wt/wt, cdw) [36,37].

To further maximize substrate usage and optimize PHA production, mixed cultures could be an ideal alternative. Mixed culture fermentations, involving two bacteria, are characterized by the conversion of a given substrate by one bacterium to an intermediate metabolite which is subsequently used by the other to generate PHA. For example, *Lactobacillus delbrueckii* and *Cupriavidus necator* can produce PHB concentrations of 12 g/L by converting glucose to lactate, and lactate to P(3HB) [38-42]. Mixed cultures may confer minimal costs, simpler facility construction, and easy recovery of material from wastes, higher growth rate of cells, decrease culture contamination and PHA accumulation  $\geq 62\%$  cdw [43-45].

In this study, we investigate the suitability of saponified *V. galamensis* oil and mixed microbial cultures under batch and fed-batch fermentations, for PHA production.

## Materials and Methods

### Microbial culture and media

Cultures of *A. latus* (ATCC 29712), *Cupriavidus necator* (formerly *R. eutropha*, ATCC 17699), and *P. oleovorans* (ATCC 29347) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and *E. coli* DH5 $\alpha$  (recombinant strain with PHA operon, from Genetic Stock Center (New Heaven, Ct, USA)). These bacteria were sub-cultured in Trypticase Soy Broth (TSB) and stock cultures of each maintained on trypticase soy agar (TSA) at 4°C. Media were prepared as outlined by the manufacturer.

The Mineral Salt Medium (MSM) used for PHA production was prepared as described elsewhere [38]. Briefly, the medium contained the following: 1.1 g (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 5.8 g K<sub>2</sub>HPO<sub>4</sub>, 3.7 g KH<sub>2</sub>PO<sub>4</sub>, 10 mL (100 mM) MgSO<sub>4</sub> solution, 1 mL microelement solution, and distilled water to make a final volume of one liter. The following were the constituents of the micronutrient solution (g/L): 2.78 g FeSO<sub>4</sub>·7H<sub>2</sub>O, 1.98 g MnCl<sub>2</sub>·4H<sub>2</sub>O, 2.81 g CoSO<sub>4</sub>·7H<sub>2</sub>O, 1.67 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.17 g CuCl<sub>2</sub>·2H<sub>2</sub>O and 0.29 g ZnSO<sub>4</sub>·7H<sub>2</sub>O. The medium was sterilized for 15 min at 121°C/15 psi, and stored at 4°C until required. Saponified *V. galamensis* oil (0.98 g/L) was utilized as the sole carbon source. Saponification of *V. galamensis* oil was done as mentioned elsewhere with modifications [1]. About 100 mL methanol and 4.95 g (0.124 mol) sodium hydroxide were added to a 500 mL round bottom flask equipped with a magnetic stirring bar and a water-jacketed condenser. The mixture was refluxed until the sodium hydroxide dissolved. A mass of 10 g of *V. galamensis* oil (0.011 moles, based on molecular weight of vernolic triacylglycerol) was added to the alkaline solution and refluxed with continuous stirring for 30 min. The resulting hot mixture was then slowly added to 50 g ice:50 g water and the resulting off-white semi-solid crushed, filtered and air dried.

### Batch/fed-batch fermentation, extraction and purification of PHA

The isolates *A. latus*, *C. necator*, *P. oleovorans* and *E. coli* DH5 $\alpha$  (recombinant strain with PHA operon) were utilized under batch and fed-batch fermentation. Under batch fermentation, aliquots of 100 mL Mineral Salt Medium (MSM) were inoculated with 5 mL of  $2 \times 10^8$  CFU/mL of each bacterium and fermentation conducted at 25°C, 120 rpm

for 96 h on a Lab-line Environ<sup>®</sup> incubator (USA) or a gyrotory shaker (New Brunswick, N.J., USA). Cells were harvested via centrifugation, lyophilized, weighed, and the polymer extracted and analyzed. Fed-batch fermentation was carried out as previously outlined for 24 h with subsequent additions of 20 mL MSM every 12 h for 96 h and, the resulting fermentation broths treated as before. All batch and fed batch fermentations were repeated five times. Furthermore, the effect of pH (4.5 and 8.5) and degree of agitation (90 and 240 rpm) on productivity was determined. Fermentations were carried out as mentioned previously under batch and fed-batch conditions and the resulting fermentation broths treated accordingly.

Throughout fermentations, 500  $\mu$ l broth was evaluated for PHA accumulation using Nile blue A and the hypochlorite assay [46-48]. Smears were stained with Nile blue A (1%) at 55°C for 10 min and slides washed with water and 8% acetic acid for 1 min then evaluated. Similarly, for the hypochlorite assay, a 200  $\mu$ l broth was mixed with 4.8 mL of 5.25% sodium hypochlorite (Clorox) then incubated at 38°C for 40 min. Lipids in solution were determined from the optical density at 436 nm.

Fermentation broths were centrifuged (Sorvall RC-5, Dupont Instruments, Newtown, CT) for 15 min at 15,000 rpm, 4°C and pellets re-suspended by washing once with 20 mL Tris-HCl buffer (pH 7.2). Pellets were lyophilized (-50°C and 25 mmHg) (LABCONCO Freeze Dryer 5, Kansas City, Missouri), and 100 mg refluxed with 100 mL chloroform for 3 h, then filtered (Whatman cellulose extraction thimble, 43×123 mm single thickness, Aldrich, Milwaukee, WI, USA). The resulting oily film was purified using methanol and chloroform and the precipitate dried and stored (-4°C) for further analysis.

The purity of the polymer was determined as mentioned previously [46]. Briefly, 0.1 mg polymer was mixed with 10 mL concentrated sulphuric acid (98%) and the mixture incubated at 97°C for 10 min. Optical density was measured between 220 to 250 nm, and melting point determined by using a Fisher Johns melting point apparatus (Fisher Scientific Company, USA).

### PHA analyses

The purified material was analyzed using Matrix Assisted Laser Desorption-Time of Flight Mass Spectrometry (MALDI-TOF MS), Gas Chromatography/Mass Spectrometry (GC/MS), Fourier Transform Infrared Spectroscopy (FTIR) and Gel Permeation Chromatography (GPC).

### MALDI-TOF MS

All samples (mixed and commercial) were base transesterified by reacting 1 mg sample with 100  $\mu$ l dichloromethane, vortexing for 1 min, and 4  $\mu$ l of 25% sodium methoxide in methanol and 5  $\mu$ l glacial acetic acid added. The reaction was allowed to proceed for 30 min. 2,5-dihydrobenzoic acid (2,5 DHB, 99%), the matrix, was prepared by dissolving 162 mg 2,5 DHB in 1 mL tetrahydrofuran (THF). Cationized oligomers were analyzed by mixing 20  $\mu$ l of the mixture with 50  $\mu$ l matrix, and applying 1  $\mu$ l to MALDI target (Bruker Reflex III MALDI-TOF mass spectrophotometer). Runs were performed with the following parameters: accelerating voltage, 20 kV; grid voltage, 56 to 99%; guide wire voltage 0 to 0.3%; positive high resolution reflective mode with summation of 150 transients. Three runs were performed for each sample.

### GC/MS

GC/MS analysis of samples was done by base transesterification

to their monomers as outlined before [1]. An Agilent Technologies 6890N Network GC System (CA, USA) interfaced directly to an Agilent Technologies 5973 Inert Mass Selective Detector was used to generate data. Electronic pressure through a capillary column was used to maintain a constant helium flow of 35 cm/s. The following parameters were included in the GC program: temperature was held at 50°C for 2 min then increased to 300°C at a rate of 20°C/min, then held at 300°C for a total of 14 min. The auxiliary temperature was set at 250°C and the column flow rate at 1.2. A solvent delay time of 4 min was applied to each sample. About 1 µl of the transesterified sample was injected into the GC/MS instrument and a Hewlett-Packard PC integration program used to calculate the peak areas and percentages of the monomeric components of the polymer. Data analysis was performed using the NIST02 database.

### Fourier Transform Infrared Spectroscopy (FT-IR)

FTIR (Perkin Elmer Spectrum 100, Version 6.3.2) was used for analysis of PHA. Samples for analysis were purified as mentioned before, dissolved in 100 µl chloroform and 20-30 µl added to sample crystal window. The following parameters were included in the instrument program: resolution, 4.0 cm<sup>-1</sup>, scan speed 0.2 cm/s, range 4000-650 cm<sup>-1</sup> and 4 scans for each run. Runs were done in duplicate.

### Gel Permeation Chromatography (GPC)

Gel permeation chromatography was used to ascertain the apparent number-average ( $M_n$ ), weight-average ( $M_w$ ), molecular masses and polydispersity (PD,  $M_w/M_n$ ) of the PHA. Samples (1 mg) were dissolved in 50:50 tetrahydrofuran (THF, HPLC grade) chloroform (HPLC grade), and filtered (0.2 µm). A Waters™ 2690 GPC separation module equipped with a Waters 2410 refractive detector and Polymer laboratories C-Linear mixed-bed size exclusion columns (2×300 mm/7.5 mm) was used. The degasser was set at continuous with a pressure of 0.5 psi, and the lines at 100°C. Polystyrene standards A with peak molecular weight range of 5460 to 96,000 and B, 2930 to 50,400 (Polyscience Corp. Warrington, PA, USA) with low polydispersity were used to generate a chromatogram and calibration curve. THF was used as the eluant at a flow rate of 0.7 mL/min at 35°C.

## Results

### Fermentation, extraction and purification of PHA

The resulting dry cell weight and PHA yield under standard conditions, variations in pH and dissolved oxygen, and the resulting melting points and peak molecular weights are shown in Table 1. Higher yields were observed under batch compared to fed-batch fermentations.

The Nile blue A and hypochlorite assays indicated the accumulation of PHA during fermentations. For the former assay, the characteristic

orange fluorescence was visible at a wavelength of 460 nm (data not shown). Furthermore, a maximum absorbance at 235 nm (data not shown), was shown for the crotonic acid assay of the purified PHA.

### PHA Analyses

**MALDI-TOF MS:** MALDI-TOF mass spectrometry was used to identify the oligomers resulting from base trans-esterification of mixed culture polymers (Figure 1). Generally, mass spectra showed mostly sodium [M+Na]<sup>+</sup> and potassium [M+K]<sup>+</sup> adducts attached to the oligomeric chains of the PHA. The mass difference between adjacent peaks was an average of 86 (Da) and indicated the presence of the PHA repeat unit [-OCH(CH<sub>3</sub>)CH<sub>2</sub>CO-]. These are clusters of isotopically resolved peaks of the same oligomer, but consisting of different end groups. The ion at  $m/z$  1156.58 is identified as a sodiated dodecamer with an olefinic end group i.e. [CH<sub>3</sub>CH=CHC(O)OCH<sub>3</sub>(CH<sub>3</sub>)CH<sub>2</sub>C(O)<sub>12</sub>OCH<sub>3</sub>-Na]<sup>+</sup>. The potassium adduct of this oligomer is shown at  $m/z$  1174.1.

**Gas chromatography/ mass spectrometry (GC/MS):** GC/MS was used for the identification of the monomeric/oligomeric composition of the isolated polymers. Overall, the gas chromatograms illustrate the various signature peaks for the methyl-β-hydroxybutyrate produced from base hydrolysis of the polymer (Figure 2). The electron impact mass spectrum shows ions at  $m/z$  85, 100 and 117 (+1). These were identified as {CH<sub>3</sub>CHCH<sub>2</sub>COO-}, {CH<sub>3</sub>CHCH<sub>2</sub>COOCH<sub>3</sub>} and {CH<sub>3</sub>CH[OH]CH<sub>2</sub>COOCH<sub>3</sub>} respectively. These signatures were identical to those produced from the base hydrolyzed commercial P(3HB) with average elution time of 6.6 min. Identity was confirmed by the NIST02 database.

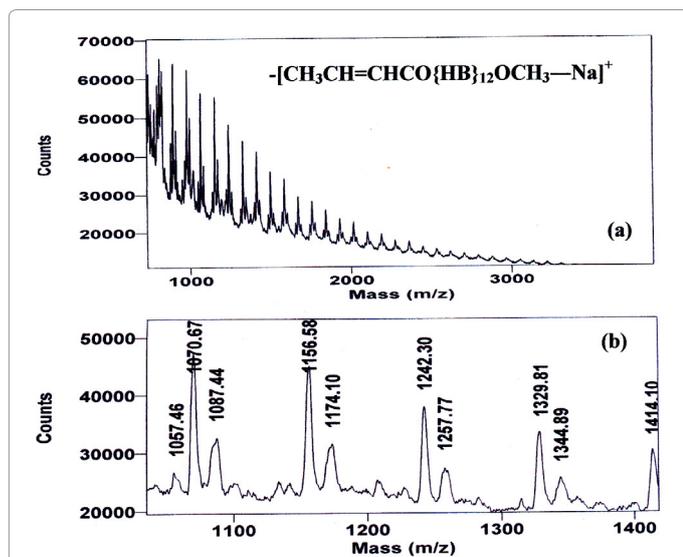
**Fourier Transform Infrared Spectroscopy (FTIR):** FTIR was utilized for the determination of functional and/or other groups found within PHA (Figure 3). The observed infrared absorption for the mixed cultures of *A. latus* and *Cupriavidus necator* were 3019.98 (=C-H and =CH<sub>2</sub>), 2917.41, 2849.44 (CH<sub>3</sub>, CH<sub>2</sub> and CH), 1740.66 (C=O); *P. oleovorans* and *Cupriavidus necator*, 2849.32 and 2917.26 (CH<sub>3</sub>, CH<sub>2</sub>, CH), 1261.41 (O-C); and pure cultures of *P. oleovorans*, 3019.83 (=C-H and =CH<sub>2</sub>), 2921.43, 2850.85 (CH<sub>3</sub>, CH<sub>2</sub> and CH), 1728.85 (C=O), 1459.24 (CH<sub>2</sub>, CH<sub>3</sub>); *Cupriavidus necator*, 2849.89, 2918.07 (CH<sub>3</sub>, CH<sub>2</sub> and CH), 1740.66 (C=O), 1261.14 (O-C) and 1017.89 (-OH). The FTIR spectrum for the commercial P(3HB), (-COCH<sub>2</sub>CHCH<sub>3</sub>)<sub>n</sub>, identified infrared absorption at cm<sup>-1</sup> 3020.12 (straight, =CH), 1724.07 (saturated aldehyde, C=O), 1281.25 (acids straight, O-C), 1133.04, 1057.16, 980.40 (OH).

**Gel-Permeation Chromatography (GPC):** GPC was used to determine the weight number average mass ( $M_n$ ), peak molecular weight (MP), molecular weight ( $M_w$ ) and polydispersity ( $M_w/M_n$ ) of the polymer produced from saponified *V. galamensis* oil (Table 1).

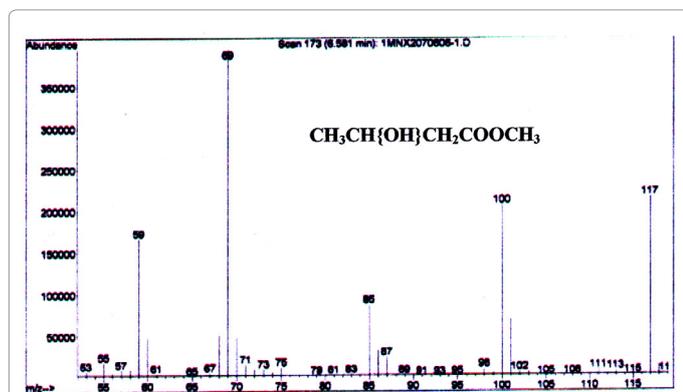
	<i>A. latus</i> + <i>C. necator</i> cdw(mg/L) PHA(%wt/wt, cdw)		<i>P. oleovorans</i> + <i>C. necator</i> cdw(mg/L) PHA(%wt/wt, cdw)		<i>E. coli</i> + <i>C. necator</i> cdw(mg/L) PHA(%wt/wt, cdw)	
pH 7	920/820	11/12	1160/860	10.3/11.6	1320/620	19/16
pH4	382/520	7.8/3	264/460	2.3/2.2	286/560	0.3/2.7
pH8	496/500	2/2	536/560	2.6/2.3	444/540	2.7/18
DO 90	240/240	2/3	280/320	3.2/2.2	180/320	3.3/3
DO 240	302/220	0.5/0.4	18/10	6/10	304/280	1.6/1.7
<sup>d</sup> PM (Da)	1.2 × 10 <sup>5</sup>		3.7 × 10 <sup>5</sup>		3.9 × 10 <sup>4</sup>	
<sup>e</sup> T <sub>m</sub> (°C)	80-90		61-65		61-70	

<sup>a</sup>Culture, *A. latus* (ATCC 29712), *C. necator* (ATCC 17699), *E. coli* (DH5α) and *P. oleovorans* (ATCC 29347); <sup>b</sup>batch fermentation, cell dry weight, cdw; <sup>c</sup>Fedbatch fermentation; <sup>d</sup>PM(Da), Peak molecular weight in Daltons; <sup>e</sup>T<sub>m</sub>(°C), melting temperature of the polymer in Celsius. Polystyrene standards A, with molecular weight 1300-377,400 Da, and standard B, with molecular weight 500-210,500 Da, were used to derive standard curve

**Table 1:** Yields and properties for PHA produced by mixed bacterial cultures grown on saponified *V. galamensis* oil under batch and fed-batch fermentation.



**Figure 1:** (a) Representative positive ion MALDI-TOF mass spectra of partially transesterified saponified *V. galamensis* oil PHA produced by mixed culture of *Escherichia coli* and *Ralstonia eutropha* (b) Expanded view of 'a' between  $m/z$  1057 and 1400. Peak at  $m/z$  1156.58 indicate the sodiated 3-HB oligomer  $m/z$  1156.58  $[\text{CH}_3\text{CH}=\text{CHCO}\{\text{HB}\}_{12}\text{OCH}_3-\text{Na}^+$  and the potassium adduct of this oligomer is shown at 1174.10  $[\text{CH}_3\text{CH}=\text{CHCO}\{\text{HB}\}_{12}\text{OCH}_3-\text{K}^+$ . Runs were done in reflection mode with 2,5-dihydrobenzoic acid (2,5 DHB, 99%) and 120 transients. Analysis was accomplished using a Bruker Reflex III MALDI-TOF mass spectrophotometer with accelerating voltage, 20 kV, positive high resolution reflective mode with 150 transients.



**Figure 2:** Electron-impact mass spectrum of trans esterified polymer produced by mixed culture cultivated on saponified *Vernonia galamensis* oil. The methylester of hydroxybutyric acid is indicated by the ion at  $m/z$  117  $[\text{CH}_3\text{CH}(\text{OH})\text{CH}_2\text{COOCH}_3]$ . The ions at  $m/z$  100  $[\text{CH}_3\text{CHCH}_2\text{COOCH}_3]$  and 86  $[\text{CH}_3\text{CHCH}_2\text{COO}-]$  are fragment from the ion at  $m/z$  117. Data was ascertained using an Agilent Technologies 6890N Network GC System (CA, USA) interfaced directly to an Agilent Technologies 5973 Inert Mass Selective Detector with program: temperature was held at 50°C for 2 min then increased to 300°C at a rate of 20°C/min, then held at 300°C for a total of 14 min. The auxiliary temperature was set at 250°C and the column flow rate at 1.2.

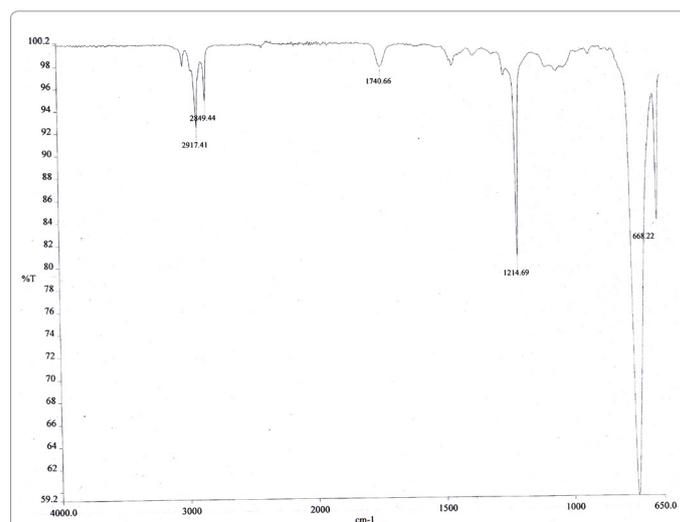
The peak molecular weights for polystyrene standard A ranged from 1300 to 377 400 Da while that of standard B ranged from 580 to 210 500 Da were automatically used to generate a standard curve (data not shown). Analysis of all chromatogram illustrates peak molecular weight between  $3.8 \times 10^3$ - $1.12 \times 10^6$  Da.

## Discussion

In the current study, saponified *V. galamensis* oil was utilized by

mixed bacterial cultures under batch and fed-batch fermentations to produce 19% (wt/wt, cdw) PHA with peak molecular weight in range  $10^3$  to  $10^6$  Da. This yield shows some improvement over previous studies wherein between 5.7 to 34.4% PHA was produced by pure culture of *Pseudomonas spp.* cultivated on rice, canola, sunflower, soybean, corn and hydrolyzed linseed oils [6,18]. Polymer accumulation, indicated by Nile blue A (Nile blue sulphate, basic blue) is consistent with previous reports and was a good indicator since stained PHA granules are easily identifiable by their characteristic orange fluorescence [16,47]. Neutral lipids do not affect identification of PHA since these lipids are liquids at the staining temperature (55°C) and have no affinity for Nile red (the oxidized form of Nile blue); therefore, cell membranes and other lipid containing cell components do not absorb enough dye to yield a detectable fluorescence at a wavelength of 460 nm [46]. Although this data was supported by the hypochlorite assay, the assay has been associated with several disadvantages which include severe degradation of polymer during digestion; only polymer in the native form of the lipid can be measured; the temperature and incubation time of the cell/hypochlorite mix must be precisely controlled and the suspension must be homogeneous [49-52].

Nevertheless, the methods were good predictors for the optimal points for PHA accumulation and cell dry weight (cdw). The resultant cdw was inversely proportional to PHA yield under both batch and fed-batch fermentation for all mixed cultures, and was dependent on *Cupriavidus necator* which is known to accumulate PHA when nutrients such as nitrogen and phosphorus are completely depleted from the environs [44]. The resulting lower PHA contents under batch and fed-batch modes indicates that bacterial growth rate under alkaline conditions is characterized by a logarithmic increase in viable cells [50]; therefore, an initial pH of 6.0 to 7.5 is ideal for microbial growth [52]. Similarly, variations in agitation had a significant effect on the growth of all mixed cultures and subsequently the PHA yields under batch and fed-batch fermentations. This further confirms the observation that high bacteria growth was inversely proportional to PHA yield. The consumption of oxygen is related to  $\text{CO}_2$  production and ATP generation; therefore, manipulating the availability of oxygen



**Figure 3:** Representative Fourier Transform Infrared Radiation (FTIR) spectrum of PHA, produced by mixed culture of *A. latus* (ATCC 29712) + *R. eutropha* (ATCC 17699), cultured with saponified *Vernonia galamensis* oil as sole carbon source. The following parameters were included in the instrument program: resolution, 4.0  $\text{cm}^{-1}$ , scan speed 0.2  $\text{cm}/\text{s}$ , range 4000-650  $\text{cm}^{-1}$  and 4 scans for each run. Runs were done in duplicate at RTP.

will contribute to overall fermentation yields i.e. PHB concentration, content and productivity, but not cell concentration [53,54]. Oxygen limitation (which can be generated by increasing the rpm) was previously shown to increase the PHB contents in fed-batch cultures containing recombinant *E. coli* with a PHB content of 80% [55]. Likewise, a cdw of 54 g/L (46% PHB) was produced by *Azotobacter chroococcum* under oxygen limitation, and 71 g/L dcw (20% PHB) under non-limiting oxygen conditions [56]. Deficiencies in oxygen, i.e., low oxidative capacity, could increase the rate of P(3HB) production in *Cupriavidus necator* and other microbes [53]. Assimilative activities such as protein and glycogen synthesis, and other cellular components are enhanced when oxygen supply is sufficient; however, if oxygen supply is curtailed, such activities will be minimized, thereby, contributing to increase production of PHA [5]. Suppression of assimilative activities contributes to the accumulation of NADH which inhibits the enzymes citrate synthase and isocitrate dehydrogenase in the tricarboxylic acid cycle (TCA) [35]. As a result, acetyl-CoA no longer enters the TCA cycle at the same rate and is instead converted to acetoacetyl-CoA by 3-ketothiolase. Therefore, increasing agitation speeds will limit the dissolved oxygen in the fermentor and contributes to increased PHB concentration, content and productivity, but not cell concentration [55].

The identity and purity of the extracted polymeric material was initially confirmed by spectrometric measurement (235 nm) of the  $\alpha$ ,  $\beta$ -unsaturated bond of crotonic acid (2-butenic acid), produced by the reaction of PHA with sulphuric acid [46]. Further analyses indicated  $T_m$  between 61 to 90°C for all mixed culture PHA, compared to that derived from commercial material, 135 to 150°C. It has been suggested that the usual  $T_m$  for a PHA consisting primarily of the homopolymer, P(3HB), occurs between 174 to 179°C; however, this decreases dramatically if the homopolymer is coupled with varying percentages of other hydroxyacids such as hydroxyvalerate (HV), hydroxyhexanoate (HHx), among others [4,35]. For example, a copolymer containing 30 mol% 3HV had a  $T_m$  of 143°C [21,39]; and  $T_m$  range of 163 to 174°C (maximum at 172°C), for the copolymer, P(3HB-co-1mol% HV), produced using *C. necator* on saponified *V. galamensis* oil [1].

Further analysis of mixed culture PHA using MALDI-TOF MS identified only the HB oligomeric unit at  $m/z$  1156.40  $[\text{CH}_3\text{CH}=\text{CHCO}\{\text{HB}\}_{12}\text{OCH}_3\text{-Na}]^+$  with isotopic fragments containing sodium and potassium adducts at 1174.1  $[\text{CH}_3\text{CH}=\text{CHCO}\{\text{HB}\}_{12}\text{OCH}_3\text{-K}]^+$  respectively. This observation was further confirmed by GC/MS i.e., only the 3-HB oligomer was identified in the polymer. This finding is unusual, since *P. oleovorans* is a known producer of medium chain length PHA. Thus, copolymers, if produced, may have been utilized by the isolate for growth and/or concentrations were beyond the threshold of the instrument. FTIR identified the PHA marker absorption band at 1740.66  $\text{cm}^{-1}$  i.e., the carbonyl (C=O) ester bond stretching vibration [57]. However, absorption at 1740.66  $\text{cm}^{-1}$  and 1738.28 were previously shown to be representative of medium chain length hydroxyacids (mclHA), while the absorption at 1728.85 and 1724.07  $\text{cm}^{-1}$ , observed for the commercial homopolymer (HB) in this study, for the HB unit and was similar to the previous study [58]. The evaluation of polymers by GPC indicated a peak molecular weight range of  $1.2 \times 10^5$  to  $1.12 \times 10^6$  Da. It was not possible to determine the uniformity of the polymers, since their polydispersities were not generated by the instrument. Other fragments eluted above 20 min had peak molecular weights in range 19 to 1000 Da and polydispersities between 1 and 1.4. Although immediate evidence is lacking, these fragments may be impurities, the lower molecular weight polymer called complexed PHA or other fragments from polymer

deterioration. The environmental conditions, method of isolation and the microbe used for fermentation, can adversely affect the molecular weight of polymers [35]. For example, *Cupriavidus necator* has been shown to accumulate PHB with peak molecular weight in the range  $6 \times 10^5$  to  $1 \times 10^6$  Da, while *Pseudomonas* spp.  $5 \times 10^4$  to  $6 \times 10^4$  Da [5,36]. The biosynthesis of mcl-PHA or other PHA copolymers is problematic for *E. coli*, due to the difficulty of drawing PHA precursors from the fatty acid metabolic pathways. Nevertheless, the current data suggests that the molecular weight of resultant polymers was dependent on bacteria used and culture conditions.

The current data illustrate that non-edible oils such as *V. galamensis* and mixed bacterial cultures could be optimized to generate PHAs with ideal melting points and molecular weights. Further studies are underway to evaluate other non-edible oils with these mixed cultures.

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