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Production of poly-(β-hydroxybutyrate) from saponified *Vernonia galamensis* oil by *Alcaligenes eutrophus*

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Saponified vernonia oil was converted exclusively to poly(β-hydroxybutyrate) (PHB) by *Alcaligenes eutrophus* in a single-stage batch culture. After harvesting, centrifugation followed by lyophilization, the resulting dried cells contained up to 42.8 wt% PHB having a peak molecular mass of 381 863 Da, weight-average molecular mass of 308 390 Da, and a polydispersity of 1.1. The PHB had a melting point (T_m) range of 163–174°C with a maximum at 172°C (lit. T_m, 175°C), and heat of fusion of 18.43 cal g^{-1}. Fermentation performed under varying conditions of nitrogen limitation indicated that there was no significant effect of nitrogen concentration on the molecular mass of PHB produced from vernonia oil by *A. eutrophus*.

Keywords: *Alcaligenes eutrophus*; biodegradable plastics; poly(β-hydroxybutyrate); vegetable oil; *Vernonia galamensis*; vernolic acid

Introduction

Current and growing concern about the environmental fate of plastics and polymeric materials has generated much interest in the development of biodegradable and biocompatible materials. Thus, various investigations have focused on the biosyntheses of a class of polyesters, known as poly(β-hydroxyalkanoates) (PHAs). PHAs are naturally occurring, optically active polyesters that are produced metabolically from the bioconversion of alkanes and alkanoic acids by a number of bacterial strains [9,15,17]. Functioning as fat does in mammals, the PHAs accumulate as inclusion bodies in bacteria as a result of a nutrient imbalance that occurs when the environment contains an excess of carbon and energy sources [7,16]. Bacterial strains that are known to accumulate PHAs include: *Pseudomonas oleovorans*, *Alcaligenes eutrophus*, *Bacillus megaterium*, *Rhodospirullum rubrum*, *Pseudomonas extorquens*, and *Pseudomonas cepacia*.

Depending on the bacterial strain and the nature of the carbon source, PHAs with varied alkyl chain length may be produced. For example, *Pseudomonas oleovorans* can produce longer-chain PHAs when grown on alkanoic acids that contain six or more carbon atoms [7]. *Alcaligenes eutrophus* is capable of producing both the most common PHA, poly-(β-hydroxybutyrate) (PHB) using glucose; and the copolymer of PHB and poly(β-hydroxyvalerate) (PHV) using glucose and propanoic acid [8,10].

PHB and the copolymer PHB-co-PHV have attracted much attention primarily because of their biodegradability and biocompatibility. They are degraded by bacteria, fungi, and other components of the physiological environment [13]. The soil bacterium *Pseudomonas lemoignei* is capable of degrading PHB by producing extra-cellular depolymerases that hydrolyze PHB to hydroxybutyrate and its dimeric acid; and an intracellular hydrolase that degrades the dimer [5]. Additionally, PHB and PHB-co-PHV have good thermoplastic properties. PHB is often compared to polypropylene because of its similar physical properties [13]. They differ in that PHB is too brittle for most applications, with an extension-to-break ratio nearly two orders of magnitude less than that of polypropylene [13]. However, PHB-co-PHV is more flexible, and thus, more desirable for most applications.

Monsanto (St Louis, MO, USA) manufactures PHB and PHB-co-PHV on a scale of metric tons, and the material is marketed under the tradename BIOPOL, and used in cosmetic bottles, packaging for personal-care products and motor oils. Furthermore, there are potential applications in biomedical devices [13]. However, despite the obvious ecological advantage of the use of PHB and PHB-co-PHV, their widespread commercial use is being handicapped by production cost. For example, in 1995, the wholesale price of the PHAs in Europe was about US $17.00 per kg, and future cost projection is estimated at US $5.00/kg [12,13]. Such a price is not competitive with petrochemical-based plastics, which are currently about US $1.00/kg [13]. Thus, because of the need to reduce production cost of PHAs, two approaches are being investigated by various research groups. In one approach, MONSANTO recently purchased Zeneca Bio Products in order to explore the expression of PHB biosynthetic genes in oilseed crops such as rapeseed and soybean [13]. The second approach is to reduce the production cost of the bacterial fermentation process. One of several means of achieving the latter goal is to increase the efficiency of the fermentation process using readily available and inexpensive carbon sources or developing novel processes. For instance, with vegetable oils valued at about US $0.60/kg, if the fermentation process can achieve an efficiency of 2.5 kg carbon source to 1.0 kg PHA, then the economics might become more attractive, particularly if this is coupled with increased demand to reduce plastic-waste pollution of the environment.

In the search for novel and relatively more efficient car-

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bon substrates, recent investigations have focused on the use of triglyceride oils and derivatives. Cromwick and coworkers [6], using *Pseudomonas* spp reported on the bacterial conversion of tallow acids (mixture of C16–C18:1) to PHAs copolymers in which the monomer units consist mostly of C6–C14:1. In another study with *Pseudomonas aeruginosa*, and using castor oil (containing 12-hydroxy-*cis*-9-octadecenoate, ricinoleate), and euphorbia oil (containing *cis*-12,13-epoxy-*cis*-9-octadecenoate, vernolate) as sole carbon sources, Eggink and coworkers [9] reported on the isolation of complex PHA copolymers. With euphorbia oil, the PHA copolymers consist of monomeric units ranging from C14–C18 in which the epoxy functionality is still present. On the other hand, Akiyama and coworkers [1], using an *Alcaligenes* strain, reported on the production of PHB and PHB-co-PHV when vegetable oils and long-chain fatty acids were used as carbon sources. They demonstrated the ability of long-chain fatty acids (C6–C22) to generate short-chain PHAs using *Alcaligenes eutrophus*. They also concluded that fatty acids with even-numbered carbons yielded PHB homopolymer, while those with odd-numbered carbons gave PHB-co-PHV. This finding was consistent with the results of Doi *et al* [8] who established that *Alcaligenes eutrophus*, when fed butyric acid gave PHB homopolymer, while pentanoic acid produced PHB-co-PHV.

In an effort to study further the feasibility of using a naturally epoxidized fatty acid for the biosynthesis of PHAs, we used vernonia oil (VO) and derivatives as carbon substrate for *Alcaligenes eutrophus*. Hydrolyzed vernonia oil contains 80% vernolic (*cis*-12,13-epoxy-*cis*-9-octadecenoic) acid, 12% linoleic acid, 4% oleic acid, 2% stearic acid, and 2% palmitic acid [4]. VO is a naturally expoxidized triglyceride oil from the seed of *Vernonia galamensis*, a new industrial crop that is currently cultivated in Zimbabwe, Kenya and Ethiopia. Numerous studies have demonstrated the potential applications of VO in commercial products ranging from plastics to paints because of its unique chemistry [3]. We recently demonstrated the ability of the oil to promote growth of *Acinetobacter* and *Pseudomonas* [14]. However, in the study there was no attempt to isolate any bioconversion product. Thus, in the present communication, we report the bioconversion of base-hydrolyzed vernonia oil to poly-(β-hydroxybutyrate) by *Alcaligenes eutrophus*.

Materials and methods

Crude VO was purchased from IXTT Corporation (Culver, IN, USA). An authentic sample of poly-(β-hydroxybutyrate) was obtained from Sigma Chemical Company (St Louis, MO, USA). GC/MS analysis of transesterified PHB was performed with an HP MS Engine 5989A interfaced with HP 5890 Series II gas chromatograph (Hewlett Packard Co, Roseville, CA, USA). The interface line was maintained at 300°C, and the ionizer temperature was set at 200°C, operating the mass spectrometer in the electron impact (EI) mode with electron energy at 70 eV. High-resolution capillary gas chromatography was conducted with a Supelco fused-silica SPB-1 (30 m, 0.32 mm i.d., 0.25 μm film) column (Bellefonte, PA, USA), oven temperature was programmed from 40°C to 300°C at 12°C min^{-1}, and helium was used as the carrier gas with a head pressure of 3 psi. Injector temperature was set at 240°C. ^{13}C nuclear magnetic resonance (cnmr) and proton nuclear magnetic resonance (pnmr) spectra were recorded on a GE NMR model QE-300 spectrometer (Fremont, CA, USA), with chloroform-d ($CDCl_3$) as solvent and internal standard. Desorption chemical ionization/mass spectrometry (DCI/MS) was performed with a sector mass spectrometer (JEOL JMS-HX 110, Tokyo, Japan). The melting point (T_m) temperatures and heat of fusion (H_m) were measured on a Perkin Elmer DSC 7. Typically, about 1 mg of sample was heated from 20°C to 200°C, then cooled, and scanned a second time using the same conditions as the first scan.

Saponification of VO

A 500-ml round-bottomed flask, equipped with a magnetic stirring bar, was charged with 100 ml methanol, and 4.95 g (0.124 mol) sodium hydroxide. The flask was then equipped with a water-condenser, and the mixture was heated to reflux until the sodium hydroxide had dissolved. To the hot alkaline solution was added 20.02 g of VO (0.022 mol, based on MW 926 for the vernolic acid triacylglycerol). The resulting brownish solution was refluxed with continuous stirring for 30 min, after which the hot mixture was slowly transferred into a beaker that contained about 50 g water and 50 g ice. After about 10 min, the resulting semi-solid was broken into fine particles in the ice/water (5°C), vacuum filtered, and then air-dried to afford an off-white solid (19.93 g, 95.0% yield based on sodium vernolate). This vernonia-oil soap was ground into a powder, and subsequently used to prepare a 3-mM stock solution that was used as the source of carbon in the culture medium.

Bacterial strain and culture conditions

Alcaligenes eutrophus strain 17699 was obtained from the American Type Culture Collection (ATCC), and was subcultured in trypticase soy broth. Stock cultures were maintained on trypticase soy agar at 4°C with transfer every 14 days. The fermentation medium was a slight modification of the E medium [11] containing: 1.1 g $(NH_4)_2HPO_4$, 5.8 g K_2HPO_4, 3.7 g KH_2PO_4, 10 ml of a 100-mM $MgSO_4$ solution, 1 ml of a microelement solution, and distilled water to make a final volume of one liter. The microelement solution consists of (g L^{-1} of 1 N HCl): 2.78 g $FeSO_4 \cdot 7H_2O$, 1.98 g $MnCl_2 \cdot 4H_2O$, 2.81 g $CoSO_4 \cdot 7H_2O$, 1.67 g $CaCl_2 \cdot 2H_2O$, 0.17 g $CuCl_2 \cdot 2H_2O$, 0.29 g $ZnSO_4 \cdot 7H_2O$. A stock solution containing 0.96 g vernonia-oil soap was added as the carbon source and the medium was autoclaved. After autoclaving, the medium was inoculated with 1.0×10^6 CFU. Fermentation was carried out in batch culture at 24°C in a temperature-controlled shaker at 120 rpm for 7 days. Cell growth was determined by standard plate count and dry-weight determination, while PHB synthesis was monitored by both phase-contrast and electron microscopy.

Isolation of biomass

Afer the 7-day growth and polymer storage phases, cells were harvested by centrifugation at 4°C (16 300 × *g*). The resulting pellet was resuspended and washed once with 10 mM tris-hydrochloride buffer (pH 7.0). The harvested cells were then lyophilized and transferred into a screw-cap scintillation vial, and stored in a freezer at −18°C until further processing. The biomass of the lyophilized cells was determined gravimetrically.

Extraction of PHB from biomass

The biomass (*ca* 200 mg) was suspended in 200 ml refluxing chloroform for a 20-h period, after which the hot mixture was filtered through a Whatman cellulose extraction thimble (Aldrich, Milwaukee, WI, USA). The chloroform was then evaporated to give a translucent film. The polymeric film was then washed 3× with 25 ml methanol. The resulting polymer was allowed to dry prior to gravimetric analysis. The PHB was then redissolved in 15 ml chloroform, subsequently evaporating the chloroform to give a transparent polymeric film of PHB.

Transesterification of PHB

Both the PHB produced in our laboratory and the commercial PHB were similarly transesterified. A 5-mg sample of the PHB was transferred into an 8-ml glass vial, and then 0.5 ml dichloromethane was added to dissolve the polymer. To the solution was added 20 μl 25 wt% sodium methoxide in methanol, mixing the suspension for about 1 min, after which 25 μl glacial acetic acid was added, and the mixture was shaken for an additional 1 min. One microliter of this sample was injected in the gas chromatograph for gc/ms analysis [2].

Characterization of PHB

The apparent number-average (M_n) and weight-average (M_w) molecular masses and polydispersity (PD, M_w/M_n) were determined by gel-permeation chromatography (GPC) [16], using a system consisting of a LC 1120/1150 HPLC pump (GBC Scientific Equipment, Victoria, Australia), equipped with Knauer refractive-index detector model K-2300/A2010 equipped with Polymer Laboratories C-Linear mixed-bed size exclusion columns (2 × 300 mm/7.5 mm). Polystyrene standards (Polyscience Corp, Warrington, PA, USA) with low polydispersity were used to generate a calibration curve. Chloroform (HPLC grade) was used as eluant (1 ml min^{-1}) at 30°C. The sample concentration and injection volume were 1 mg ml^{-1} and 10 μl, respectively. ^{13}C NMR was used to establish the polymer backbone. Analytical samples were prepared with 25 mg PHB in 0.5 ml deuterated chloroform. DCI/MS was used to further confirm the gc/ms analysis of the transmethylated PHB. Samples (0.5 μl of 1 μg μl^{-1}) were placed on the DCI probe's filament, air-dried to remove the solvent, and inserted directly into the source. Isobutane was used as the chemical ionization gas, and the filament heated from 0–500 mA at 20 mA s^{-1}. Samples were scanned over a range of 75–700 amu.

Results and discussion

Saponification of VO

The isolation yield of the vernonia soap was 95.0% based on trivernolin. Because vernonia soap is water-soluble at ambient temperature, a mixture of ice and water (5°C) was needed to ensure efficient precipitation. Thus the high yield will be greatly reduced if an excess amount of water is used during precipitation of the soap. Furthermore, an attempt to reduce the volume of methanol used as a solvent during hydrolysis resulted in premature precipitation of the soap during refluxing, thereby preventing reaction completion. Hence the procedure reported here is the optimum for producing vernonia soap via sodium hydroxide hydrolysis, using methanol as solvent.

Cell growth and PHB formation

A. eutrophus was able to grow and utilize hydrolyzed vernonia oil as its sole source of carbon within the 7-day fermentation period. Cell growth reached a stationary phase within 48 h, and a few cytoplasmic inclusion granules were apparent during this period. Analyses of these cell extracts after lyophilization revealed mainly derivatives of the epoxidized carbon substrate. These intermediate products remain unknown, however, gc/ms analysis indicated the absence of vernolic acid, while ^{13}C NMR analysis revealed the lack of an epoxy group. These results suggest that the epoxy functionality on the carbon substrate may have been reduced prior to polymer formation. Electron micrographic analysis of the cells during the polymer storage phase was used to establish the optimum harvest time. At the seventh day, most of the cells contained PHA inclusions (Figure 1). Attempts were made to increase the biomass, and subsequently PHA synthesis by varying the concentration of the nitrogen source. Table 1 summarizes the results of such attempts in which 1.10, 0.83, 0.55 and 0.0 g L^{-1} of $(NH_4)_2HPO_4$ yielded 0.28, 0.36, 0.27, 0.34 and 0.15 g L^{-1} of dry cells. The wt% PHB in the biomass were 31.9, 28.3, 42.8, 22.7 and 0.0 respectively. However, these data do not seem to demonstrate any apparent trend, particularly as they relate to the percent conversion of carbon substrate to PHB. These results are consisent with studies which employed other carbon substrates in the production of PHA by *A. eutrophus*. For example, Doi and coworkers [8] reported

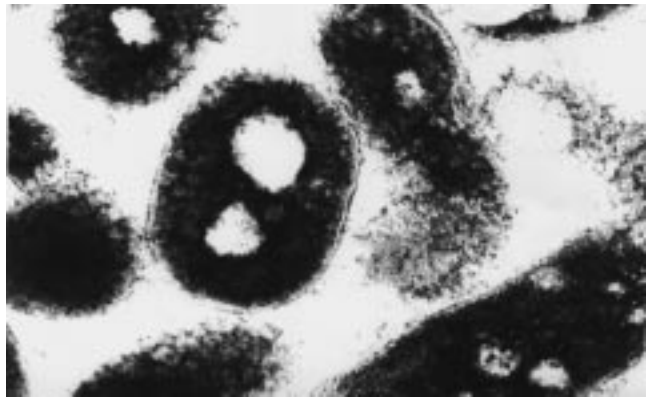

Figure 1 Transmission electron micrograph of *Alcaligenes eutrophus* showing inclusion granules after 7-day cultivation on hydrolyzed vernonia oil as sole carbon source.

Table 1 Effect of ammonium concentration on conversion of hydrolyzed vernonia oil to PHB by *Alcaligenes eutrophus*

$(NH_4)_2HPO_4$ (g L^{-1})	Cell yield dry wt (g L^{-1})	PHB yields	
		(g L^{-1})	% for cell dry wt
1.10	0.28	0.09	0.03
0.83	0.36	0.10	0.03
0.55	0.27	0.11	0.04
0.28	0.34	0.08	0.02
0.0	0.15	0.0	0.0

31–54 wt% of PHA in dried cells depending on the carbon source.

Characterization of PHB

The PHB had a melting (T_m) range of 163–174°C with a maximum at 172°C (literate T_m, 175°C) [8], and heat of fusion of 18.43 cal g^{-1}. DCI/MS of the isolated PHB gave a series of ions that include (m/z): 87, 173, 259, 345, 431, 517, 603, and 689, suggesting the combination of monomer unit (m/z 86) that represents the butyrate moiety. This was further confirmed by the gc/ms analysis of the transesterified PHB which gave only the methyl 3-hydroxybutanoate. Figure 2 shows the ^{13}C NMR spectrum of the PHB biosynthesized. The signal at 169.1 is due to the carbonyl carbon; the peak at 67.6 represents the carbon beta to the carbonyl, methine carbon; the peak at 40.8 ppm is assigned to the carbon alpha to the carbonyl; and the signal at 19.7 ppm is attributed to the methyl group. This spectrum is in agreement with previously reported spectra for poly(β-hydroxybutyrates) [8]. Table 2 gives the molar masses and polydispersity of the PHB produced from the bioconversion of hydrolyzed vernonia oil by the *A. eutrophus* strain used in this project. The weight-average molar mass of 308 390 Da is in the same range as those obtained by previous studies [1] using a different strain of *A. eutrophus* on C_2–C_5 alkanoic acids, however, the polydispersity of 1.1 in our results are much lower than those of earlier studies. On the other hand, earlier studies [1] using C_{18} alkanoic acids resulted in PHB with a M_w of 986 000 Da and a PD of 1.9. The differences in these results is a further confirmation that the molecular masses of PHB produced by *A. eutrophus* depend on the bacterial strain as well as the carbon substrates. The latter conclusion is in contrast to that reached by Taidi and coworkers [16] in which they suggest that the M_w of PHB produced by *A. eutrophus* is generally unaffected by the type of carbon substrates.

We also made some attempt to examine any relationship between the nitrogen concentration and the molecular masses of the PHB. As shown in Table 2, there is no apparent correlation, and remarkably, the PHB from the different experiments gave a M_w of about 300 000 Da with low PD. Research on the bioconversion of vernonia oil and derivatives is on-going in our laboratory, and we are currently investigating the physical properties of the PHB from this study. In addition, we intend to use different bacterial strains on vernonia oil, and undertake comparative studies to determine the efficiency of vernonia oil relative to other vegetable oils in the bacterial production of PHAs.

Finally, we note the contrast between the results of this study and those reported by Eggink and coworkers [9], in which they isolated complex mixtures of PHAs from *Pseudomonas aeruginosa* that was cultivated on euphorbia oil containing 64% vernolate, 9% linoleate and 19% oleate. In the present investigation, only PHB was isolated from *A. eutrophus* cultivated on vernonia oil acids. Since the chemistry of euphorbia oil is similar to that of vernonia oil, the difference in results may be attributed to the inability of *P. aeruginosa* to produce PHB from long-chain fatty acids. The present study constitutes the first report of the use of vernonia oil to produce PHB by bacteria.

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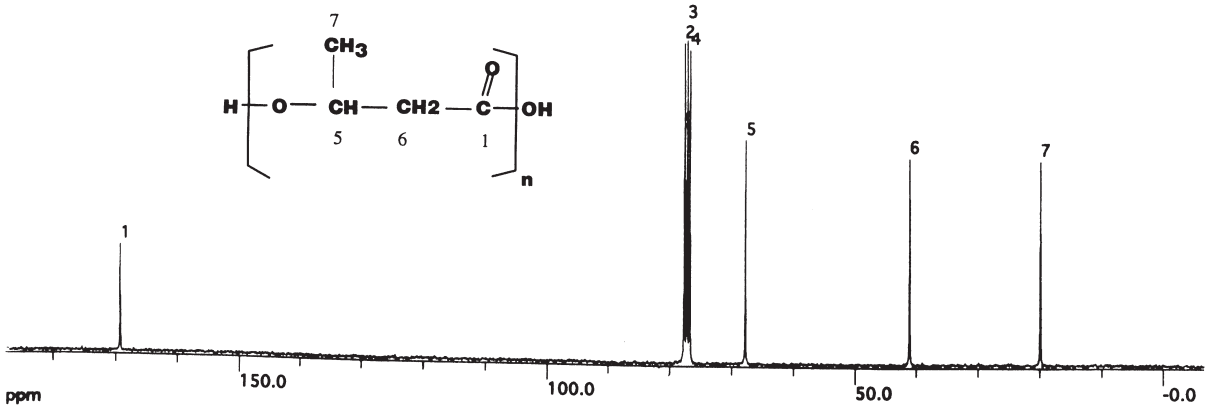

Figure 2 Proton-decoupled ^{13}C nuclear magnetic resonance spectrum of poly(β-hydroxybutyrate) produced from hydrolyzed vernonia oil by *Alcaligenes eutrophus*. Peaks are referenced to $CDCl_3$ at 77.000 ppm. Samples were made to 15 wt% in $CDCl_3$. Operating frequency was 75.6 MHz. Chemical shift (= ppm): 1 = 169.1, carbonyl carbon; 2,3,4 = $CDCl_3$; 5 = 67.6, methine carbon; 6 = 40.8, methylene carbon alpha to carbonyl group; 7 = 19.7, methyl carbon.

Table 2 Effect on ammonium concentration on the molecular weight of PHB from hydrolyzed vernonia oil by *Alcaligenes eutrophus*

$(NH_4)_2HPO_4$ (g L^{-1})	Peak molecular wt	Weight average molecular wt (M_w)	Number average molecular wt (M_n)	PD[a] (M_w/M_n)
1.10	378 332	300 845	250 204	1.2
0.83	378 673	285 085	216 505	1.3
0.55	381 863	308 390	272 280	1.1
0.28	381 628	293 280	234 335	1.3

[a]Polydispersity.

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