# Poly-3-hydroxyalkanoates Produced from *Pseudomonas oleovorans* Grown with ω-Phenoxyalkanoates

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ABSTRACT: Poly-3-hydroxyalkanoates (PHAs) produced by *Pseudomonas oleovorans* grown solely with 6-phenoxyhexanoate, 8-phenoxyoctanoate, and 11-phenoxyundecanoate were investigated. Weight fractions of PHAs in dry cells were ~10%, regardless of the carbon substrate used. DSC thermograms of these polymers showed no crystalline melting endotherm. Glass transition temperatures were ~20 °C, and number-average molecular weights were ~65 000, with polydispersity indexes of 2.7 for all PHAs. GC/MS analysis showed that 3-hydroxy-5-phenoxypentanoate was the main repeating unit in the PHA produced from 11-phenoxyundecanoate, and 3-hydroxy-4-phenoxybutyrate was the main repeating unit in PHAs produced from 6-phenoxyhexanoate and 8-phenoxyoctanoate.

## Introduction

Production of poly-3-hydroxyalkanoates (PHAs) from *Pseudomonas oleovorans* has been investigated extensively. This microorganism utilizes relatively long chain carbon substrates for PHA production. Carbon substrates smaller than hexanoate and larger than hexadecanoate do not support PHA production by *P. oleovorans*.[1]

PHAs produced by *P. oleovorans* grown with *n*-alkanoates are elastomers.[2] These polymers melt between 30 and 60 °C and possess glass transitions below −20 °C. Production of PHAs having various unusual groups has been reported.[3−7] PHAs bearing bromo, olefin, phenyl, and cyano groups are the most interesting of these polymers. Some of these polymers are produced by cometabolism. A PHA produced by *P. oleovorans* grown with 5-phenylvalerate is a homopolymer, poly(3-hydroxy-5-phenylvalerate), having a glass transition temperature as high as 0 °C.[6,7] Every repeating unit in the PHA produced by *P. oleovorans* grown with 10-undecenoate contains an unsaturated group.[5]

We have been interested in production of PHAs using carbon substrates bearing phenoxy groups. PHAs produced from these carbon substrates were expected to have repeating units substituted with phenoxy groups. PHAs containing phenoxy groups would have improved physical properties. PHAs having unusual properties like liquid crystalline behavior might be produced from *P. oleovorans* grown with carbon substrates bearing substituted phenoxy groups, such as cyanophenoxy and alkylphenoxy groups.

It might also be possible to biosynthesize PHAs containing smaller alkoxy groups, such as methoxy and ethoxy groups. PHAs bearing methoxy or ethoxy groups would have increased water compatibility.

Gross and collaborators investigated PHA production by *P. oleovorans* grown with mixtures of *n*-alkanoates and (4-cyanophenoxy)alkanoates. The fraction of cyanophenoxy groups incorporated into the polymer was low.[8]

**Table 1. Dry Cell and PHA Yields from *Pseudomonas oleovorans* Grown with ω-Phenoxyalkanoates**

| carbon source | dry cell, mg/L | PHA, mg/L | PHA content, % |
|---|---|---|---|
| 6-phenoxyhexanoate | 950 | 100 | 10.5 |
| 8-phenoxyoctanoate | 820 | 90 | 11 |
| 11-phenoxyundecanoate | 150 | 15 | 10 |

Ritter and Grafin Von Spee investigated PHA production by *P. oleovorans* grown with various phenoxyalkanoates. They isolated a PHA only from *P. oleovorans* grown with 11-phenoxyundecanoate. No PHA was isolated from *P. oleovorans* grown with 8-phenoxyoctanoate and 6-phenoxyhexanoate.[9] The PHA isolated from *P. oleovorans* grown with 11-phenoxyundecanoate was reported to contain 3-hydroxy-9-phenoxynonanoate and 3-hydroxy-5-phenoxypentanoate units as main repeating units.

We have been independently investigating PHA production by *P. oleovorans* grown with 6-phenoxyhexanoate, 8-phenoxyoctanoate, and 11-phenoxyundecanoate to obtain results different from Ritter's. All these carbon substrates supported PHA production by *P. oleovorans*. The repeating unit composition of the PHA isolated from *P. oleovorans* grown with 11-phenoxyundecanoate was different from that reported by Ritter and collaborators. The PHAs produced from *P. oleovorans* grown with 6-phenoxyhexanoate, 8-phenoxyoctanoate, and 11-phenoxyundecanoate were characterized by gel permeation chromatography, $^{13}$C NMR spectroscopy, and differential scanning calorimetry (DSC). Repeating unit compositions of these PHAs were determined by GC/MS analysis of the methanolyzed samples.

## Results and Discussion

Dry cell weights and PHA yields are listed in Table 1. The weight percentages of PHA in dry cells were ~10%, regardless of the carbon source. The low yield of dry cell, and the subsequent low yield of PHA, are most likely related to the poor solubilities of carbon substrates in the growing media, which results in poor cell growth. Purified PHAs were relatively hard at room

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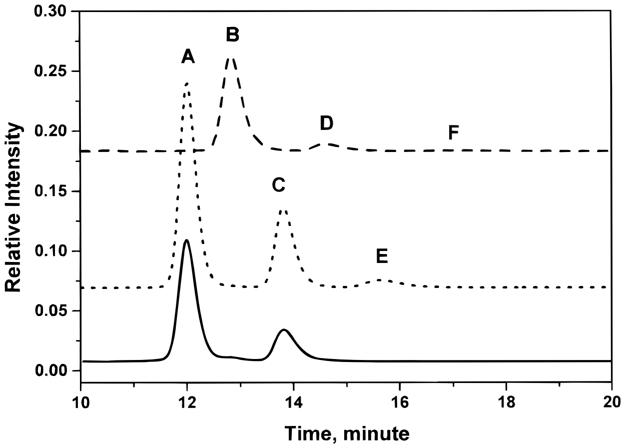


**Figure 1.** Gas chromatograms of methanolyzed samples of PHAs. (- - -) PHA(11-phenoxy); (···) PHA(8-phenoxy); and (—) PHA(6-phenoxy).

**Table 2. Peak Area Percent of Each Methyl Phenoxy-3-hydroxyalkanoate in Figure 1**

| PHA | peak area percent |
|---|---|
| PHA(11-phenoxy) | B, 90.5; D, 9.5; F, trace |
| PHA(8-phenoxy) | A, 65.1; C, 30.0; E, 4.9 |
| PHA(6-phenoxy) | A, 75.8; C, 24.2; E, not detected |

temperature. DSC thermograms of these polymers did not show any crystalline melting endotherm. Glass transition temperatures of these polymers were ~20 °C. Number-average molecular weights of these PHAs were ~65 000, and polydispersity indexes were ~2.7.

Gas chromatograms of methanolyzed samples of PHAs are shown in Figure 1. Area percentages of peaks in Figure 1 are listed in Table 2. Figure 1 and Table 2 show that the PHAs from 6-phenoxyhexanoate (PHA(6-phenoxy)) and from 8-phenoxyoctanoate (PHA(8-phenoxy)) had similar compositions. Peak E (see Figure 1) was not detected in the methanolyzed sample of PHA(6-phenoxy). Methanolyzed samples of PHA(11-phenoxy) contained three components, B, D, and F (trace amount).

Molecular weight peaks and *m/z* values of main peaks obtained from CI and EI mass spectroscopy measurements are listed in Table 3. The phenoxy group was well fragmented ($m/z = 94$) from the main body, and characteristic *m/z* peaks (71, 43, 103) from methyl 3-hydroxyalkanoates were detected. The molecular weight of each peak in Figure 1 was confirmed by CI mass spectroscopy. The *m/z* values listed in Table 3 correspond to the values of ion fragments produced from methyl 3-hydroxy-$\omega$-phenoxyalkanoates. The characteristic ion fragments are produced by the loss of phenoxy grouop ($m/z = 93$), $CH_2COOCH_3$ (73), phenoxy group/$CH_3OH$ (125), and phenoxy group/$CH_3COOCH_3$ (167). The results of mass spectroscopy show clearly that peaks A−F in Figure 1 are methyl 3-hydroxy-4-phenoxybutyrate, methyl 3-hydroxy-5-phenoxypentanoate, methyl 3-hydroxy-6-phenoxyhexanoate, methyl 3-hydroxy-7-phenoxyheptanoate, methyl 3-hydroxy-8-phenoxyoctanoate, and methyl 3-hydroxy-9-phenoxynonanoate, respectively. Only phenoxy ion was detected for F by EI mass spectroscopy due to its low concentration. The molecular weight of F was determined by CI mass spectroscopy. Trace amounts of B were detected in methanolyzed samples of PHA(6-phenoxy) and PHA(8-phenoxy) by GC/MS analysis.

$^{13}C$ NMR spectra of PHA(8-phenoxy) and PHA(11-phenoxy) are shown in Figures 2 and 3, respectively. The $^{13}C$ NMR spectrum of PHA(6-phenoxy) was almost identical to that of PHA(8-phenoxy).

The chemical makeup of polymers produced from *P. oleovorans* grown with phenoxyalkanoates shows a trend similar to that for polymers produced from *P. oleovorans* grown with *n*-alkanoates. Repeating units found in PHAs biosynthesized by *P. oleovorans* grown with *n*-alkanoates are 3-hydroxyalkanoates containing the same number of carbons as the carbon source and 3-hydroxyalkanoates containing more or fewer carbons by $2n$ ($n$ = integer). Trace amounts of 3-hydroxyalkanoates containing one more or one less carbon were also found. Formation of these repeating units was most likely related to production of acetate and carbon dioxide.

The main repeating units in PHAs biosynthesized by *P. oleovorans* grown with *n*-alkanoates having an even number of carbons and an odd number of carbons are 3-hydroxyoctanoate and 3-hydroxynonanoate, respectively. Production by *P. oleovorans* of polymers containing the main repeating units of 3-hydroxy-4-phenoxybutyrate and 3-hydroxy-5-phenoxypentanoate from carbon substrates used in this study might be explained by the following: structures of fully extended 3-hydroxy-4-phenoxybutyrate and 3-hydroxy-5-phenoxypentanoate are similar to those of 3-hydroxyoctanoate and 3-hydroxynonanoate, respectively, and these are the preferred monomer repeat units. Production of copolymers from $\omega$-phenoxyalkanoates is illustrated in Scheme 1.

It is remarkable that the oxygen atom at C-5 did not suppress PHA production by *P. oleovorans*. The presence of a polar group close to the carboxyl group of the organic acid has been reported to have unfavorable effects on PHA production and cell growth.[1]

The results of this study suggest that carbon substrates bearing an oxygen atom close to the carboxyl group of the carbon source would support PHA production by *P. oleovorans*. Thus, PHAs containing various aryloxy and alkoxy groups may be biosynthesized by *P. oleovorans*.

## Experimental Section

**Synthesis of Carbon Sources.** 6-Phenoxyhexanoic acid, 8-phenoxyoctanoic acid, and 11-phenoxyundecanoic acid (Aldrich) were synthesized by refluxing mixtures of ethanol, potassium hydroxide (3 equiv), phenol (2 equiv), and the appropriate $\omega$-bromoalkanoic acid (1 equiv). The mixture was

**Table 3. Results Obtained from GC/MS Analysis of Methanolyzed Samples of PHAs**

| peak in Figure 1 | main *m/z* (EI, 70 eV) | MW[a] | structure |
|---|---|---|---|
| A | 210, 192, 161, 137,[b] 117[c] 103, 94, 85,[d] 77, 71, 43[e] | 210 | methyl 3-hydroxy-4-phenoxybutyrate |
| B | 224, 151,[b] 131[c], 103, 99,[d] 94, 71, 57,[e] 43 | 224 | methyl 3-hydroxy-5-phenoxypentanoate |
| C | 238, 189, 165,[b] 145,[c] 113,[d] 103, 94, 77, 71,[e] 43 | 238 | methyl 3-hydroxy-6-phenoxyhexanoate |
| D | 159,[c] 127,[d] 94, 85,[e] 43, 77, 71 | 252 | methyl 3-hydroxy-7-phenoxyheptanoate |
| E | 173,[c] 141,[d] 103, 99,[e] 94, 81, 77, 71, 43 | 266 | methyl 3-hydroxy-8-phenoxyoctanoate |
| F | 94 | 280 | methyl 3-hydroxy-9-phenoxynonanoate |

[a] Determined by chemical ionization. $CH_4$ was used for ionization. [b] $M - 73$ ($CH_2COOCH_3$). [c] $M - 93$ ($C_6H_5O$, phenoxy). [d] $M - 125$ (phenoxy + $CH_3OH$). [e] $M - 167$ (phenoxy + $CH_3COOCH_3$).

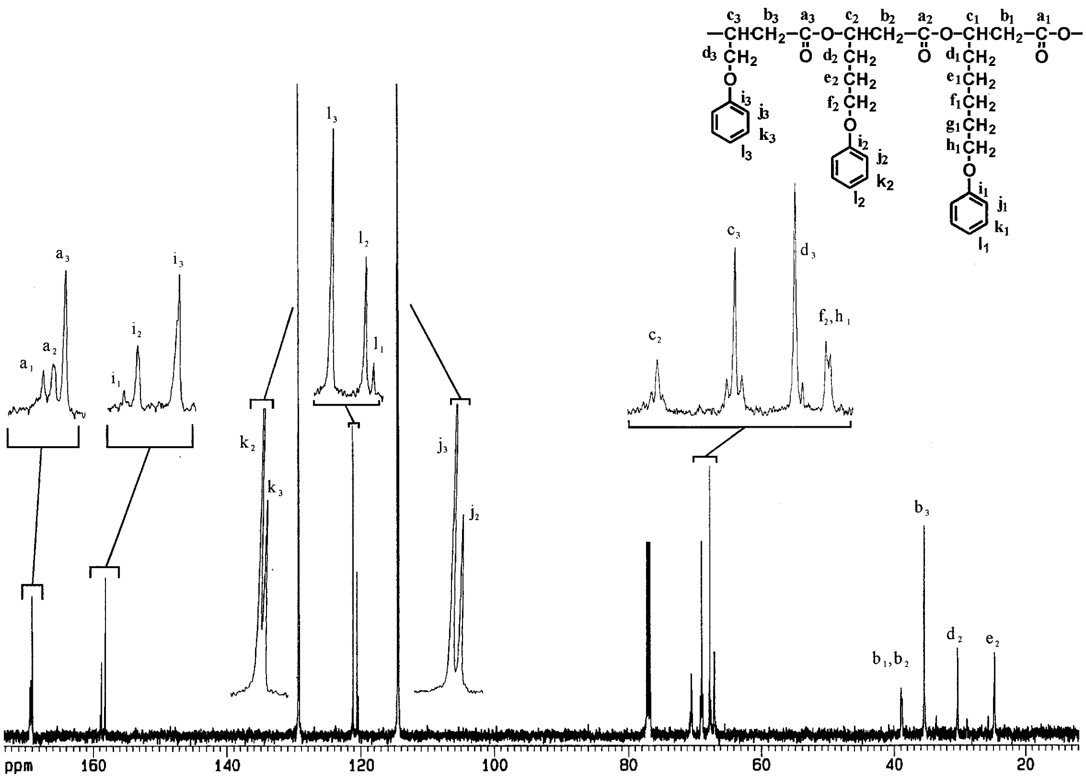


Figure 2. $^{13}$C NMR spectrum of PHA(8-phenoxy).

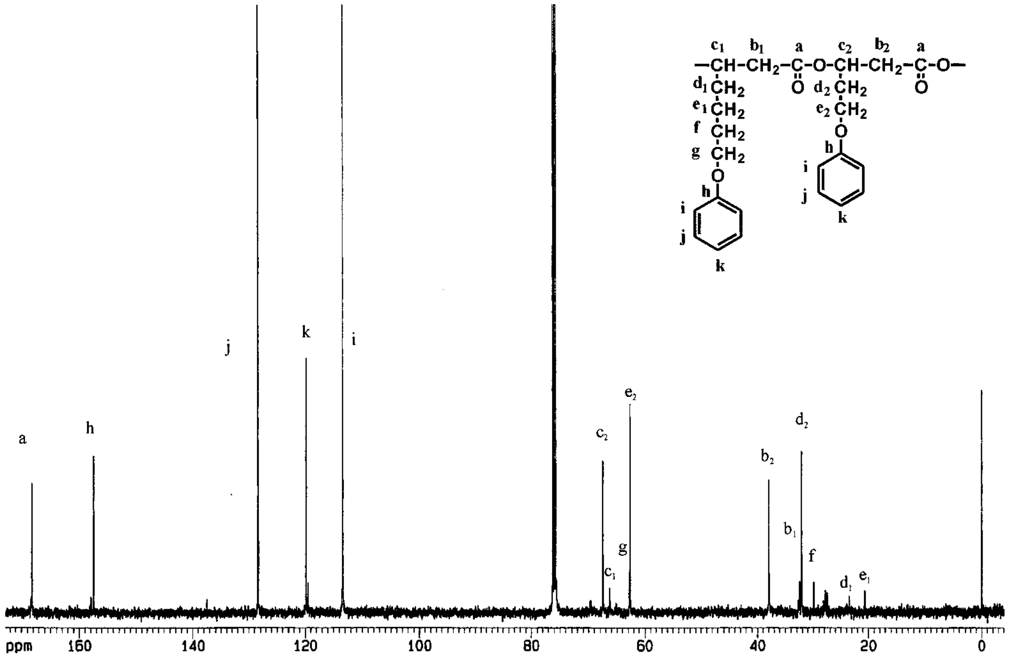
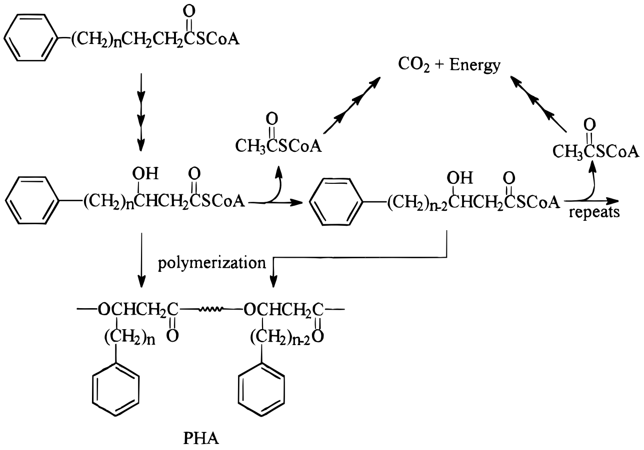


Figure 3. $^{13}$C NMR spectrum of PHA(11-phenoxy).

refluxed for 18 h and then acidified with concentrated HCl. The mixture was concentrated and poured into a large amount of water to obtain solid products. Phenoxyalkanoic acids were isolated and purified by recrystallization from ethyl alcohol (yields, ~60%). The structure was confirmed by NMR spectroscopy, and the purity was checked by TLC.

**Biosynthesis of PHA.** *P. oleovorans* was grown in a 1 L medium. The growth medium was prepared and treated as described elsewhere.[10] Growth medium was prepared to give 10 mM solutions of each carbon substrate. However, solubilities of carbon substrates used in this study were too low to obtain homogeneous growth media. Significant amounts of carbon substrates remained undissolved throughout the fermentation process. Thus, the concentration of carbon substrate in the growth medium was unknown, but the growth medium was saturated with the carbon substrate. Monitoring cell growth by measuring optical density was not possible due to the floating particles of undissolved carbon sources. A small amount of the growing culture was taken, and the supernatant was observed with a microscope to confirm cell growth. Induction periods were ~4 days for growth with 6-phenoxyhexanoate and 6 days for growth with 8-phenoxyoctanoate and 11-phenoxyundecanoate. Induction periods were estimated by shaking flask experiments in 50 mL scales. Fermentation was stopped, and cells were harvested after ~2 weeks. The pH of the medium was adjusted to ~6 just before harvesting. Centrifugation of the culture yielded cells contaminated with remaining carbon sources. The solid mixture was freeze-dried

**Scheme 1. PHA Synthesis by *Pseudomonas oleovorans* Grown with Phenoxyalkanoates**



and then washed with methanol to remove remaining carbon sources. Cells were recovered by filtration and then agitated in chloroform (300 mL for 1 g of cells) at room temperature for 18 h. The mixture was filtered, and the filtrate was concentrated. PHA was precipitated by dropping the concentrated filtrate into vigorously stirred hot ethanol. The precipitation process was repeated twice to obtain final PHA products.

**Analysis of PHAs.** Compositions of PHAs were investigated by gas chromatography analysis of methanolyzed samples. Methanolysis was carried out as described elsewhere.[10] A Young-In GC 680A equipped with a FID detector and a HP 20M capillary column (Hewlett-Packard) was used for GC analysis. The oven temperature was raised from 70 °C to 170 °C at a ramp of 10 °C/min.

Identification of repeating units was carried out by GC/MS analysis. A HP 5988 GC/MS system equipped with a SE30 nonpolar capillary column (0.32 mm $\times$ 30 m $\times$ 0.12 $\mu$m) was used. The ionization energy was 70 eV for electron impact ionization (EI), and methane was used for chemical ionization (CI).

**Miscellaneous.** Differential scanning calorimetry measurement was carried out using a Perkin-Elmer DSC 7. The temperature was scanned from −100 °C to 180 °C at a ramp of 20 °C/min. NMR spectra were recorded using a Bruker AM 300 or a Bruker AMX 500.

Molecular weights of PHAs were determined using a gel permeation chromatography system equipped with a Waters 6000 solvent delivery system, RI detector, and U6K injector. Linear Ultrastyragel, $10^3$ Å, and $10^4$ Å Waters Styragel columns were used. A standard curve was established with standard polystyrene samples. Samples were prepared in concentrations of ~0.3 wt % in chloroform. Approximately 100 $\mu$L was injected, and chloroform was used as the eluent.

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