# Bacterial Poly(3-hydroxyalkanoates) Bearing Carbon−Carbon Triple Bonds

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ABSTRACT: Production of poly(3-hydroxyalkanoates), PHAs, by *Pseudomonas oleovorans* (*P. oleovorans*) and *Pseudomonas putida* (*P. putida*) grown with mixtures of nonanoic acid, NA, and 10-undecynoic acid, 10-UND(≡), were investigated. Both microorganisms produced PHAs containing carbon−carbon triple bonds in fractions from 0 to 100%, depending on the composition of the carbon substrate mixture. The amounts of unsaturated repeating units in PHAs produced by *P. oleovorans* were higher than those in PHAs produced by *P. putida* grown with the same carbon substrates. The repeating units containing carbon−carbon triple bonds were 3-hydroxy-8-nonynoate, 3HN(≡), and 3-hydroxy-10-undecynoate, 3HUD-(≡). 3HN(≡) was the major repeating unit formed from 10-UND(≡). The relative amounts of 3HN(≡) and 3HUD(≡) in PHAs produced by *P. putida* were slightly different from those in PHAs produced by *P. oleovorans*. The number average molecular weights of PHAs produced in this study were approximately 50 000, and polydispersity indices were approximately 2.5 as determined by gel permeation chromatography. The molecular weight distribution and the relative amounts of 3HN(≡) and 3HUD(≡) were not affected by either growth time or the composition of the carbon substrate. PHAs bearing triple bonds were soft and differential scanning calorimetry thermograms of these polymers showed very small melting endotherms at approximately 60 °C. The glass transition temperatures were in the range of −33 to −21 °C.

## Introduction

Poly(3-hydroxyalkanoates), PHAs, are biodegradable polyesters accumulated by a wide variety of bacteria as a reserve of carbon and energy.[1] Over 90 genera of bacteria, encompassing Gram-positive and Gram-negative species, have been found to produce PHAs.[2] PHAs can be subdivided in two groups. Short-chain-length (scl) PHAs contain 3-hydroxybutyrate and/or 3-hydroxyvalerate, while medium-chain-length (mcl) PHAs contain 3-hydroxyalkanoates larger than 3-hydroxyvalerate. scl-PHAs are thermoplastics with a high degree of crystallinity, and mcl-PHAs are elastomers with a low degree of crystallinity.

Bacteria producing PHAs can be similarly subdivided into two groups. One group, including the bacterium *Alcaligenes eutrophus*, produces scl-PHAs, while a distinct group, including *Pseudomonas oleovorans*, produces mcl-PHAs.

*Pseudomonas oleovorans* (*P. oleovorans*) and *Pseudomonas putida* (*P. putida*) are the most investigated microorganisms among those producing mcl-PHAs. *P. oleovorans* produces PHAs containing various functional groups when grown with carbon substrates bearing the corresponding functional groups.[3−8] Some of these PHAs were produced only when the carbon substrate containing a functional group was cofed with an *n*-alkanoic acid such as nonanoic acid, NA.[9−11] Recent results in our laboratory show that *P. putida* cannot produce all of PHAs containing functional groups produced by *P. oleovorans*.[12]

PHAs bearing functional groups are of great interest as some functional groups can be chemically modified to yield PHAs containing more useful functional groups that cannot be directly produced by microorganisms. It is also to be noted that microorganisms produce polymers containing functional groups that cannot be easily synthesized by chemical methods. For example, PHAs bearing desired amounts of olefin groups in the side chain can be easily biosynthesized by feeding *P. oleovorans* with mixed carbon substrates of NA and undecylenic acid.[6]

In this study we have investigated PHAs produced by *P. oleovorans* and *P. putida* grown with 10-undecynoic acid, 10-UND(≡), and mixtures of NA and 10-UND-(≡). PHAs produced by both microorganisms contained carbon−carbon triple bonds. Many polymers containing conjugated carbon−carbon triple bonds in the polymer backbone have been synthesized to prepare electrically conducting polymers.[13−15] However, there are a few reports on soluble polymers bearing triple bonds in the side chain.[16,17]

## Results and Discussion

The growth curves of *P. putida* grown with various mixtures of NA and 10-UND(≡) are plotted in Figure 1. Figure 1 shows that the growth was not affected by 10-UND(≡) when the concentration of NA was 50 mol % or higher. When the concentration of NA was 25 mol % and lower, the growth curve shifted to the right with the final optical density (OD) value decreased significantly.

The IR spectrum of the PHA produced by *P. putida* grown with only 10-UND(≡) is shown in Figure 2. The absorption peaks at 3291 and 2116 $cm^{-1}$ in Figure 2 are characteristic peaks for ≡CH and C≡C stretching, respectively. Gas chromatograms of methanolyzed samples of PHAs produced from carbon substrates containing 10-UND(≡) showed two new peaks with

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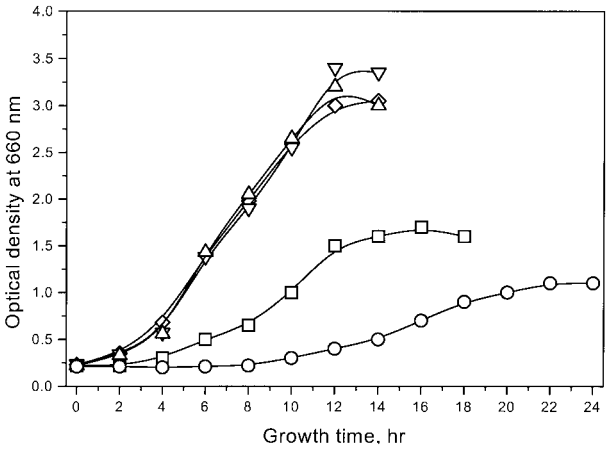


**Figure 1.** Optical density vs growth time for *Pseudomonas putida* grown with various mixtures of NA and 10-UND(≡): (○) 100 mol % 10-UND(≡); (□) 25 mol % NA + 75 mol % 10-UND(≡); (+), 50 mol % NA + 50 mol % 10-UND(≡) (▽) 75 mol % NA + 25 mol % 10-UND(≡); (◇) 100 mol % NA.

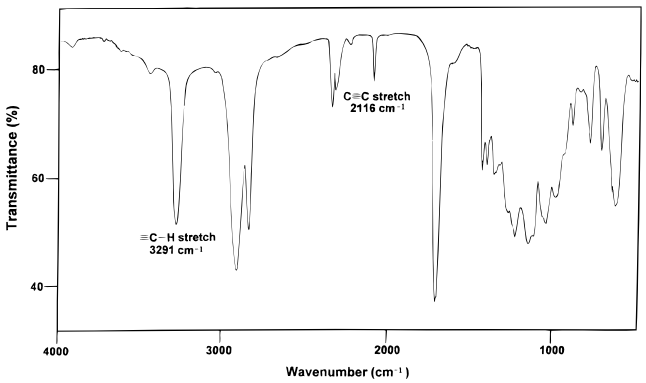


**Figure 2.** IR spectrum of a PHA isolated from *P. putida* grown with only 10-UND(≡).

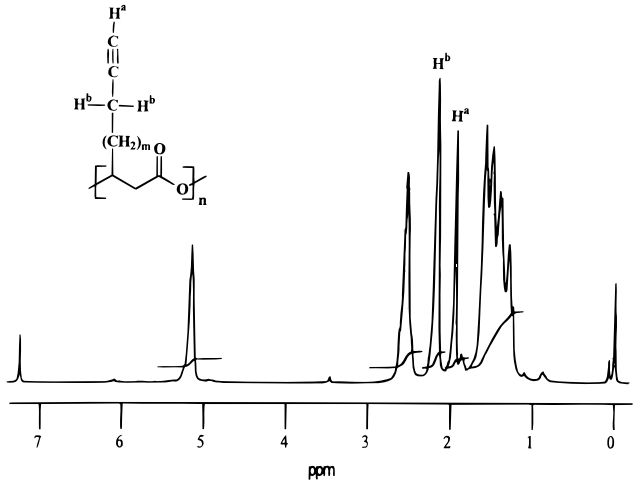


**Figure 3.** $^{1}$H NMR spectrum of a PHA isolated from *P. putida* grown with only 10-UND(≡).

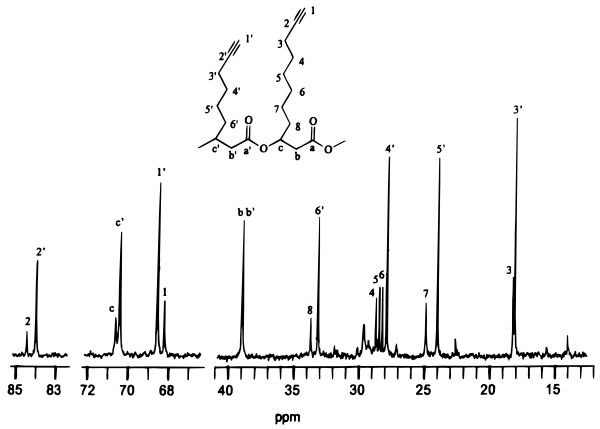


**Figure 4.** $^{13}$C NMR spectrum of a PHA isolated from *P. putida* grown with only 10-UND(≡).

retention times that did not correspond to that of any methyl 3-hydroxyalkanoate and methyl 3-hydroxyalkenoate known. The electron impact mass spectra of these new peaks contained ion fragments with *m*/*z* values of 103, 74, and 43 which are characteristic ones produced from methyl 3-hydroxyalkanoates. The molecular weights of these new peaks were 184 and 212 as determined by a chemical ionization mass spectrometer. These molecular weights correspond to those of methyl 3-hydroxy-8-nonynoate, 3HN(≡), and methyl 3-hydroxy-10-undecynoate, 3UND(≡), respectively.

$^{13}$C NMR and $^{1}$H NMR spectra of a PHA produced by *P. putida* grown solely with 10-UND(≡) and are shown in Figures 3 and 4, respectively. Chemical shifts and patterns of peaks in Figures 3 and 4 correspond to those expected from a PHA consisting of 3HN(≡) and 3HUD(≡). These results confirm that the repeating units produced from 10-UND(≡) were 3HN(≡) and 3HUD(≡).

The PHAs produced from by *P. oleovorans* grown with carbon substrates containing 10-UND(≡) also contained 3HN(≡) and 3HUD(≡). The relative amounts of repeating units in PHAs produced by *P. oleovorans* and *P. putida* grown with various mixtures of NA and 10-UND(≡) were determined from the areas of the peaks in the gas chromatograms from methyl esters of repeating units produced by methanolysis of the PHAs, which are listed in Table 1. Table 1 shows that the relative amounts of 3HN(≡) and 3HUD(≡) in PHAs produced by these two microorganisms were slightly different. However, the relative amounts of these two repeating units in PHAs produced by the same microorganism did not change significantly regardless of the growth time and the composition of the carbon substrate mixture. It is to be noted that Table 1 shows that the relative amount of 3HN and 3HHp did not change significantly in PHAs produced by *P. putida*, while that in PHAs produced by *P. oleovorans* changed significantly depending on the amount of 10-UND(≡) in the carbon substrate. The ratio of the amount of 3HN to 3HHp decreased from 2.9 to 1.1 as the fraction of 10-UND(≡) increased from 100 to 25 mol %. This is the first example that cofeeding a carbon substrate affected the composition of 3-hydroxyalkanoates formed from NA in PHAs produced by *P. oleovorans*.

The total fractions of unsaturated repeating units in the PHAs produced by *P. oleovorans* and *P. putida* are plotted against the mole percent of 10-UND(≡) in the carbon substrate mixtures in Figure 5. Figure 5 shows that the total amount of unsaturated repeating units in PHAs produced by *P. oleovorans* was higher than that in PHAs produced by *P. putida* grown with the same carbon substrates.

As both *P. oleovorans* and *P. putida* produced PHAs containing the same repeating units, PHAs produced by *P. oleovorans* were not investigated further in this study. However, it is to be noted that PHA production behaviors of these two microorganisms were signifi-

**Table 1. Relative Amounts of Repeating Units in PHAs Produced by *P. putida* and *P. oleovorans* Grown with Various Mixtures of 10-UND(≡) and NA**

| concn of carbon source (mM) | | relative amount[a] of repeating units in PHAs | | | | | | | | thermal transition temp of PHAs produced by *P. putida* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | produced by *P. oleovorans* | | | | produced by *P. putida* | | | | | |
| 10-UND(≡) | NA | 3HN(≡) | 3HUD(≡) | 3HHp | 3HN | 3HN(≡) | 3HUD(≡) | 3HHp | 3HN | $T_g$ | $T_m$ |
| 10 | 0 | 74 | 26 | 0 | 0 | 72 | 28 | 0 | 0 | −21 | |
| 7.5 | 2.5 | 58 | 23 | 9 | 10 | 47 | 18 | 10 | 25 | −25 | |
| 5 | 5 | 40 | 15 | 20 | 25 | 29 | 12 | 16 | 43 | −30 | sb[b] |
| 2.5 | 7.5 | 23 | 9 | 24 | 44 | 14 | 6 | 23 | 57 | −33 | sb[b] |
| 0 | 10 | 0 | 0 | 26 | 74 | 0 | 0 | 25 | 75 | −31 | 57 |

[a] GC area percent. [b] Small bump at approximately 60 °C.

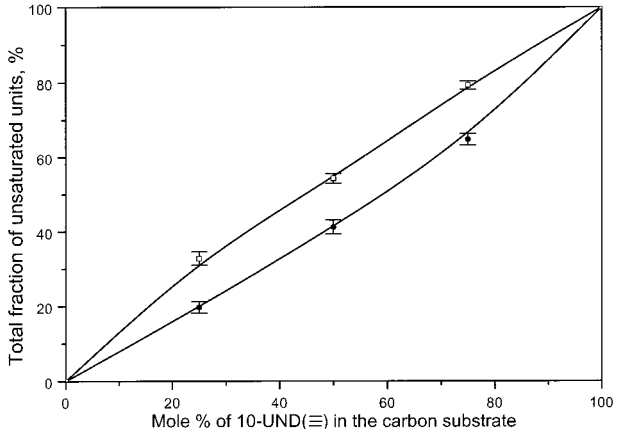


**Figure 5.** Total fraction of unsaturated repeating units in PHAs produced by *P. oleovorans* and *P. putida* against the mole percent of 10-UND(≡) in carbon substrate mixtures: (□) *P. oleovorans*; (●) *P. putida*.

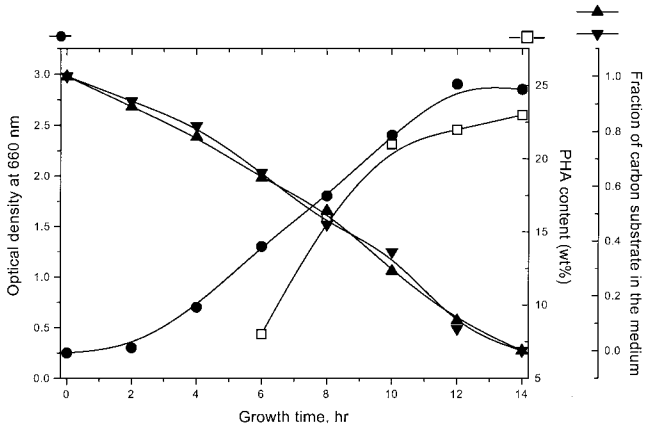


**Figure 6.** Kinetics of PHA production by *P. putida* grown with an equimolar mixture of NA and 10-UND(≡): (▲) the fraction of NA remaining in the medium; (▼) the fraction of 10-UND(≡) remaining in the medium; (□) PHA content (wt %); (●) optical density at 660 nm.

cantly different, which are under further investigation in our laboratories.[12]

The fractions of carbon substrates remaining in the medium and PHA yields obtained from a batch fermentation of *P. putida* grown with an equimolar mixture of 10-UND(≡) and NA are plotted against the growth time in Figure 6. Figure 6 shows that NA and 10-UND(≡) were consumed by the microorganism at the same rate. The compositions of PHAs remained constant throughout the growth.

Fermentation results for *P. putida* grown with various mixtures of NA and 10-UND(≡) are listed in Table 2. The yields of saturated units and unsaturated units in Table 2 were calculated by dividing the total weights of saturated units and unsaturated units by the initial weights of NA and 10-UND(≡) added to the medium, respectively. In this calculation, the fraction of a repeating unit determined by GC analysis was assumed to be proportional to the weight fraction of the repeating unit. Table 2 shows that cell yield and PHA content decreased as the fraction of 10-UND(≡) in the carbon substrate was increased. Dry cell weight and polymer yields from both 10-UND(≡) and NA increased significantly as the concentration of NA in the carbon substrate increased from 2.5 (25 mol %) to 5 mM (50 mol %). These results indicated that NA induced PHA production from 10-UND(≡) when NA was present in the medium in a concentration higher than a critical value. Similar results were obtained in a study biosynthesizing PHAs from *P. oleovorans* grown with NA and bromoalkanoic acids.[18] The effect of the concentration of 10-UND(≡) on the growth mentioned above might be explained similarly. It is also to be noted that PHA production from NA was suppressed by 10-UND(≡) when the concentration of 10-UND(≡) was high.

Glass transition temperatures and melting temperatures determined by differential scanning calorimetry (DSC) analysis are listed in Table 1. PHAs containing carbon−carbon triple bonds showed only negligible melting endotherms in DSC thermograms. It is interesting that the glass transition temperature increased as the fraction of carbon−carbon triple bond increased. These results are in contrast to those from PHAs containing an olefin group. The glass transition temperature of the PHA decreased as the fraction of olefin group increased for PHAs prepared from NA and undecylenic acid.[6] These results might be related to the fact that the carbon−carbon triple bond has a rigid structure that might stack easily to form ordered structure.

Polymers containing triple bonds cross-linked easily by gentle heating or irradiating UV light. Modification of these polymers including a cross-linking study are under investigation.

## Experimental Section

**Biosynthesis of PHAs.** *P. putida* KCTC 2407 and *P. oleovorans* ATCC 29347 were grown as described elsewhere.[6,19] 10-Undecynoic acid and nonanoic acid were used as purchased. The total concentration of carboxylic acids in the growth medium was 10 mM. Inocula were prepared by growing each microorganism in 100 mL of media containing nonanoic acid in a concentration of 10 mM. The cell growth was monitored by measuring the optical density at 660 nm. Fermentation was stopped approximately 2 h after the growth reached stationary phase. Cells were harvested by centrifugation and then lyophilized. Polymers were isolated from lyophilized cells

**Table 2. Fermentation Results for *P. putida* Grown with Various Mixtures of 10-UND(≡) and NA**

| amount of carbon source (g/L) | | incubation time, h | dry cell wt (g/L) | PHA content (% wt) | fraction of unsaturated units (%) | PHA (g/L) | wt of unsaturated units (g/L) | yield of unsaturated units (%) | wt of saturated units (g/L) | yield of saturated units (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| 10-UND(≡) | NA | | | | | | | | | |
| 1.82 | 0 | 24 | 0.50 | 3.4 | 100 | 0.017 | 0.017 | 0.9 | 0 | |
| 1.37 | 0.40 | 18 | 0.65 | 9.6 | 65 | 0.062 | 0.041 | 3.0 | 0.02 | 5.5 |
| 0.91 | 0.79 | 14 | 1.11 | 21.0 | 41 | 0.233 | 0.095 | 10.4 | 0.138 | 17.5 |
| 0.46 | 1.19 | 14 | 1.13 | 22.1 | 20 | 0.250 | 0.051 | 11.1 | 0.199 | 16.8 |
| 0 | 1.58 | 14 | 1.09 | 28.3 | 0 | 0.309 | | 0 | 0.389 | 19.5 |

by extraction with hot chloroform using a Soxhlet extractor and then purified by repeated precipitation from methanol.

**Polymer Compositions.** The relative amounts of repeating units in PHAs obtained in this study were determined from the areas of the peaks of the methyl esters of each repeating unit in the gas chromatograms of the products of methanolysis of the samples. The total mole percent of carbon−carbon triple bonds in the PHAs determined by $^1$H NMR corresponded to that calculated from gas chromatography within a few percent. Polymer compositions reported in this study are average values of at least three polymers prepared from separate fermentation. Methanolysis was carried out as described in our previous paper.[6] A Hewlett-Packard 5890 or a Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detector and a HP-1 capillary column was used for gas chromatography analysis. The oven temperature was initially maintained at 80 °C for 4 min and then was raised to 230 °C at a ramp of 10 °C/min.

**Analysis of Residual Carbon Substrates in the Medium.** Approximately 100 mL of the culture was taken at different growth times and centrifuged. A 10 mL aliquot of the supernatant of the culture was taken, and 2 mL of 0.8 wt % benzoic acid solution in chloroform was added as an internal standard. The mixture was acidified with a small amount of 2 N HCl solution. To this mixture, 5 mL of chloroform was added, and then the mixture was agitated vigorously. The organic layer was taken and dried over a small amount of anhydrous $MgSO_4$. A 1 $\mu$L aliquot of the chloroform solution was injected into a gas chromatograph. The amounts of carbon substrates were calculated by the internal standard method.[4]

**Miscellaneous.** DSC analysis was carried out using a Perkin-Elmer DSC 7 from −70 to +150 °C. NMR and IR spectra were recorded using a Bruker 500 NMR spectrometer and a Bomem 102 FT-IR spectrometer, respectively. GC/MS analysis was carried out using a HP 5988 GC/MS system as described in our previous paper.[20] Molecular weights of PHAs were determined using a gel permeation chromatography system equipped with a Waters 6000 solvent delivery system, RI detector, and a Rheodyne 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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