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Poly(β-hydroxyalkanoate) Copolymers Containing Brominated Repeating Units Produced by *Pseudomonas oleovorans*

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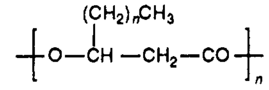
ABSTRACT: Poly(β-hydroxyalkanoate) (PHA) copolymers containing brominated repeating units were produced by *Pseudomonas oleovorans* grown with mixtures of an ω-bromoalkanoic acid and either nonanoic acid (NA) or octanoic acid (OA). Cells grown solely with an ω-bromoalkanoic acid did not produce any PHA. The mole percent of brominated units in the PHAs obtained varied from 2% to 37% depending on the nonbrominated carbon substrate used as cosubstrates and on the ratio of the two substrates. The mole fractions of brominated units in the PHAs obtained from cells grown with mixtures of OA and an ω-bromoalkanoic acid were lower than those in the PHAs obtained from cells grown with mixtures of NA and an ω-bromoalkanoic acid, but the molecular weights of the PHAs were essentially the same regardless of carbon sources used. In addition, the molecular weights and the fractions of brominated units in the copolymers produced were constant throughout the growth.

Introduction

Poly(β-hydroxyalkanoates) (PHAs) are produced by a wide variety of microorganisms as an intracellular energy and carbon source storage material.[1,2] These biopolymers have the general structure



$$\left[O-\overset{(CH_2)_nCH_3}{\overset{|}{CH}}-CH_2-CO \right]_n$$

in which n can vary from 0 to at least 9 depending on the carbon substrate used for growth and the microorganism. PHAs are a highly reduced form, and being virtually insoluble in water, they exert a negligible osmotic pressure; therefore, these polymers are an ideal storage material.

Pseudomonas oleovorans is one of the most versatile microorganisms in its ability to produce PHAs from a variety of carbon substrates, including many different *n*-alkanes, *n*-alkenes, alkanoic acids, and alkenoic acids,[3-7] but the type of carbon substrates used is important in regulating both the amount of PHA product and the rate and amount of cell growth.[7] Production of PHAs containing functional groups has been investigated recently, and PHAs containing functional groups such as phenyl,[8,12] olefin,[3,6,7] ester,[7] chloride,[9] nitrile,[7] and fluoride[10] groups have been reported.

In this laboratory *P. oleovorans* has been grown with various types of organic compounds to evaluate the potential of new carbon substrates for producing nonnatural PHAs, including those with functional groups, and the carbon substrates evaluated have been classified according to the results obtained for cell growth and PHA production.[7] The classifications include (1) carbon substrates that support cell growth and PHA production, type A; (2) carbon substrates that support cell growth without PHA production, type B; and (3) carbon substrates that do not support cell growth, type C. Of the alkanoic acids in type A substrates, nonanoic acid (NA) and octanoic acid (OA) gave the highest PHA and cell yields. Most alkanoic acids that contained a functional substituent such as a nitrile, bromine, or alcohol group belonged to type B, while alkanoic acids containing very polar substituents, such as either an amino or a second alkanoic acid, belonged to type C. Type B substrates were evaluated in this study, including 6-bromohexanoic acid (6BRHA), 8-bromooctanoic acid (8BROA), and 11-bromoundecanoic acid (11BRUA) for PHA production. Mixtures of these ω-bromoalkanoic acids with either NA or OA were used to produce polymers and to determine whether the PHAs so produced contained brominated repeating units.

Results and Discussion

The growth curves for *P. oleovorans* with either 6BRHA, 8BROA, or 11BRUA are shown in Figure 1. It is seen in Figure 1 that 11BRUA gave the highest growth rate and cell density indicated by optical density. In fact, the growth rate with 11BRUA was as fast as that with either NA or OA. In contrast, 6BRHA did not support growth for the first 80 h, and the optical density increased to 0.3 only after 200 h. These results suggested that a substantial separation between the bioreactive group (COOH) and the bromo substituent group is necessary for the utilization of the carbon compound and for cell growth. However, no PHA granules were observed in cells grown with any of these ω-bromoalkanoic acids, so mixtures of these compounds with known PHA-producing substances were evaluated.

To investigate PHA production by *P. oleovorans* grown with mixtures of ω-bromoalkanoic acids and PHA-producing substrates, NA and OA were chosen for the latter, and initially each ω-bromoalkanoic acid was mixed with an equimolar amount of NA or OA. The growth curves of *P. oleovorans* with the NA mixtures are presented in Figure 2. As seen in Figure 2, very surprisingly growth was faster when the substrate mixture contained shorter ω-bromoalkanoic acid. This result was opposite to our expectation because *P. oleovorans* grew fastest with 11BRUA as seen in Figure 1. The increase of the optical density of the culture in the late stationary phase grown with an equimolar mixture of NA and 11BRUA might be caused by the observed change of the color of the culture,

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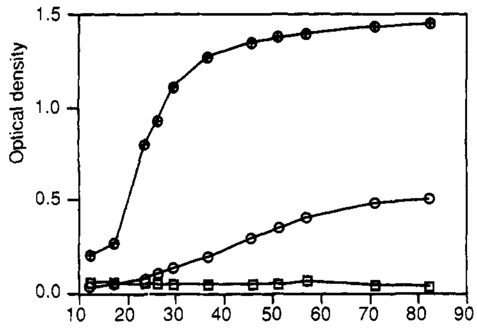

Figure 1. Growth curves for *P. oleovorans* grown with various ω-bromoalkanoic acids. (⊗) 11BRUA; (○) 8BROA; (□) 6BRHA.

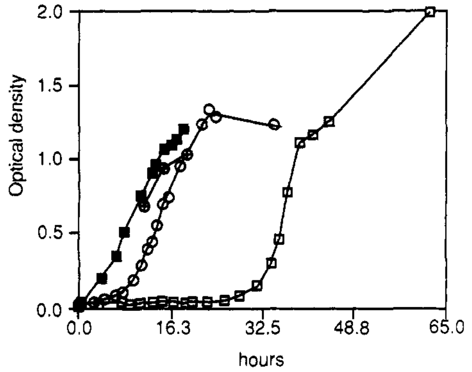

Figure 2. Growth curves for *P. oleovorans* with mixtures of NA and ω-bromoalkanoic acids. (■) 2:1 mixture of NA with 8-bromooctanoic acid; (⊗) equimolar mixture of NA with 6BRHA; (○) equimolar mixture of NA and 8BROA; (□) equimolar mixture of NA and 11BRUA.

Table I
Preparation and Bromine and Brominated Unit Contents in the PHAs Obtained from Cells Grown with Mixtures of NA and ω-Bromoalkanoic Acids

ω-bromo-alkanoic acid	mixture ratio[a]	biomass yield, g/L	PHA yield, g/L	PHA content, wt %	brominated units,[b] mol %	bromine, wt %
11BRUA	1:1	0.68	0.10	14.0	37.5	21.67
11BRUA[c]	1:1	0.69	0.18	26.1	3.7	ND[d]
8BROA	1:1	0.63	0.08	13.0	25.0	10.35
8BROA	2:1	0.55	0.21	38.2	4.2	<2.00
6BRHA	1:1	0.42	0.02	4.5	25.0	15.30

[a] Mole ratio of NA/ω-bromoalkanoic acid in the carbon source. [b] Determined by ^{1}H NMR spectra. [c] NA and 11BRUA were fed in sequence. [d] Not determined.

both of liquid phase and the cells, from light yellow to dark brown, which would decrease transmittance of the light used for optical density measurement.

Cells grown with all three ω-bromoalkanoic acid mixtures contained PHA granules, and the data for biomass and PHA yields so obtained are collected in Table I. The highest PHA and biomass yields were obtained from the culture grown with an equimolar mixture of NA and 11BRUA, and the lowest yields were obtained from the culture grown with the 6BRHA mixtures.

All of the PHAs prepared from cells grown with mixtures of NA and a ω-bromoalkanoic acid were soft and tacky in feel, but the PHA obtained from cells grown with shorter bromoalkanoic acid mixture was harder. The DSC thermograms of the PHAs prepared from mixtures of NA with either 11BRUA or 8BROA are presented in FIgure 3. As seen in this figure, the PHA prepared using the 11BRUA mixture had a much lower heat of fusion and a lower melting temperature than the PHA prepared using 8BROA mixture. The heat of fusion and melting temperature of the PHA from the equimolar 6BRHA mixture was almost identical to that from the 8BROA mixture. All of the PHA copolymers had only a single glass transition temperature and a single melting temperature, indicating that these PHAs were random copolymers.

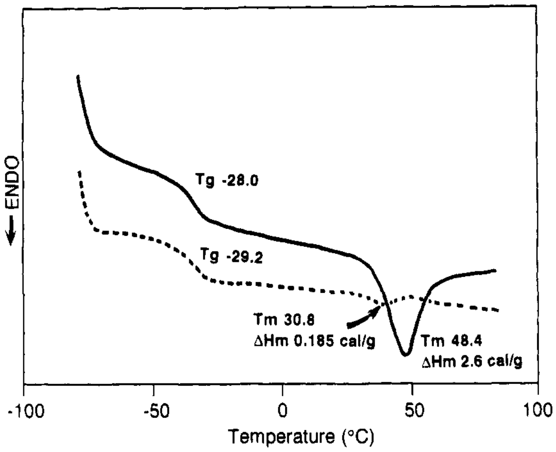

Figure 3. DSC thermograms of PHAs obtained from equimolar mixtures of either NA with 8BROA (solid line) or NA with 11BRUA (dashed line).

The weight fractions of bromine in these PHAs were determined by elemental analysis, and the mole fractions of the brominated units were also estimated from their ^{1}H NMR spectra, with the results in Table I. The data in Table I show that the equimolar mixture of NA with 11BRUA produced the copolymer with the highest amount of brominated units.

The ^{13}C NMR spectra of the PHAs produced from equimolar mixtures of NA and either 6BRHA or 8BROA are shown in Figures 4 and 5, respectively. The spectra indicate that the PHA from the 6BRHA mixture contained both 6-bromo-3-hydroxyhexanoate (BRHH) and 4-bromo-3-hydroxybutyrate (BRHB) units, as well as the units expected from NA, while the PHA from the 8BROA mixture contained both 8-bromo-3-hydroxyoctanoate (BRHO) and 6-bromo-3-hydroxyhexanoate (BRHH) units with the NA units. The ^{13}C NMR spectrum of the PHA from an equimolar mixture of NA with 11BRUA was too complicated to be analyzed precisely, but it is likely that this PHA contained mainly 9-bromo-3-hydroxynonanoate (BRHN) and 7-bromo-3-hydroxyheptanoate (BRHP) units along with the NA units on the basis of gas chromatography analyses.

The gas chromatography/atomic emission detector (GC/AED) chromatograms of the methanolyzed samples of the PHAs produced from either equimolar mixtures of NA with 6BRHA, NA with 8BROA, or NA 11BRUA are presented in Figures 6, 7, and 8, respectively. Figures 6a, 7a, and 8a were obtained by monitoring the intensity of the light emitted with a wavelength of 495.7 nm, which is from carbon atoms, and Figures 6b, 7b, and 8b were obtained by monitoring the intensity of the light emitted with a wavelength of 478.6 nm, which is from bromine atoms. All of these chromatograms show peaks for methyl 3-hydroxyheptanoate (HH) and methyl 3-hydroxynonanoate (HN) units. If the methyl 3-hydroxyundecanoate unit (HU) was present, the amount was too small to be detected. The retention times of repeating units in Figure 8 are different from those in Figures 6 and 7 because analysis conditions were different.

Figure 6 shows that the chromatogram of the methanolyzed sample of the PHA obtained from the 6BRHA mixture with NA contained one new peak which is not a simple 3-hydroxyalkanoate. The new peak is designated

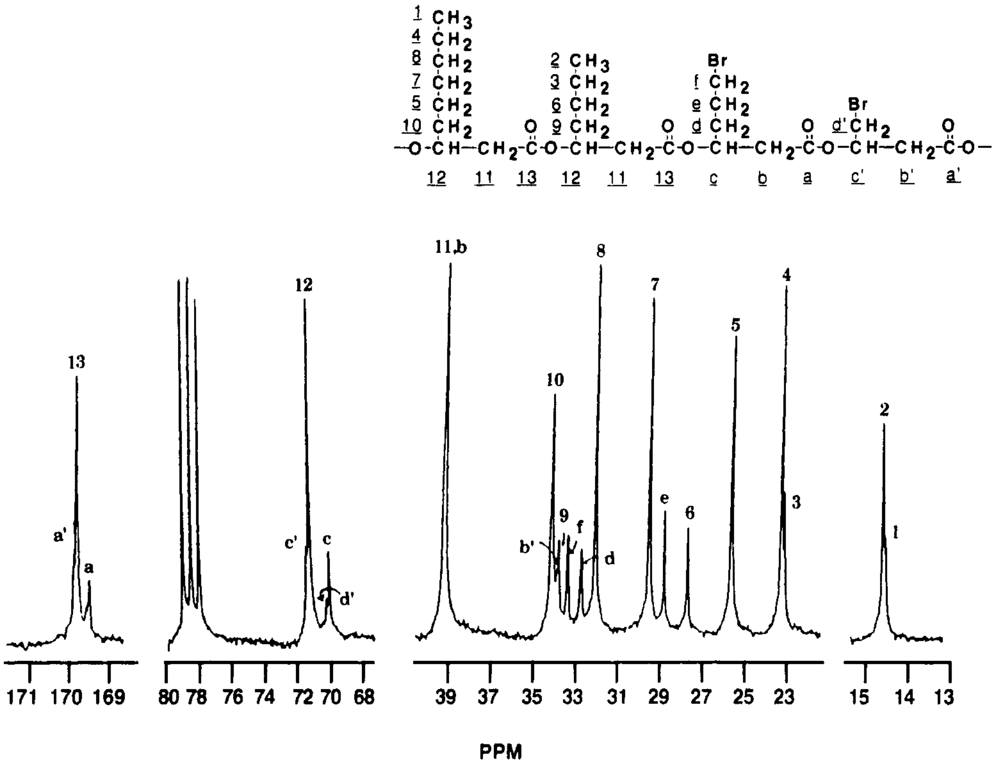

Figure 4. ^{13}C NMR spectrum of the PHA obtained from an equimolar mixture of NA with 6BRHA.

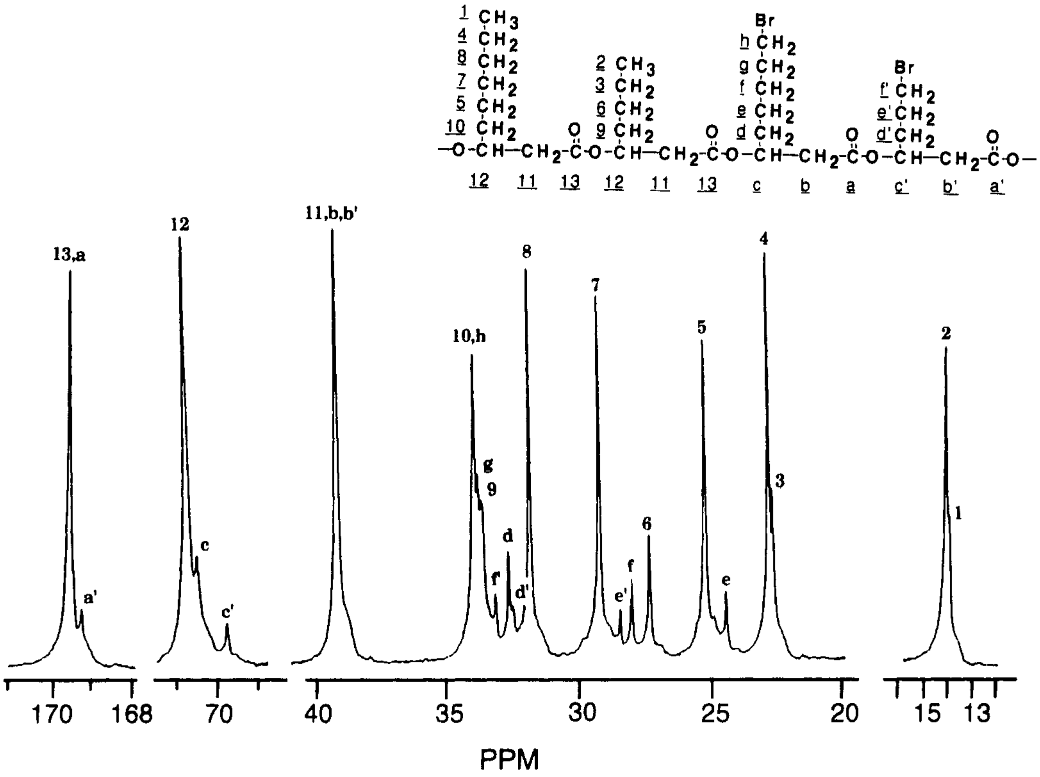

Figure 5. ^{13}C NMR spectrum of the PHA obtained from an equimolar mixture of NA with 8BROA.

X, and FIgure 6b shows that this new peak did not contain bromine. The GC/MS spectrum of X did not show an ion fragment with an *m/z* of 103, which is characteristic for the methyl 3-hydroxyalkanoates. The same peak was also detected in the gas chromatogram of the methanolyzed products of the PHAs from mixtures of either NA with 8BROA (Figure 7) or OA with 8BROA. The presence of this peak in the PHAs obtained from carbon source mixtures containing either 6BRHA or 8BROA suggests that X may be a product of an unknown reaction of BRHH which may occur during methanolysis. Figures 7 and 8 show that brominated units longer than BRHP did not undergo such reaction during methanolysis and contained bromine.

A similar unknown peak, labeled X, is seen in Figure 7. This peak is believed to represent the product of the reaction of BRHP during the methanolysis. Hence, the X peaks may be products of an intramolecular, nucleophilic substitution reaction on bromine by the hydroxyl group, which could form either five-membered or six-

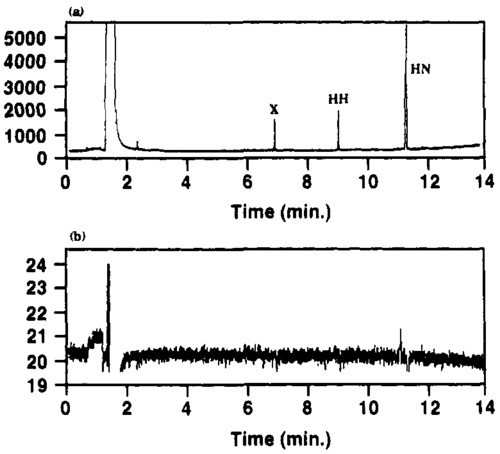

Figure 6. Gas chromatography/atomic emission detector (GC/AED) chromatogram of the methanolyzed sample of the PHA obtained from an equimolar mixture of NA with 6BRHA. (a) GC/AED chromatogram of carbon-containing peaks; (b) GC/AED chromatogram of bromine-containing peaks.

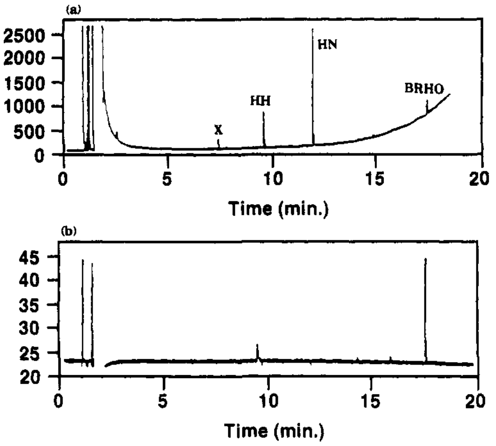

Figure 7. Gas chromatography/atomic emission detector (GC/AED) chromatogram of the methanolyzed sample of the PHA obtained from an equimolar mixture of NA with 8BROA. (a) GC/AED chromatogram of carbon-containing peaks; (b) GC/AED chromatogram of bromine-containing peaks.

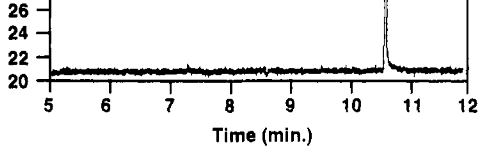

Figure 8. Gas chromatography/atomic emission detector (GC/AED) chromatogram of the methanolyzed sample of the PHA obtained from an equimolar mixture of NA with 11BRUA. (a) GC/AED chromatogram of carbon-containing peaks; (b) GC/AED chromatogram of bromine-containing peaks.

membered cyclic compounds from BRHH and BRHP, respectively.

To examine the relationship between the composition of the mixture of carbon sources and the amount of brominated units incorporated into the PHA, *P. oleovorans* was also grown with a 2:1 mixture of NA and 8BROA. The growth curve for this mixture is presented in Figure 2. Both the PHA yield and the PHA content obtained from cells grown with this mixture were significantly higher than those obtained from the equimolar mixture as shown in Table I, but the mole percent of brominated repeating units was only approximately 4% (the weight percent of bromine in the polymer was less than 2%). It is to be expected that the content of the brominated units in the PHA would decrease as the amount of bromoalkanoic acid in the carbon source was decreased, but the decrease of mole percent of brominated units in the PHA from 25% to 4% was unexpectedly large considering the decrease in the mole percent of 8BROA in the carbon source was only from 50% to 33%.

A growth experiment in which *P. oleovorans* was grown with NA and 11BRUA fed in sequence was also carried out. The initial concentration of NA was 5 mM, and when the growth reached the deceleration phase, a neutral aqueous solution of 60 mmol of 11BRUA was added to the 12-L culture so that the total amount of both carbon sources used in this sequential feeding experiment was the same as the amount of the carbon sources used with the equimolar mixture of the two carboxylic acids.

The growth rate resumed as soon as the 11BRUA solution was added to the culture, indicating that *P. oleovorans* utilized the 11BRUA as soon as it was available. The amount of biomass obtained by harvest of this culture at the second stationary phase was not significantly different from the amount of biomass obtained with the equimolar mixture of these carbon sources, but the PHA yield was almost twice as much as that from the culture grown with an equimolar mixture of the carbon sources. Nevertheless, the mole fraction of the brominated units in the PHA was only 3.7% even though the yield of cellular materials and the PHA yield were higher than for the culture grown with an equimolar mixture of these acids. Apparently, therefore, a smaller amount of 11BRUA was utilized for production of the PHA than when NA was a cosubstrate with 11BRUA.

PHA production by *P. oleovorans* grown with mixtures of OA and 11BRUA or 8BROA was also investigated. It was surprising that there was no growth for 48 h when *P. oleovorans* was exposed to an equimolar mixture of OA with 11BRUA. The growth experiment was stopped after 48 h.

An equimolar mixture of OA with 8BROA supported cell growth, and granules were observed in the cells as expected. Cell growth reached the stationary phase 22 h after inoculation, and the optical density in the stationary phase was 1.15. From 1 L of the culture grown with this mixture, 0.31 g of biomass and 0.11 g of PHA were obtained. The mole percent of the brominated units and the weight percent of bromine in the PHA were approximately 2% and 1%, respectively, which are much lower than those in the PHA obtained from an equimolar mixture of NA and 8BROA. These results are quite surprising considering the results from our previous study on the production of PHA from *P. oleovorans* using an equimolar mixture of OA and NA as the carbon source.[7] The PHA obtained in that study contained 46 mol % of the repeating units produced from OA and 54 mol % of the repeating units produced from NA, and the copolymer so obtained was a random copolymer. These results indicated that when OA and NA were cosubstrates, *P. oleovorans* did not prefer one of these carboxylic acids over the other either for growth or for polymer production.

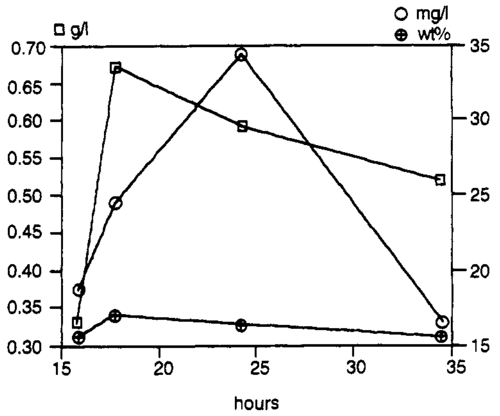

Figure 9. Biomass yield, weight percent of bromine in the PHA, and PHA yield from cells grown with an equimolar mixture of NA and 8BROA vs growth time. (□) Biomass yield (g/L); (⊗) wt % of bromine; (○) polymer yield (mg/L).

This difference in the utilization of NA or OA was also observed when *P. oleovorans* was grown with mixtures of either NA or OA with either 11-cyanoundecanoic acid or 11-aminoundecanoic acid. In both cases, OA gave lower PHA yields and lower incorporation of the functional carbon substrate.

The effect of the amount of 8BROA in the substrate mixture was also evaluated in an attempt to obtain PHAs with higher brominated unit contents. For this purpose, a 2:1 mixture of 8BROA and OA was used as the carbon source. The yields of biomass and PHA from the cells grown with this mixture were 0.37 and 0.08 g/L, respectively. The mole percent of brominated units and the weight percent of bromine were only approximately 4% and 2%, respectively, which was still low but higher than that in the PHA from the equimolar mixture.

The changes in the weight percents of bromine in the PHA, in the biomass yields, and in the PHA yields obtained from cultures grown with an equimolar mixture of NA with 11BRUA are plotted against growth time in Figure 9. As explained under Experimental Section, the solubility of 11BRUA was so low that there were particles of 11BRUA undissolved in the medium. The biomass obtained after 17 h contained these 11BRUA particles which may be responsible for the higher biomass yield at 17 h than at 24 h. After 24 h, there was no 11BRUA undissolved in the medium. Figure 9 shows that the PHA yield decreased after 24 h but the weight percent of bromine in the PHA remained unchanged. The gas chromatographic analysis and NMR spectroscopic analysis of the PHA also showed that the composition of repeating units from NA remained constant throughout the growth. The ratio of the amount of HH units and the amount of HN units was 21:79 as calculated from the GC peak areas, which was the same as that found in other PHAs obtained from carbon sources containing NA. The mole percent of brominated units in the PHA was 25% as determined by NMR spectroscopy.

The molecular weights of PHA samples used for plotting Figure 9 were constant. This result showed that the molecular weights of the polymer accumulated in the cells do not change according to growth time. The number-average molecular weight was approximately 50 000, and the polydispersity index was approximately 2.0 in all cases.

Experimental Section

Biosynthesis of PHA. Biosynthesis of PHAs was carried out in 12-L culture scale at 30 °C as described in our previous papers[4,8] except for the growth experiment reported in Figure 1, which was carried out using 500-mL cultures. The 500-mL cultures were incubated in a shaker maintained at 30 °C. Sterilization of 12-L media containing brominated alkanoic acids was carried out only for 40 min, and the media were cooled to room temperature immediately after sterilization to minimize the possible hydrolysis reaction. The initial concentrations of ω-bromoalkanoic acids in the growth media were 10 mM. However, the solubility of 11BRUA was so low that the medium prepared with 11BRUA was a suspension of a partially dissolved 11BRUA solution and undissolved particles of 11BRUA. A medium prepared with equimolar mixtures of 11BRUA with either OA or NA in concentrations of 5 mM for each carbon substrate contained some undissolved 11BRUA particles. Therefore, the actual concentration of 11BRUA in the medium prepared with 10 mmol of 11BRUA/L was lower than 5 mM. However, the undissolved 11BRUA particles eventually dissolved and were consumed by the cells as they grew.

For the sequential feeding experiment of NA and 11BRUA, *P. oleovorans* was grown initially on a 12-L E* medium containing 10.5 mL of 98% NA. When growth reached the stationary phase, a 500-mL aqueous solution of 16 g of 11BRUA was added to the culture. The pH of the 11BRUA solution was preadjusted to 7.

Cells were harvested when growth reached the second stationary growth state. PHAs were isolated from lyophilized cells by extraction with hot chloroform in a Soxhlet extractor. The crude polymer was dissolved in chloroform and reprecipitated by addition to rapidly stirred methanol. The precipitation was usually repeated three times.

Composition of PHA. The mole fraction of brominated units was determined by NMR spectroscopy. The peak areas of protons on the brominated carbons (3.4 ppm) and protons on the carbon at the β position to carbonyl group (5.2 ppm) were used to calculate the mole fraction. The weight percentages of bromine in the PHA were determined by elemental analysis, which was carried out in the Micro-Analysis Laboratory at the University of Massachusetts. All new PHAs were methanolyzed and gas chromatographed according to the procedure described in our early papers.[4,8] The same samples were analyzed for the presence of bromine using a Hewlett-Packard 5890A gas chromatograph equipped with a Hewlett-Packard 5921A atomic emission detector (AED). Emissions of light with wavelengths of 478.6 and 495.7 nm were monitored for the detection of bromine and carbon, respectively.

Others. DSC study was carried out using a Du Pont differential scanning calorimeter between temperatures of −100 and 100 °C. The heating rate was 20 °C/min. PHA samples were stored at room temperature for approximately 2 months before DSC measurement. DSC thermograms shown in Figure 3 were obtained from the first heating cycle.

Molecular weight was determined by a gel permeation chromatography system equipped with a Waters 6000 solvent delivery system, RI detector, U6K injector, 200 A Styragel column, and linear Ultrastyragel column. A standard curve established with standard polystyrenes was used. The eluent was chloroform; the concentration of the sample was approximately 10 mg/mL, and 30–40 μL was injected. For the study of molecular weight change with respect to growth time, the molecular weights of the crude extracts from biomasses harvested at different growth times were analyzed to prevent possible molecular weight change caused by purification procedure.

NMR spectra were taken using a Varian XL-200 or a Varian XL-300 NMR spectrophotometer at 17 °C. Approximately 10 mg of a PHA was dissolved into 1 mL of chloroform-*d* for NMR spectrum measurement.

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