Preparation and Characterization of Poly(β-hydroxyalkanoates) Obtained from *Pseudomonas oleovorans* Grown with Mixtures of 5-Phenylvaleric Acid and *n*-Alkanoic Acids

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ABSTRACT: *Pseudomonas oleovorans* was grown with various mixtures of 5-phenylvaleric acid, PA, and either *n*-nonanoic acid, NA, or *n*-octanoic acid, OA. The resulting polymers contained the 3-hydroxyalkanoate units produced from either NA alone or OA alone and 3-hydroxy-5-phenylvalerate (HPV) units from PA. The mole fraction of HPV units in the polymeric products increased as the mole fraction of 5-phenylvaleric acid was increased in the feed, but close investigation of substrate consumption, cell growth, and polymer production revealed that *P. oleovorans* utilized 5-phenylvaleric acid and the *n*-alkanoic acid independently to produce mixtures of poly(β-hydroxy-5-phenylvalerate) (PHPV) and the poly(β-hydroxyalkanoate) (PHA), produced from either *n*-alkanoic acid alone. The molecular weights of the two types of PHAs produced were independent of the ratio of two organic acid substrates and of the growth time.

Introduction

Pseudomonas oleovorans produces poly(β-hydroxyalkanoates) (PHAs) as an energy and carbon storage material when it is grown with medium-length alkanes or alkanoic acids (hexanoic acid to dodecanoic acid), which are the most frequently used carbon sources for PHA preparation by *P. oleovorans*.[1,2] The PHAs produced from *n*-alkanoic acids are elastomers with glass transition temperatures between −30 and −50 °C and melting transition temperatures between 40 and 55 °C. The PHA prepared from the octanoic acid, OA, has the highest melting transition temperature of the PHAs produced by *P. oleovorans* from such substrates, but the highest PHA yield was obtained when nonanoic acid, NA, was used as the sole carbon source.[2]

One of the objectives of the research program in this laboratory on the production of PHAs by bacteria is to obtain polymers that contain functional groups in the side chain. To this end, *P. oleovorans* has been grown with carbon substrates containing various substituents, and the PHAs isolated from the biomass were evaluated for the presence of that functionality. By this procedure PHAs containing various functional groups such as olefin, phenyl, bromine, nitrile, and methyl ester groups have been obtained in our laboratory.[3,4,6,7] The PHA containing the phenyl group was obtained from *P. oleovorans* grown with only 5-phenylvaleric acid, PA.[4] This polymer was a homopolymer of 3-hydroxy-5-phenylvalerate (HPV) units, that is, poly(β-hydroxy-5-phenylvalerate) (PHPV). PHPV had a higher glass transition temperature than the PHAs prepared from the higher *n*-alkanoic acids, approximately 18 °C, but a very low degree of crystallinity as indicated by the endothermic peak for the melting transition in the DSC thermogram.

PHAs produced by *P. oleovorans* grown with mixtures of NA and OA were random copolymers, which contained all of repeating units expected for the polymers produced with either alkanoic acid alone,[5] and, in general, PHAs produced by this bacteria from mixtures of two carbon sources are random copolymers.[7] Therefore, the growth of *P. oleovorans* with mixtures of organic substrates is a new and useful method for the preparation of PHAs having new monomer compositions.

In this study the production of PHAs by *P. oleovorans* grown with mixtures of PA and either NA or OA was investigated. One goal of this program was the preparation of PHAs from *n*-alkanoic acids which have higher glass transition temperatures as a result of the random incorporation of HPV units.

Experimental Section

Production of PHAs. The cell growth and PHA production experiments were carried out as described in our previous study.[5] The concentration of the alkanoic acids in the growth medium was 10 mM.

Substrate Composition. A culture grown with an equimolar mixture of NA and PA was used for monitoring the amount of NA and PA remaining in the medium after different growth times. Sixty-milliliter samples of the culture were taken at different growth times, cells were removed by centrifugation, and exactly 25 mL of the aqueous solution supernatant was taken for analysis. To this solution was added 2.0 mL of 20 mM aqueous sodium octanoate solution as the internal standard. The solution was acidified with 2 mL of concentrated HCl by using litmus paper to confirm the acidity. The alkanoic acids in the solution were extracted with two portions of 4 mL of chloroform, the chloroform solution was dried over anhydrous magnesium sulfate, and the chloroform was evaporated until the total volume of the solution became approximately 4 mL. One milliliter of the dry chloroform solution and 1 mL of 3% (v/v) H_2SO_4 solution in methanol were mixed in a Pyrex tube with a screw cap, the cap was hand tightened, and the tube was placed in an oil bath, which was preheated to 100 °C. After 4 h, the tube was cooled to room temperature and the reaction mixture was washed with 2 mL of distilled water. The organic layer was dried over anhydrous magnesium sulfate, and 1–2 μL of this solution was injected into a Perkin-Elmer 8500 gas chromatograph equipped with a Durabond-Carbowax-M15 megabore capillary column and a flame ionization detector. The fractions of remaining acids were calculated from the areas of the peaks in the gas chromatograms by using the peak of methyl octanoate as the internal standard.

PHA Composition. A culture grown with a medium containing an equimolar mixture of NA and PA was used for this analysis. One or two liters of the growing culture was harvested

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Table I
Cell Growth and PHA Production for *P. oleovorans* Grown with Mixed Substrates

alkanoic acid (mol:mol)	growth time, h	o.d. at harvest	biomass yield, g/l	PHA yield, g/l	PHA content,[b] (% DW)	HPV units, mol %
NA:PA (1:0)	18	2.3	1.10	0.43	39.1	0.0
NA:PA (2:1)	15.5	2.0	0.78	0.29	37.2	12.6
NA:PA (1:1)	17.5	2.0	0.77	0.29	37.7	25.7
NA:PA (1:2)	15.0	1.7	0.50	0.16	31.6	40.6
NA:PA (2:1)[a]	12.0	1.2	0.83	0.13	15.1	14.9
OA:PA (1:0)	18.8	2.2	1.01	0.36	36.0	0.0
OA:PA (1:1)	13.5	1.5	0.51	0.20	39.2	24.3

[a] Aeration rate and agitation rate were 2 L/min and 100 rpm for the first 10 h and then increased to 6 L/min and 200 rpm for 2 h before harvesting. [b] Weight percent of polymer based on the dry weight, DW, of the biomass.

at different times, and the biomass was collected by centrifugation followed by freeze drying.[5] The PHA was extracted from the dry biomass by stirring with an excess of chloroform (10 mL of chloroform for 1 g of biomass) at room temperature. The extraction was carried out for 12 h, after which residual cell materials were removed by filtration. The filtered cell materials contained less than 1% of PHN (PHA produced on NA) as determined by gas chromatography. The PHA product was obtained by precipitating the crude polymer twice from methanol.[5] The mole fraction of phenyl groups in the PHA was determined by ^{1}H NMR spectroscopy on solutions of the polymers in acetone-d_6. The peak areas of the aromatic protons and the protons in the β position from the ester group were used for the calculation.

Fractionation. Fractionation was carried out on a polymer mixture isolated from cells grown with a 2:1 mixture of NA and PA. This PHA contained 13 mol % of HPV units. One gram of the polymer was dissolved into 10 mL of chloroform, and *n*-hexane was added to the vigorously stirred solution until the solution became cloudy. The precipitated polymer was recovered by centrifugation and the unprecipitated polymer by evaporation of the solvent. The mole fraction of HPV units in the precipitated fraction was about 60% while the unprecipitated fraction contained less than 5% of HPV units. The amount of the precipitated fraction that was recovered from this analysis was approximately 0.2 g.

Analyses. NMR spectra were recorded on a Varian XL 200 or a Varian XL 300 NMR spectrometer at 18 °C. The IR spectra were recorded on a Perkin-Elmer 1600 series FTIR in the form of a coated film on a NaCl cell. A Du Pont DSC 100 differential scanning calorimeter was used for glass transition and melting transition measurements between the temperature range of −100 and +100 °C at a heating rate of 20 °C/min. Molecular weights were determined with a gel permeation chromatography (GPC) system consisting of a Waters 6000A solvent delivery system, a U6K injector, an RI detector, an Ultrastyragel 200-Å column, and an Ultrastyragel linear column. Chloroform was used as the eluent. A standard curve was established with standard polystyrene samples. Methanolysis and gas chromatography analysis were carried out as described in our previous study.[5]

Results and Discussion

The results from the PHA preparation by *P. oleovorans* using various mixtures of PA and either NA or OA are presented in Table I. As described previously, the cells were harvested when their growth reached the stationary phase to obtain the maximum PHA yield.[5] Therefore, the harvesting time in Table I is the time taken for the growth to reach the stationary phase after inoculation. The induction time for the growth of *P. oleovorans* with PA was 20 h, and it took 50 h for the growth to reach the stationary phase with this substrate,[4] but it took only 18 h for the growth to reach the stationary phase when NA was the only carbon source.

The time taken to reach the stationary phase was shorter when PA was a cosubstrate with NA than when the latter was alone. This result differs from that obtained when 10-undecenoic acid and NA were cosubstrates for *P. oleovorans*. For each of those substrates used alone, the induction time and the time taken to reach the stationary phase were both longer with 10-undecenoic acid than with NA, and for mixed substrates of these two compounds the time to reach the stationary phase was increasingly longer when the fraction of 10-undecenoic acid was increased in the carbon source.[7]

Table II
Copolymer Compositions of PHAs Obtained with Different Substrates

alkanoic acid (mol:mol)	repeating units in polymers produced,[a] mol %							
	C5	C6	C7	C8	C9	C10	C11	HPV[b]
NA:PA (1:10)	1.7	c	25.3		70.1		2.9	
NA:PA (2:1)	1.7		21.7		60.7		3.3	12.6
NA:PA (1:1)	1.3		17.3		54.2		1.5	25.7
NA:PA (1:2)	0.6		16.0		41.1		1.7	40.6
NA:PA (2:1)[d]	1.5		21.3		60.3		2.0	14.9
OA:PA (1:0)		10.7		83.3		6.1		
OA:PA (1:1)		7.3		64.5		3.9		24.3

[a] C*n* represents the number of carbon atoms *n* in the β-hydroxyalkanoate units in the polymer. [b] β-Hydroxy-5-phenylvalerate unit. [c] Not measurable amounts. [d] Aeration rate and agitation rate were 2 L/min and 100 rpm for the first 10 h and then increased to 6 L/min and 200 rpm for 2 h before harvesting.

The earlier cessation of cell growth when PA was present as cosubstrate may be caused by the production of 3-hydroxy-3-phenylpropionic acid, which could be formed by deacetylation of 3-hydroxy-5-phenylvaleric acid, and the former would not be expected to support cell growth because our investigations have shown that 4-phenylbutyric acid did not.[9] However, the presence of 3-hydroxy-3-phenylpropionic acid in the growth medium was not investigated in this study.

Table I lists the biomass yield and PHA yield, both of which decreased significantly as the ratio of NA to PA in the carbon source was changed from 1:0 to 2:1. This result is to be expected based on the low biomass and PHA yields obtained when only PA was used as the carbon source.[4]

Table I also shows that the difference between biomass yield and PHA yield from cells grown solely with either NA or OA was not significant. Nevertheless, an equimolar mixture of PA and OA gave a significantly lower biomass yield and PHA yield than the equimolar mixture of NA and PA. These results indicate that utilization of OA and NA by *P. oleovorans* is apparently different when they are present as cosubstrates. In other studies in this laboratory, different results from OA and NA were also obtained when these carbon sources were mixed with either bromoalkanoic acids[3] or aminoalkanoic acids.[7]

The repeating unit compositions of the PHAs obtained using various carbon source mixtures are presented in Table II. When PA was used either as a substrate or as a cosubstrate, 3-hydroxy-5-phenylvalerate (HPV) was the only phenyl-containing unit present in the polymer as determined by gas chromatography analysis and ^{13}C NMR spectroscopy. The mole fraction of HPV units determined by NMR spectroscopy increased smoothly with an increase in the mole fraction of PA in the carbon source mixture as shown in Figure 1. The number-average molecular weight of the polymers produced was always approximately 50000, and the polydispersity index was approximately 2.0, regardless of the composition of the carbon source.

The relative amounts of repeating units from NA and OA were calculated by ignoring the presence of HPV units. As seen in Table III, the amounts of all the units present, from the 3-hydroxypentanoate (C5) to 3-hydroxyunde-

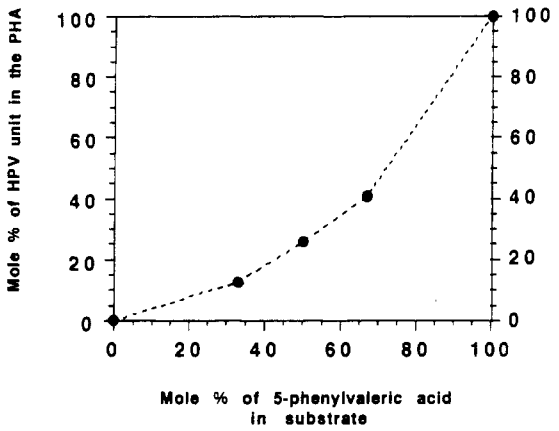

Figure 1. Mole fraction of HPV units in the PHA as a function of the fraction of PA in the substrate mixture with NA.

Table III
Relative Amounts of NA and OA Units in PHA Copolymers Produced on Mixed Substrates

	repeating units in polymers produced,[a] mol %						
carbon source	C5	C6	C7	C8	C9	C10	C11
NA:PA (1:0)	1.7	[b]	25.3		70.1		2.9
NA:PA (2:1)	2.0		24.8		69.5		3.8
NA:PA (1:1)	1.8		23.3		73.0		2.0
NA:PA (1:2)	1.0		26.9		69.2		2.9
NA:PA(2:1)[c]	1.8		25.0		70.8		2.4
OA:PA (1:0)		10.7		83.3		6.0	
OA:PA (1:1)		9.6		85.2		5.2	

[a] See footnotes in Table II. [b] Not measurable amounts. [c] Aeration rate and agitation rate were 2 L/min and 100 rpm for the first 10 h and then increased to 6 L/min and 200 rpm for 2 h before harvesting.

Table IV
Effect of Growth Time on Copolymer Composition with a 1:1 NA:PA Substrate Mixture

harvesting time, h	repeating units in polymers produced,[a] mol %		
	C5	C7	C9
6.2	1.8	29.9	68.3
9.7	1.8	29.5	68.7
13.7	1.6	25.0	73.5
15.0	1.7	25.1	73.2
16.5	2.1	25.6	72.3
18.5	1.8	23.2	75.1
24.7	1.9	24.8	73.3

[a] See footnotes in Table II.

canoate (C11) units, were constant regardless of the presence and the amount of PA. Also, the relative amount of C5, C7, and C9 units (see footnote *a* in the Table II) in the PHA obtained from cells grown with an equimolar mixture of NA and PA was not affected by the growth time as shown in Table IV. The constant ratio of repeating units derived from a given carbon source regardless of the presence of another carbon source has been observed with other combinations of carbon sources in our studies.[3,7] The molecular weights of the PHAs isolated from cells harvested at different times were also constant.

The appearnace of the PHA obtained from mixtures of PA and NA changed according to the harvesting time. That is, the PHA isolated from cells harvested during the exponential phase was transparent, and the PHA obtained became increasingly more cloudy with the increasing growth time at which the cells were harvested. This result suggested that the PHAs obtained from these substrate mixtures might not be random copolymers, so the relative amounts of NA and PA remaining in a culture grown with an equimolar mixture of these two substrates were

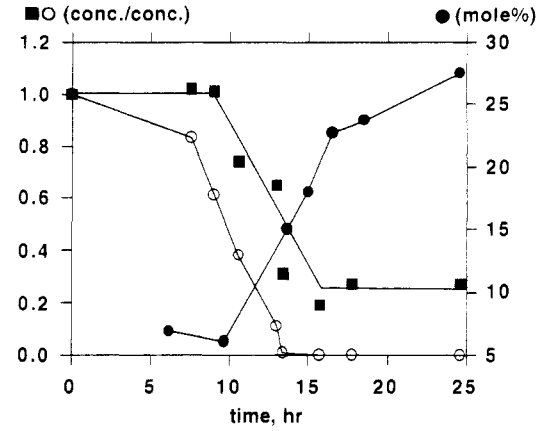

Figure 2. Polymer composition and substrate content as a function of growth time for an initial equimolar mixture of NA and PA: ●, mole percent of HPV units in the polymer; ■, fraction of initial PA remaining in the medium; O, fraction of initial NA remaining in the medium.

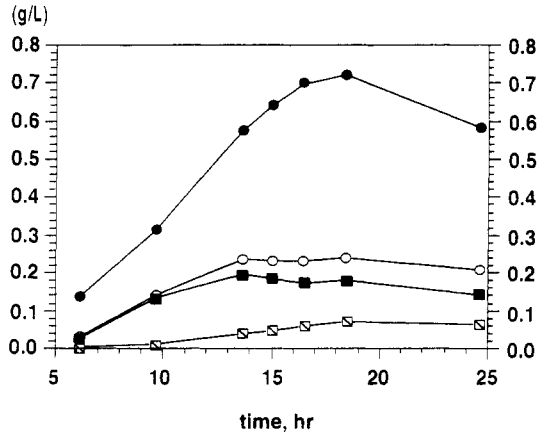

Figure 3. Biomass yield and polymer yield as a function of growth time: ●, biomass yield (g/L); O, PHA yield (g/L); ■, PHN yield (g/L); ⧅, PHPV yield (g/L).

determined as a function of growth time, and the results are shown in Figure 2. The mole fraction of NA in the culture decreased to 0.6 after 8 h, but PA had not started to be consumed at that time, and this selective consumption of NA by *P. oleovorans* was reflected in the mole fraction of HPV units in the PHA. The amount of HPV units in the PHAs increased according to the growth time as presented in Figure 2. In previous studies in this laboratory, in which random copolymers were produced from *P. oleovorans* grown with mixed carbon sources, both of the carbon sources were consumed at the same rate, and the monomer composition of PHA was constant regardless of the harvesting time.[6,7]

In Figure 3, the biomass yield, PHA yield, yield of polymer obtained from NA alone (PHN), and PHPV yield are shown as a function of growth time. The PHN and PHPV yields were calculated assuming that these PHAs were mixtures of pure PHN and PHPV. Yields of both of these polymers changed smoothly with time. The PHN yield, the fraction of NA remaining in the medium, and the optical density of the culture grown with a 5 mM NA medium are shown as a function of growth time in Figure 4. The growth plot presented in Figure 5 was obtained from a separate growth experiment. The growth reached the stationary phase as NA was depleted, and the amount of PHN began to decrease when the NA was depleted. These results are similar to those obtained from our early investigations on the growth behavior of *P. oleovorans* grown with NA.[7] Therefore, it appears most likely that

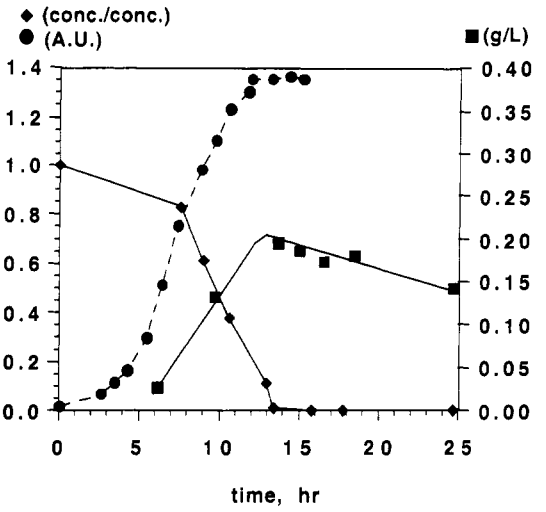

Figure 4. Cell growth, polymer yield, and substrate utilization during growth with an equimolar mixture of NA and PA (5 mM each): ●, fraction of nonanoic acid remaining in the medium; ●, optical density (A.U.); ■, PHN yield (g/L).

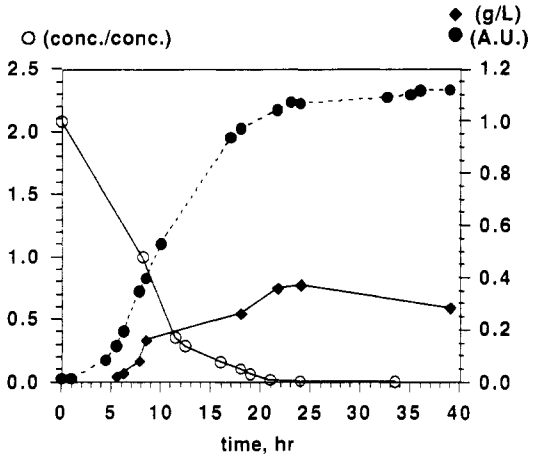

Figure 5. Polymer yield and substrate utilization during growth with 10 mM NA: ●, optical density (A.U.); ◆, PHA yield (g/L); O, fraction of NA remaining in the medium.

P. oleovorans consumes NA and PA independently for energy and polymer production.

None of the PHAs listed in Table I were transparent over the temperature range from room temperature to 70 °C, which is higher than the melting transition temperatures of both PHN and PHO (45 and 55 °C, respectively). The DSC thermogram of the PHA obtained from cells grown with a 1:2 mixture of NA and PA is shown in Figure 6. Two glass transitions, one at approximately −31 °C and one at approximately +5 °C, are seen, and these transitions are close to the glass transition temperatures of the polymer produced with NA alone (PHN) and PHPV. The melting transition temperature in Figure 6 is also identical with that of PHN. The DSC thermograms of the PHA prepared from an equimolar mixture of OA and PA gave similar results.

All of these results indicate that the PHAs produced by *P. oleovorans* grown with cosubstrates consisting of PA and either NA or OA were not random copolymers but were mixtures of two PHAs. Solvent fractionation of the two components was carried out on the PHA obtained from cells grown with a 2:1 mixture of NA and PA. The mole fraction of HPV units in this PHA as determined by NMR was 12.6%. From 1 g of this PHA, approximately 0.8 g of a PHN-rich fraction and 0.2 g of a PHPV-rich fraction were recovered. The PHN-rich fraction contained

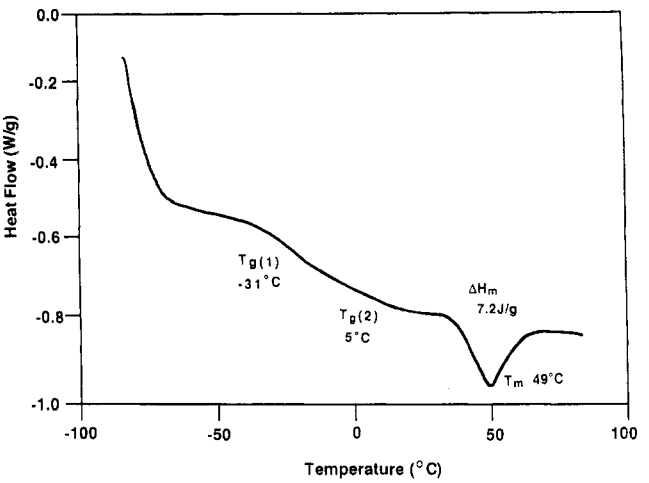

Figure 6. DSC thermogram of the PHA isolated from cells grown with a 2:1 mixture of PA to NA.

less than 5 mol % of HPV units, and the PHPV-rich fraction contained about 60 mol % of HPV units. These results clearly demonstrate that the PHA produced is a mixture of two PHAs.

In the late stationary phase, when the carbon source is depleted, *P. oleovorans* degrades the stored PHA for both energy and cell material production. As a result, a significant number of PHA granules in the cells disappear. In our previous investigation, the repeating unit compositions of the PHAs produced from various alkanoic acids were not affected by this type of degradation[5,7] and those PHAs were random copolymers. Therefore, the constant repeating unit composition, even after degradation of significant amounts of PHA granules, occurs because these PHAs were random copolymers.

The effect of digesting PHA molecules on the repeating unit composition of the PHA produced from cells grown with a 2:1 mixture of NA and PA was investigated. In this experiment, the degradation was facilitated by increasing the aeration rate after a sufficient amount of polymer was produced.[7,8] That is, the cells were grown under normal conditions for the first 10 h, and then the aeration and agitation rates were increased from 2 L/min and 100 rpm to 6 L/min and 200 rpm. Visual observation by a light microscope revealed that a significant number of PHA granules disappeared during these 2 h of increased air supply. The fermentation results for this experiment are shown in the fifth row of Table I. There was a sharp decrease in the PHA yield and PHA content after this procedure was followed, but the amount of cellular materials excluding PHA [(biomass) − (PHA)] increased from 0.49 to 0.7 g/L.

These results showed clearly that under these conditions *P. oleovorans* produced cellular materials as well as energy at the expense of a PHA initially produced. The mole fraction of HPV units slightly increased during the PHA degradation, which suggested that PHN was degraded earlier than PHPV. These observations correspond to the results discussed earlier.

The production of mixtures of PHAs by *P. oleovorans* grown with mixtures of PA and NA was an unexpected result because both of these substrates support cell growth and PHA production when used as sole carbon sources. Our previous investigations showed that with other substrate mixtures random copolymers were obtained,[5-7] but in the present case the substrates were much different in structure, and this difference could be responsible for the result obtained. It has been recently demonstrated that in both *P. oleovorans* and *Alcaligenes eutrophus*

there are present two DNA sequences coding for PHA polymerases.[10,11] The results reported in this work would tend to substantiate that there are two different polymerase enzymes, which simultaneously produce two different polymers. Whether or not these two polymerase systems are present in the same cell or in separate cells remains to be determined.

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Registry No. PA, 2270-20-4; NA, 112-05-0; OA, 124-07-2; PHPV (homopolymer), 134736-36-0; PHPV (SRU), 129645-03-0; PHNA (homopolymer), 120659-39-4; PHNA (SRU), 128482-62-2; PHOA (homopolymer), 120659-38-3; PHOA (SRU), 86175-71-5.