Polyesters Produced by *Pseudomonas oleovorans* Containing Cyclohexyl Groups

Marianela Andújar, María A. Aponte,* and Emilio Díaz

Department of Chemistry, University of Puerto Rico, Mayagüez, Puerto Rico 00681

Eduardo Schröder

Department of Agronomy, University of Puerto Rico, Mayagüez, Puerto Rico 00681

Received August 2, 1996; Revised Manuscript Received December 10, 1996[⊗]

ABSTRACT: Polymers containing cyclohexyl units were isolated when *Pseudomonas oleovorans* was grown with 5-cyclohexylvaleric (CHV) or 4-cyclohexylbutyric acid (CHB). This was opposed to the case where shorter chain acids were used as carbon sources. Growth experiments using homologous cycloalkylalkanoic acids as carbon sources were carried out with varying ratios of the acids to nonanoic acid. A concentration of 90% CHV (20 mM total concentration) afforded a polymer in which monomeric units containing a cyclohexyl ring (up to 24% of units) were identified by NMR and GC−MS. The polymeric material isolated in about 9% of the dry cell weight was a beige, sticky material similar in texture to poly(3-hydroxynonanoate). The polymer's intrinsic viscosity was 0.77 dL/g, and its weight-average molecular weight 52 000, as determined by GPC. Thermal analysis indicated the presence of two T_g values: $T_{g,1} = -16.3$ °C and $T_{g,2} = -32.6$ °C, which suggest the presence of two polymers, one containing more cyclohexyl rings than the other. This observation was sustained by TLC analysis.

Introduction

Synthetic polymers which are not easily degraded tend to accumulate in the environment, causing an ecological problem. Several approaches have been suggested to deal with this problem, including burying, incinerating, or recycling the material, making currently used polymers more degradable, and developing new biodegradable polymers. In this context, many synthetic or natural biodegradable polymers, some of the latter produced by microorganisms, have been studied.[1]

The quest for new biodegradable polyesters exhibiting different properties has included the study of the capability of the bacterium *Pseudomonas oleovorans* to produce polymers containing unusual substituted groups. In general, it has been observed that when alkanes and alkanoic acids are fed to this microorganism, it produces poly(hydroxyalkanoate) (PHA) copolymers having 3-hydroxyalkanoate units with various alkyl pendant groups ranging in size from methyl to nonyl residues.[2] In addition, it has been reported that PHA with either branched,[3] unsaturated,[4] or phenyl[5] groups can be obtained from *P. oleovorans* if the bacteria are forced to grow with organic substrates containing such groups. These polymers have different properties compared to the most common homolog, poly(3-hydroxybutyrate). Bacterial polyesters containing more exotic groups, such as halogen substituents, have also been isolated. Polymeric units containing fluorine were detected in polymers characterized by Hori and co-workers[6] and others containing bromine have also been reported by Kim.[7]

Since bacterial polyesters containing aromatic ring substituents have been isolated in the past by Kim and co-workers,[5] the purpose of the present study was to investigate if new polymers of this kind could be obtained which contained instead aliphatic rings as substituents. It was of particular interest to vary the distance of the ring (cyclohexyl or cyclopentyl) from the carboxylic end of the carbon source fed to *P. oleovorans* to study if (1) the bulky aliphatic group could be incorporated into the polymer as well as the planar phenyl group and (2) there is a constraint on the proximity of the substituent to the active end of the molecule. There was also interest in examining the physical properties of the polymer itself to determine its usefulness.

Experimental Section

Growth Studies. A preculture of *Pseudomonas oleovorans* (ATCC 29347) was obtained by growing the bacteria in 50 or 100 mL of E* media.[8] This medium contained, per liter, the following: 1.58 g of nonanoic acid, 1.1 g of $(NH_4)_2HPO_4$, 5.8 g of K_2HPO_4, 3.7 g of KH_2PO_4, 10 mL of a 100 mM solution of $MgSO_4$, and 1.0 mL of a microelement solution containing, per liter of 1 N HCl: 2.78 g of $FeSO_4 \cdot 7H_2O$, 1.98 g of $MnCl_2 \cdot H_2O$, 2.81 g of $CoSO_4 \cdot 7H_2O$, 1.67 g of $CaCl_2 \cdot 2H_2O$, 0.17 g of $CuCl_2 \cdot 2H_2O$, and 0.29 g of $ZnSO_4 \cdot 7H_2O$. Cultures of *P. oleovorans*, prepared by inoculating modified E* media with the preculture, were grown in which cyclohexylacetic acid (CHA), 3-cyclohexylpropionic acid (CHP), 4-cyclohexylbutyric acid (CHB), 5-cyclohexylvaleric acid (CHV), or 3-cyclopentylpropionic acid (CPP) were used as the unusual carbon source (UCS), either pure or in varying proportions with nonanoic acid (NA) as the cofeed, all at 20 mM total concentration. The alkanoic acids were purchased from Aldrich and used as received.

After inoculating the media with 0.3 mL/100 mL of preculture, the bacteria were grown aerobically with shaking at 150−200 rpm and at 28 °C for up to 2 weeks. Two series of cultures were prepared; small cultures: 50 or 100 mL (in 125 mL Erlenmeyer flasks), and large cultures: 1 or 2 L (in 4 L Erlenmeyer flasks). The growth was monitored by determining the optical density at 650 nm daily. The small cultures were carried out to obtain growth curves of experiments where the unusual carbon sources were fed to the bacteria.

Isolation of the Polymeric Material. The 1-L cultures were used to isolate the polymeric material. After achieving the desired growth, the cultures were processed to extract the polymer by centrifuging the cells at 12 000 rpm for 30 min at 4 °C, drying them in a vacuum oven at room temperature overnight, and extracting the polymeric material by refluxing overnight in about 200 mL of chloroform. The mixture obtained was filtered, and the solution reduced in volume to about 1 mL with a Büchi rotary evaporator. The viscous liquid obtained was poured slowly into methanol with stirring to yield a sticky, beige-colored material. This material was isolated

* To whom correspondence should be addressed.

[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1997.

S0024-9297(96)01163-1 CCC: $14.00

by decanting the methanol and drying in a vacuum oven at room temperature overnight. It was then weighed and the percentage of polymer dry cell weight was determined.

Cellular Observations. The bacterial samples were studied using an Olympus BH-2 fluorescence microscope equipped with a blue filter and a camera after being stained[9] with Nile Blue A to observe the inclusion bodies, if present.

Spectral Analyses. Proton and carbon nuclear magnetic resonance (NMR) studies, including COSY analysis, were carried out using a Varian Gemini 300 FT-NMR spectrometer to help determine the nature of the polymer obtained. The samples were dissolved in deuterated chloroform, and tetramethylsilane was used as the internal standard.

Composition Analyses. Gas chromatography/mass spectrometry (GC−MS) analyses were done on a Hewlett-Packard 5890 Series II system equipped with a Hewlett-Packard DB-225 column to identify the units present in the polymers. For this study, either the isolated polymeric material or just the dry cells were methanolized by reacting the material in Teflon-lined screw-cap tubes containing 0.85 mL of methanol, 1.0 mL of chloroform, and 0.15 mL of concentrated sulfuric acid.[10] The tubes were heated for 140 min in a thermostated oil bath at 100 °C. After this period, the samples were washed with 1 mL of water, and the organic phase was isolated, dried over anhydrous sodium sulfate, filtered, and stored for analysis. A synthetic standard was used to identify the polymeric units obtained after methanolysis. It was prepared according to Fritzsche[3] by alkylating methyl acetoacetate with cyclohexylmethyl bromide and later reducing the obtained product with potassium borohydride.

Determination of the Molecular Weight. Molecular weight was determined by gel permeation chromatography (GPC) using a Waters Millipore 410 refraction index detector, a 746 data module, and a 600E system controller equipped with linear and 10^3 Å Ultrastyragel columns placed in series. The polymer was extracted from 6 L of culture grown with 20 mM 90% CHV for 261 h. Injections of 0.5 μL of 0.2% (w/v) solutions were used in each case. The intrinsic viscosity was determined at 30 °C with a Ubbelohde semimicro apparatus, using chloroform as the solvent. A calibration curve, where the product of the intrinsic viscosity and M_w was plotted against elution time, was prepared using polystyrene standards.

Thin-Layer Chromatography Studies. Thin-layer chromatography was carried out to roughly determine possible separation of polymers. The samples were applied to silica gel plates with a fluorescent indicator, and a solution of 1:4 acetone/*n*-hexane was used as the eluent.[8] Resublimed iodine was employed to reveal the plates.

Thermal Analysis. Thermal analysis was carried out on a 2910 TA Instruments differential scanning calorimeter (DSC) by heating the samples in closed aluminum pans from −50 to +100 °C with a ramp of 10 degs/min. Quenching the samples at about −100 °C and carrying out a second run allowed the determination of the glass transition temperature.

Results and Discussion

Growth Studies. Small-scale growth experiments of *P. oleovorans* were carried out in E* media as described by Antoun[8] containing different concentrations of CHA, CHP, CHB, CHV, or CPP as the unusual carbon source (UCS) in varying proportions along with nonanoic acid (NA) as the cosubstrate. Growth curves (optical density vs hours) were obtained in each case. All showed that the bacteria were capable of growing in the presence of the unusual acids, although decreased in this ability as the amount of NA decreased. The bacteria grew with 100% UCS only when these were CHV and CHB. However, in the latter case, growth was possible only after 400 h. Figure 1 shows the results obtained when CHV and NA were the carbon sources at 20 mM total concentration.

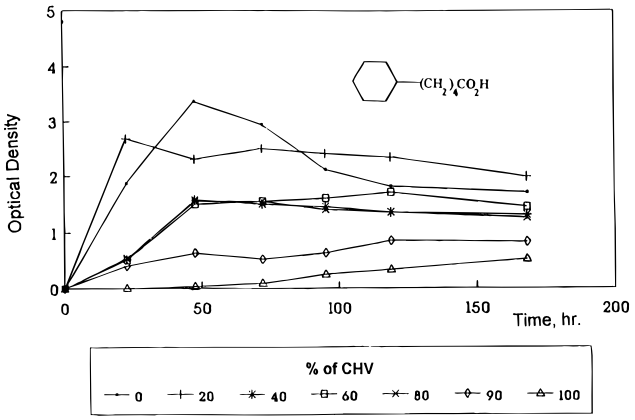

Figure 1. Growth of *P. oleovorans* as measured by optical density using CHV as the unusual carbon source and NA as the usual carbon source in varying proportions (20 mM total concentration).

The results indicate that *P. oleovorans* grows more easily in media that contains UCS with cyclohexyl groups remote from the carboxyl group. They are comparable with previous studies in which *P. oleovorans* was grown with octanoic acids having methyl groups in different positions of the chain, where as the distance of the methyl group to the carboxyl group increased, larger bacterial growth was obtained, as well as higher polymeric production.[3] These results also compare with those carried out by Fritzsche,[11] in which *P. oleovorans* grew in the presence of 5-phenylvaleric acid at different concentrations. In this case, the lower the acid concentration, the higher and faster the bacterial growth. On the contrary, when the source was 3-phenylpropionic acid, the cells did not grow until past 6 weeks. In addition, in studies done with bromine as the substituent group in the UCS, the highest and fastest growth was possible when that group was as far as possible from the carboxyl group.[7,13]

Cellular Observations. *P. oleovorans* cells grown with different carbon sources as well as the isolated polymeric material were stained with Nile Blue A and observed under a fluorescence microscope. This was carried out to determine the presence of polymer in the cell, since orange fluorescence is evidence of the presence of polymer and a green fluorescence is autofluorescence of the bacteria and thus indicates absence of polymer.[9] It was observed that (1) the unstained cells showed green autofluorescence, independently of whether they contained polymer or not, (2) the isolated and stained polymer gave a strong orange fluorescence, and (3) orange fluorescence gave evidence of the presence of polymer in cells that used as carbon sources NA and (NA + CHV), respectively.

Isolation of the Polymeric Material. The biomass (dry cell weight) and polymer yield results of the cultures of *P. oleovorans* grown with different UCS at different concentrations carried out on a large scale with 20 mM total concentration of carbon sources are reported in Table 1. The monomer units detected, as characterized by NMR, are reported in the table.

The following may be observed from Table 1: (1) Only poly(3-hydroxynonanoate) (PHN) was formed when the UCS had short aliphatic chains (2 and 3 carbons), even when using 90% CHA and CHP (a−c). (2) The cyclohexyl ring was incorporated only when the UCS had chain lengths of more than 3 carbons (e−g). In these trials, however, both polymer and cellular yield were always low. (3) The experiments where the UCS had 3

Table 1. Polymers Produced by *P. oleovorans* per Liter of Culture Using Several Unusual Carbon Sources

run	carbon source	dry cell wt (mg)	polym wt (mg)	polym yield[a] (%)	monomer units detected[b]
a	90% CHA	65.1	4.75	7.3	NA
b	90% CHP	176.5	28.5	16.1	NA
c	60% CPP	375.9	85.0	22.6	NA
d	50% CHB	756.0	89.1	11.8	NA/CHB
e	90% CHB	132.8	19.3	14.5	NA/CHB
f	90% CHV	438.0	38.2	8.7	NA/CHV
g	100% CHV	249.1	7.9	3.2	NA/CHV
h	100% NA[c]	1500.0	700.0	46.7	NA

[a] Percentage of polymer per dry cell weight. [b] Determined by NMR. [c] Reported by Gross[2] with 10 mM total concentration.

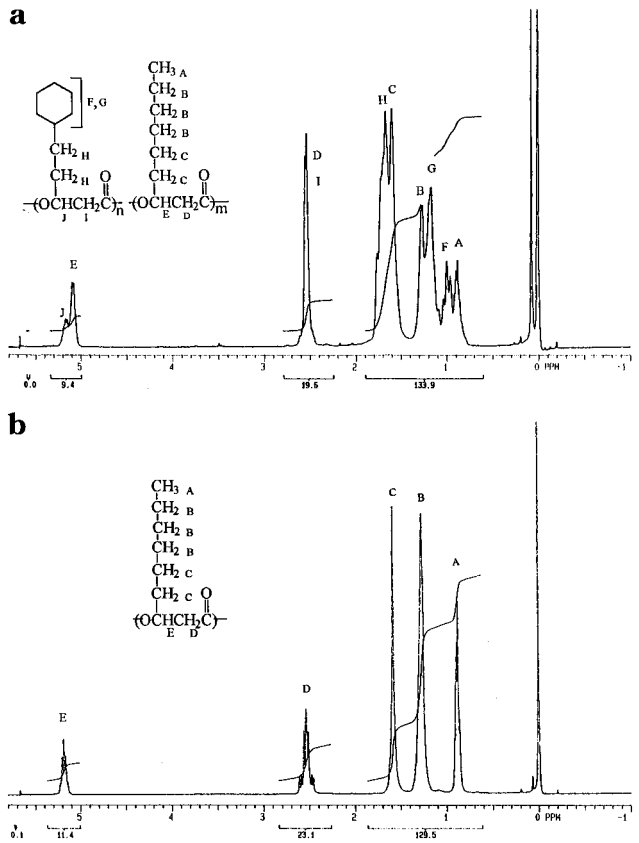

Figure 2. (a) Proton NMR spectrum of polymer synthesized by *P. oleovorans* with 90% CHV + 10% NA (20 mM total concentration) as the carbon sources; harvested after 214.5 h of growth. (b) Proton NMR spectrum of polymer synthesized by *P. oleovorans* using NA as the only carbon source.

chain carbons and either the five or six member ring (CHP and CPP) produced polymer with no ring included in its structure (b and c). (4) It was observed that using a higher concentration of the UCS yielded less dry cells (d vs e and f vs g). Item h presents the values previously reported for the case of nonanoic acid for comparison.[2]

These results suggested that the best condition to grow *P. oleovorans* at a larger scale (2 L) in order to isolate enough polymer containing the saturated ring is using 90% CHV at 20 mM. This large-scale growth experiment was carried out three times to obtain enough polymeric material for different characterization and physical properties tests.

The isolated material was beige and sticky. It was relatively easy to extract and its content in the dry cells was always close to 9.0% (Table 1). Figure 2a shows the proposed structure of the polymer units where the values of *n* and *m* could vary. This was confirmed by

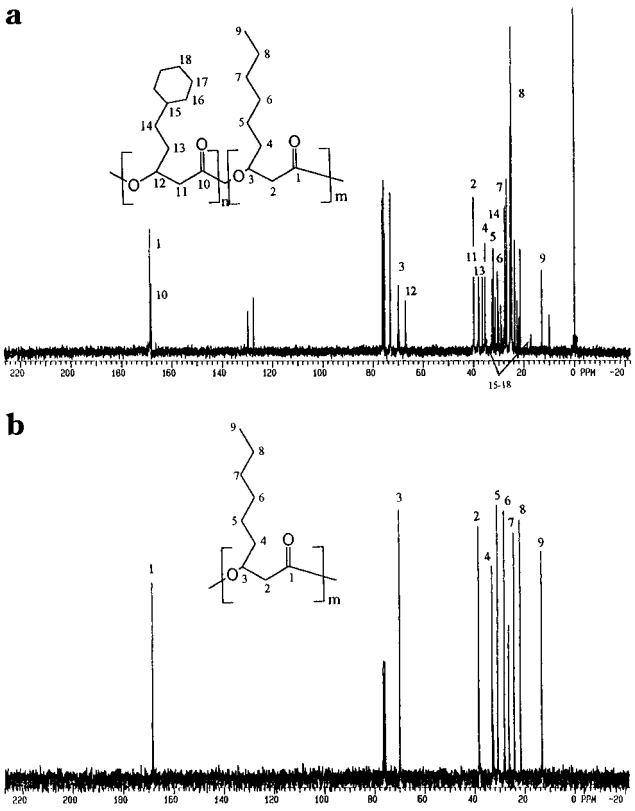

Figure 3. (a) Carbon NMR spectrum of polymer synthesized by *P. oleovorans* with 90% CHV + 10% NA (20 mM total concentration) as the carbon sources; harvested after 214.5 h of growth. (b) Carbon NMR spectrum of polymer synthesized by *P. oleovorans* using NA as the only carbon source.

characterization with GC−MS and proton and carbon NMR analyses, as discussed in the next sections.

Spectral Analyses. When 90% CHA, 90% CHP, and 60% CPP were the UCS, the NMR signals observed corresponded closely to those of the previously reported[2,6,12] spectrum of PHN (Figure 2b), evidencing that only PHN was formed without including the cyclohexyl ring in the polymer. The carbon NMR spectra of the mentioned samples confirmed this result.

Figures 2a and 3a correspond respectively to the proton and carbon NMR spectra of the polymer synthesized by the bacteria grown with 90% CHV (20 mM total concentration), which was found to contain 24% of cyclohexyl units according to GC−MS analysis. These spectra are very similar to those obtained from the polymer isolated from the culture grown with CHB (not shown), which agree with previous results reported by Kim,[13] indicating that production of poly(3-hydroxy-4-cyclohexylbutyrate) is possible. Figures 2b and 3b present respectively the proton and carbon NMR spectra of PHN, which are included for comparison. The peaks in the spectra obtained for all PHA were partly assigned following a previous analysis reported by Fritzsche and co-workers.[3]

The proton NMR spectrum in Figure 2a shows two signals in the area of the methine protons between 5.0 and 5.4 ppm. The downfield extra signal is assigned to the methine proton of the monomeric units with the saturated cyclohexyl ring. In addition, the signal of the methylene protons in the 1.2−1.7 ppm region is more complicated than that corresponding to the spectrum of PHN, suggesting the presence of the cyclohexyl unit.

The carbon NMR spectrum in Figure 3a shows complicated regions due to the presence of the carbons

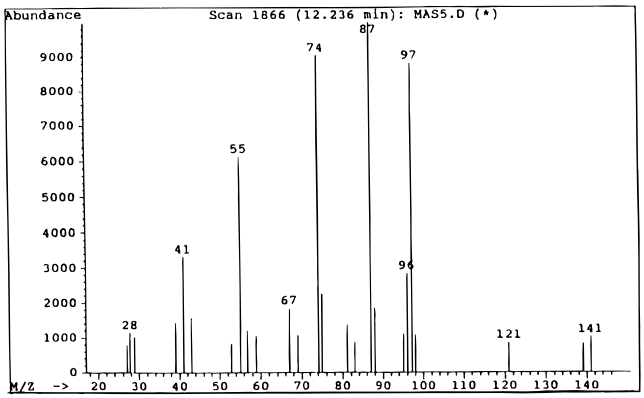

Figure 4. Mass spectrum of the signal at $t_R = 12.24$ min of the chromatogram of the methanolysis product of the dry cells of *P. oleovorans* grown with 90% CHV + 10% NA (20 mM total concentration) extracted after 171.5 h of growth.

from both units, the one with the saturated ring and the one without it. The zone between 20 and 40 ppm, assigned to the methylene carbons, contrasts sharply with the relatively simple region corresponding to the spectrum of PHN (Figure 3b). In addition, two discrete signals close to 168 ppm are also observed, which can be assigned to different carbonyl carbons.

In addition to these NMR analyses, a homonuclear correlation in two dimensions (COSY) was carried out. The analysis showed the coupling of the methylene and methyl protons of the straight-chain substituent, of the methylene protons among themselves, of the diasterotopic methylenes with the methine proton, and of the methine proton with the methylene of the chain closer to it. These NMR results strongly suggest that the cyclohexyl ring is present in the polymer.

Composition Analyses. In order to use the GC−MS technique to determine the composition of the polymer, it was necessary first to methanolize to small volatile units the isolated material or the dry cells containing polymer. Since the polymer was expected to contain cyclohexyl units in the backbone, which would produce methyl 5-cyclohexyl-3-hydroxypentanoate on methanolysis, this compound was synthesized through a route reported by Fritzsche[3] as a standard. Thus, GC−MS analysis was carried out on both this standard and the methanolized polymer or dry cells in order to confirm the presence of the cyclohexyl unit.

A signal at $t_R = 12.84$ min in the chromatogram was assigned to the synthesized standard as confirmed with the mass spectrum obtained. The most important fragments were $m/z = 141$ (fragment of the saturated ring with the hydroxyl group included), $m/z = 96$ and 97 (fragment of the saturated ring and the proximal methylene group), $m/z = 83$ (fragment of the saturated ring), and $m/z = 55$, 41, and 29 (fragments due to ring fragmentation).

A chromatogram of the methanolysis product of the dry cells of *P. oleovorans* grown with 90% CHV, extracted after 171.5 h of growth was studied. A signal at $t_R = 12.24$ min was attributed to the units containing the saturated ring. This was confirmed by studying its fragmentation pattern (Figure 4). The most important fragments at $m/z = 141$, 97, 83, 55, 41, 29, and 28 compare closely with those of the standard. Signals at $t_R = 17.03$ and 17.64 min corresponded to methyl 3-hydroxyalkanoates, as identified by the library. This confirmed that the polymer also contained open-chain carbon units, mostly nonanoate fragments.

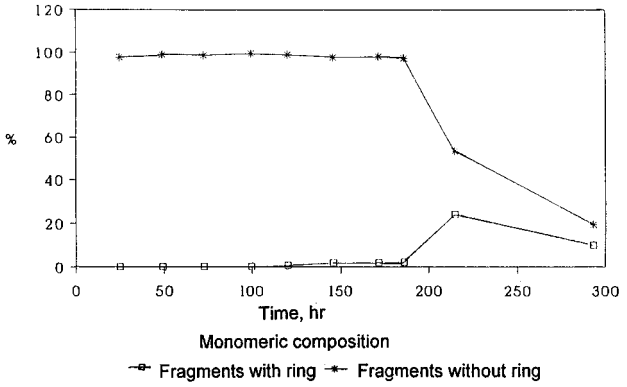

Figure 5. Variation of monomeric composition of the polymer synthesized by *P. oleovorans* using 90% CHV + 10% NA (20 mM total concentration) versus time determined by GC−MS.

Bacterial Production vs Time. In order to determine if the production of the polymer followed a particular pattern for incorporation of the cyclohexyl ring, culture samples were isolated after different times of growth and GC was performed on each one. The areas of the signals corresponding to the polymeric fragments with and without the ring were calculated, and the results are shown in Figure 5. This shows that the cyclohexyl ring fragment was not included initially in the synthesized polymer, but its incorporation reached a maximum after 200 h. This result suggests that the bacteria metabolize CHV only after they have consumed NA, which would be their primary carbon source.

Determination of the Molecular Weight. The molecular weight of the polymer was determined by GPC. The intrinsic viscosity and the molecular weight were found to be, respectively, 0.77 dL/g and 52 000. The M_w of this bacterial polymer compares with that reported by Kim[7] for a polymer synthesized by *P. oleovorans* with carbon sources that contain bromine as the substituent, which was 50 000.

Thin-Layer Chromatography Studies. This method was carried out on polymers extracted from dry cells of *P. oleovorans* that grew in different carbon sources to attempt to distinguish between the presence of one or several polymers in the isolated polymeric product. These studies showed the following: (1) The $R_f = 0.24$ of PHN is very close to the one reported[10] previously (0.20). (2) The $R_f = 0.24$ of the polymer synthesized by the bacteria with 60% CPP is similar to that reported (0.20), which is in agreement with the results obtained from NMR and DSC analysis. All methods confirm that with this carbon source the bacteria only utilizes NA to synthesize PHN. (3) The polymer synthesized by the bacteria with 90% CHV exhibits a value of $R_f = 0.22$, also similar to the one reported[8] for PHN. These findings suggest that this polymer is actually either a mixture of polymers, most richer in nonanoate than in 5-cyclohexylvalerate units, or a copolymer with very few cyclohexyl rings, which make it similar to PHN.

Thermal Analyses. A thermogram of PHN (Figure 6b) used as control exhibited a melting temperature (T_m) = 53.3 °C and a glass transition temperature (T_g) = −35.0 °C, indicating that the polymer is semicrystalline. These values compare well with other studies done with the same bacteria and identical carbon source.[2,5] The thermograms of the polymers synthesized by *P. oleovorans* grown with 90% CHA, 90% CHP, and 60% CPP (at 20 mM total concentration) as UCS were similar to

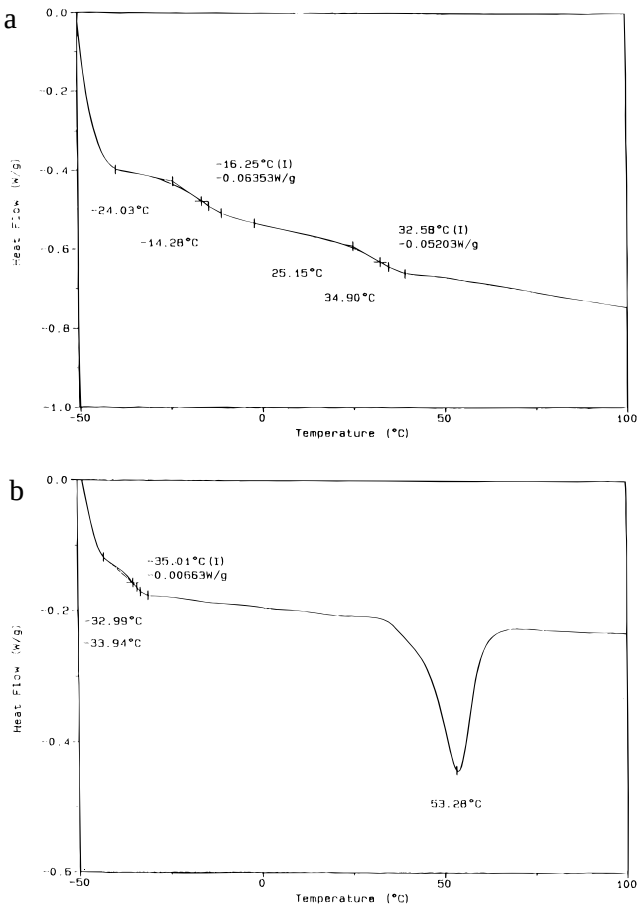

Figure 6. (a) DSC thermogram of polymer synthesized by *P. oleovorans* with 80% CHV + 20% NA (20 mM total concentration) as the carbon sources; harvested after 283.5 h of growth. (b) DSC thermogram of polymer synthesized by *P. oleovorans* using NA as the only carbon source.

that of PHN. These results were very well in accord with the proton and carbon NMR that indicated that only PHN is produced when the mentioned carbon sources are used by *P. oleovorans*.

A very different situation to the one described above is observed in Figure 6a, corresponding to the DSC thermogram of the polymer synthesized by the bacteria with 80% CHV (20 mM total concentration), extracted after 283.5 h of growth. A T_m signal is not observed, so it is assumed that this polymer is mostly amorphous. Two well-differentiated values of T_g, $T_{g,1} = -16.3$ °C and $T_{g,2} = 32.6$ °C, may also be seen in the thermogram, which suggests the presence of two polymers. This is not surprising when comparing this result with that reported previously by Antoun[8] for poly(3-hydroxy-5-phenylvalerate), where a very small endotherm covering from 50 to 65 °C was observed, as well as a $T_g = 17-20$ °C. This was also true in previous studies reported, which were carried out with NA and 5-phenylvaleric acid as carbon sources and *P. oleovorans* as the chosen bacteria. These studies concluded that the bacteria produced a mixture of two PHA and not just one copolymer.[5,14]

Conclusion

The results reported in this work indicate that it is possible to incorporate a bulky cyclohexyl ring into a PHA, although only if the distance from it to the carbonyl end of the alkanoic acid which contains it is at least four carbons long. The polymer composition was observed to vary drastically with growth time. Apparently, the optimum conditions to incorporate the ring moiety in the polymer are when 90% CHV + 10% NA (20 mM total concentration) are the carbon sources and the harvesting time is about 200 h. On the other hand, the thermal analyses and TLC results suggest that what was actually obtained was a mixture of two polymers, one richer in cyclohexyl ring units than the other.

Acknowledgment. This work was funded in part by Grant HRD-910667 from the RIMI−NSF program. We would like to acknowledge the help of Irving Vega with the preparation of the GC−MS standard.

References and Notes

(1) (a) Doi, Y.; Fukuda K. *Biodegradable Plastics and Polymers*; Elsevier: New York, 1994. (b) Lenz, R. W. *Advances in Polymer Science*; Springer-Verlag: Berlin, 1993; Vol. 107.
(2) Gross, R. A.; DeMello, C.; Lenz, R. W.; Brandl, H.; Fuller, R. C. *Macromolecules* **1989**, *22*, 1106.
(3) Fritzsche, K.; Lenz, R. W.; Fuller, R. C. *Int. J. Biol. Macromol.* **1990**, *12*, 92.
(4) Fritzsche, K.; Lenz, R. W.; Fuller, R. C. *Int. J. Biol. Macromol.* **1990**, *12*, 85.
(5) Kim, Y. B.; Lenz, R. W.; Fuller, R. C. *Macromolecules* **1991**, *24*, 5256.
(6) Hori, K.; Soga, K.; Doi, Y. *Biotechnol. Lett.* **1994**, *16*, 501.
(7) Kim, Y. B.; Lenz, R. W.; Fuller, R. C. *Macromolecules* **1992**, *25*, 1852.
(8) Antoun, S.; Grizzi, I.; Lenz, R. W.; Fuller, R. C. *Chirality* **1991**, *3*, 492.
(9) Ostle, A. G.; Holt, J. G. *Appl. Environ. Microbiol.* **1982**, *44*, 238.
(10) Brandl, H.; Gross, R. A.; Lenz, R. W.; Fuller, R. C. *Appl. Environ. Microbiol.* **1988**, *54*, 1977.
(11) Fritzsche, K.; Lenz, R. W.; Fuller, R. C. *Makromol. Chem.* **1990**, *191*, 1957.
(12) De Koning, G. J. M.; Van Bilsen, H. M. M.; Lemstra, P. J.; Hazenberg, W.; Witholt, B.; Preusting, H.; Van der Galien, J. G.; Schirmer, A.; Jendrossek, D. *Polymer* **1994**, *35*, 2090.
(13) Lenz, R. W.; Kim, Y. B.; Fuller, R. C. *FEMS Microbiol. Rev.* **1992**, *103*, 207.
(14) Curley, J. M.; Hazer, B.; Lenz, R. W. *Macromolecules* **1996**, *29*, 1762.

MA9611634