Bacterial Polyesters Produced by *Pseudomonas oleovorans* Containing Nitrophenyl Groups

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Received September 18, 1998; Revised Manuscript Received February 19, 1999

ABSTRACT: Polymers containing nitrophenyl groups were isolated when *P. oleovorans* was grown with 5-(2′,4′-dinitrophenyl)valeric acid (DNPVA). Growth experiments were carried out containing variable ratios of this and nonanoic acid (NA). These revealed a loss of the 2′-nitro group as the experiment proceeded. The bacteria produced polymeric materials with 1.2%−6.9% of repeating units containing 4′-nitro and/or 2′,4′-nitrophenyl rings, depending on the DNPVA:NA molar ratio and total concentration of these carbon sources, according to NMR analysis. The polymeric material isolated from approximately 15% dry cell weight when using a 7:3 DNPVA:NA molar ratio at 10 mM total concentration was a yellow, elastic substance. Thermal analysis indicated the presence of two T_g values, $T_{g,1} = -35.95$ °C and $T_{g,2} = 28.74$ °C, and one T_m value of 56.42 °C. These data suggest the presence of two polymers, one which contains nitrophenyl rings and the other being the copolymer of nonanoic acid.

Introduction

Recently, there has developed a great deal of interest among scientists to find solutions to the environmental problems resulting from significant accumulation of plastics in dump sites. Since these materials are practically indestructible, they are very difficult to eliminate.

Many microorganisms can, under certain conditions, synthesize biodegradable polymers which are very similar to synthetic plastics. Polyesters produced in this manner are known as poly(3-hydroxyalkanoates) (PHA). It has been found that these bacteria are capable of producing several types of PHA, each of which contains different units along the polymer chain. It is notable that both the individual microbial species and the substrate it feeds upon are factors that affect the molecular identity of the chain produced.[1]

Studies related to the incorporation of unusual substituents in PHA have been carried out where bacteria have been exposed to several substrates.[2] In particular, units with cyclohexyl,[3,4] methyl ester,[5] chlorine, bromine,[6] fluorine,[7] nitrile, and hydroxyl groups[8] have been incorporated. To achieve this, the bacteria were grown with both the desired substrate and the cosubstrate nonanoic or octanoic acid.

In 1990, a homopolymer containing the phenylethyl side group was synthesized by *Pseudomonas oleovorans* when 5-phenylvaleric (PVA) and nonanoic acid (NA) were used as the carbon sources.[9] This material was the first example of a PHA containing an aromatic substituent produced by a microorganism. Analysis revealed that, instead of a new copolymer, a mixture of two PHAs was obtained. This mixture contained the homopolymer poly(3-hydroxy-5-phenylvalerate) and the copolymer of nonanoic acid (PHN). The chemical modification of PVA was carried out by Curley[10] with the introduction of methyl, ethyl, and phenyl groups in its ring. The new substrates and the NA cosubstrate were fed to the bacteria. However, only the methyl group derivative favored polymer production. The polymer obtained was a mixture of two PHAs: poly[3-hydroxy-5-(4-tolyl)valerate] and PHN.

In 1995 Kim and co-workers reported that *Pseudomonas putida* formed PHAs whose composition was highly sensitive to the second-stage cultivation time. It was found that the percent incorporation of 3-hydroxy-6-(*p*-cyanophenoxy)hexanoate groups increased dramatically at extended culture times.[8]

The principal objective of this work was to obtain new bacterial polyesters, produced by *Pseudomonas oleovorans,* using the substrates nonanoic acid and a modified version of PVA. This latter molecule contained the nitro group.

Materials and Methods

Synthesis of 5-(2′,4′-Dinitrophenyl)valeric Acid (DNPVA). A beaker containing 20.0 mL of concentrated sulfuric acid was cooled on ice. Then 10 g (0.06 mol) of PVA was added to the beaker. A cold nitrating mixture containing 7.0 mL of concentrated sulfuric acid and 7.0 mL of concentrated nitric acid was added in a dropwise manner and thoroughly mixed with the beaker solution. Subsequently, 15 drops of concentrated nitric acid were added, and the beaker was removed from the ice and left at room temperature for 2 h under continuous agitation. The solution was slowly poured over approximately 84 g of crushed ice (produced from distilled water). It was slowly stirred until a pasty, yellow solid formed. After this period, the mixture was left to stand for 15 min.

The isolated solid was filtered and washed several times with distilled water. It was then dried under a vacuum for 12 h. The solid was recrystallized from a 9:1 mixture of 2-propanol:water to produce a light yellow solid in 65% yield. Mp = 80−83 °C. ^{1}H NMR: 7.6, 8.4, and 8.8 ppm (aromatic protons); 1.8, 2.4, and 3.1 ppm (methylene protons). ^{13}C NMR: 120−150 ppm (six aromatic carbons), 180 ppm (carbonyl carbon). FT-IR: 1527 and 1348 cm^{-1} (nitro groups), 1604 cm^{-1} (ortho/para substitution in the aromatic ring), 1705 cm^{-1} (carboxyl group), 3482 cm^{-1} (carboxyl group), and 2939−3090 cm^{-1} (aromatic stretching vibrations). Elemental analysis:

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10.1021/ma981482q CCC: $18.00
Published on Web 04/15/1999

Table 1. Composition of the Cultures Prepared with DNPVA:NA

total molar concn (mM)	DNPVA:NA (molar ratio)	% DNPVA	vol of culture (L)
5	100% NA	0	0.1
10	1:1	50	0.1
20 and 30	100% NA	0	0.1
20 and 30	1:9	10	0.1
20 and 30	3:7	30	0.1
20 and 30	1:1	50	0.1
20 and 30	7:3	70	0.1
10	1:1	50	2
20	1:1	50	2
10	7:3	70	2
10	PVA:NA = 1:1	50% PVA	0.1

theory (C, 49.21; H, 4.47; O, 35.80; N, 10.45), found (C, 49.09; H, 4.42; O, 36.11; N, 10.38).

It should be noted that although no special attempt was carried out to obtain a dinitrated compound, the analyses confirmed two nitro groups, ortho and para to the carbon chain, to be present on the ring. Thus, this dinitrated compound was used as the unusual carbon source.

Bacterial Growth. *Pseudomonas oleovorans* was chosen for this study because, in addition to being a nonpathogenic organism that is found commonly in the soil, it also produces high amounts of PHA, including those containing aromatic units.[9] The strain used was ATCC 29347, from the American Type Culture Collection. For the preculture, 50 mL of modified E* solution was placed in a 125 mL Erlenmeyer flask. This medium was prepared as previously reported.[4] The preculture was incubated in a New Brunswick shaker model PsycroTherm NBS at 150 rpm and at 29 °C for 48 h.

Cultures of 0.1 and 2.0 L were prepared containing different proportions of DNPVA or PVA to NA (including DNPVA or PVA as the sole carbon source) as shown in Table 1, at a total concentration of 5, 10, 20, and 30 mM. Each liter of culture was inoculated with 3 mL of the preculture. Culture growth was monitored by determining the optical density at 650 nm. For example, when following the polymerization over time, two 2 L cultures were prepared at a total substrate concentration of 10 mM. Aliquots of 200 mL were then removed from each culture at predetermined times.

Analysis of the Supernatants. The 200 mL aliquots were centrifuged for 30 min at 12 000 rpm and 4 °C. The cellular material was resuspended in approximately 100 mL of a 10 mM neutral Tris-HCl buffer solution and centrifuged again under these same conditions.[11] The centrifuged cells were dried under vacuum at room temperature for 12 h and refrigerated for further analysis, as will be discussed in the following section.[11]

From each sample, 25.00 mL of supernatant was removed and 2.00 mL of 20 mM sodium octanoate (to serve as internal standard) was added. The solution was acidified with sufficient 6 M HCl to protonate the acid carboxylates and extract them with 2 × 5 mL of chloroform. The organic phase was removed and dried with anhydrous magnesium sulfate. After filtration, this phase was evaporated to 3 mL.

Methanolysis of the samples was carried out by placing 1.00 mL of the concentrated chloroform solution in a screw-cap test tube. To this were added methanol (0.85 mL) and sulfuric acid (0.15 mL). Each tube was placed in an oil bath maintained at 100 °C for 140 min.[12] Upon reaction completion, the solution was washed with 1 mL of distilled water and agitated vigorously for 1 min. The organic phase was extracted and dried with anhydrous magnesium sulfate. After filtration, a total of 0.1 μL of this organic phase was injected into the GC-MS.

A quantitative study, using a Hewlett-Packard 5890 series II gas chromatograph/mass spectrometer (GC-MS) in conjunction with a 5971A selective mass detector, followed in order to analyze the presence of any remaining carbon substrates (DNPVA and NA) in the supernatant, in the form of their methyl esters. The program of analysis consisted of the following: initial temperature of 85 °C for 5 min, temperature ramp of 70 °C/min up to 250 °C for 30 min. The temperatures of the injector and detector were 230 and 275 °C, respectively. The fractions of the carbon sources remaining at harvest times were calculated using the areas of each peak of interest. These values were compared with the areas of the octanoic acid standard, NA, and DNPVA peaks of the supernatant extracted at zero hours of incubation.

Analysis of the Centrifuged Cells. The dry cells obtained from the 200 mL aliquots were weighed to determine biomass. The polymeric material in the cells was extracted by refluxing them in chloroform (15 mL of chloroform per gram of biomass) at 60 °C for 12 h.[12] Following this process, the mixture was filtered and evaporated down to 2 mL. It was poured dropwise into cold methanol (10 mL of methanol/mL of chloroform) to precipitate the polymer.[7] The precipitated material was isolated by decantation and dried under vacuum at room temperature for 24 h, weighed, and reprecipitated. The resulting material was quantitatively and qualitatively analyzed with NMR.

Characterization of the Polymers. Each 1 or 2 L culture was harvested when it reached maximum optical density. The cells were isolated as described above. Biomass and polymer weights were determined, and characterization of the material was achieved through NMR, FT-IR, and thermal analysis.

NMR Spectroscopic Analysis. Nuclear magnetic resonance analysis was carried out using a Varian 300 FT-NMR spectrometer. In each case, 5 mg samples were dissolved in 0.7 mL of deuterated chloroform, and tetramethylsilane was used as the internal standard.

FT-IR Spectroscopic Analysis. Samples were first dissolved in chloroform and then run. The resulting spectrum confirms the presence of the following functional groups: 1519 and 1379 cm^{-1} (nitro group), 3771−3122 cm^{-1} (aromatic ring), 1738 and 1215 cm^{-1} (ester group).

DSC Thermal Analysis. The analyses of samples (7−10 mg) were carried out using a TA Instruments model 2910/Thermal Analyst 2100 differential scanning calorimeter (DSC). Aluminum cells without covers and an indium standard were employed. The analysis was programmed to run at an initial temperature of −60 °C with a warming ramp of 10 °C/min for up to 100 °C.

Results and Discussion

Measurements of Optical Density. Small-scale cultures of *P. oleovorans* with DNPVA, PVA, and NA as the sole carbon sources (0.1 L at 10, 20, and 30 mM) were studied. Optimal growth with all carbon sources was evidenced at 20 mM. Very limited growth with DNPVA (maximum average OD = 0.83) was observed. This was in contrast to growth carried out with PVA (maximum average OD = 1.2) and with NA (maximum average OD = 2.3).

Cultures containing DNPVA or PVA and NA in varying molar proportions at 10, 20, or 30 mM (total carbon source concentration) exhibited less growth than cultures having NA as the only carbon source. In these mixed media, optimum growth was obtained using 1:1 DNPVA:NA (30 mM) and 7:3 DNPVA:NA (20 mM). The growth curve for the 10 mM (1:1) study is shown in Figure 1. This figure also demonstrates the growth curve when NA (at 5 mM), for comparison purposes, was used as the only carbon source. It may be observed that the growth curve for the culture with DNPVA shows the lower maximum OD than any other curve. Thus, the presence of the nitrophenyl group appears to be detrimental to *P. oleovorans* growth.

The growth curve for 20 mM DNPVA:NA cultures at different molar proportions is shown in Figure 2. In cultures containing the lower DNPVA concentrations (DNPVA:NA = 1:9 and 3:7), it was observed that maximum growth took place after 28 h. This was also

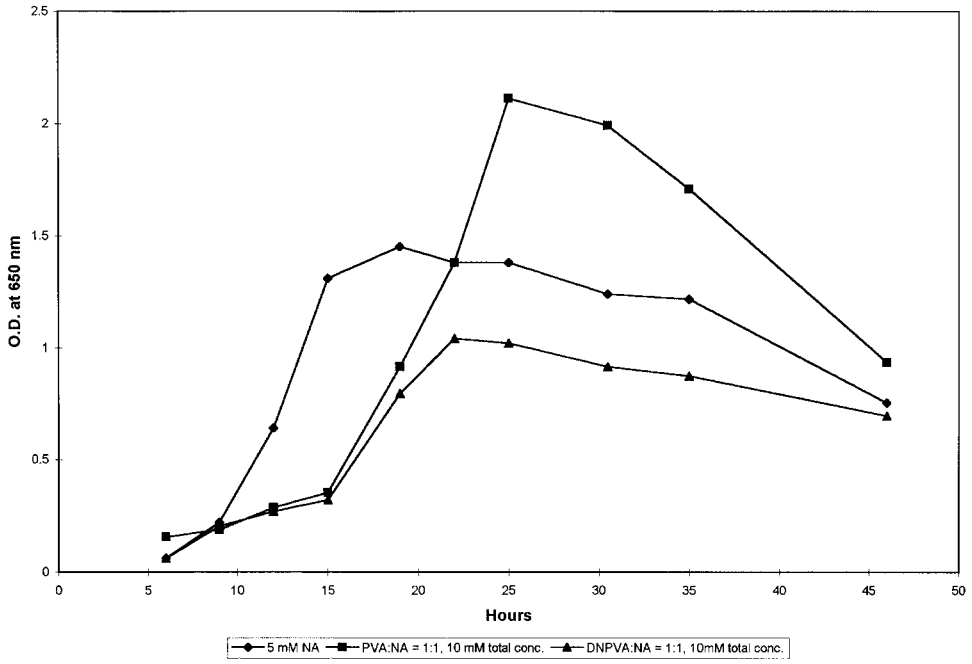

Figure 1. Growth curves of *P. oleovorans* with PVA or DNPVA and NA (1:1 molar ratio, 10 mM total concentration) as the carbon sources. The growth curve of the culture containing only NA (5 mM) is included for comparison.

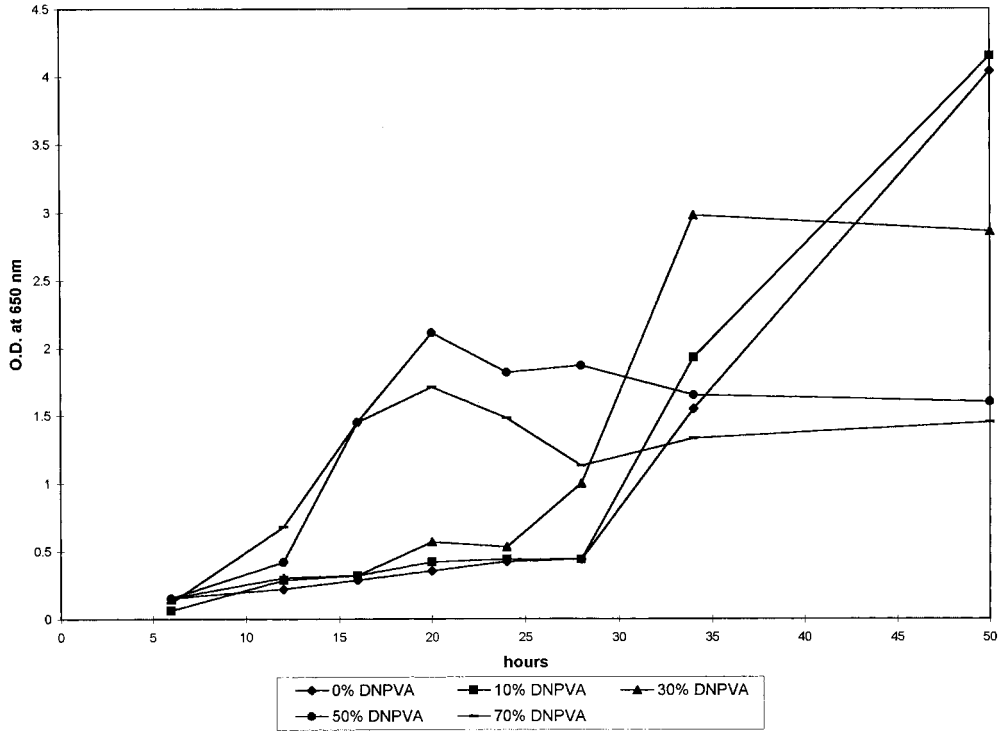

Figure 2. Growth curves of *P. oleovorans* with DNPVA and NA (20 mM total concentration) as the carbon sources at varying molar proportions.

found to be the case for the 10 mM NA control culture. However, cultures with higher DNPVA concentrations (DNPVA:NA = 1:1 and 7:3) reached maximum growth levels at 20 h, with no growth thereafter. In general, it can be stated that as the percentage of DNPVA was increased in the culture medium, less time was required to reach maximum growth. It should be mentioned that although higher cell production was obtained when lower concentrations of DNPVA were present (and longer incubation periods were allowed), very little polymer containing phenyl units was produced. In addition, PHA production seems to occur slightly faster than cell growth (as monitored by OD), as may be observed from the results shown in Table 2.

Analysis of the Consumption of the Carbon Sources vs Time. To meet this study's goals, the supernatants and extracted polymers from nine cultures grown with 1:1 DNPVA:NA (10 mM) were analyzed (see Table 2). Figure 3 presents the remaining percentage of each carbon source in the supernatant as a function of growth time. The results indicate that consumption of the carbon sources NA and DNPVA began immediately after culture inoculation. Although little consumption of DNPVA was detected, following 40.5 h

Table 2. Composition of the Polymer Produced with 1:1 DNPVA:NA (10 mM, 200 mL aliquots) at Different Harvest Times

harvest time (h)	OD max. (at 650 nm)	biomass (g/L)	total PHA (wt %)	aromatic units (%)	MDNPVA (%)[a]	DDNPVA (%)[a]
11	0.39	0.108	20.4	3.0	0.0	3.0
16	0.75	0.229	27.3	n.d.[b]	n.d.	n.d.
20	1.19	0.354	56.6	n.d.	n.d.	n.d.
24	1.43	0.467	40.5	2.1	2.0	0.054
33	1.58	0.499	43.8	2.3	2.1	0.25
40	1.50	0.467	28.9	3.9	3.9	0.0
45	1.43	0.500	39.0	2.8	2.8	0.0
58	1.40	0.416	25.2	2.1	2.1	0.0
65	1.36	0.350	23.1	2.0	2.0	0.0

[a] MDNPVA = mononitrated groups, DDNPVA = dinitrated groups, as identified by integration with NMR. [b] n.d. = not determined.

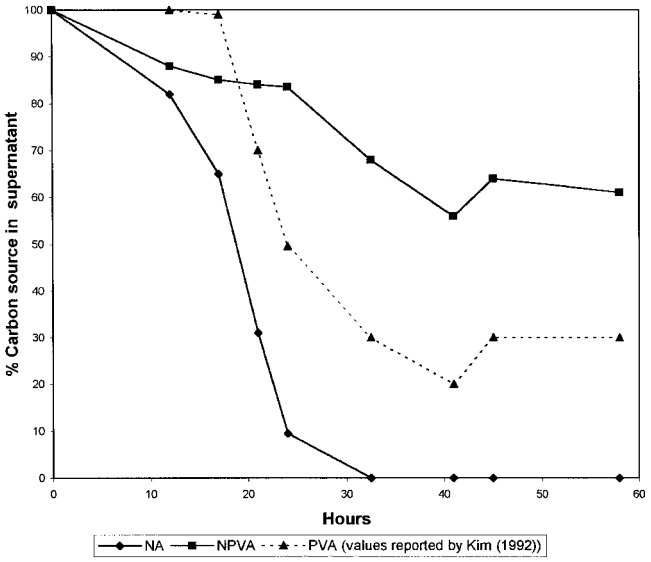

Figure 3. Variation of percentage of carbon sources present in supernatant of *P. oleovorans* culture with DNPVA and NA (1:1 molar ratio, 10 mM total concentration) versus time.

only 60% of this carbon source remained. At the same time, NA was removed completely after only 32 h of growth. It should be mentioned that DNPVA did not show any change from dinitro to mononitro substitution in the supernatant over time, as evidenced by the GC-MS study.

A similar study by Kim[6] reported that NA consumption began immediately. However, almost 100% PVA still remained in the supernatant while NA medium levels had been reduced to 60% at 20 h. (The corresponding curve is included for comparison in Figure 3.) This seemed to indicate that since both components were not being incorporated at the same time, a mixture of two polymers was being biosynthesized. This might also be the case in the present study.

Study of the Composition of the Polymers Produced vs Time. The polymers used to carry out this analysis were extracted from the centrifuged cells of a 1:1 DNPVA:NA (10 mM) culture harvested at different times (see Table 2). The isolated material was dissolved in chloroform, precipitated with methanol, and decanted. This process was performed twice to ensure that no carbon source remained. The NMR spectrum of each sample was run to calculate the percentage of nitrated aromatic units in the polymeric chain by determining the relative areas under the aromatic and methine peaks (7.4−8.8 and 5.2−5.4 ppm, respectively). Interestingly, these spectra indicate a change in aromatic peak pattern over time. This finding suggests that the extracted polymer contained modified aromatic groups. Figure 4 presents expansions of the aromatic region (7.4−9.0 ppm) of the spectra obtained for the polymers

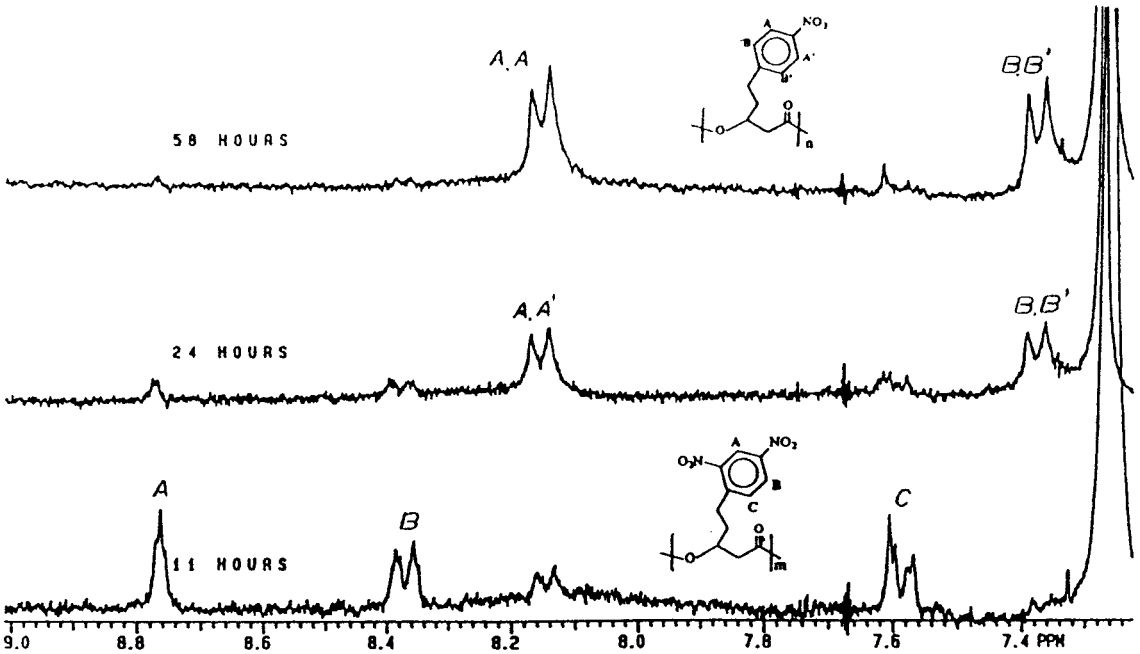

Figure 4. Partial proton NMR spectra of polymeric material produced by *P. oleovorans* with DNPVA:NA (7:3 molar ratio, 10 mM total concentration) at different harvest times.

Table 3. Polymer Isolation Study Carried out with Mixed DNPVA:NA 2 L Cultures

DDNPVA:NA	total concn (mM)	OD max. at 650 nm	harvest time (h)	biomass (g/L)	total PHA (%)	aromatic units (%)[a]
1:1	10	1.31	33	0.403	40	1.2
1:1	20	1.26	27	0.419	23	3.1
7:3	10	1.00	24	0.359	15	6.9

[a] As identified by integration with NMR.

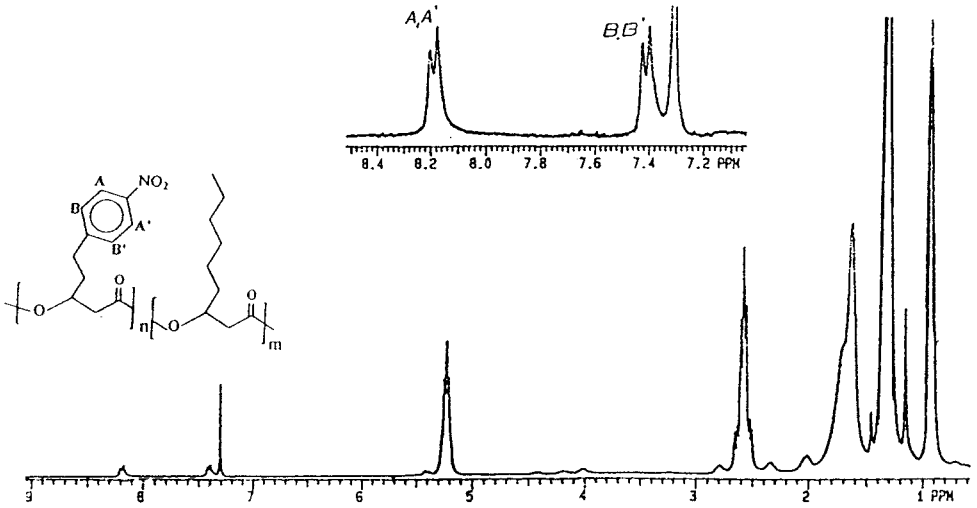

Figure 5. Proton NMR spectrum of polymeric material produced by *P. oleovorans* with DNPVA:NA (7:3 molar ratio, 10 mM total concentration) at 24 h harvest time.

extracted at 11, 24, and 58 h of growth. It was observed that at 11 h of growth the three signals corresponding to the three aromatic protons (A, B, and C) characteristic of the ortho/para substitution in DNPVA are clearly present. This was also noted when pure DNPVA was fed to the bacteria. However, after 24 h of growth, two additional signals were noted to be present. One of these was detected at 7.4 ppm and the other at 8.2 ppm. These are two doublets, corresponding to aromatic substitution at the para position. After 58 h of growth, these two doublets can be observed more clearly, but only residual A, B, and C peaks are present. At this time, there is little evidence of the original ortho/para aromatic substitution pattern. Accordingly, it appears as though a nitro group is removed from the ring at the ortho position, either when DNPVA is incorporated into the bacteria or later when the molecule is being polymerized. This conclusion is warranted as the supernatant contained only DNPVA (with no mononitro derivative present) as confirmed by the GC-MS analysis. Thus, it may be suggested that the bacteria is first synthesizing a polymer that contains aromatic groups with ortho/para substitution. Later, as the time of growth increases, the polymer being synthesized contains aromatic groups with para substitution only. Table 2 presents a summary of the information obtained from the polymer production study.

Maximum incorporation of modified aromatic groups (present in 3.9% of repeating units) took place at 40 h of growth, a time occurring immediately after maximum OD (1.50) was achieved. At this point, 0.467 g/L of biomass had been isolated. The results obtained by Kim[6] for PVA:NA cultures demonstrated a maximum production of polymer at 20 h with a total polymer production of 38%. The biomass reported was 0.77 g/L and the percentage of aromatic units 26%. Comparing the values obtained in our laboratory with these results, it can be seen that the incorporation of aromatic units into the polymer in the present case was much less than that achieved in Kim's study.

Characterization of the Polymers. Even though the amount of nitrophenyl units introduced in the polymer was very small, the isolated material was physically very different from that polymer obtained when NA is utilized as the only carbon source. While PHN is whitish and has a sticky texture, the isolated polymers appeared light yellow and elastic.

Table 3 summarizes the information gathered from the mixed DNPVA:NA 2 L cultures. The incorporation of aromatic groups increased as the proportion of DNPVA to NA increased. At the same time, OD, harvest time, and PHA decreased. The 7:3 DNPVA:NA (10 mM) culture afforded the maximum amount of aromatic units (present in 6.9% of repeating units). However, it yielded the lowest production of biomass.

NMR Spectroscopic Analysis. Figure 5 shows the complete proton NMR spectrum for the 7:3 DNPVA:NA (10 mM, 24 h harvest time) polymeric material exhibiting aromatic signals between 7.4 and 8.2 ppm. These data indicate substitution at the para position of the ring. An expansion of this region allows one to more clearly observe the two doublets characteristic of this substitution pattern. Such a pattern is very different from the polymer isolated after 11 h of growth, as noted previously (Figure 4). This figure also shows that the material contains not only aromatic units but also the aliphatic pattern characteristic[4] of PHN, suggesting that this polymer (or its copolymers) is also present. Figure 6 exhibits the carbon NMR spectrum for the same polymer with signals at 124 and 130 ppm. The results suggest that para substitution has occurred in the ring and again indicate the presence of PHN.

FT-IR Spectroscopic Analysis. The analysis indicates the presence of nitro and ester groups, as well as the aromatic ring by the appearance of the bands mentioned previously.

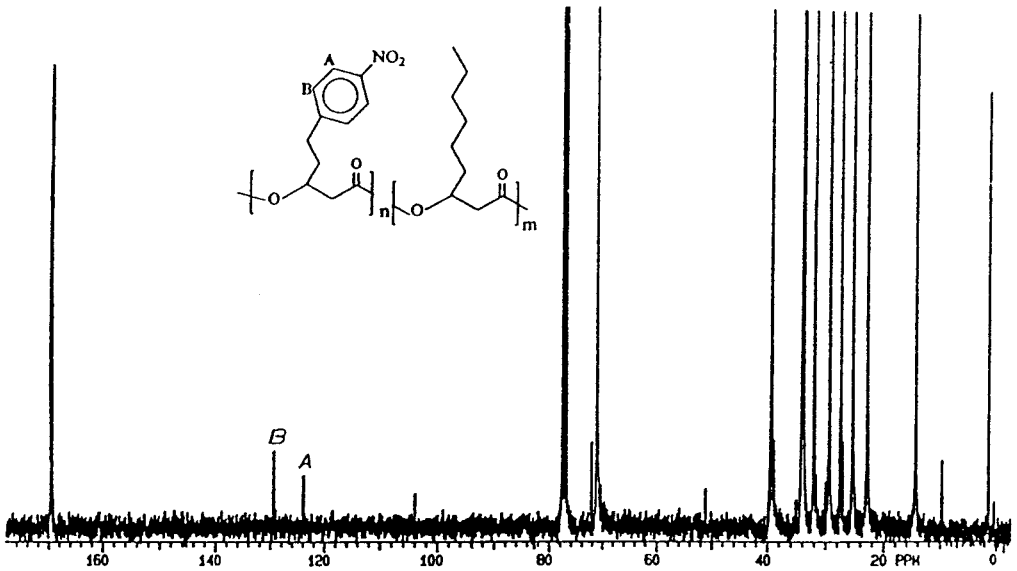

Figure 6. Carbon NMR spectrum of polymeric material produced by *P. oleovorans* with DNPVA:NA (7:3 molar ratio, 10 mM total concentration) at 24 h harvest time.

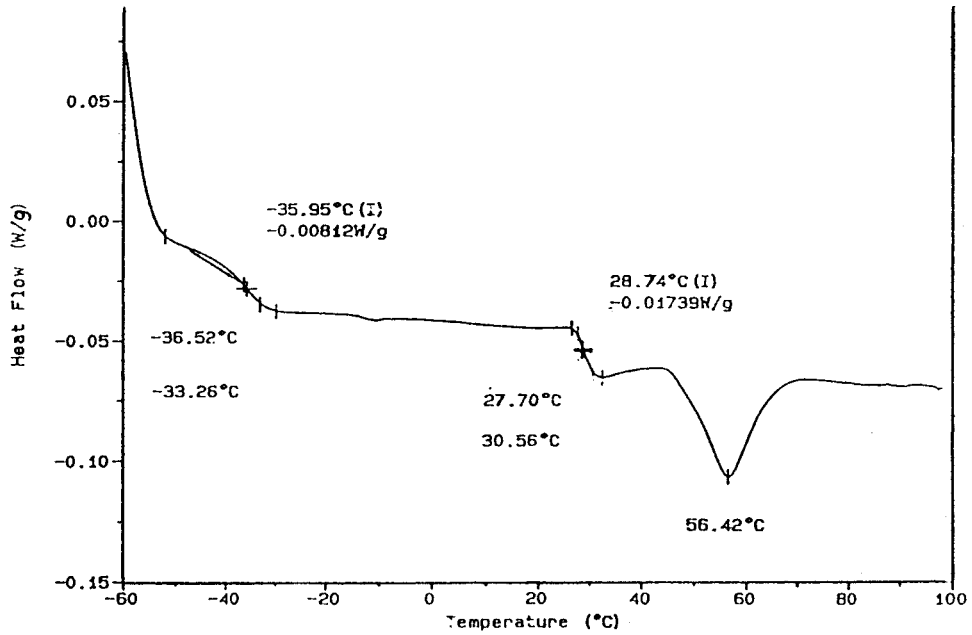

Figure 7. DSC thermogram of polymeric material produced by *P. oleovorans* with DNPVA:NA (7:3 molar ratio, 10 mM total concentration) at 24 h harvest time.

DSC Thermal Analysis. Figure 7 presents the thermogram of the 7:3 DNPVA:NA (10 mM, 24 h harvest time) polymer. The presence of one T_m and two T_g values is evident. The T_m at 56.42 °C and the T_g at −35.95 °C may be assigned to PHN. However, it is presumed that the T_g value at 28.74 °C corresponds to the newly synthesized polymer. The existence of two values of T_g seems to indicate that a mixture of two polymers is present. The fact that only the T_m of PHN is observed may indicate that the new polymer does not contain regions with crystalline domains. This agrees with previous results reported by Curley[10] and Kim,[13] which indicated the presence of two polymers being produced when a PVA/NA mixture was used as the carbon source for *P. oleovorans*.

Conclusions

Pseudomonas oleovorans was capable of growing in cultures prepared with varied proportions of DNPVA and NA. However, in cultures containing only DNPVA, the bacteria grew little if at all. This seems to indicate that the carbon source is rather toxic to the bacteria. *P. oleovorans* incorporated nitrophenyl groups. Upon studying the production of polymer vs time in cultures prepared with 1:1 DNPVA:NA (10 mM), it was observed that as the time of growth increased, the aromatic substitution pattern changed drastically. The material isolated after a few hours of growth showed incorporation of dinitrated groups. However, as time passed, the resulting material exhibited the NMR pattern characteristic of mononitrated groups. The GC-MS study carried out indicated that 40% of DNPVA was removed from the medium and that no mononitrated substrate was present. However, only 4−7% of the polymeric units contained nitrophenyl groups. This seems to indicate that the bacteria consumes the nitrophenyl carbon source for some other purpose in addition to polymer production. The maximum incor-

poration of modified units was observed for the 7:3 DNPVA:NA (10 mM) culture, which also exhibited the lowest polymer production. These results are similar to those reported by Curley and co-workers.[14] The latter studies also demonstrate that the production and degradation of bacterial polyesters can be monitored directly using the carbon NMR spectra of growing cultures.

Thermal analyses as well as NMR spectra suggest that the isolated polymers may actually constitute a mixture of two polymers, one which contains nitrophenyl rings and the other being the copolymer of nonanoic acid. This appears likely as two T_g transitions were observed in the DSC thermogram. Since no new T_m value was observed, it may be said that the polymer containing nitrophenyl units is amorphous, thus lacking crystalline domains. In terms of the physical appearance of this new material, it is yellow and elastic and therefore very different from PHN, which is whitish and sticky by comparison.

Acknowledgment. The authors acknowledge the help of Berlinda Torres, Odalyse Cedeño, Iris Alvarado, and Henry Terc during the final stages of this work and of Dr. John Uscian in the preparation of the document.

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MA981482Q