Growth and Polymer Incorporation of *Pseudomonas oleovorans* on Alkyl Esters of Heptanoic Acid

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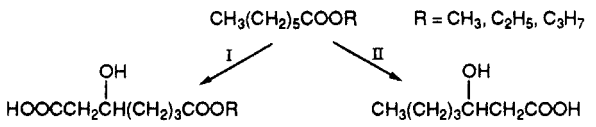
Introduction

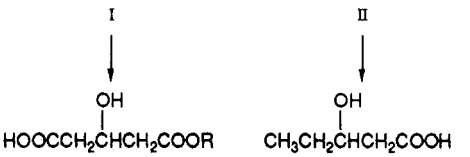
A wide variety of microorganisms is capable of producing intracellular carbon and energy storage materials. These intracellular incorporations exist in forms of chemically different polymers and vary depending on the specific microorganism, e.g., polysaccharides, polyphosphates, and poly(β-hydroxyalkanoates) (PHAs). PHAs are thermoplastic polyesters which are biodegradable and potentially biocompatible. The polymers are optically active, exhibiting absolute *R*-configuration. These biopolyesters are produced by bacterial biosynthesis from a broad variety of carbon sources.[1] *Pseudomonas oleovorans*, the bacterium used in the present study, can produce a variety of poly(β-hydroxyalkanoates) on carbon sources having at least six-carbon units.[1–4] *P. oleovorans* can grow on carbon sources which contain unsaturated groups,[5] on branched alkyl compounds,[6] and on alkyl compounds containing bromo groups,[7] phenyl groups,[8,9] and ester groups.[10] During the conversion of these substrates to form PHAs, the functional groups in the substrate can remain unattacked to a certain degree and will be present in the side chains of the polymers.

In addition to their biodegradability, some PHAs are also biocompatible so the functionalized polymers could be of interest for biomedical applications.[11] That is, by the introduction of a functional group on the side chains of the biopolymer during its synthesis in the cell, it may be possible to subsequently attach covalently active compounds to the polymer, such as drugs for medical treatment. By slow degradation of that polymer a controlled drug release could be achieved, so these polymers could be of interest in delivery systems and in the general field of controlled release of active substances.

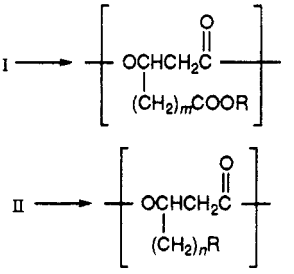
In the present investigation alkyl esters of heptanoic acid as well as the acid alone were evaluated as substrates for the biosynthesis of functionalized PHAs by *P. oleovorans*. As previously described[10] *P. oleovorans* is capable of growing on methyl, ethyl, and *tert*-butyl esters of octanoic, nonanoic, and decanoic acid, and in the metabolic cycle for monomer synthesis in the cell the substrate can be converted by either of two different β-oxidation routes, I and II, to form the monomer, as shown for the utilization of the esters of heptanoic acid:



In each case, as in the utilization of *n*-alkanoic acids directly,[1–3] further modification of the substrate can also occur to form monomers containing two carbon atoms less than the original substrate, as follows:



Route I results in the formation of the ester of 3-hydroxypimelic acid, and this monomer could polymerize to maintain the ester group in the side chain. Conversely, if the cell utilizes the substrate from the end which contains the ester group by route II, presumably by first converting the ester to the alkaonic acid, then the ester group will not be present as a terminal group on the β-substituent in the polymer. The final product of the biosynthesis would then be a copolymer containing units of varying side-chain length, with or without terminal ester pendant groups, as shown:



In the present study methyl, ethyl, and propyl esters of heptanoic acid were separately used as substrates in order to determine the growth behavior of *P. oleovorans* on these substrates. Of special interest was the degree of retention of the ester pendant groups in the polymer formed.

Experimental Section

Fermentations. Stock cultures of *P. oleovorans* (ATCC 29347) were used throughout the experiments. The strain was maintained at 4 °C on nutrient agar plates using a modified E* medium,[12] with 10 mmol concentration of either sodium octanoate or sodium nonanoate, as the carbon source. The cultures were cultivated in a mineral medium (modified E* medium) consisting of 1.1 g/L of $(NH_4)_2HPO_4$, 5.8 g/L of K_2HPO_4, 3.7 g/L of KH_2PO_4, 10.0 mL/L of a 100 mM $MgSO_4$ solution, and 1.0 mL/L of a microelement solution. The latter is a 1 N HCl solution containing 2.78 g/L of $FeSO_4 \cdot 7H_2O$, 1.67 g/L of $CaCl_2$ and $2H_2O$, 0.17 g/L of $CuCl_2 \cdot 2H_2O$, 0.29 g/L of $ZnSO_4 \cdot 7H_2O$, 1.98 g/L of $MnCl_2 \cdot 4H_2O$, and 2.81 g/L of $CoSO_4 \cdot 7H_2O$. The esters of alkanoic acids were added at concentrations of 20, 50, and 100 mM. The pH was adjusted to 7.0.

For the preculture, 200 mL of the modified E* medium, using sodium octanoate as the carbon source, was autoclaved at 120 °C (18 lbs/in.) for 20 min (Amsco Laboratory autoclave). After the culture solution was cooled down to 30 °C, bacteria were inoculated from the plate by a sterile procedure. After 14–17 h the preculture was ready to use as an inoculum for the cultures. The growth of all cultures was examined by recording the optical density of the solution spectrometrically with a Spectronic 20 Bausch and Lomb at a layer thickness of 1 cm, referenced to distilled water.

Fermentations were carried out under aerobic, oxygen-limiting conditions using 2.8-L Fernbach flasks for 1-L cultures and 500-mL Erlenmeyer flasks for 250-mL cultures. The E* medium was autoclaved for 30 min under the same conditions as described above. After the flasks were cooled to 30 °C, the carbon source and inoculum were added. Ester carbon sources were not autoclaved in order to prevent hydrolysis of the ester group. 100 mL of the preculture solution was added to the cultures. Oxygen-limiting conditions during the fermentation were achieved by using a cotton plug and alumina foil to cover the flasks. In

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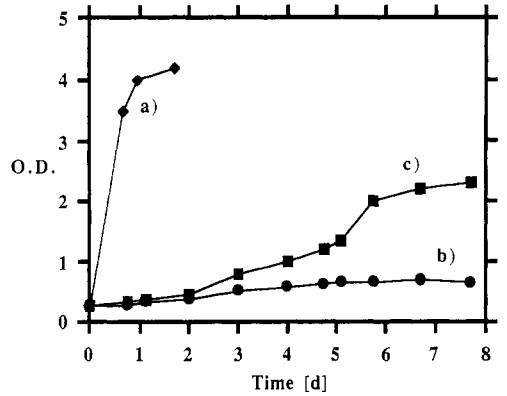

Figure 1. Growth of *P. oleovorans* on heptanoic acid and methyl heptanoate: (a) 25 mM heptanoic acid; (b) 20 mM methyl heptanoate; (c) 100 mM methyl heptanoate.

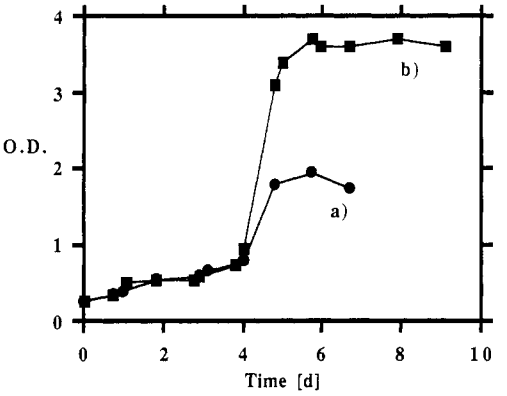

Figure 2. Growth of *P. oleovorans* on ethyl heptanoate: (a) 20 mM ethyl heptanoate; (b) 100 mM ethyl heptanoate.

order to guarantee aeration of cultures, the flasks were continuously shaken in a lab-line incubator shaker at 250 rpm and 30 °C.

The polymer was extracted from the lyophilized cells by refluxing in 100–150 mL of chloroform for 12 h. After the cellular material was filtered, the solvent was removed by evaporation until a remaining residue of a concentrated polymer solution of 1–5 mL was obtained. The polymer was precipitated into 50–70 mL of vigorously stirred methanol, collected, and dried at room temperature under vacuum for 12 h.

Polymer Characterization. The polymer was characterized by using ^1H NMR. Spectra were obtained using chloroform-*d* solutions of the polymer with a Bruker AC 200 spectrometer at 200 MHz referenced to tetramethylsilane.

For analysis of the repeating units of the polymers by gas chromatography (GC) after methanolysis, 3–4 mg of the polymers, dissolved in 1.0 mL of chloroform, was converted into the methyl esters with 1.0 mL of 15% H_2SO_4 in MeOH for 3 h at 100 °C. The solution was washed with 1.0 mL of distilled water by rapidly stirring for 20 s. The chloroform layer was separated and analyzed by GC (Perkin-Elmer 8500; D-B WAX; capillary column, 15 m × 0.53 mm; carrier gas, He; 20 mL/min; temp program, 35 °C for 2.0 min, ramp 15 °C/min, 200 °C for 3.0 min).

Results

Influence of the Substrate Concentration on Cell Multiplication. *P. oleovorans* was grown on methyl, ethyl, and propyl heptanoate and heptanoic acid in order to investigate growth behavior and the extent of incorporation of pendant ester groups in the polymer. The ester substrates were applied in concentrations of 20 and 100 mM. As shown in Figures 1–3 the growth curves exhibit considerable differences upon varying the substrate concentration. That is, by using 100 mM ester substrate as the carbon source the bacterial cultures reached considerably higher optical densities. The type of ester

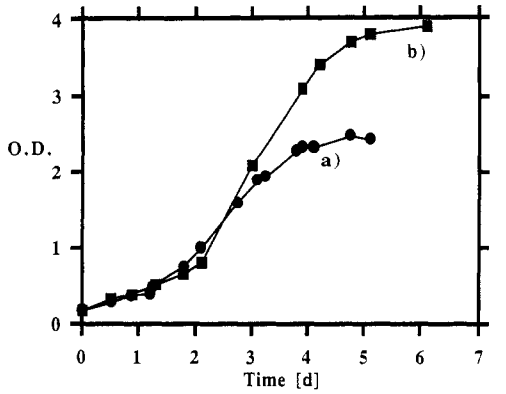

Figure 3. Growth of *P. oleovorans* on propyl heptanoate: (a) 20 mM propyl heptanoate; (b) 100 mM propyl heptanoate.

influenced the multiplicity value by which the optical density is increased. The greatest difference was achieved with methyl esters; using 100 mM methyl heptanoate resulted in a 3-fold increase in the optical density. By using higher esters the differences in the maximum optical density reached by feeding 100 and 20 mM substrate became smaller. Feeding 100 mM ethyl heptanoate resulted in a 2-fold increase in the optical density compared with 20 mM. In the case of propyl heptanoate only a 1.6 increase in the cell density was achieved by applying the higher substrate concentration compared with the other esters. The growth behavior of methyl esters depended strongest on the applied concentration of the feed. This ester showed the slowest growth, and obviously only a large excess of this carbon source in the medium forced the bacteria to utilize it, that is, its transformation into monomer and a subsequent polymerization and incorporation of the polymer. The bacteria grew better on the higher esters and did not depend to the same extent on the concentration of the carbon source. While the substrate concentration influenced the cell density, it had no influence on the lag time. This is the time which the bacteria need to adopt to the new environment. As seen in Figures 1–3 and Table 1, the lag time depended only on the type of ester but not its concentration. While there was no lag time for heptanoic acid, it decreases from methyl to propyl heptanoate.

Influence of the Type of Ester on the Growth Behavior and Yield. *P. oleovorans* grew best on heptanoic acid alone. The growth on this carboxylic acid resulted in the highest cell density (OD 4.2) and polymer yield (0.16 g/L) and in the highest polymer incorporation of 8.3 wt % of dry cells. The growth on heptanoic acid started immediately after the inoculation; there was no adaption of the cells, which were pregrown on sodium octanoate.

None of the investigated esters of heptanoic acid could reach the results achieved by the plain heptanoic acid. Comparing the different ester substrates reveals that bacteria grew best on propyl heptanoate, exhibiting a comparatively short lag time of 2 days and reaching reasonable cell densities (OD 3.9) even if the lower substrate concentration of 20 mM was applied. However, as shown in Table 1 the polymer yield was very poor, reaching only 4 mg/L which corresponds to 0.27 wt % polymer of the dry cell yield after feeding 100 mM propyl heptanoate.

When fed ethyl heptanoate, the bacteria responded stronger to differences in the concentration of the carbon source. The lag time was twice as long as that for propyl heptanoate, 4.0 days; however, the growth resulted finally in almost the same cell density, (OD 3.7). The polymer

Table 1. Growth Behavior of *P. oleovorans* on Heptanoic Acid and Ester Derivatives of Heptanoic Acid

		methyl heptanoate		ethyl heptanoate		propyl heptanoate	
	heptnaoic acid 25 mM	20 mM	100 mM	20 mM	100 mM	20 mM	100 mM
growth time (days)	1.0	6.7	7.7	5.0	5.5	4.5	5.0
cell yield (g/L)	1.9	nd	1.3	1.1	2.0	1.3	1.5
polymer yield (mg/L)	0.1	0.01	0.09	0.005	0.008	0.003	0.004
polymer incorp. (%)	5.2		6.9	0.45	0.4	0.23	0.27
max OD	4.0	0.7	2.3	1.8	3.7	2.4	3.9
lag time	0	4.8	4.8	4.0	4.0	2.0	2.0

Table 2. Copolymer Composition, Determined by Methanolysis/GC[a]

	repeating unit (%)						
substrate	PHV	PHH	PHO	PHN	-COOR	-COOR[b]	other
C_7	1.7	91	1.0	5.3			1.5
MeC_7	2.6	89	2.0	1.7	3.4[c]	2.5[c]	1.8
EtC_7[e]	2.7	86		11.0			
PrC_7	nd	nd	nd	nd	nd	60[d]	

[a] PHV: poly(β-hydroxyvalerate. PHH: poly(β-hydroxyheptanoate). PHO: poly(β-hydroxyoctanoate). (PHN: poly(β-hydroxynonanoate). -COOR: ester derivative of carboxylic acid. C_7: heptanoic acid. MeC_7: methyl heptanoate. EtC_7: ethyl heptanoate. PrC_7: propyl heptanoate. [b] By ^1H NMR. [c] R = CH_3. [d] R = C_3H_7. [e] Polymer obtained consisted of copolymer from the fermentation of ethyl heptanoate and of copolymer transferred with the inoculation of the preculture; the latter was not taken into consideration regarding the copolymer composition.

yield was again very low, reaching only 5 mg/L; this is 0.4 wt % polymer of the dry cell weight. As shown in Table 1 the percentage of incorporated polymer based on dry cell weight is determined only by the type of ester and is independent of the concentration of the carbon source (ethyl heptanoate, 0.4 wt %; propyl heptanoate, 0.3 wt %).

The fermentation of methyl heptanoate had a long lag time of 4.75 days, after which growth reached an optical density of 0.7 (20 mM) and 2.3 (100 mM). Despite these low cell densities, a considerably higher polymer incorporation could be achieved, 90 mg/L polymer which corresponds to 7.0 wt % of dry cells. These data are in good agreement with previous results obtained from the fermentation of ester derivatives of octanoic, nonanoic, and decanoic acid. As seen before,[10] *P. oleovorans* grew better on higher esters, representing a shorter lag time and reaching higher optical densities. However, these higher cell densities did not correspond to higher amounts of incorporated polymer. Even the opposite was the case; the organisms grew quite well on the higher esters, but their tendency of incorporation of storage material decreased. The higher the ester, the more they tended to be considered according to Lenz[13] as substrates of class B, meaning organic compounds that support cell growth but not PHA production.

Copolymer Composition. Methanolysis/gas chromatography was used to determine the individual repeating units which formed the copolyester. The polymer obtained from the fermentation of heptanoic acid was a copolymer consisting of a main fraction of poly(β-hydroxyheptanoate) (PHH), 90.5 mol % accompanied by small amounts of poly(β-hydroxynonanoate) (PHN) and poly(β-hydroxyvalerate) (PHV), 5.3 and 1.7 mol %, respectively, and 1.0 mol % of poly(β-hydroxyoctanoate) (PHO), as shown in Table 2. The PHO was most likely transferred into the culture by the inoculation of the preculture, which was cultivated on sodium octanoate. These data, however, were in contrast to the results found by Lavegeen,[14] who got a PHH homopolymer from the fermentation of heptanoic acid by *P. oleovorans*.

The fermentation of the methyl and propyl ester containing substrates resulted in copolymers with pendant ester groups. The copolymer obtained from the fermentation of methyl heptanoate consisted of 88.5 mol % PHH, 2.6 mol % PHV, 1.7 mol % PHN, and 3.4 mol % of repeating units containing pendant methyl ester groups; additionally 2.0 mol % PHO were determined (Table 2). A methyl ester content of 2.5 mol % was determined from the NMR characterization. The data of GC and NMR are in good agreement. As shown before[10] the ester content depends significantly on the oxygen supply to the medium. An increase of the available oxygen during the fermentation process results in higher amounts of incorporated pendant ester groups in the side chains of the PHA. In the present study the oxygen supply was limited by using cotton stoppers and alumina foil, but it was not quantified.

The fermentation of propyl heptanoate resulted in very low yields. Due to these low amounts of polymeric material obtained, characterization was carried out by ^1H NMR only. The fermentation of propyl heptanoate resulted in the highest amount of incorporated pendant propyl ester groups, of 60 mol %. The biopolymer obtained in different experiments was gathered and purified by reprecipitation; thus, inclusion of monomer within the polymer is unlikely. It was apparently difficult for the microorganisms to recognize the carboxylic structure of the propyl ester group in the substrate as a potential oxygen supplier, which could be transformed into the carboxylic acid to form the monomer for the polymerization reaction (route II). Instead the bacteria utilized the substrate mainly like an alkane.

The fermentation of ethyl heptanoate resulted always in the formation of unsubstituted PHA without pendant ester groups. The copolymer composition differs from that of the plain heptanoic acid. The copolymer obtained from ethyl heptanoate was characterized by a higher amount of PHN (11.0 mol %). Furthermore, the copolyester consisted of 86.3 mol % PHH and 2.7 mol % PHV. The microorganisms removed the ethyl ester group utilizing the remaining carboxylic acid group of heptanoic acid for further oxidation processes and also added C_2 groups to the substrate.

Conclusion

From a comparison of the results obtained with the methyl, ethyl, and propyl esters of heptanoic acid used as substrates for *P. oleovorans*, it can be seen that the cell densities reached during the fermentations depended strongly on the substrate concentrations. However, the lag time which the microorganisms needed for the adaptation to the new environment did not depend on the substrate concentration; it was determined only by the type of ester used. It was observed that the cells grew fastest on the propyl ester substrate, and the slowest growth was obtained with the methyl ester substrate. However, the methyl ester substrates gave the best results for the incorporation of ester groups as the terminal position in the side chain of the PHA produced. In the fermentation

experiments with the ethyl ester substrates, unsubstituted PHAs were obtained. The fermentation of the propyl ester substrate resulted in very low yields. However, a maximum of incorporated repeating units carrying pendant ester groups was achieved. The fermentation of heptanoic acid, which was used as a control, exhibited the fastest growth with the highest incorporation of storage polyesters. These data could not be achieved by the fermentation of any of the ester substrates.

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