APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1991, p. 625–629
0099-2240/91/030625-05$02.00/0

Vol. 57, No. 3

# Continuous Production of Long-Side-Chain Poly-β-Hydroxyalkanoates by *Pseudomonas oleovorans*

BRUCE A. RAMSAY,[1] ILIE SARACOVAN,[2] JULIANA A. RAMSAY,[1]* AND ROBERT H. MARCHESSAULT[2]

*Department of Chemical Engineering, Ecole Polytechnique de Montréal, C.P. 6079, Succursale "A," Montreal, Quebec H3C 3A7,[1] and Chemistry Department, McGill University, Montreal, Quebec H3A 2A7,[2] Canada*

Received 16 October 1990/Accepted 20 December 1990

**Shake flask experiments showed that *Pseudomonas oleovorans* began to be growth inhibited at 4.65 g of sodium octanoate liter$^{-1}$, with total inhibition at 6 g liter$^{-1}$. In chemostat studies with 2 g of ammonium sulfate and 8 g of octanoate liter$^{-1}$ in the feed, the maximum specific growth rate was 0.51 h$^{-1}$, and the maximum specific rate of poly-β-hydroxyalkanoate (PHA) production was 0.074 g of PHA g of cellular protein$^{-1}$ h$^{-1}$ at a dilution rate ($D$) of 0.25 h$^{-1}$. When the specific growth rate ($\mu$) was <0.3 h$^{-1}$, the PHA composition was relatively constant with a $C_4/C_6/C_8/C_{10}$ ratio of 0.1:1.7:20.7:1.0. At $\mu > 0.3$ h$^{-1}$, a decrease in the percentage of $C_8$ with a concomitant increase in $C_{10}$ monomers as $\mu$ increased was probably due to the effects of higher concentrations of unmetabolized octanoate in the fermentor. At $D = 0.24$ h$^{-1}$ and an increasing carbon/nitrogen ratio, the percentage of PHA in the biomass was constant at 13% (wt/wt), indicating that nitrogen limitation did not affect PHA accumulation. Under carbon-limited conditions, the yield of biomass from substrate was 0.76 g of biomass g of octanoate$^{-1}$ consumed, the yield of PHA was 0.085 g of PHA g of octanoate$^{-1}$ used, and 7.9 g of octanoate was consumed for each gram of $NH_4^+$ supplied. The maintenance coefficient was 0.046 g of octanoate g of biomass$^{-1}$ h$^{-1}$. Replacement of sodium octanoate with octanoic acid appeared to result in transport-limited growth due to the water insolubility of the acid.**

Poly-β-hydroxyalkanoates (PHAs) are accumulated as an intracellular storage material by a wide variety of bacteria. This family of copolymers may be divided into two major groups: the short-side-chain (SSC) PHAs (such as poly-β-hydroxybutyrate and poly-β-hydroxybutyrate-co-β-hydroxyvalerate), produced under appropriate conditions by many bacteria (1, 12a, 16, 22, 23), and the long-side-chain (LSC) PHAs, produced by the fluorescent pseudomonads when grown on alkanes or alkanoic acids (1, 5, 12, 13). Since SSC PHAs have properties similar to conventional thermoplastics and were the first to be discovered, they were the first to find commercial application. Uses for LSC PHAs are less obvious but may be more numerous because of their chemical diversity. For example, their monomers may serve as a source of chiral building blocks for stereochemical syntheses or the polymers themselves may be mixed with SSC PHAs to alter their mechanical properties.

Although the precise properties of LSC PHAs depend on the number of carbons in their side chain (18), in general, the stress-strain curve, the glass-transition temperature, and the degree of crystallinity are comparable to those of thermoplastic elastomers. *Pseudomonas oleovorans* produces these materials by using substrates such as linear and branched alkanes, 1-alkenes, and alkanoates with chain lengths ranging from $C_6$ to $C_{12}$ (2, 7, 8, 10, 13, 17). The composition of PHA is related to the number of carbons in the substrate (13) such that most of the monomers have the same carbon chain length as the substrate (2, 13, 17). Furthermore, the presence of monomers containing two or four carbon atoms less than the substrate indicates the occurence of β-oxidation (10, 13). Monomeric units with two additional carbon atoms are also detected (2, 13). These may be due to a condensation reaction of intermediates in the biosynthetic pathway (12). Side chains suitable to further modifications (by chemical or biological means or both) can be introduced into the biopolyester by the addition of an appropriate carbon substrate during PHA accumulation (7, 8, 17). This opens up new avenues in the development of biopolyesters with an even wider variety of properties.

Unfortunately, the use of and even the study of LSC PHAs is greatly limited by the difficulty involved in their production. Methods do not presently exist for the production and isolation of large amounts of LSC PHAs. A step toward the development of such methods is to understand how physiological conditions affect their accumulation. In many SSC-producing bacteria, limitation by elements such as nitrogen or phosphate stimulates the specific production rate of PHA by affecting the intracellular concentrations of key regulatory metabolites such as $NADH_2$ and acetyl-coenzyme A (4). However, the mechanism of PHA synthesis in the fluorescent pseudomonads is very different. For example, β-ketothiolase is a key regulatory enzyme for SSC PHA synthesis by *Alcaligenes eutrophus* (20), but this enzyme may play little or no role in the PHA synthetic pathway of *P. oleovorans* (17). Since the pathways for the synthesis of LSC and SSC PHAs are different, the mechanisms which trigger accumulation of SSC PHAs may not affect accumulation of LSC PHAs.

Although it is known that the microbial strain and the chemical structure of the carbon substrate are important (1, 2, 7, 8, 10, 12, 13, 17), little is known about the effect of growth rate, production rate, and substrate concentration on LSC PHA composition. These parameters have been shown to affect the end products formed in other fermentations (11, 15, 19, 24). In this work, the effects of the specific growth rate, specific production rate, substrate concentration (i.e., octanoate), and carbon/nitrogen ratio on the production and the composition of LSC PHA produced by *P. oleovorans* in a chemostat are described.

* Corresponding author.

## MATERIALS AND METHODS

**Microorganism and growth medium.** *P. oleovorans* ATCC 29347 was grown on a mineral salts medium containing the following components per liter: 3.7 g of $Na_2HPO_4 \cdot 7H_2O$, 0.83 g of $KH_2PO_4$, 2.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 60 mg of ferrous ammonium citrate, 10 mg of $CaCl_2 \cdot 2H_2O$, and 1 ml of microelement solution. Each liter of microelement solution contained 0.3 g of $H_3BO_3$, 0.2 g of $CoCl_2 \cdot 6H_2O$, 0.1 g of $ZnSO_4 \cdot 7H_2O$, 30 mg of $MnCl_2 \cdot 4H_2O$, 30 mg of $NaMoO_4 \cdot 2H_2O$, 20 mg of $NiCl_2 \cdot 6H_2O$, and 10 mg of $CuSO_4 \cdot 5H_2O$. Sodium octanoate of 99% purity (Omega Chemical Co., Quebec, Canada) or octanoic acid (Aldrich Chemical Co., Milwaukee, Wis.) was used as the carbon source.

**Fermentation conditions.** Abbreviations relating to fermentation parameters are as follows: $X$, $S$, and $P$, concentrations of biomass, substrate, and PHA, respectively, expressed as grams per liter; $D$, dilution rate, expressed per hour; $\mu$, specific growth rate, $(1/X)$ $(dX/dt)$, expressed per hour; $\mu_{max}$, maximum specific growth rate, expressed per hour; $q_p$, specific PHA production rate, $(1/X)$ $(dP/dt)$, expressed as grams per gram per hour; $q_{p,max}$, maximum specific PHA production rate, expressed as grams per gram per hour; $Y_{X/S}$, yield of biomass per gram of substrate consumed, expressed as grams per gram; $Y_{P/S}$, yield of PHA per gram of substrate consumed, expressed as grams per gram.

Continuous fermentations were performed in a Multigen F-2000 2-liter fermentor (New Brunswick Scientific, Edison, N.J.). The working volume was kept at 1.5 liters, with the agitation rate at 900 rpm and the aeration rate at 1.5 liter $h^{-1}$. The temperature and the pH were maintained at 30°C and 7.0, respectively. The contents of the feed reservoir were continuously stirred to obtain a homogeneous feed into the fermentor. To study the effect of substrate concentration, the sodium octanate in the feed was added to the reactor at a dilution rate of 0.24 $h^{-1}$. In the dilution rate experiments, 9.28 g of sodium octanoate $liter^{-1}$ (i.e., 8 g of octanoate $liter^{-1}$) or 8 g of octanoic acid $liter^{-1}$ and mineral salts concentrations were kept constant in the medium feed throughout the fermentation. Samples were taken after at least 6 fermentor volumes of medium had passed through the reactor at the previously lower substrate concentration or dilution rate. For all chemostat experiments, the sodium octanoate concentration was calculated based on the octanoate ion concentration and PHA content is reported as the percentage of PHA per dry weight of biomass.

**Shake flask experiment.** The initial pH of 100 ml of sterile mineral salts medium containing different concentrations of sodium octanoate in 500-ml Erlenmeyer flasks was adjusted to 7.0. After the addition of the *P. oleovorans* inoculum, the contents of the flasks were incubated on a New Brunswick G10 Gyrotory shaker (New Brunswick Scientific Co., Edison, N.J.) at 250 rpm and 30°C for 48 h.

**Biomass and protein.** For biomass measurements, 20 ml of culture broth was centrifuged and the pellet was resuspended and washed in distilled water. The pelleted biomass was then transferred into an aluminum dish and dried to a constant mass at 105°C (23). The centrifuged biomass from 10 ml of culture broth was analyzed for protein content by the biuret reaction (26), using 1.0% (wt/vol) bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as a standard.

**Analysis of medium components.** The ammonium ion concentration in the culture broth supernatant was quantified by a modified Berthelot reaction (25). The presence of unassimilated octanoate was determined by gas chromatography. Samples were prepared by adding 2 ml of methanol containing 0.5% (vol/vol) pentanoic acid (as an internal standard) and 2% (vol/vol) concentrated $H_2SO_4$ to 1 ml of supernatant. This mixture was heated for 1 h at 105°C in a screw-cap tube. After cooling to room temperature, 2 ml of distilled water was added and the methyl esters were extracted into 2 ml of ether. Subsequent analysis was performed with a 5890 gas chromatograph (Hewlett-Packard Co., Palo Alto, Calif.) equipped with a flame ionization detector and a 25-m-long HP5 capillary column. The helium flow rate through the column was 0.5 ml $min^{-1}$. The injection port and detector temperatures were 200 and 250°C, respectively. The temperature-time profile used was as follows: initial temperature of 80°C, initial time of 1 min, rate of temperature increase of 8°C $min^{-1}$, final temperature of 150°C, and final time of 0.5 min. Injections of 1 μl were made.

**PHA analysis.** To quantify the intracellular PHA, biomass samples were harvested and analyzed by the method of Braunegg et al. (3). The extracted methyl esters (2 μl) of the PHA monomers were injected into the same gas chromatograph as above. The injection port and the detector were maintained at 250 and 275°C, respectively. The following temperature-time profile was used: initial temperature of 90°C, initial time of 1 min, temperature ramp of 7°C $min^{-1}$, final temperature of 160°C, final time of 1 min, a second temperature ramp of 5°C $min^{-1}$, a second final temperature of 180°C, a second final time of 0.50 min, a third temperature ramp of 20°C $min^{-1}$, a third final temperature of 250°C, and a third final time of 10 min. The retention times of the methyl esters of β-hydroxybutyrate ($C_4$), β-hydroxyhexanoate ($C_6$), β-hydroxyoctanoate ($C_8$), and β-hydroxydecanoate ($C_{10}$) were 3.84, 6.81, 10.92, and 15.54 min, respectively. Standards were obtained from purified, solvent-extracted PHA (8) whose composition was verified by nuclear magnetic resonance spectroscopy.

## RESULTS

**Growth inhibition by octanoate.** The growth of *P. oleovorans* was partially inhibited when the initial concentration of sodium octanoate in the shake flask was higher than 4.65 g $liter^{-1}$ and totally inhibited at concentrations higher than 6.0 g $liter^{-1}$.

**Effect of dilution rate.** To determine the effect of growth rate on the production and composition of the PHA produced by *P. oleovorans*, a dilution rate experiment was performed with initial ammonium and sodium octanoate concentrations of 2 and 8 g $liter^{-1}$, respectively. In chemostat culture, the specific growth rate ($\mu$) can be controlled by and is equal to the dilution rate ($D$). As $D$ increased to 0.3 $h^{-1}$, there was a slight increase in biomass, while the PHA concentration decreased from 20.5 to 14.5% (wt/wt) (Fig. 1). As $D$ was increased beyond 0.3 $h^{-1}$, the PHA content of the cells diminished rapidly. This corresponded to an increase in unmetabolized octanoate. In contrast, no ammonium was detected in the fermentor until $D = 0.4\ h^{-1}$. At $D > 0.4\ h^{-1}$, the cellular protein concentration fell, and the decrease in the biomass concentration was accelerated as $D$ approached the washout value of 0.51 $h^{-1}$. The specific PHA production rate ($q_p$) increased with $D$ until a maximum value of 0.074 g of PHA g of cellular $protein^{-1}$ $h^{-1}$ was reached at $D = 0.25\ h^{-1}$

When octanoic acid was used in place of sodium octanoate, the kinetics were different such that, at $D > 0.13\ h^{-1}$, the biomass concentration dropped steadily as $D$ was increased (Fig. 2). Unconsumed ammonium ions were de-

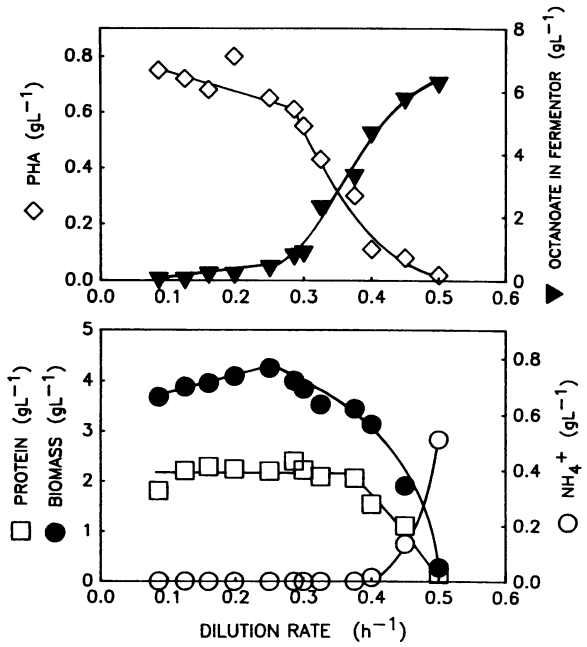


FIG. 1. Effect of growth rate on production of PHA by *P. oleovorans*, using 2 g of ammonium sulfate and 8 g of octanoate (i.e., 9.28 g of sodium octanoate) liter$^{-1}$ in the feed. The maximum specific growth rate, $\mu_{max}$, was 0.51 h$^{-1}$, while the maximum specific PHA production rate, $q_{p,max}$, was 0.074 g of PHA g of cellular protein$^{-1}$ h$^{-1}$ at a dilution rate ($D$) of 0.25 h$^{-1}$. PHA accumulation (as percentage of biomass) decreased as $D$ increased.

tected in the medium at $D > 0.22$ h$^{-1}$. For both sodium octanoate and octanoic acid the maximum amount of PHA found in the biomass was about 20% (wt/wt).

Below a specific growth rate ($\mu$) of 0.3 h$^{-1}$, the composition of the PHA was relatively constant, with the $C_4/C_6/C_8/C_{10}$ ratio being 0.1:1.7:20.7:1.0 (Table 1). Although the $C_4$ and $C_6$ monomers/total PHA ratio remained unchanged above a $D$ of 0.3 h$^{-1}$, the percentage of $C_{10}$ monomers increased and the percentage of $C_8$ monomers decreased as $\mu$ increased. At $D > 0.38$ h$^{-1}$, the unmetabolized octanoate concentration in the reactor was >5 g liter$^{-1}$ and the $C_8/C_{10}$ ratio was 3.8:1.0. The results were similar when octanoic

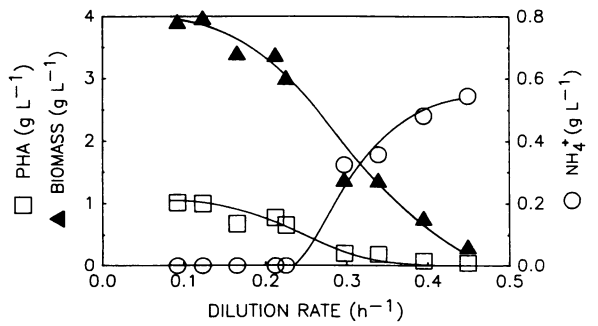


FIG. 2. Effect of growth rate on production of PHA by *P. oleovorans*, using 2 g of ammonium sulfate and 8 g of octanoic acid liter$^{-1}$ in the feed. At a dilution rate ($D$) >0.13 h$^{-1}$, biomass concentration and PHA concentration decreased as $D$ increased, with a maximum of 20% (wt/wt) PHA in the biomass at low $D$. The relationship between biomass and $D$ indicates transport-limited growth, as is often found with water-insoluble substrates.

TABLE 1. Effect of dilution rate on PHA monomer composition for *P. oleovorans* grown on sodium octanoate

| Dilution rate (h$^{-1}$) | % of: | | | |
|---|---|---|---|---|
| | β-Hydroxy-butyrate | β-Hydroxy-hexanoate | β-Hydroxy-octanoate | β-Hydroxy-decanoate |
| 0.086 | 0.3 | 8.1 | 88.4 | 3.1 |
| 0.125 | 0.3 | 8.9 | 89.0 | 2.6 |
| 0.160 | 0.3 | 7.6 | 88.0 | 3.7 |
| 0.198 | 0.3 | 7.9 | 89.0 | 2.7 |
| 0.250 | 0.3 | 7.8 | 88.6 | 3.4 |
| 0.286 | 0.3 | 7.2 | 87.9 | 3.6 |
| 0.300 | 0.4 | 8.2 | 86.5 | 4.0 |
| 0.325 | 0.5 | 8.4 | 85.4 | 5.1 |
| 0.375 | 0.7 | 8.0 | 81.7 | 6.7 |
| 0.400 | 1.8 | 6.4 | 75.5 | 20.9 |
| 0.450 | 1.3 | 7.5 | 72.8 | 18.8 |

acid was used as the carbon source except that the accumulation of a higher percentage of $C_{10}$ monomer occurred at a lower $D$.

**Effect of carbon/nitrogen ratio.** Keeping $D$ constant at 0.24 h$^{-1}$, the concentration of sodium octanoate in the feed was increased to determine its effect on growth and the production of PHA. As the substrate concentration increased, the biomass concentration increased to a maximum of 4.6 g liter$^{-1}$ when there was 7 g of octanoate liter$^{-1}$ in the feed (Fig. 3). Although ammonium became limiting at 4.2 g of octanoate liter$^{-1}$, the biomass continued to increase until a maximum level was attained at 7 g of octanoate liter$^{-1}$. After the onset of ammonium limitation, an accumulation of unmetabolized octanoate began to occur while the phosphate concentration in the reactor (which had decreased from an initial value of 1.89 to 0.92 g liter$^{-1}$) remained relatively constant. At 7 to 13 g of octanoate liter$^{-1}$ in the feed, the

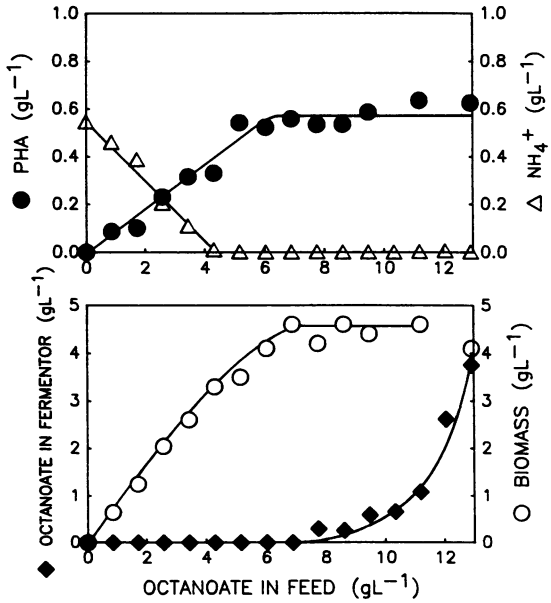


FIG. 3. Effect of octanoate concentration in the feed on PHA accumulation in a chemostat at a dilution rate of 0.24 h$^{-1}$. As octanoate concentration increased, biomass and PHA concentration increased to maximum values at 7 g of octanoate liter$^{-1}$ in the feed, although ammonium became limiting at 4.2 g of octanoate liter$^{-1}$.

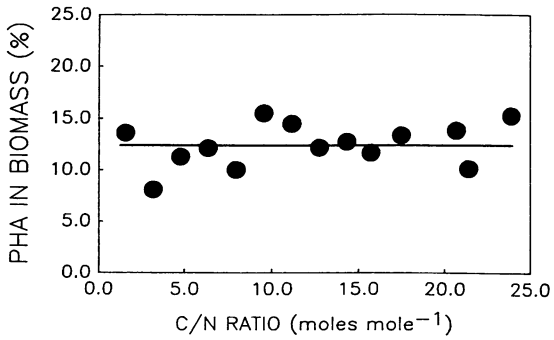


FIG. 4. Effect of carbon/nitrogen ratio on amount of PHA contained in *P. oleovorans* biomass. The data are from the same experiment as Fig. 3 and show that nitrogen limitation had no significant effect on PHA accumulation under these conditions.

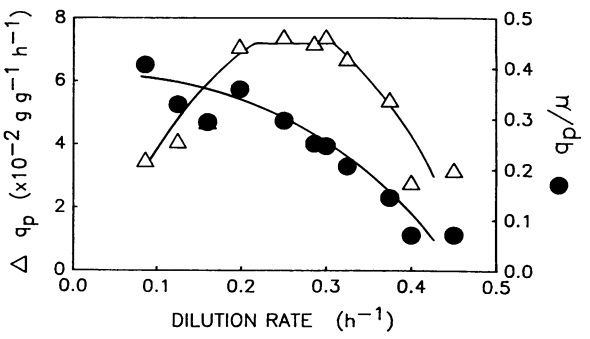


FIG. 5. Relationship between specific PHA production rate ($q_p$) and dilution rate ($D$) in a nitrogen-limited chemostat (data from Fig. 1). Although $q_p$ increased with $D$ at low $D$ values, $q_p/\mu$ always decreased, resulting in less PHA being accumulated per gram of biomass.

phosphate concentration in the reactor fell from 0.81 to 0.70 g liter$^{-1}$.

At this $D$ of 0.24 h$^{-1}$, the percentage of PHA in the biomass was relatively constant at 13% (wt/wt) at all C/N ratios (Fig. 4). No significant change in the PHA monomeric composition was evident as the octanoate concentration in the feed was varied.

**Yields.** Under carbon-limited conditions at $D$ = 0.24 h$^{-1}$, the yield of biomass from substrate ($Y_{X/S}$) was 0.76 g of biomass g of octanoate$^{-1}$ used, while the yield of PHA ($Y_{P/S}$) was 0.085 g of PHA g of octanoate$^{-1}$ consumed. $Y_{X/S}$ and $Y_{P/S}$ diminished somewhat when octanoate was in excess. The maintenance coefficient and the "true growth yield" (i.e., biomass produced per substrate consumed for biomass production) of *P. oleovorans* was calculated from data taken from Fig. 1 and 3, respectively. The cells were found to require 0.046 g of octanoate g of biomass$^{-1}$ h$^{-1}$ for maintenance, with the true growth yield being 0.89 g of biomass g of octanoate$^{-1}$consumed. For each gram of $NH_4^+$ supplied, 7.9 g of octanoate was consumed under carbon-limited conditions.

## DISCUSSION

One of the difficulties in the production of LSC PHAs is the nature of the carbon source. While alkanes, alkanoates, alkanoic acids, or similar structures ($C_6$ to $C_{12}$) can be used, the alkanes and alkanoic acids are immiscible in the aqueous growth medium. Except for the alkanes (which are highly flammable), the other carbon sources are toxic to bacteria at relatively low concentrations. When grown on the water-insoluble octanoic acid, the graph of biomass concentration versus dilution rate (Fig. 2) was similar to the typical curve of transport-limited growth found in hydrocarbon fermentations (6, 14). Since octanoic acid is only weakly acidic, it does not readily ionize and remains water insoluble even when added to a fermentor containing sodium and potassium ions at pH 7.0. The addition of certain surfactants to the growth medium may help in its dispersion (9), but substitution of the alkanoate salt for the acid completely eliminated the apparent effects of transport limitation (Fig. 1) as the salt was completely soluble in the nutrient feed. If octanoate were to be used for the batch culture of *P. oleovorans*, the initial concentration must be below the toxic concentration (i.e., 6 g liter$^{-1}$ or less). Since $Y_{X/S}$ is about 76% (wt/wt), a theoretical maximum of 4.6 g liter of biomass$^{-1}$ could be produced in such a system. Therefore, a chemostat or fed-batch culture must be used to produce more substantial quantities of biomass from alkanoates.

As the specific growth rate (as controlled by $D$) increased, $q_p$ increased until $D$ = 0.2 h$^{-1}$ and then diminished at $D$ > 0.3 h$^{-1}$ (Fig. 5). However, the PHA content continually decreased when expressed as a percentage of the biomass. This is because the increase in the rate of growth is more rapid than the increase in the rate of PHA production as seen by the ratio of $q_p/\mu$, which decreased as $D$ increased (Fig. 5). Therefore, the highest concentration of PHA in terms of percentage of biomass is obtained at low growth rates.

It is well known that the monomer composition of LSC PHA is directly related to the structure of the carbon source (2, 17). The dilution rate study showed that $\mu$ had very little effect on monomer composition until a high $D$ was attained (Table 1). At higher $D$ values when the unmetabolized octanoate concentration in the fermentor was >5 g liter$^{-1}$, there were less $C_8$ monomers and more $C_{10}$ in terms of the percentage of total PHA. The same effect was observed at a lower $D$ (i.e., $\mu$) when octanoic acid was used as the carbon source. With increasing octanoate concentration in the feed, there was little change in the PHA monomeric composition, but then the octanoate concentration in the fermentor was <5 g liter$^{-1}$. It is therefore probable that neither $\mu$ nor $q_p$ has much influence on monomer composition but rather the presence of the excess carbon or nitrogen or both in the fermentor at higher $D$ values is of greater importance.

The maintenance coefficient for *P. oleovorans* (based on total biomass, i.e., PHA and non-PHA biomass) grown on sodium octanoate is quite low. Most of the carbon source consumed goes to making non-PHA biomass, with only an estimated 4% of the total biomass being turned over each hour. (The turnover rate of the biomass was calculated as the product of the maintenance coefficient and the true growth yield (21).)

Although these cells are reasonably efficient at converting substrate to biomass, the existence of a physiological trigger which can stimulate high PHA accumulation is not evident. At $D$ = 0.24 h$^{-1}$, nitrogen limitation had little effect on the percentage of PHA in the biomass of *P. oleovorans* (Fig. 4). This is entirely contrary to previous reports for the same strain grown in batch culture on octane (17). For SSC PHA production, maximum accumulation usually occurs when a surplus of utilizable carbon and energy is available but growth is limited due to a shortage of other nutrients such as ammonium or phosphate (4). In the above chemostat study,

$\mu$ was controlled by $D$. By Monod kinetics, $\mu$ decreases to very low values in nutrient-limited batch culture. As shown above, $q_p$ increases as $\mu$ increases (for low $D$ values), but the overall effect is a decrease in the percentage of PHA in the biomass as $\mu$ increases. From a close examination of the batch data of Lageveen et al. (17), it can be hypothesized that, as nitrogen becomes limiting and its concentration falls to zero, $\mu$ simultaneously decreases to zero. Meanwhile, $q_p$ (which does not directly depend on nitrogen) decreases more gradually, permitting PHA accumulation when there is little or no growth. However, in the chemostat study, although nitrogen is limiting, enough is continuously provided to maintain a certain rate of growth but, at all levels of limiting nitrogen concentration tested, there was no increase in the percentage of PHA accumulated in the biomass. Thus, the impression that nitrogen limitation stimulates PHA accumulation in batch culture may result from a decrease in $\mu$ rather than an increase in $q_p$. It would appear that nitrogen limitation in batch culture results in a lower reactor productivity but a higher percentage of PHA in the biomass.

Although potentially valuable, the exploitation of LSC PHA copolymers is limited by their high cost of production, which is related to a general lack of knowledge concerning their biosynthesis and isolation. An understanding of the factors governing the metabolic control of the biosynthesis of LSC PHAs could aid in the development of fermentation systems which can provide the appropriate physiological conditions for maximum production.

## ACKNOWLEDGMENTS

We acknowledge the financial support of the Biotechnology Research Institute of Montreal and the Pulp and Paper Research Institute of Canada.

## REFERENCES

1. **Anderson, A. J., G. W. Haywood, and E. A. Dawes.** 1990. Biosynthesis and composition of bacterial poly(hydroxy-alkanoates). Int. J. Biol. Macromol. **12**:102–105.
2. **Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller.** 1988. *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as a biodegradable polyester. Appl. Environ. Microbiol. **54**:1977–1982.
3. **Braunegg, G., B. Sonnleitner, and R. M. Lafferty.** 1978. A rapid method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur. J. Appl. Microbiol. Biotechnol. **6**:29–37.
4. **Dawes, E. A., and P. J. Senior.** 1973. The role and regulation of energy reserve polymers in micro-organisms. Adv. Microb. Physiol. **10**:135–266.
5. **deSmeet, M. J., G. Eggink, B. Witholt, J. Kingma, and H. Wynberg.** 1983. Characterization of intracellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. J. Biotechnol. **154**:870–878.
6. **Erickson, L. E., and A. E. Humphrey.** 1969. Growth models of cultures with two phases. III. Continuous cultures. Biotechnol. Bioeng. **11**:489–515.
7. **Fritzche, K., R. W. Lenz, and R. C. Fuller.** 1990. Production of unsaturated polyesters by *Pseudomonas oleovorans*. Int. J. Biol. Macromol. **12**:85–91.
8. **Fritzche, K., R. W. Lenz, and R. C. Fuller.** 1990. Bacterial polyesters containing branched poly(β-hydroxyalkanoates) units. Int. J. Biol. Macromol. **12**:92–101.
9. **Gerson, D. F., D. G. Cooper, B. Ramsay, and J. E. Zajic.** 1980. The effect of substrate on inhibition of *Corynebacterium lepus* by isonicotinic acid hydrazide (Isoniazid). Can. J. Microbiol. **26**:1498–1500.
10. **Gross, R. A., C. deMello, R. W. Lenz, H. Brandl, and R. C. Fuller.** 1989. The biosynthesis and characterization of poly(β-hydroxyalkanoates) produced by *Pseudomonas oleovorans*. Macromolecules **22**:1106–1115.
11. **Harder, W., and L. Dijkhuizen.** 1983. Physiological responses to nutrient limitation. Annu. Rev. Microbiol. **37**:1–23.
12. **Haywood, G. W., A. J. Anderson, and E. A. Dawes.** 1989. A survey of the accumulation of novel polyhydroxyalkanoates by bacteria. Biotechnol. Lett. **11**:471–476.

12a. **Holmes, P. A., S. H. Collins, and W. F. Wright.** April 1987. European patent 69,497.

13. **Huisman, G. W., O. de Leeuw, and G. Eggink.** 1989. Synthesis of poly-3-hydroxyalkanoates is a common feature of fluorescent pseudomonads. Appl. Environ. Microbiol. **55**:1949–1954.
14. **Humphrey, A. E., and L. E. Erickson.** 1972. Kinetics of growth on aqueous-oil and aqueous-solid dispersed systems. J. Appl. Chem. Biotechnol. **4**:125–147.
15. **Jarman, T. R., L. Deavin, S. Slocombe, and R. C. Righelato.** 1978. Investigation of the effect of environmental conditions on the rate of exopolysaccharide synthesis in *Azotobacter vinelandii*. J. Gen. Microbiol. **107**:59–64.
16. **Kunioka, M., Y. Kawaguchi, and Y. Doi.** 1989. Production of biodegradable copolyesters of 3-hydroxybutyrate and 4-hydroxybutyrate by *Alcaligenes eutrophus*. Appl. Microbiol. Biotechnol. **30**:569–573.
17. **Lageveen, R. G., G. W. Huisman, H. Preusting, P. Ketelaar, G. Eggink, and B. Witholt.** 1988. Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(*R*)-3-hydroxyalkanoates and poly-(*R*)-3-hydroxyalkenoates. Appl. Environ. Microbiol. **54**:2924–2932.
18. **Marchessault, R. H., C. J. Monasterios, F. G. Morin, and P. R. Sundararajan.** 1990. Chiral poly(β-hydroxyalkanoates): an adaptable helix influenced by the alkane side-chain. Int. J. Biol. Macromol. **12**:158–165.
19. **Matin, A., C. Veldhuis, V. Stageman, and M. Veenhuis.** 1979. Selective advantage of a *Spirillum* sp. in a carbon-limited environment. Accumulation of poly-β-hydroxybutyric acid and its role in starvation. J. Gen. Microbiol. **112**:349–355.
20. **Oeding, V., and H. G. Schlegel.** 1973. β-Ketothiolase from *Hydrogenomonas eutrophus* H16 and its significance in the regulation of poly-β-hydroxybutyrate metabolism. Biochem. J. **134**:239–248.
21. **Pirt, S. J.** 1975. Principles of microbes and cell cultivation. Blackwell Scientific Publications, London.
22. **Ramsay, B. A., K. Lomaliza, C. Chavarie, B. Dubé, P. Bataille, and J. A. Ramsay.** 1990. Production of poly-(β-hydroxybutyric-co-β-hydroxyvaleric) acids. Appl. Environ. Microbiol. **56**:2093–2098.
23. **Ramsay, B. A., J. A. Ramsay, and D. G. Cooper.** 1989. Production of poly-β-hydroxyalkanoic acid by *Pseudomonas cepacia*. Appl. Environ. Microbiol. **55**:584–589.
24. **Seviour, R. J., and B. Kristiansen.** 1983. Effect of ammonium ion concentration on polysaccharide production by *Aureobasidium pullulans* in batch culture. Eur. J. Appl. Microbiol. Biotechnol. **17**:178–181.
25. **Sonnleitner, B., E. Heinzle, G. Braunegg, and R. M. Lafferty.** 1979. Formal kinetics of poly-β-hydroxybutyric acid (PHB) production in *Alcaligenes eutrophus* H16 and *Mycoplana rubra* R14 with respect to the dissolved oxygen tension in ammonium-limited batch cultures. Eur. J. Appl. Microbiol. Biotechnol. **7**:1–10.
26. **Stickland, L. H.** 1951. The determination of small quantities of bacteria by means of the biuret reaction. J. Gen. Microbiol. **43**:159–271.