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Biosynthesis of Poly-β-Hydroxyalkanoates from Pentoses by *Pseudomonas pseudoflava*

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The potential of *Pseudomonas pseudoflava* to produce poly-β-hydroxyalkanoates (PHAs) from pentoses was studied. This organism was able to use a hydrolysate from the hemicellulosic fraction of poplar wood as a carbon and energy source for its growth. However, in batch cultures, growth was inhibited completely at hydrolysate concentrations higher than 30% (vol/vol). When *P. pseudoflava* was grown on the major sugars present in hemicelluloses in batch cultures, poly-β-hydroxybutyric acid (PHB) accumulated when glucose, xylose, or arabinose was the sole carbon source, with the final PHB content varying from 17% (wt/wt) of the biomass dry weight on arabinose to 22% (wt/wt) of the biomass dry weight on glucose and xylose. Specific growth rates were 0.58 h^{-1} on glucose, 0.13 h^{-1} on xylose, and 0.10 h^{-1} on arabinose, while the specific PHB production rates based on total biomass ranged from 0.02 g g^{-1} h^{-1} on arabinose to 0.11 g g^{-1} h^{-1} on glucose. PHB weight-average molecular weights were 640,000 on arabinose and 1,100,000 on glucose and xylose. The absolute amount of PHB in the cells decreased markedly when nitrogen limitation was relaxed by feeding ammonium sulfate at the end of the PHB accumulation stage of the arabinose and xylose fermentations. Copolymers of β-hydroxybutyric and β-hydroxyvaleric acids were produced when propionic acid was added to shake flasks containing 10 g of glucose $liter^{-1}$. The β-hydroxyvaleric acid monomer content attained a maximum of 45 mol% when the initial propionic acid concentration was 2 g $liter^{-1}$.

Many species of microorganisms can accumulate poly-β-hydroxybutyric acid (PHB) as intracellular granules. This crystalline biopolymer represents a storage form of excess carbon and energy, and its synthesis is controlled by the culture conditions. PHB is also a thermoplastic whose properties resemble those of polypropylene, and it has the advantage of being biodegradable (10, 38). Exogenous PHB can be attacked by pseudomonads, streptomycetes, and *Bacillus* species (13). Recent investigations have focused on the possibility of blending PHB with nonbiodegradable commercial polymers (1, 18) in the hope of producing a generation of plastics that will be degraded after disposal.

With most bacteria that can synthesize PHB, the accumulation of the polymer is triggered by limitation of a nutrient such as oxygen (31) or nitrogen (25, 30). This limitation leads to an excess of NADH or NADPH (31), which in turn represses the enzymes of the tricarboxylic acid cycle. The acetyl coenzyme A produced by oxidation of the carbon source is then shunted to PHB synthesis, assuming the role of an electron sink. At least one microorganism, *Azotobacter vinelandii*, has been shown to accumulate PHB during balanced growth because of a deficient NADH oxidase (27). *Alcaligenes latus* also has this ability of accumulating PHB constitutively, although nitrogen limitation will enhance the synthesis of the biopolymer (6).

Most bacteria that accumulate PHB can also produce different types of poly-β-hydroxyalkanoate (PHA) copolymers (9, 11, 16, 17, 29, 37). Copolymers of β-hydroxybutyric and β-hydroxyvaleric acids [P(HB-co-HV)] are far less brittle than the homopolymer of β-hydroxybutyric acid. By varying the β-hydroxyvaleric acid content of this random copolymer, one can control properties such as its melting point (2, 24). P(HB-co-HV) can be produced by adding propionic acid and glucose to nitrogen-limited cultures of *Alcaligenes eutrophus* (P. A. Holmes, W. F. Wright, and S. H. Collins, European patent 69,497, April 1987). The fluorescent pseudomonads can synthesize elastomeric PHAs which have longer side chains than those of *A. eutrophus* (5, 14, 19). Other PHA copolymers and terpolymers can also be produced biologically (15, 22).

The cost of PHA production is largely dependent on the carbon source used (8). PHA production is usually a two-step process in which high-protein biomass is first produced, followed by PHA accumulation. Replacing the traditional carbon sources in one or both of these stages by an inexpensive substrate such as hemicellulosic sugars could reduce the cost significantly.

While lignocellulosic biomass contains 40 to 50% (wt/wt) cellulose, 20 to 50% (wt/wt) of this biomass is hemicellulose (20). Cellulose and hemicellulose represent a large volume of inexpensive fermentation substrate. For example, it was estimated that the biomass potentially available as a fermentation substrate from U.S. forests in 1980 was 375 million tons, while the agricultural residue was 385 million tons (21). Xylose, arabinose, glucose, and mannose are found in different proportions in hemicellulose, depending on the plant species. White birch, a hardwood, consists of 36% (wt/wt) five-carbon sugars (23), typically present as xylans (32), while softwoods, such as the Douglas fir, may contain hemicellulose with more hexoses than pentoses, with the dominant polymers being mannans (32). Despite the heterogeneous nature of hemicellulose as compared with the homopolymeric cellulose, hemicellulosic sugars have potential uses as fermentation feedstock in the production of ethanol (21, 23), butanediol (21), and acetone and butanol (36).

Although many microorganisms have been shown to produce PHA from hexoses, methanol, ethanol, organic acids, and mixtures of hydrogen, carbon dioxide, and air (8), there have been no reports of PHA production from pentoses or

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hemicellulose. Producing PHA from pentoses and, more importantly, from hemicellulose, would represent a tremendous savings in terms of substrate costs. Although the effect of the carbon source on the accumulation of the PHB homopolymer or PHA copolymers by *Pseudomonas pseudoflava* was unknown, this organism was chosen for this study because of its nutritional versatility and its ability to accumulate PHB (34). This paper describes the production of PHA by *P. pseudoflava* with glucose, xylose, and arabinose in batch cultures, as well as the growth of *P. pseudoflava* on the hemicellulosic fraction of *Populus deltoïdes*, a hardwood.

MATERIALS AND METHODS

Microorganism and growth media. *P. pseudoflava* ATCC 33668 was used in all the experiments. The preinocula for the fermentation and shake flask experiments were prepared in Erlenmeyer flasks containing 0.8% (wt/vol) nutrient broth (Oxoid Ltd., London, England) and 0.5% (wt/vol) glucose and incubated at 34°C for 18 h. Five milliliters of this culture was added to 100 ml of the following defined medium: 10 g of the carbon source, 2 g of $(NH_4)_2SO_4$, 6.8 g of $Na_2HPO_4 \cdot 7H_2O$, 1.5 g of KH_2PO_4, 100 mg of $CaCl_2 \cdot 2H_2O$, 60 mg of NH_4-Fe(III) citrate, 200 mg of $MgSO_4 \cdot 7H_2O$, and 1 ml of trace element solution liter^{-1}. The trace element solution contained 100 mg of $ZnSO_4 \cdot 7H_2O$, 30 mg of $MnCl_2 \cdot 4H_2O$, 300 mg of H_3BO_3, 200 mg of $CoCl_2 \cdot 6H_2O$, 20 mg of $CuSO_4 \cdot 5H_2O$, 20 mg of $NiCl_2 \cdot 6H_2O$, and 30 mg of $NaMoO_4 \cdot 2H_2O$ liter^{-1}.

Batch fermentation. Multigen F-2000 2-liter fermentors (New Brunswick Scientific Co., Inc., Edison, N.J.) containing 1,500 ml of defined medium with 40 g of the carbon source liter^{-1} were inoculated with 100 ml of inoculum. The temperature of all fermentations was 35 ± 1°C, and the pH was maintained at 7.0 ± 0.2. The airflow rate was 1 vvm ± 15% (volume of air per working volume of reactor per minute), and agitation was initially 550 rpm with three Rushton turbines.

Biomass and cellular protein measurement. Culture broth samples were centrifuged for 5 min at 4,000 × *g*. The pellet was suspended in 5 ml of distilled water and centrifuged. The pellet was transferred to a preweighed aluminum dish and dried to a constant weight at 105°C to obtain the total dry biomass. For protein measurements, culture samples were centrifuged as described above and the pellets were analyzed for protein content by the biuret reaction (35) with 1% (wt/vol) bovine serum albumin as a standard (Sigma Chemical Co., St. Louis, Mo.).

PHA analysis. PHA samples were prepared as described by Braunegg et al. (7). The resulting methyl esters of the PHA monomers were quantified with a model 5890 gas chromatograph coupled to a model HP-3396A integrator (both from Hewlett-Packard Co., Palo Alto, Calif.). The chromatographic procedure was performed with a 25-mm HP5 capillary column (Hewlett-Packard) as previously described (29).

Molecular weight determination. After the centrifuged biomass was washed in 10 volumes of acetone for 24 h, it was constantly stirred in 10 volumes of chloroform for 24 h. PHA was precipitated with cold ethanol, and the solid was redissolved in chloroform to yield a concentration of 0.5% (wt/vol). The gel permeation chromatographic analysis procedure and parameters were the same as those previously described (29).

Sugar analyses. The carbon source concentration was determined by high-pressure liquid chromatography with an Aminex 87-P column (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) at 80°C with water as the mobile phase. A model R-401 refractive index detector (Waters Associates, Inc., Miford, Mass.) was used to monitor the separation.

TABLE 1. Growth and PHB production of *P. pseudoflava* on various carbon sources at an initial pH of 6.7 in a shake flask experiment

Substrate	Final biomass (g liter^{-1})	Substrate consumed (g liter^{-1})	PHB		Final pH
			g liter^{-1}	%	
Glucose	1.04	5.7	0.06	5.7	3.0
Lactose	0.19	0.6	0.00	0.0	6.7
Galactose	0.80	4.7	0.02	2.5	2.8
D-Xylose	2.08	4.9	0.41	19.7	3.6
L-Arabinose	1.74	2.4	0.43	24.7	5.5
Mannose	0.00	0.2	0.00	0.0	6.7

Hemicellulosic hydrolysate. The aqueous hemicellulosic fraction of *P. deltoïdes* was a gift from E. Chornet. This fraction was acidified with 0.2 N sulfuric acid and heated in an autoclave at 121°C for 30 min. The hydrolysate was rapidly cooled and neutralized by adding sterile 10 N NaOH. The resulting solution was analyzed by high-pressure liquid chromatography as described above. The hydrolysate was found to contain about 7.6 g of pentoses liter^{-1} (mostly xylose) and trace amounts of glucose.

Propionic acid. One milliliter of methanol and 0.2 ml of concentrated sulfuric acid containing 0.1% (wt/vol) β-hydroxybutyric acid were added to 1 ml of culture broth supernatant and heated at 55°C for 30 min. The methyl esters were extracted with 2 ml of dichloromethane after the addition of 1 ml of water. The gas chromatographic analysis was performed with the same instrument and column as those used in the PHA analysis but with the following temperature-time profile: initial temperature, 40°C; gradient, 2.5°C min^{-1}; final temperature, 60°C. The injector and detector temperatures were 170 and 200°C, respectively.

Ammonium ion. The ammonium ion concentration was quantified with a gas ammonia probe (Orion Research Laboratories, Cambridge, Mass.) or a modified Berthelot reaction (33) and with $(NH_4)_2SO_4$ solutions as standards.

Oxygen. A quadrupole mass spectrometer (VG Quadrupoles, Middlewich, England) was used for on-line analysis of the gas at the outlet of the fermentor. The flow rate of the gas entering the spectrometer was maintained at between 100 and 150 ml min^{-1}, and the settling time for analysis was 60 s. The air at the inlet of the fermentor was used as a reference to compute the oxygen consumption.

RESULTS

To determine whether substrates such as cheese whey and hemicelluloses could be used as carbon sources for PHB production, we performed shake flask experiments with *P. pseudoflava* grown on various sugars (which are found in cheese whey and hemicelluose) in a nitrogen-limited medium. Although this strain did not utilize lactose or mannose, it grew on glucose, galactose, xylose, and arabinose (Table 1). PHB accumulation varied from 2.5% (wt/wt) on galactose to 24.7% (wt/wt) on arabinose on a dry-biomass basis.

When propionic acid was added to flasks containing 10 g of glucose liter^{-1}, *P. pseudoflava* synthesized a copolymer of

TABLE 2. Effect of propionic acid on growth and copolymer synthesis of *P. pseudoflava* at an initial pH of 7.1 in a shake flask experiment[a]

Initial propionate concn (g liter^{-1})	Biomass (g liter^{-1})	PHA (g liter^{-1})	β-Hydroxy-valeric acid monomer (mol%)	Final [NH_4^+] (mg liter^{-1})	Final pH
0.0	0.68	0.15	0.0	0	3.5
0.1	0.81	0.35	1.2	0	3.6
0.3	1.50	0.57	3.5	0	4.1
1.0	0.72	0.14	20.3		3.6
2.0	0.47	0.08	45.2	26	3.9
3.0	0.39	0.00	0.0	36	4.2
5.0	0.25	0.00	0.0	60	4.6
7.0	0.28	0.00	0.0	75	5.3
10.0	0.23	0.00	0.0		5.7

[a] Incubation was done for 48 h at 29°C. The initial ammonium ion concentration was 400 mg liter^{-1}. The initial glucose concentration was 10 g liter^{-1}.

P(HB-co-HV) (Table 2). Growth and P(HB-co-HV) production were optimum at a propionic acid concentration of 0.3 g liter^{-1}, and the copolymer contained 3.5 mol% of the β-hydroxyvaleric acid monomer. At increasing concentrations of propionic acid, both growth and PHA accumulation were inhibited. Inhibition of PHA production began at propionic acid concentrations higher than 1 g liter^{-1} and was complete at a propionic acid concentration of 3 g liter^{-1}.

P. pseudoflava was grown on glucose, xylose, and arabinose in batch cultures with an initial carbon/nitrogen ratio of 44 mol mol^{-1}. On glucose, up to 22% (wt/vol) of the biomass dry weight was accumulated as PHB in about 10 h (Fig. 1).

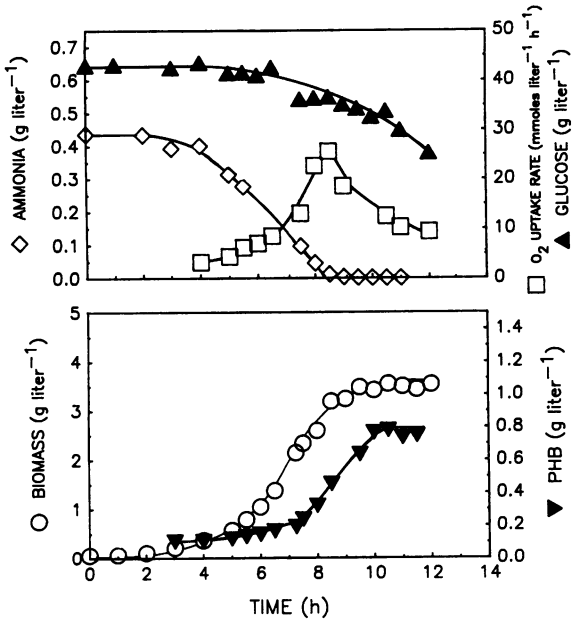

FIG. 1. Batch fermentation of *P. pseudoflava* done in a 2-liter Multigen fermentor with an initial carbon/nitrogen ratio of 44 mol mol^{-1} and glucose as the sole carbon source. At the onset of ammonia limitation (at about 8.5 h), the oxygen uptake rate peaked and the rate of PHB accumulation increased to yield a maximum of 22% (wt/wt) PHB in the biomass at 10 h. The maximum specific growth rate (μ_{max}) was 0.58 h^{-1}, and the maximum specific PHB production rate ($q_{p,max}$) was 0.11 g g^{-1} h^{-1}.

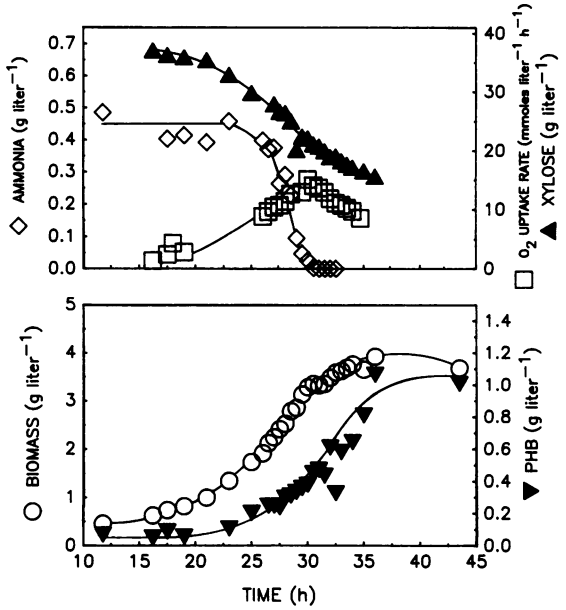

FIG. 2. Batch fermentation of *P. pseudoflava* done in a 2-liter Multigen fermentor with an initial carbon/nitrogen ratio of 44 mol mol^{-1} and xylose as the sole carbon source. Ammonia limitation (at 30 h) coincided with the peak in the oxygen uptake rate. However, the PHB accumulation rate had already started to increase at 25 h to yield a final concentration of 22% (wt/vol) PHB in the biomass. The maximum specific growth rate (μ_{max}) was 0.13 h^{-1}, and the maximum specific PHB production rate ($q_{p,max}$) was 0.03 g g^{-1} h^{-1}.

The maximum specific growth rate (μ_{max}) under these conditions was 0.58 h^{-1}, while the maximum specific PHB production rate ($q_{p,max}$) was 0.11 g of PHB g of biomass^{-1} h^{-1}. The maximum oxygen consumption rate was 9.2 mmol g of biomass^{-1} h^{-1}, while the yield of biomass over substrate ($Y_{x/s}$) was 0.36. The weight-average molecular weight of the polymer at the end of the fermentation was 1,045,000.

When *P. pseudoflava* was grown on xylose or arabinose as the sole carbon source (Fig. 2 and 3, respectively), specific growth and PHB production rates were four to five times slower than with glucose, while the amount of biomass obtained per gram of substrate was half that observed with glucose. However, the final PHB content was similar to that measured for the glucose fermentation. The weight-average molecular weights of the polymer from xylose and arabinose were 1,145,000 and 640,000, respectively. The kinetic parameters and yields are summarized in Table 3.

At the end of the accumulation phase of duplicate fermentations on xylose and arabinose, a pulse of ammonium ions was added to yield a concentration of 6 g of ammonium sulfate liter^{-1} (data not shown). The absolute amounts of PHB decreased from 0.80 to 0.20 g liter^{-1} in the culture on arabinose and from 0.74 to 0.67 g liter^{-1} in the culture on xylose.

P. pseudoflava was grown in shake flasks containing different concentrations of a hemicellulosic hydrolysate from poplar wood, 5 g of additional xylose liter^{-1}, and a nonlimiting concentration of ammonium sulfate. The hemicellulosic hydrolysate had been pretreated as described in Materials and Methods to yield optimal concentrations of free xylose and to limit the production of decomposition products such as furfural and 5-hydroxymethyl furfural. When the hydrolysate fraction was greater than 30% (vol/vol) in batch cultures, growth was totally prevented by the presence of some

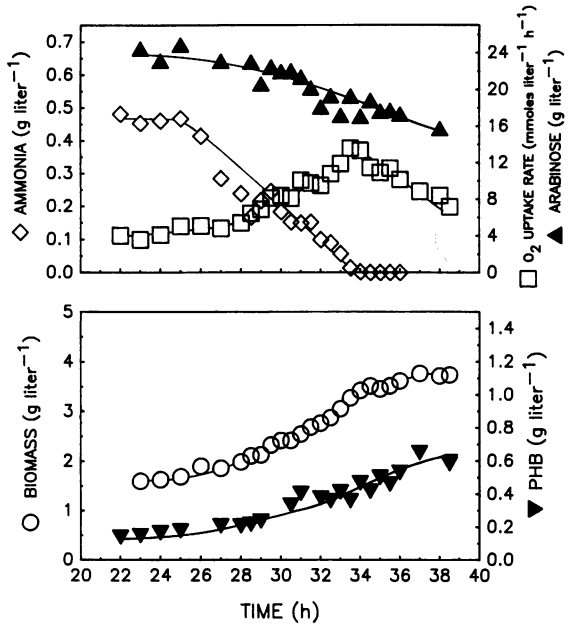

FIG. 3. Batch fermentation of *P. pseudoflava* done in a 2-liter Multigen fermentor with an initial carbon/nitrogen ratio of 44 mol mol^{-1} and arabinose as the sole carbon source. The final PHB content of the biomass was 17% (wt/vol). The maximum specific growth rate (μ_{max}) was 0.10 h^{-1}, and the maximum specific PHB production rate ($q_{p,max}$) was 0.02 g g^{-1} h^{-1}.

inhibitory compound. In chemostat cultures, *P. pseudoflava* was able to use the hydrolysate as a sole carbon and energy source.

DISCUSSION

P. pseudoflava was able to produce significant amounts of PHB from glucose, galactose, arabinose, or xylose in shake flask experiments. Lactose, however, was not used because the bacterium evidently did not produce β-galactosidase. When propionic acid was supplied to shake flasks containing glucose, the industrially more interesting P(HB-co-HV) copolymer was produced. Inhibition of both growth and production of the biopolymer began at 1 g of propionic acid liter^{-1}, with total inhibition at 3 g liter^{-1}. A concentration of 1 g of propionic acid liter^{-1} has been reported to inhibit polymer synthesis in *A. eutrophus* (8), which is the organism most commonly cited for possible use in commercial PHA production. Hence, the feeding regime of the organic acid must be precisely controlled in a fermentation process for copolymer synthesis. In shake flasks, growth and PHB accumulation are usually limited by the lack of pH control. Hence, for the most promising substrates, glucose, arabinose, and xylose, fermentations were subsequently performed in a bioreactor which had pH control to better study the kinetics of growth and PHB production.

TABLE 3. Kinetic and yield constants derived from batch cultures of *P. pseudoflava* grown on glucose, xylose, or arabinose as the sole carbon source

Substrate	% PHB	μ_{max} (h^{-1})	$q_{p,max}$ (g g^{-1} h^{-1})	$q_{O_2,max}$[a] (mmol g^{-1} h^{-1})	$Y_{x/s}$	MW, 10^3
Glucose	22	0.58	0.11	9.2	0.36	1,045
Xylose	22	0.13	0.03	4.8	0.17	1,145
Arabinose	17	0.10	0.02	3.9	0.19	640

[a] $q_{O_2,max}$, Maximum oxygen consumption rate.

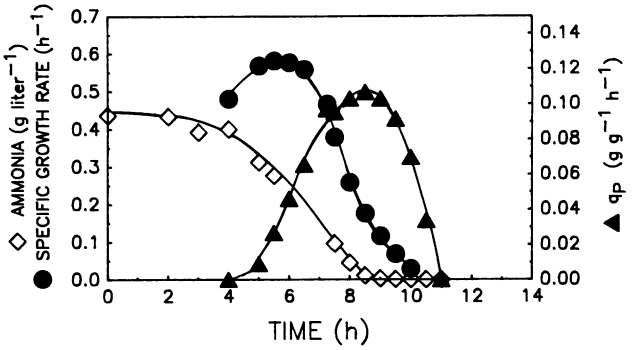

FIG. 4. Ammonia concentration, specific growth rate (μ), and PHB production rate (q_p, based on biomass) in a batch fermentation of *P. pseudoflava* with glucose as the sole carbon source.

The batch fermentation data in the bioreactor showed that both growth and PHB production rates were slower on pentoses than on glucose. The weight-average molecular weights of the polymers produced from glucose and xylose were comparable (1,100,000), but the weight-average molecular weight was only 640,000 for arabinose. It was previously shown that PHB from cultures of *A. eutrophus* had a weight-average molecular weight on the order of 1,200,000 (4). Since the recommended molecular weight for commercial PHA is greater than 50,000 (Holmes et al., European patent 69,497, April 1987), the polymers synthesized by *P. pseudoflava* on pentoses are of potential industrial use.

Treatment of the data obtained for the batch fermentation of *P. pseudoflava* on glucose showed that PHB production was not growth associated, since the specific PHB production rate (q_p) was slightly lower than one-third the maximum specific PHB production rate ($q_{p,max}$) when μ_{max} was achieved (Fig. 4). $q_{p,max}$ occurred at a fermentation time of 8.5 h, which corresponded to an ammonium concentration of zero. $q_{p,max}$ was maintained for a relatively short period of time after NH_4^+ exhaustion, suggesting that the feeding of small quantities of ammonium ion may be necessary to extend the period of maximum productivity. The distinct separation of growth and PHB accumulation has been described by Oeding and Schlegel (25), who reported that PHB accumulation in *A. eutrophus* was initiated by a nitrogen source limitation. This distinction was not as evident on xylose and arabinose.

When the nitrogen limitation was relaxed at the end of the accumulation phase, there was a large decrease in the PHB content for the arabinose and xylose fermentations. Although there was sugar in the medium, the cells still degraded some of the polymer, indicating that PHB may be turned over continually in *P. pseudoflava*. This result contradicts observations reported in a recent review by Dawes (12) in which it was suggested that PHB is left intact unless carbon limitation takes place. It is possible that PHB is a preferred carbon source over pentoses but not over glucose.

The carbon source was always provided in excess to allow maximum PHB accumulation in the biomass. However, under nonoptimized conditions, the total accumulation of PHB in *P. pseudoflava* never exceeded 22% (wt/wt) of the biomass dry weight. Other organisms, such as *A. eutrophus*, have been reported to accumulate up to 90% (wt/wt) of the biomass dry weight as PHB (12). The cessation of PHB

accumulation in *A. eutrophus* seems to result from a physical space limitation (2). This is probably not the case for *P. pseudoflava*, and studies should be done to optimize growth and PHB accumulation and to identify and modify the control mechanism(s) that limits accumulation in organisms that produce little PHB.

Fed-batch fermentation would be preferred for the production of PHA from a hemicellulosic hydrolysate, since organism growth and PHA production were affected by inhibitory substances, possibly furans, when the organism was grown in batch cultures. Degradation of monosaccharides to furfural and 5-hydroxymethyl furfural during acid hydrolysis is common. However, the kinetics of the process can be controlled because the rate of release of monosaccharides from the oligomers is faster than the rate of decomposition of the monosaccharides to furfural and 5-hydroxymethyl furfural (3). Enzymatic degradation of hemicelluloses would produce a better carbon source than would chemical treatment. However, such a process may not be feasible, since it would involve the combined action of many enzymes, such as xylanases, mannanases, glucuronidases, acetylesterases, and xylosidases, if hemicelluloses were to be degraded to their monomeric components.

Hemicellulosic sugars represent an interesting alternative to commonly used carbon sources because of their abundance. There are processes which can selectively remove the hemicellulosic sugars from hardwoods and limit the formation of degradation products (3). Mild acid hydrolysis, for example, greatly enhances the release of desirable end products (21). Hemicellulosic sugars which are presently wasted may be processed into higher-value products such as biodegradable thermoplastics.

Although *P. pseudoflava* is the first microorganism which has been shown to use pentoses from hemicellulosic sugars to produce PHAs, many others are likely to possess this ability, and their rate of PHB production and PHB yield may be much higher. For organisms such as *P. pseudoflava* which produce little PHB, treatment with mutagens followed by a selection process such as density gradient centrifugation (28) or replica plating and then staining with Nile blue A (26) may allow the development of commercially useful strains. Bulk polypropylene costs \$0.88 kg^{-1} (Chemical Week, 20 June 1990, p. 29), while P(HB-co-HV) has been reported by Imperial Chemical Industries to cost \$4.40 kg^{-1} when a full-scale production unit is used with glucose as a substrate (T. J. Galvin, Proc. 1st Int. Sci. Consensus Workshop on Degradable Materials, 1989, p. 39–53). If organisms which produce much PHB by utilizing pentose are supplied with a hemicellulosic substrate generated from cheap and efficient hydrolysis processes, the cost of PHA production could be lowered to make it much more competitive with the production of nonbiodegradable plastics.

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LITERATURE CITED

1. **Avella, M., and E. Martuscelli.** 1988. Poly-*D*(−)(3-hydroxybutyrate)/poly(ethylene oxide) blends: phase diagram, thermal and crystallization behaviour. Polymer **29**:1731-1737.
2. **Ballard, D. G. H., P. A. Holmes, and P. J. Senior.** 1987. Formation of polymers of β-hydroxybutyric acid in bacterial cells and a comparison of the morphology of growth with the formation of polyethylene in the solid state, p. 293–314. *In* M. Fontanille and A. Guyot (ed.), Recent advances in mechanistic and synthetic aspects of polymerization. D. Reidel Publishing Co., Dordrecht, The Netherlands.
3. **Baugh, K. D., and P. L. McCarthy.** 1988. Thermochemical pretreatment of lignocellulose to enhance methane fermentation: I. Monosaccharide and furfurals hydrothermal decomposition and product formation rates. Biotechnol. Bioeng. **31**:50–61.
4. **Berger, E., B. A. Ramsay, J. A. Ramsay, and C. Chavarie.** 1989. PHB recovery by hypochlorite digestion of non-PHB biomass. Biotechnol. Tech. **3**:227–232.
5. **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 biodegradable polyesters. Appl. Environ. Microbiol. **54**:1977–1982.
6. **Braunegg, G., and B. Bogensberger.** 1985. Zur Kinetik des Wachstums und der Speicherung von Poly-D(−)-3-hydroxybuttersaüre bei *Alcaligenes latus*. Acta Biotechnol. **5**:339–345.
7. **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.
8. **Byrom, D.** 1987. Polymer synthesis by microorganisms: technology and economics. Trends Biotechnol. **5**:246–250.
9. **Capon, R. J., R. W. Dunlop, E. L. Ghisalberti, and P. R. Jeffries.** 1983. Poly-3-hydroxyalkanoates from marine and freshwater cyanobacteria. Phytochemistry **22**:1181–1184.
10. **Chowdury, A.** 1963. Bacteria and exoenzymes capable of degrading poly-β-hydroxybutyric acid. Arch. Mikrobiol. **47**:167–200.
11. **Davis, J. B.** 1964. Cellular lipids of a *Nocardia* grown on propane and *n*-butane. Appl. Microbiol. **12**:301–304.
12. **Dawes, E. A.** 1988. Polyhydroxybutyrate: an intriguing biopolymer. Biosci. Rep. **8**:537–547.
13. **Dawes, E. A., and P. J. Senior.** 1973. The role and regulation of energy reserve polymers in microorganisms. Adv. Microb. Physiol. **10**:134–266.
14. **de Smet, M. J., G. Eggink, B. Witholt, J. Kingma, and H. Wynbert.** 1983. Characterization of intracellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. J. Bacteriol. **154**:870–874.
15. **Doi, Y., A. Tamaki, M. Kunioka, and K. Soga.** 1987. Biosynthesis of terpolyesters of 3-hydroxybutyrate, 3-hydroxyvalerate, and 5-hydroxyvalerate in *Alcaligenes eutrophus* from 5-chloropentanoic and pentanoic acids. Makromol. Chem. Rapid Commun. **8**:631–635.
16. **Findlay, R. H., and P. C. White.** 1983. Polymeric β-hydroxyalkanoates from environmental samples of *Bacillus megaterium*. Appl. Environ. Microbiol. **45**:71–78.
17. **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.
18. **Holmes, P. A.** 1985. Applications of PHB—a microbially produced biodegradable thermoplastic. Physics Technol. **16**:32–36.
19. **Huisman, G. W., O. de Leeuw, G. Eggink, and B. Witholt.** 1989. Synthesis of poly-3-hydroxyalkanoates is a common feature of fluorescent pseudomonads. Appl. Environ. Microbiol. **55**:1949–1954.
20. **Janshekar, H., and A. Fiechter.** 1983. Lignin: biosynthesis, application, and biodegradation. Adv. Biochem. Eng. Biotechnol. **27**:119–178.
21. **Jeffries, T. W.** 1983. Utilization of xylose by bacteria, yeasts, and fungi. Adv. Biochem. Eng. Biotechnol. **27**:1–32.
22. **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.
23. **Magee, R. J., and N. Kosaric.** 1985. Bioconversion of hemicellulosics. Adv. Biochem. Eng. Biotechnol. **32**:61–94.

24. **Mitomo, H., P. J. Barham, and A. Keller.** 1988. Temperature dependence of mechanical properties of poly(β-hydroxybutyrate-β-hydroxyvalerate). Polymer Commun. **29:**112–115.
25. **Oeding, V., and H. G. Schlegel.** 1973. β-Ketothiolase from *Hydrogenomonas eutropha* H16 and its significance in the regulation of poly-β-hydroxybutyrate metabolism. Biochem. J. **134:**239–248.
26. **Ostle, A. G., and J. G. Holt.** 1982. Nile blue A as a fluorescent stain for poly-β-hydroxybutyrate. Appl. Environ. Microbiol. **44:**238–241.
27. **Page, W. J.** 1989. Production of poly-β-hydroxybutyrate by *Azotobacter vinelandii* strain UWD during growth on molasses and other complex carbon sources. Appl. Microbiol. Biotechnol. **31:**329–333.
28. **Pedros-Alio, C., J. Mas, and R. Guerrero.** 1985. The influence of poly-β-hydroxybutyrate accumulation on cell volume and buoyant density in *Alcaligenes eutrophus*. Arch. Microbiol. **143:**178–184.
29. **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.
30. **Schlegel, H. G., G. Gottschalk, and R. von Bartha.** 1961. Formation and utilization of poly-β-hydroxybutyric acid by Knallgas bacteria (*Hydrogenomonas*). Nature (London) **191:** 463–465.
31. **Senior, P. J.** 1984. Polyhydroxybutyrate, a specialty polymer of microbial origin, p. 266–271. *In* A. C. R. Dean, D. C. Ellwood, and C. G. T. Evans (ed.), Continuous culture, vol. 8. Biotechnology, medicine, and the environment. Ellis Horwood, Chechester, United Kingdom.
32. **Sjöström, E.** 1981. Wood chemistry: fundamentals and applications, p. 51–66. Academic press, Inc., New York.
33. **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.
34. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:**159–271.
35. **Stickland, L. H.** 1951. The determination of small quantities of bacteria by means of the biuret reaction. J. Gen. Microbiol. **5:**689–703.
36. **Volesky, B., and T. Szczesny.** 1983. Bacterial conversion of pentose sugars to acetone and butanol. Adv. Biochem. Eng. Biotechnol. **27:**101–118.
37. **Wallen, L. L., and R. K. Rohwedder.** 1974. Poly-β-hydroxyalkanoate from activated sludge. Environ. Sci. Technol. **45:**576–579.
38. **Williams, D. F., and N. D. Miller.** 1987. The degradation of polyhydroxybutyrate (PHB). Adv. Biomater. **7:**471–476.