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# Methanogenic Degradation of Poly(3-Hydroxyalkanoates)

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**Poly(3-hydroxybutyrate) and the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) were fermented to methane and carbon dioxide within 16 days by an anaerobic sewage sludge consortium. The cultures adapted quickly to metabolize these polymeric compounds, and between 83 and 96% of the substrate carbon was transformed to methane and carbon dioxide.**

Poly(3-hydroxyalkanoates) (PHAs) are produced by a variety of bacteria and function as intracellular storage polymers for carbon and energy (2). These polyesters accumulate in cells as distinct granules that can be isolated by hypochlorite treatment (3). Of the PHAs, poly(3-hydroxybutyrate) (PHB) is the most prevalent. Some bacteria are also capable of forming copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate [P(HB-co-HV)] (2). When extracted from the cells, PHB and P(HB-co-HV) have been shown to be biodegradable, biocompatible thermoplastics (12). Indeed, shampoo bottles made of these polymers were recently test marketed in Europe.

Extracellular depolymerases from several aerobic bacteria capable of degrading PHAs have been isolated and investigated (20, 23). Thus, the basic mechanisms of aerobic PHA degradation are understood (2, 6). Anaerobic degradation of PHAs, on the other hand, has not been well documented, although it has been suggested that PHAs can be degraded in an anaerobic environment such as sewage sludge (24). In this article, we present data demonstrating the methanogenic biodegradation of PHB and copolymers of P(HB-co-HV) in sewage sludge.

PHB and P(HB-co-HV)s were produced in shake flask cultures of *Azotobacter vinelandii* UWD (17). The copolyesters were formed by controlled feeding of sodium valerate to the glucose- and ammonium acetate-containing culture during the active PHB production phase (unpublished data). Organic acid feeding is a well-documented method for promoting copolymer formation among PHA-forming bacteria (2). The polyester-containing granules were extracted from the cells with commercial bleach and purified (13). The resulting acetone-insoluble polyester powders were used for chemical analyses and were added as the carbon source supplement in the test medium. The polymer was analyzed after methanolysis (4), and the β-hydroxy-carboxylic acid methyl esters were separated on a fused silica capillary column (Nukol; 15-m length, 0.53-mm inner diameter; Supelco) housed in a Hewlett Packard 5890A gas chromatograph. Calculation of methyl ester peak areas was done with a Hewlett Packard 3390A integrator, and the compositions of the copolymers were determined to be P(HB-co-13%HV) and P(HB-co-20%HV).

Anaerobic cultures were prepared in triplicate serum bottles by a variation of the Hungate technique (16). The cultures consisted of 10 ml of culture fluid in 59-ml serum bottles. A prereduced defined anaerobic mineral medium was prepared as previously described (10), except that no vitamin B mix was added and the additional carbon substrates were PHAs added at a concentration of 500 mg/liter (5 mg per bottle) as dry powders. Anaerobically digested domestic sewage sludge was used as the source of the methanogenic inoculum. The cultures were adjusted to ambient atmospheric pressure (9) before incubation in the dark at 35°C.

Methane production in the culture bottles was measured by gas chromatography (9). The observed $CH_4$ concentrations (in percent by volume) were corrected for the presence of water vapor. Gas volumes were determined with a pressure transducer (Micro Switch 142 PC 30G; Honeywell, Freeport, Ill.) (19). The measured volumes were corrected for the presence of water vapor and adjusted to atmospheric pressure, thus allowing a calculation of dry $CH_4$ amounts (9). The net $CH_4$ production in the sample bottles was obtained by subtracting the amount of $CH_4$ produced in the unamended control cultures from that in the test bottles. All data were statistically analyzed by the method of Dunnett (7) or by Duncan's multiple range test (21).

Molecular weights of the PHAs were determined by gel permeation chromatography using a Shimadzu LC-6A HPLC system containing a Phenogel-10μ 10-μm particle size, $10^5$-nm pore size (Phenominex) column followed by a Progel-TSK G5000-H6 (Supelco) column and a refractive index detector, with chloroform (flow rate, 1 ml/min) as the elution solvent. PHA samples were dissolved in chloroform (0.5%, wt/vol) and 50 μl was analyzed within 24 h of sample preparation. Polystyrene molecular weight standards dissolved in chloroform (0.1%, wt/vol) were used to construct the calibration curve.

The weight-average molecular weights of the PHAs are shown in Table 1. These were used to determine the empirical formulae of the biopolymers, which subsequently were used to calculate the carbon content of the PHAs. The proportions of carbon in the PHB and P(HB-co-20%HV) preparations were determined by the Microanalytical Laboratory of the Department of Chemistry, University of Alberta. The measured carbon contents agreed closely with the calculated values.

Figure 1 illustrates the cumulative production of $CH_4$ in the anaerobic cultures over a 21-day period. Fermentable substrates accompanied the anaerobic sludge inoculum, and these resulted in the $CH_4$ observed in the unamended control cultures. The amounts of $CH_4$ in the PHA-containing cultures and in the control cultures were the same after 1 day of incubation. By the third day of incubation, the amounts of $CH_4$ in the test samples were significantly greater ($P < 0.05$)

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TABLE 1. Characteristics of the PHAs used in this study

| PHA | Mol wt | Empirical formula | % Carbon | |
|---|---|---|---|---|
| | | | Calculated[a] | Found[b] |
| PHB | $1.0 \times 10^6$ | H $(C_4H_6O_2)_{12,000}$ OH | 55.8 | 55.5 |
| P(HB-co-13%HV) | $1.4 \times 10^6$ | H $(C_4H_6O_2)_{14,000}$ $(C_5H_8O_2)_{2,100}$ OH | 56.4 | NA[c] |
| P(HB-co-20%HV) | $1.5 \times 10^6$ | H $(C_4H_6O_2)_{14,000}$ $(C_5H_8O_2)_{3,500}$ OH | 56.8 | 56.3 |

[a] From formula.
[b] By microanalysis.
[c] NA, not analyzed.

than those found in the unamended cultures. Of the various polymers tested, cultures containing P(HB-co-13%HV) had the greatest $CH_4$ production by day 3, while PHB cultures generated the smallest amount of $CH_4$. On day 4, the $CH_4$ production in the culture containing P(HB-co-20%HV) did not differ significantly ($P < 0.05$) from that in the P(HB-co-13%HV) culture. By the eighth day of incubation, no significant differences ($P < 0.05$) in $CH_4$ production among the test samples were detected. Clearly, the presence of PHAs in the test cultures yielded rapid $CH_4$ production at a level significantly higher than that in the control cultures.

On the basis of the empirical formulae in Table 1 and Buswell and Mueller's formula (5), $C_nH_aO_b + [n - a/4 - b/2]H_2O \rightarrow [n/2 - a/8 + b/4]CO_2 + [n/2 + a/8 - b/4]CH_4$, the theoretical amounts of gas produced from the degradation of 5 mg of the polymers were calculated (Table 2). Measurements taken on the 16th day of incubation revealed that for all the polyesters, the measured values were near the theoretical values. The total amount of gas produced was 87, 96, and 83% of the expected values for PHB, P(HB-co-13%HV), and P(HB-co-20%HV), respectively. The theoretical amount of $CH_4$ produced from each substrate was within the range of the observed mean ± 1 standard deviation. These data showed that PHB and copolymers of P(HB-co-HV) were degraded under methanogenic conditions. The substrates were rapidly fermented to $CH_4$ and $CO_2$ during the 21-day test period. The $CO_2$ and $CH_4$ yields were close to the expected yields, and more than 80% of the substrate carbon was converted to $CH_4$.

Studies of the extracellular depolymerase and oligomer hydrolases from *Alcaligenes faecalis* have shown that the mechanism of PHB depolymerization under aerobic conditions is a two-step process (20). First, the water-insoluble PHB substrate is hydrolyzed into water-soluble oligomers and traces of monomers by a PHB depolymerase (23). Second, the slightly water-insoluble PHB oligomers are reduced to monomers by an oligomer hydrolase (20). The monomers can then be absorbed and utilized by the microorganisms.

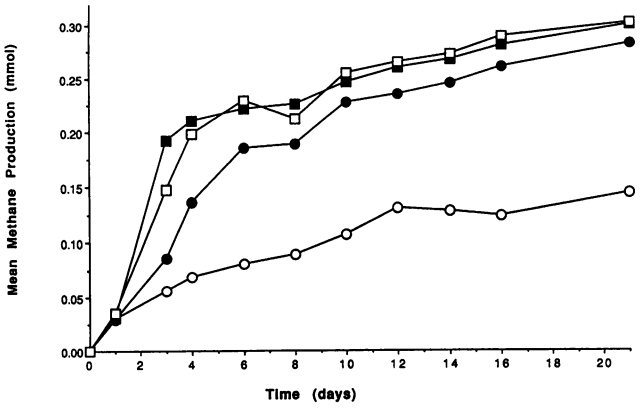


FIG. 1. Methane production in sewage sludge cultures incubated with PHB and P(HB-co-HV) copolymers. Symbols: ○, control (unamended) culture; ●, PHB-containing culture; ■, P(HB-co-13%HV)-containing culture; □, P(HB-co-20%HV)-containing culture.

PHB accumulation has been found in cells of the anaerobic syntrophic bacterium *Syntrophomonas wolfei* (1). This bacterium can also degrade $C_4$ to $C_8$ straight-chain fatty acids to acetate and $H_2$ or to acetate, propionate, and $H_2$ (25). These reactions, however, are energetically unfavorable unless the $H_2$ concentration is maintained at a very low level by $H_2$-using methanogens (15).

Overall, the biodegradation of PHB and P(HB-co-HV) under methanogenic conditions would most likely involve the initial hydrolysis of PHA to its monomeric units. The conversion of the monomers (for example, 3-hydroxybutyrate) to acetate and $H_2$ by $H_2$-producing acetogenic bacteria (such as *S. wolfei*) in syntrophic association with $H_2$-using methanogens (14) would follow. Ultimately, methanogens would complete the degradation of acetate derived from the PHAs, yielding $CH_4$ and $CO_2$.

Figure 2 shows that conversion of 3-hydroxybutyrate and acetate to $CH_4$ proceeds very rapidly. Indeed, the rate of $CH_4$ production in cultures incubated with these substrates was significantly greater ($P < 0.05$) than the rate of $CH_4$ production in the unamended controls during the first day of incubation. Surprisingly, those cultures incubated with butyric acid showed no significant increase in $CH_4$ production over the control cultures (Fig. 2), even after 21 days of incubation (data not shown). Wolin (26) states that $\Delta G^{\circ\prime} = +48.1$ kJ for the oxidation of butyric acid to acetic acid and hydrogen and $\Delta G^{\circ\prime} = -35.1$ kJ for the oxidation of 3-hydroxybutyric acid to acetic acid and hydrogen. Thus, 3-hydroxybutyric acid would be more amenable to oxidation than butyric acid.

A 3-hydroxybutyrate-fermenting bacterium, *Ilyobacter polytropus*, has been observed in low numbers in anaerobic digestor sludge (22). This bacterium ferments 2 mol of 3-hydroxybutyrate to 2 mol of acetate and 2 mol of butyrate. It is unlikely that this type of fermentation is significant in our cultures. If butyrate was produced, it would probably accumulate in the medium because of the inability of the sludge inoculum to produce $CH_4$ from this acid (Fig. 2). Thus, at most only 50% of the carbon from PHB would be converted to gas. However, the results in Table 1 show that 87% of the carbon from PHB was recovered as gas.

In the experiment whose results are shown in Fig. 2, the enhanced $CH_4$ production from 3-hydroxybutyrate was evident after 1 day of incubation, indicating that this monomer was readily fermented to $CH_4$. In contrast, after 1 day of incubation, the amount of $CH_4$ in the PHA-containing cul-

TABLE 2. Gas production from PHB, P(HB-co-13%HV), and P(HB-co-20%HV) substrates plus carbon mass balance after 16 days of incubation

| Substrate[a] | Total production (mmol) of: | | | | Conversion of substrate carbon to gas (%) |
|---|---|---|---|---|---|
| | Methane | | Gas | | |
| | Measured[b] | Theoretical | Measured[b] | Theoretical | |
| PHB | 0.14 ± 0.034 | 0.13 | 0.20 ± 0.12 | 0.23 | 87 |
| P(HB-co-13%HV) | 0.16 ± 0.033 | 0.14 | 0.23 ± 0.11 | 0.24 | 96 |
| P(HB-co-20%HV) | 0.17 ± 0.029 | 0.14 | 0.20 ± 0.11 | 0.24 | 83 |

[a] Each culture received 5 mg of the indicated PHA.
[b] Results are given as mean ± standard deviation.

tures was not greater than that in the unamended control culture. This indicates that the readily fermentable monomer was not present and suggests that the hydrolysis of the PHB and the P(HB-co-HV) polymers was the rate-limiting step in methanogenic degradation of the PHAs. Similarly, the hydrolysis of other biopolymers, such as cellulose, has been shown to be the rate-limiting step in anaerobic digestion (8, 11, 18).

Attempts to detect decreases in the molecular weights of the PHAs caused by depolymerization during the early stages of incubation of these methanogenic cultures have failed because of difficulties in recovering the added biopolymer from the solids that accompanied the sludge inoculum. Nonetheless, the depolymerization must occur quite rapidly, because a burst of $CH_4$ production was observed between days 1 and 3 (Fig. 1).

With civic landfills reaching their capacities, the managed biodegradation of waste plastic made from PHAs by anaerobic digestion may be a feasible solution to the waste problem, with the added benefit of energy conservation due to $CH_4$ recovery.

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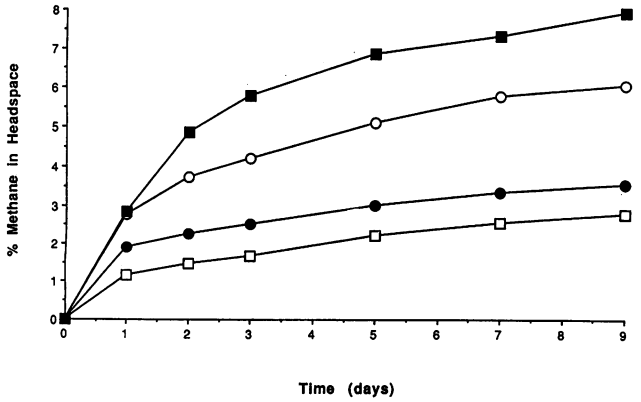


FIG. 2. Methane production by sewage sludge cultures incubated with acetate, butyrate, and 3-hydroxybutyrate. Symbols: □, control (unamended) culture; ●, butyrate; ○, acetate; ■, 3-hydroxybutyrate. All substrates were added as sodium salts to a final concentration of 400 mg/liter.

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