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Anaerobic Microbial Degradation of Poly(3-hydroxyalkanoates) with Various Terminal Electron Acceptors

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The microbial degradation of poly(3-hydroxyalkanoates) (PHAs) under anaerobic conditions with various terminal electron acceptors was examined. Nitrate-reducing consortia were established using activated sludge, and PHAs were shown to be biodegradable under these conditions. A positive correlation between carbon dioxide production and nitrate reduction was demonstrated. Nitrous oxide accumulated as the main N-containing product of nitrate reduction. The amount of PHAs in activated sludge cultures decreased approximately 20% within 40 days of incubation. Attempts were made to establish iron- and sulfate-reducing consortia from spring water, yet it could not be demonstrated that the mixed cultures were capable of degrading PHAs. Pure cultures of iron- and sulfate-reducing bacteria could not utilize PHAs as sole carbon sources. Methanogenic environments sampled included pond sediment and rumen fluid. PHAs were fermented to methane and carbon dioxide after 10 weeks by a sediment consortium, with 43 to 57% of the substrate carbon transformed to methane. Although it could not be demonstrated that PHAs were biodegraded by a rumen fluid consortium, a facultative anaerobic bacterium, identified as a *Staphylococcus* sp., that could grow on PHAs was isolated from rumen fluid.

KEY WORDS: Poly(3-hydroxyalkanoates); poly(3-hydroxybutyrate); poly(3-hydroxybutyrate-co-3-hydroxyvalerate); biodegradation; nitrate reduction; iron reduction; sulfate reduction; methanogenesis.

INTRODUCTION

Since the 1960s a plethora of research has been conducted on the bacterial storage polyesters of carbon and energy, poly(3-hydroxyalkanoates) (PHAs). It is now known that PHA accumulation occurs in a wide variety of taxonomically different groups [1]. Most of these prokaryotes are capable of accumulating PHAs from 30 to 80% of their cellular dry weight [2]. The commercial use of these polymers as thermoplastics has been aided by knowledge of the physiology and enzymology of PHA production and of the mechanical properties of PHAs both as it exists within the cell and as extracted, crystallized polymers. Notably, PHAs are being used to form such commodity products as bottles. Imperial Chemical Industries (ICI) began fermenting *Alcaligenes eutrophus* H16 on a large scale to produce poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] under the trade name Biopol. Shampoo bottles made entirely of Biopol were first test-marketed in Germany in 1990 [3]. Subsequently, several hair care companies in Japan also began producing bottles from Biopol [4].

Products made from PHAs are environmentally significant since they biodegrade to CO_2 and H_2O. Several PHA-degrading pseudomonads [5–8], *Alcaligenes faecalis* [9–14], *Comamonas* spp. [15], and *Comamonas testosteroni* [16], have been isolated and studied in detail. Although the substrate specificity varies among the bacteria, the mechanism of PHA degradation is sim-

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ilar. The bacteria secrete lipase-like extracellular depolymerases. The depolymerase of *Alcaligenes faecalis* has both a catalytic site and a hydrophobic domain as the binding site and therefore shows a two-step reaction mechanism [17]. The enzyme first adsorbs onto the surface of the PHA polymer via the binding site and the catalytic site then mediates the hydrolysis of the polymer chains into water-soluble products. The depolymerases hydrolyze only the polymer chains in the surface layers of the films and degradation proceeds via the surface, and not by internal random chain scission as in simple chemical hydrolysis [18, 19].

Studies by Doi *et al.* [18] showed that the rate of film degradation strongly depended on the composition of the polymers, with polymers of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] degrading more rapidly than P(3HB-co-3HV) films. The rapid erosion of P(3HB-co-4HB) films by the depolymerase may be due to the fact that the 4-hydroxybutyrate (4HB) units are less sterically bulky than 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) units, thus allowing easy access of the enzyme towards the ester groups of the polymer chains. Crystalline regions in PHA retard microbial degradation [19, 20] and subsequently amorphous regions are degraded selectively or preferentially [20].

Only recently have studies been reported on the biodegradation of PHAs by microbial consortia collected from the environment and brought to the laboratory or by direct introduction of PHAs into the specific environment being tested. The majority of the samples have been from aerobic environments and few specific attempts were made to subject the samples to anaerobic conditions. PHA biodegradation in soils [21, 22], activated sludge [23, 24], compost [25–27], and marine [27–29] and freshwater [27, 29–31] systems were analyzed. In general, PHAs were biodgraded in these environments by bacteria and fungi. Some nonenzymatic hydrolysis occurred but at higher temperatures (greater than 40°C) such as would occur in compost.

In contrast to the experiments conducted with aerobic systems, very little is known on the anaerobic biodegradation of PHAs. Janssen and Harfoot [32] isolated and identified an anaerobic bacterium, *Ilyobacter delafieldii*, from sediment that can degrade exogenous poly(3-hydroxybutyrate) [P(3HB)]. More recently, sediment containing heterotrophic fermentative bacteria as well as sulfate-reducing bacteria (SRB) were shown to be able to degrade PHAs under sulfate-reducing conditions [33]. The study of the anaerobic biodegradation of PHAs is important because it would establish that the polymers are indeed biodegradable under conditions that exist in most landfills, if sufficient moisture is present [34]. Anoxic or anaerobic conditions develop when all of the oxygen has been utilized and oxygen is no longer available in free form. Microorganisms under these conditions must use alternate electron acceptors to carrying out redox metabolic reactions. In landfills, methanogenic and possibly sulfate-reducing conditions exist. Previously we established that PHAs are biodegraded under methanogenic conditions with sewage sludge as the inoculum [35]. The objective of the present study was to determine whether various electron acceptors, including nitrate, ferric iron [Fe(III)], sulfate, and CO_2, would support PHA degradation by selected pure cultures and mixed microbial populations from various environmental sources. The cultures were primarily monitored for the production of CO_2 or CH_4 and the depletion of the electron acceptor.

MATERIALS AND METHODS

Preparation of Polyhydroxyalkanoates

P(3HB) and P(3HB-co-15%3HV) polymers were prepared in shake flask cultures of *Azotobacter vinelandii* strain UWD (ATCC 53799) [36, 37]. P(3HB-co-15%3HV) polymers were formed as described by Page *et al.* [37]. The polymer-containing granules were extracted from the cells with commercial bleach for 60 min at 45°C and purified [38]. The resulting insoluble polymer granules were washed with distilled water, allowed to dry, and ground to a fine powder using a mortar and pestle. This produced a heterogeneous mixture of PHAs <0.1 mm in diameter.

Sources of Inocula

All samples were collected from locations in Edmonton, Canada. Activated sludge and anaerobic sludge were obtained from the Goldbar Waste Water Treatment Plant, which receives primarily domestic sewage, with little industrial input. It provides conventional activated sludge treatment for an average flow of 260,000 m^3/day. Water samples and a sediment sample were taken from a spring that flows into Whitemud Creek. Sediment samples were collected during the summer from a pond at Hawrelak Park. Rumen content was taken from a fistulated dairy cow at the University of Alberta Farm.

Cultivation of Cultures Under Nitrate-Reducing Conditions

Amended nitrate-reducing medium [39], supplemented with trace metals solution [40], was used. Yeast extract was omitted and potassium nitrate was added to a final concentration of 30 mM. Cultures were prepared in triplicate with culture volumes of 50 ml in 158-ml serum bottles. P(3HB) and P(3HB-co-3HV), at 1.0 g/L, were added separately to cultures to serve as the sole source of carbon. The PHAs were added to 13 × 100-mm test tubes that were placed within the serum bottles in such a way that the mouths of the test tubes were above the culture fluid line. At the time of substrate additions, the culture bottles were tipped to allow the PHA into the culture medium. Unamended cultures (no carbon substrate added) were prepared as the negative control. No reducing agent or resazurin was added to the cultures. The medium was prepared under aerobic conditions. Methanogens and SRB were inhibited with 50 mM bromoethanesulfonic acid (BESA) and 20 mM sodium molybdate, respectively, to ensure that these bacteria would not produce CO_2 while using other appropriate electron acceptors if nitate was depleted from the medium. Activated sludge was inoculated at a volume of 10% (v/v). The cultures were incubated without shaking at room temperature (22 ± 2°C) in the dark for up to 92 days.

Nitrate-reducing activated sludge cultures amended with P(3HB) and P(3HB-co-3HV) were transferred at 20% (v/v) into corresponding fresh amended nitrate-reducing medium, prepared under anaerobic conditions, containing the PHA at 1.0 g/L. Unamended cultures were transferred into fresh, unamended medium that had also been prepared under anaerobic conditions. The headspace gas was He.

Cultivation of Cultures Under Sulfate-Reducing Conditions

Desulfococcus multivorans (DSM 2059) and *Desulfovibrio sapovorans* (DSM 2055) were cultivated in medium according to Collins and Widdel [41]. *D. sapovorans* was grown in medium that contained, per liter, 1 g NaCl, 0.4 g $MgCl_2 \cdot 2H_2O$, and 2.5 ml sodium lactate (60% syrup), whereas *D. multivorans* was grown in medium that contained, per liter, 20 g NaCl, 3 g $MgCl_2 \cdot 2H_2O$, and 0.5 g sodium benzoate. Cultures were grown with their preferred carbon source for 3 weeks at 35°C until turbid growth was visible. These cultures served as the inocula, with an inoculum volume of 9% (v/v) for *D. sapovorans* and 8% (v/v) for *D. multivorans*. P(3HB) or P(3HB-co-3HV) was provided as the sole carbon and energy source at 0.5 g/L. Also, cultures with their preferred carbon substrate and 0.5 g/L P(3HB) were set up. Cultures incubated with the preferred carbon source as sole carbon source were used as positive controls. Cultures were prepared in triplicate with culture volumes of 75 ml in 100-ml serum bottles and incubated without shaking at 35°C in the dark for 130 days.

Postgate C medium [42] was used for the cultivation of spring water cultures. Sodium lactate and yeast extract were omitted from the medium when PHAs or other substrates were tested. Cultures were prepared in triplicate and final culture volumes were 50 ml in 158-ml serum bottles. PHAs were provided at 1.0 g/L as sole carbon sources. They were added to serum bottles prior to the addition of medium and were then autoclaved in the medium to sterilize. Sodium sulfate (20 mM) served as the terminal electron acceptor. Cultures inoculated with spring water (5%; v/v) were incubated for 41 or 81 days, whereas cultures inoculated with anaerobic sewage sludge (2%; v/v) were incubated for 60 days. These incubations were at room temperature in the dark without shaking.

Cultivation of Cultures Under Iron-Reducing Conditions

Modified anaerobic citrate medium [43, 44] was used for the cultivation of *Shewanella putrefaciens* (ATCC 8071). P(3HB) and P(3HB-co-3HV) at 0.5 g/L, and the sodium salt of 3HB added to a final concentration of 15 mM, were provided singly as the sole carbon substrate. Unamended and sterile cultures were also prepared. All cultures were prepared in duplicate at a culture volume of 50 ml in a 158-ml serum bottle. Cultures were incubated without shaking at 28°C in the dark for 12 days.

The medium used for *Geobacter metallireducens* (ATCC 53774) was anaerobic citrate medium (ATCC medium 1768). Carbon substrates analyzed included P(3HB) and P(3HB-co-3HV) at a concentration of 0.5 g/L and the sodium salt of 3HB, which was added to a final concentration of 50 mM. The inoculum was 10% (v/v) *G. metallireducens* cultures grown on acetate for 2 weeks at 28°C. Cultures were prepared in triplicate and final culture volumes were 50 ml in 158-ml serum bottles. Incubation was carried out at 28°C in the dark without shaking for 17 days.

Amorphous Fe(III)oxyhydroxide [$Fe(OH)_3$], pre-

pared as per Lovley and Phillips [45] and added to a final concentration of 250 mM Fe(III), served as the iron source for spring water cultures. Iron-enrichment medium [46] was used. Triplicate cultures under a N_2 atmosphere with final culture volumes of 50 ml in 158-ml serum bottles were prepared. $Fe(OH)_3$ was added to bottles prior to medium addition. The carbon substrates tested included P(3HB) and P(3HB-co-3HV) added at 1.0 g/L. Spring water was inoculated at 5% (v/v) and the cultures were incubated at room temperature in the dark.

Enrichment cultures of a mixture of spring water and spring water sediment were prepared by incubating a 10% (v/v) inoculum in B10 medium [47] amended with yeast extract (0.1%; w/v) at room temperature for several days until turbid growth was visible. Enrichment cultures with both yeast extract and P(3HB) or P(3HB-co-3HV) were also set up and served as the inocula for cultures amended with P(3HB) and P(3HB-co-3HV). The amount of $Fe(OH)_3$ added provided 250 mM Fe(III). The cultures were incubated at room temperature for 43 days.

The same medium and substrate concentrations used in the spring water cultures were used for experiments with anaerobic sewage sludge as inoculum. Cultures were prepared in triplicate in 59-ml serum bottles at a final culture volume of 10 ml. BESA was added to give a final concentration of 50 mM in order to inhibit methanogenesis. A 2% (v/v) inoculum was used. Cultures were incubated at 35°C in the dark without shaking for 80 days and CO_2 production was measured.

Cultivation of Cultures Under Methanogenic Conditions

Pond sediment inoculated at 50% (v/v) into 59-ml serum bottles using a wide-mouth pipette. The medium of Fedorak and Hrudey [48] was used to give a final culture volume of 10 ml. Cultures were prepared in triplicate. P(3HB) and P(3HB-co-3HV) were added separately at 0.5 g/L to the serum bottles prior to the addition of medium and sediment. The sodium salt of 3HB (0.4 g/L) was also tested as the sole source of carbon. Unamended cultures were set up to measure CH_4 production from organic matter in the sediment. The cultures were incubated in the dark at 15°C after being adjusted to ambient atmospheric pressure with the manometer [35].

A rumen fluid sample was placed in an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI) and filtered first through 6 layers of cheesecloth and then twice through 12 layers of cheesecloth. The final filtrate was then removed from the chamber and stored overnight on a laboratory bench in a container with the lid lightly screwed on to allow some of the organics to be consumed to reduce background levels of CH_4. Rumen fluid was inoculated into 59-ml serum bottles at 50% (v/v). Identical media, substrates, and substrate concentrations as in the pond sediment cultures were used. The cultures were incubated in the dark at 35°C after being adjusted to atmospheric pressure with the manometer.

Sewage sludge cultures were set up as described previously [35].

Isolation of a PHA-Degrading Bacterium

To isolate PHA-degrading bacteria from rumen fluid, dilution blanks of 9 ml of 0.3 mM phosphate buffer (pH 7.0) with 0.1% (w/v) sodium thioglycolate and 0.1% (w/v) methylene blue in Hungate tubes were prepared. A dilution series of 10^{-2} to 10^{-7} was prepared with rumen fluid, and 0.1 ml from each dilution was spread onto PHA-overlay plates that were prepared as follows. The bottom layer contained, per liter, 6.6 g of Brain Heart Infusion (Difco Laboratories, Detroit, MI), 18 g of agar (Difco), and 10 ml of a 0.1% (w/v) resazurin solution. After autoclaving, it was cooled to about 50°C and 10 ml of sterile 0.1% sodium thioglycolate was added. Approximately 25 ml of the medium was poured into each plate and allowed to solidify. The overlay contained, per liter, 2 g of P(3HB) or P(3HB-co-3HV), 18 g of agar, and 10 ml of a 0.1% resazurin solution. After autoclaving it was cooled to about 50°C and 10 ml of sterile 0.1% (w/v) sodium thioglycolate was added. Approximately 10 ml of the overlay was poured over the surface of the bottom layer.

The plates were inoculated and incubated in an anaerobic chamber at 30°C, then checked periodically for isolated colonies that could clear PHA from the overlay.

Gas Measurements

CO_2, N_2O, and N_2 in culture headspaces were separated and measured using a Varian Aerograph Model 700 GC with a 3 m × 0.5-cm column packed with Poropak R. The GC was fitted with a thermal conductivity detector operated at 25°C and 150 mV. He was the carrier gas, at 110 ml/min. GC oven and injector temperatures were 60 and 24°C, respectively. An HP Model 3390A integrator was used for peak area measurements. Quantitative standards of CO_2 and N_2O were prepared by the addition of known volumes of CO_2 or N_2O to air-containing, sealed 159-ml serum bottles. The amounts

of CO_2 and N_2O measured in the cultures are reported as a percentage (by volume) of the headspace gas in the serum bottles.

When cultures were prepared under a He headspace, N_2 production, in addition of CO_2 and N_2O production, was specifically monitored. Because some N_2 was introduced with the inocula, the test cultures had different amounts of N_2 in their headspaces at the start of incubation. Therefore, 8 ml of CH_4 was injected into cultures at the time of inoculation to serve as internal standards and the relative amount of N_2 produced in the cultures was determined by comparing its peak area to the CH_4 peak area. The ratio of N_2-to-CH_4 peak area was normalized by dividing each new ratio measurement by the time 0 ratio.

The method of Fedorak and Hrudey [49] was used to analyze the headspace gases of the cultures for CH_4. The GC described previously [35] was superseded by an HP Model 5700 GC equipped with a flame ionization detector and a 6 ft × $\frac{1}{8}$-in. column packed with Chromosorb 104 (80/100). N_2 was the carrier gas, at a flow rate of 24 ml/min. The injection port, oven, and detector temperatures were 25, 25, and 200°C, respectively. Quantitative standards of CH_4 were prepared as for CO_2 and N_2O.

Nitrate Analysis

Nitrate concentrations were determined using an HP Anion Chromatography System (Hewlett Packard, Germany) adapted to fit a Waters M-45 (Millipore) HPLC system. An HP ion chromatography column (125 × 4 mm) was heated at 40°C in a Shimadzu CTO-6A column oven. The mobile phase was prepared as outlined in the HP Anion Chromatography System user's guide (HP Part No. 90027). A flow rate of 1.5 ml/min was used. The detector was a Waters 486 Tunable Absorbance Detector (Millipore) with signal polarity switching. The detection wavelength was 266 nm and a negative polarity was used.

Samples of culture fluid (0.5 ml) were removed for nitrate analyses and stored in Eppendorf tubes at −20°C until the analyses were done. Before analysis, particles were removed by centrifugation and the samples were diluted either 1:50 or 1:20 with deionized, distilled, ultrapure water (Milli-Q Reagent Water System, Millipore Continental Water Systems, Millipore Corporation, Bedford, MA). Standards of KNO_3 were prepared and used to construct calibration curves from peak areas recorded with an HP model 3390A integrator. Calibration curves were constructed each time a new batch of mobile phase was used.

Other Analytical Techniques

Iron reduction was monitored by measuring the formation of Fe(II) from the reduction of Fe(III) with a colorimetric assay using 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (Ferrozine, Sigma Chemical Company, St. Louis, MO) as described by Lovley and Phillips [45].

The amount of 3HB present in a liquid sample was determined by capillary column GC [50]. Samples of 1 ml were stored in Eppendorf tubes at −20°C until the time of analysis. Samples were subjected to methanolysis by heating the samples at 55°C for 45 min in 1 ml of methanol, containing 0.5% butyric acid as internal standard, and 200 μl of conc. sulfuric acid. The resulting 3-hydroxycarboxylic acid methyl esters were extracted in 1 ml dichloromethane. The methyl esters were separated on a fused silica capillary column [37]. Standards of 3HB were also prepared and analyzed in the same manner as the samples and used to construct calibration curves.

Identification of N_2O in culture headspace gas was done by the Mass Spectrometry Laboratory of Department of Chemistry, University of Alberta.

Statistical Methods

All data were statistically analyzed by the method of Dunnett [51] or by Duncan's multiple-range test [52].

RESULTS AND DISCUSSION

PHA Degradation by Activated Sludge Cultures Under Nitrate-Reducing Conditions

Activated sludge is known to have a high number of nitrate-reducing bacteria [53]. They are facultative anaerobic bacteria which possess the ability to reduce nitrogen oxides when O_2 becomes limiting [53]. Nitrate-reducing bacteria, therefore, do not require strict anaerobic media or procedures for their growth. Thus, cultures were inoculated into serum bottles containing unreduced medium and anaerobic conditions were allowed to develop with time in the sealed bottles.

Two groups of cultures were inoculated: one was initially supplemented with nitrate, and the other was not. CO_2 production was measured as an indication of microbial metabolism and representative results are shown in Fig. 1. To ensure that nitrate reduction, and not respiration, was coupled to PHA biodegradation, cultures were incubated initially with no added PHAs.

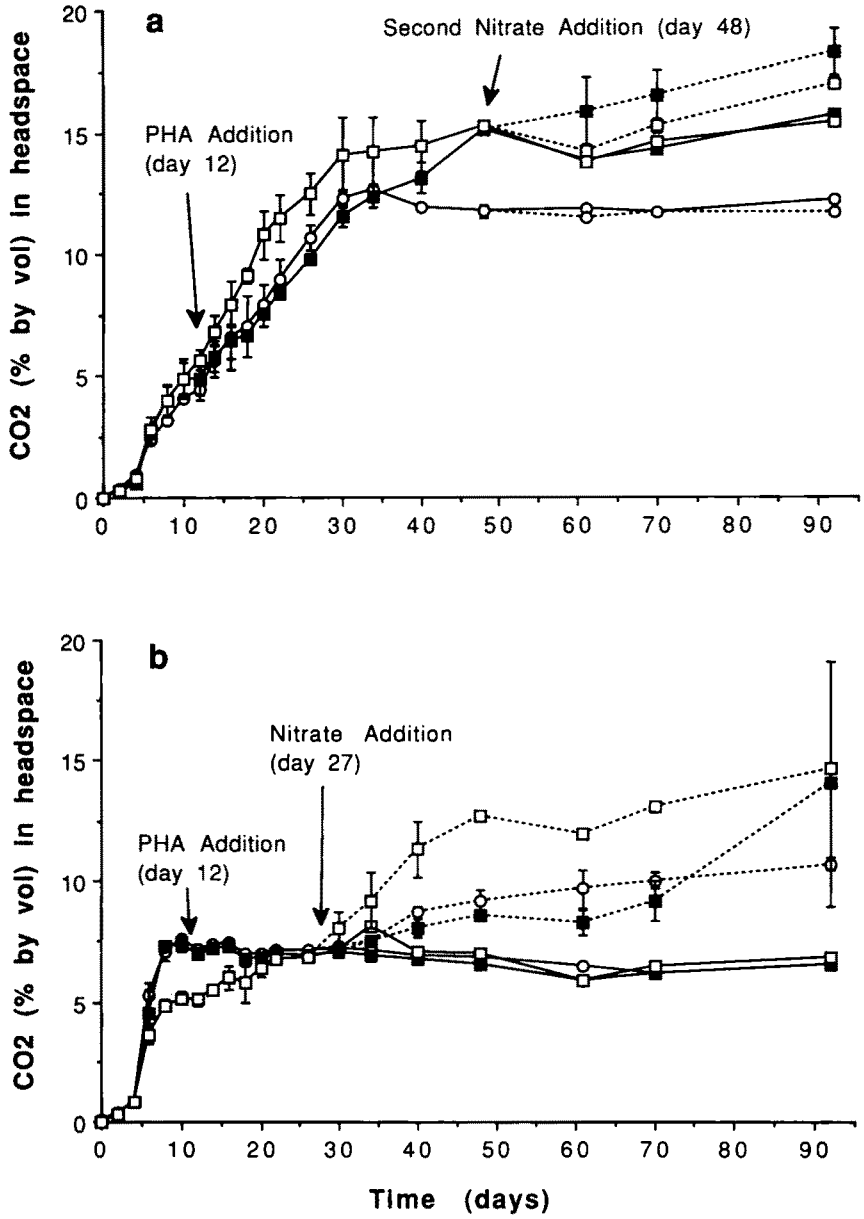

Fig. 1. Mean cumulative CO_2 production from activated sludge-containing cultures incubated under nitrate-reducing conditions: cultures that were supplemented with nitrate at the time of inoculation (a); cultures that received no nitrate at the time of inoculation (b). Unamended (○); P(3HB) (■); P(3HB-co-15%3HV) (□). Dashed lines represent the CO_2 production in two of the three replicate cultures of each test substrate given additional nitrate. Error bars show one standard deviation.

The microbial population consumed O_2 using background organic matter found in activated sludge as carbon and energy sources. Once anoxic conditions developed as indicated by a plateau in CO_2 production, the test substrates were added. Increased CO_2 production after the addition of PHAs to these molybdate- and BESA-containing cultures could then be attributed to nitrate reduction.

During the first 12 days of incubation, CO_2 production in most of the cultures was similar, as shown in Figs. 1a and b. In cultures that contained no added nitrate (Fig. 1b), there was a marked decrease in the rate

of CO_2 production at day 10. This was taken to indicate that O_2 had been depleted from the cultures, then the PHAs were added to the cultures on day 12 (Figs. 1a and b). This plateau in CO_2 production was not observed in Fig. 1a, because nitrate respiration was occurring. Figure 2 shows a slight decrease in nitrate concentration during the first 12 days of incubation, followed by an increased rate of consumption between day 12 and day 30. The depletion of nitrate from the medium coincided with a plateau in CO_2 production at day 30 (Fig. 1a). Because of the presence of organic material that accompanied the activated sludge inoculum, the CO_2 production in the unamended cultures did not completely stop until after 30 days of incubation, at which time the mean CO_2 was 12 ± 0.3%.

The addition of P(3HB-co-3HV) caused a 1.3-fold increase in CO_2 production over the unamended cultures within approximately 6 days of addition. By day 48 (Fig. 1a), CO_2 was beginning to plateau at 15 ± 0.2% for the P(3HB-co-3HV)-amended cultures. It was not until day 40 that the P(3HB)-amended cultures showed increased CO_2 production over the unamended cultures. CO_2 amounts in P(3HB)-amended cultures reached a plateau at 15 ± 0.3% after 48 days of incubation.

Also shown in Fig. 2 is the loss of nitrate in cultures that received the monomer, 3HB. These cultures showed the most rapid consumption of nitrate, and although not included in Fig. 1a, these cultures also had the highest initial rate of CO_2 evolution after the 3HB was added on day 12. Thus, the rate of mineralization of the PHAs was limited by their depolymerization.

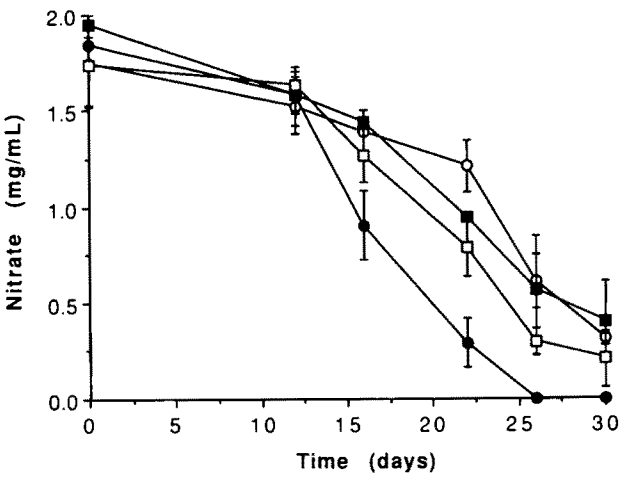

Fig. 2. Mean nitrate concentration from activated sludge-containing cultures given nitrate at the time of inoculation. Unamended (○); 3HB(●); P(3HB) (■); P(3HB-co-15%3HV) (□). Error bars show one standard deviation.

A second addition of nitrate (3 mg/ml KNO_3) was given to two of the three cultures in each triplicate set on day 48 (Fig. 1a). This was to determine whether an increase in CO_2 production would occur in the nitrate-depleted cultures if more nitrate was added. Results showed an increased CO_2 production in all cases (dashed lines in Fig. 1a) except for the unamended cultures. Increases in CO_2 production in PHA-amended cultures given extra nitrate were between 1.1- and 1.3-fold higher than in their corresponding cultures with no extra nitrate addition. The second addition of nitrate also resulted in significant nitrate reduction to occur in P(3HB)- and P(3HB-co-3HV)-amended cultures from the unamended cultures.

In the second group of cultures, which was not initially supplemented with nitrate, there was virtually no increase in CO_2 production after the addition of P(3HB), and only a slight increase in CO_2 after the addition of P(3HB-co-3HV) (Fig. 1b). The amounts of CO_2 in all three cultures without nitrate were similar from about day 22 to day 27.

The addition of nitrate on day 27 to two of the three cultures in each triplicate set resulted in increased CO_2 production (dashed lines in Fig. 1b) over that in the cultures not given nitrate. By day 40 (13 days after nitrate addition), P(3HB-co-3HV)-amended cultures given nitrate had significantly higher ($P < 0.05$) amounts of CO_2 (11 ± 1% CO_2) than the rest of the cultures and continued to have significantly greater ($P < 0.05$) CO_2 production until the end of the incubation period. After 65 days of incubation with nitrate, the CO_2 in P(3HB)-amended cultures had increased twofold, from 7 ± 0.04% at day 30 to 14 ± 5% at day 92, but there were large variations in CO_2 amounts between the two cultures. Nitrate analysis revealed that nitrate consumption occurred in all of the cultures.

The utilization of PHAs in the spring water and anaerobic sewage sludge cultures under nitrate-reducing conditions could not be demonstrated (data not shown). However, the results presented here clearly show that active nitrate-reducing consortia capable of degrading PHAs were obtained with activated sludge as inoculum.

Transfer Cultures of Activated Sludge Nitrate-Reducing Consortia

To determine the products formed from the reduction of nitrate, the activated sludge cultures initially given nitrate and described in the previous section (Fig. 1a) were transferred after 40 days of incubation and used to inoculate fresh medium (20%; v/v) prepared under a

He headspace gas. This allowed the measurement of N_2O and N_2 in the headspace of the cultures.

CO_2 production by these transferred cultures was also monitored and the results from the P(3HB-co-3HV)-amended cultures and the unamended controls are shown in Fig. 3a. The CO_2 production in the P(3HB)-amended cultures was not different from that in the controls, hence these results are not shown. CO_2 production occurred in the unamended cultures (Fig. 3a), which was most likely due to the presence of carbon substrates found in activated sludge that had accompanied the transfers. The CO_2 produced in P(3HB-co-3HV)-amended cultures was significantly greater than in the unamended cultures by day 3.

Nitrate consumption occurred in both P(3HB-co-3HV)-amended and unamended cultures (Fig. 3b). Although nitrate removal appeared to be faster in the P(3HB-co-3HV)-amended cultures than in the unamended cultures, there were no statistical differences between the samples on any sampling day because of the large variability among the replicate unamended cultures.

Because the nominal molecular weights of CO_2 and N_2O are the same, high-resolution mass spectrometry was used to confirm the presence of N_2O in the headspace gas of these cultures. The amounts of N_2O measured in the triplicate unamended cultures was extremely variable (Fig. 3c). However, the amount of N_2 in the unamended cultures remained unchanged over the 40-day period (Fig. 3d), whereas N_2 accumulated in the P(3HB-co-3HV)-amended cultures after a 10-day lag period. Clearly, in the latter culture, nitrate was reduced

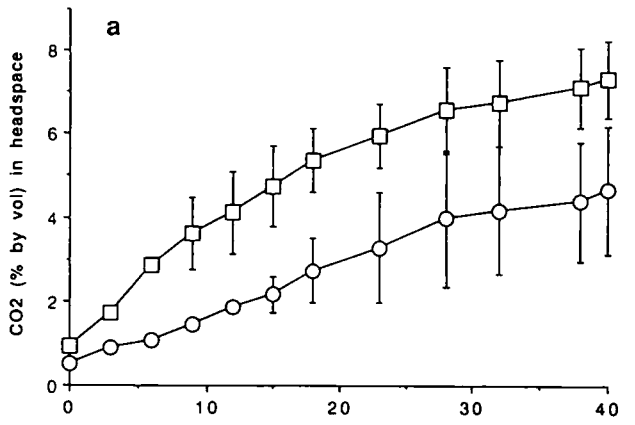

Fig. 3. Mean cumulative CO_2 production (a), nitrate removal (b), N_2O production (c), and N_2 formation relative to the amount of methane added as an internal standard and normalized to the N_2 present at time 0 (d) from activated sludge transfer cultures. Unamended (○); P(3HB-co-15%3HV) (□). Error bars show one standard deviation.

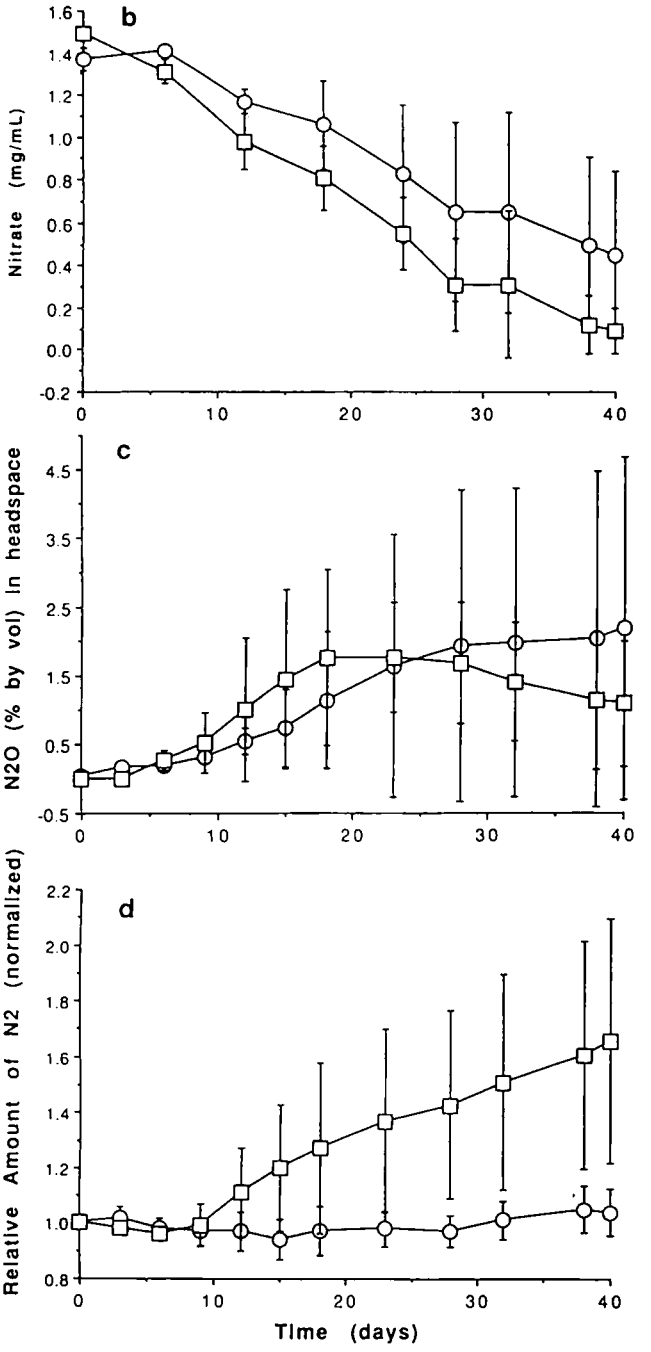

Fig. 3. Continued.

first to N_2O and then to N_2. The data in Fig. 3 show that nitrate removal was accompanied by CO_2 and N_2 production in the P(3HB-co-3HV)-amended cultures.

After 40-days of incubation, a visible biofilm could be seen covering the granules, and in most cultures the granules were aggregated together and coated with the

biofilm. The weights of polymer granules remaining at this time in the P(3HB-co-3HV)-amended cultures were determined by subjecting the granules to methanolysis and measuring the resulting 3-hydroxycarboxylic acid methyl esters by capillary column GC. The weights of granules remaining in the P(3HB-co-3HV)-amended cultures had decreased from 50 to approximately 40 mg/culture within 40 days.

There was virtually no N_2O or N_2 produced by the P(3HB)-amended cultures (data not shown). The difference between the degradation of P(3HB-co-3HV) and that of P(3HB) may be due to the fact that the P(3HB) granules were more crystalline than the copolymer granules, thus retarding enzymatic hydrolysis of the ester bonds [20]. This is consistent with the findings of Budwill *et al.* [35], which showed that copolymer P(3HB-co-3HV) was more rapidly fermented to CH_4 than P(3HB).

Attempts to Demonstrate PHA Biodegradation by Pure Cultures of Iron-Reducing and Sulfate-Reducing Bacteria

Two iron-reducing bacteria were tested for their ability to utilize PHAs under iron-reducing conditions. The first, *Shewanella putrefaciens*, a nonfermenting, facultative anaerobic bacterium [54], grew in defined medium with lactate or pyruvate as the carbon source and ferric citrate serving as terminal electron acceptor. CO_2 analyses revealed that *S. putrefaciens* could not utilize P(3HB-co-3HV), P(3HB), or 3HB. Only limited iron reduction occurred, but this was likely the result of abiotic reduction [44].

The second microorganism tested was *Geobacter metallireducens* ATCC 53774, recently characterized as a Gram-negative, strict anaerobic bacterium capable of oxidizing several short-chain fatty acids, alcohols, and monoaromatic compounds with Fe(III) as sole electron acceptor [55]. From CO_2 and Fe(II) analyses, there was no evidence that *G. metallireducens* could use P(3HB-co-3HV), P(3HB), or 3HB.

Several SRB have been reported to synthesize and store PHA internally [1]. Among these are *Desulfovibrio sapovorans* and *Desulfococcus multivorans*, which were tested for their ability to degrade P(3HB). Both species were incubated in medium with a known growth substrate in the absence and presence of P(3HB) and in medium with P(3HB) as the sole carbon source. The growth substrates were lactate for *D. sapovorans* and benzoate for *D. multivorans*. After 61 days of incubation, the CO_2 evolved from growth comprised 8 to 10% of the headspace gas in the cultures that contained the known growth substrates. However, CO_2 production in cultures that contained P(3HB) was not significantly greater than that in cultures without P(3HB). These results are consistent with previous findings that bacteria which synthesize and store P(3HB) cannot degrade exogenous P(3HB), because the extracellular depolymerase system has been shown to be distinct from the intracellular depolymerase system [56].

Attempts to Demonstrate PHA Biodegradation Under Iron-Reducing and Sulfate-Reducing Conditions with Environmental Samples

Studies have shown that the complete oxidation of complex organic material coupled to iron reduction probably involves a consortium containing iron-reducing bacteria [44]. Fermentative organisms in the consortium hydrolyze the complex organic matter to smaller compounds. These fermentation products are oxidized, with the concomitant reduction of iron by a second group of bacteria. Similarly, SRB typically use low molecular weight compounds as electron donors, and they rely on fermentative bacteria to break down complex organic matter into less complex, readily available products [57]. SRB are thus viewed as terminal degraders, much like methanogenic bacteria that form CH_4 and CO_2 as final anaerobic products.

Iron-reducing bacteria such as *S. putrefaciens* have been detected and isolated from the spring water used as the inoculum for PHA biodegradation studies (F. D. Cook, personal communication). Cultures inoculated with spring water and amended with yeast extract produced 3% CO_2 and 240 μg/ml Fe(II) after a short incubation time of 16 days. In contrast, very little iron-reducing activity was detected in spring water cultures amended with PHAs, as $<0.2\%$ CO_2 and <4 μg/ml Fe(II) were measured in these cultures after this short incubation time. Even when spring water was enriched for iron-reducing bacteria in B10 medium, little iron reduction was observed when these cultures were amended with PHAs.

These results showed that it was possible to obtain an iron-reducing enrichment culture from the spring water, but the degradation of PHAs coupled to iron reduction could not be detected.

Postgate C medium, with lactate as the electron donor, was inoculated with spring water (10%; v/v) to detect the presence of SRB. Indeed, after 10 days of incubation, the characteristic blackening of the medium

was observed, caused by the precipitation of sulfide produced by SRB. Samples of spring water and portions of the cultures grown on lactate in Postgate C medium were inoculated into fresh medium, without lactate or yeast extract, but containing PHA granules to test whether they could be degraded under sulfate-reducing conditions. These were monitored for CO_2 production for periods of 81 and 41 days, respectively. However, no enhanced CO_2 production was detected in any of these samples, indicating that the PHAs were not mineralized under these conditions.

Anaerobic sewage sludge was also used to determine whether P(3HB-co-3HV), P(3HB), or 3HB could be mineralized under sulfate-reducing conditions in BESA-containing Postgate C medium, without lactate and yeast extract. Very little CO_2 production was observed in these cultures inoculated with 2% (v/v) of sludge, and after 60 days of incubation, CO_2 comprised $<2\%$ of the headspace gas. In contrast, the headspace gas of cultures amended with yeast extract contained $9 \pm 3\%$ CO_2 after the same incubation time.

Previous most probable number enumerations of this anaerobic sludge showed that it contained about 4×10^5 SRB/ml [58]. However, in the current study, the mixed population incubated under sulfate-reducing conditions did not mineralize the PHAs or the monomer, 3HB. When anaerobic sludge from the same source was inoculated into an appropriate medium (at 50%; v/v) and incubated under methanogenic conditions [35], almost-complete conversion of the polymeric carbon to CH_4 and CO_2 was observed. As part of the current study, CH_4 and CO_2 were produced in cultures inoculated with 2% (v/v) anaerobic sludge after lag times of 6 and 8 days for P(3HB-co-3HV) and P(3HB), respectively. Thus, the sludge contained PHA-degrading bacteria, and the fermentation products were ultimately available to the methanogens in the inoculum. However, the sequence of PHA degradation and mineralization was not observed under sulfate-reducing conditions.

Recently, Urmeneta *et al.* [33] demonstrated PHA degradation and subsequent increases in sulfide concentrations in cultures inoculated with sediment from a gypsum-based karstic lake. In that lake, PHAs are synthesized by *Chromatium* spp., and these polymers are deposited into the sediment. For their studies, Urmeneta *et al.* [33] used an 83% (v/v) slurry of the sediment supplemented with commercially available P(3HB-co-3%3HV). CO_2 production was not measured, but the loss of the polymer and sulfide concentrations were monitored. The addition of sodium molybdate decreased the rate of PHA degradation and sulfide production.

Biodegradation of PHAs Under Methanogenic Conditions

Galvin [59] predicted that PHA biodegradation would occur at a faster rate in sewage sludge than in other environments such as sediments, activated sludge, soil, and seawater. The high number of fermenting bacteria in sludge should be able to break down PHAs. Methanogenic bacteria cooperate with the heterotrophic bacteria in degrading organic compounds. Anaerobic sediment from a local pond and rumen fluid were used as inocula to test whether PHAs would be biodegraded by microbial populations from these sources.

Cultures, inoculated with pond sediment (50%; v/v), were incubated at 15°C, which was the temperature of the sediment when it was collected. After 7 weeks of incubation, enhanced CH_4 production ($P < 0.05$) was detected in cultures amended with P(3HB-co-3HV), P(3HB), and 3HB (Fig. 4). Unlike the gas production data presented in Figs. 1 and 3a, the variability in CH_4 production by the triplicate cultures was very small, and the standard deviations (not shown in Fig. 4) were always $<10\%$ of the mean CH_4 production.

Each of the PHA-containing cultures inoculated with pond sediment received 5 mg of the polymer. Elemental analyses of the polymers showed that they contained 56% carbon [35]. Thus, each culture received 0.23 mmol carbon from the polymer. Based on the equation of Buswell and Mueller [60], these substrates should yield 0.23 mmol of gas in methanogenic cultures, including 0.13 mmol of CH_4 and 0.10 mmol of CO_2. After 14 weeks of incubation, the P(3HB)-containing cultures produced 0.18 mmol of gas (78% of the theoretical amount) and 0.10 mmol of CH_4 (77% of the theoretical amount). Similarly, the P(3HB-co-3HV)-containing cultures produced 0.19 mmol of gas (83% of the theoretical amount) and 0.10 mmol of CH_4 (77% of the theoretical amount). In studies with anaerobic sewage sludge used as the inoculum, the total gas yields from PHAs ranged from 83 to 96% of the theoretical amounts, and CH_4 yields were nearly 100% of the theoretical amounts [35]. The hydrolysis of PHAs was shown to be the rate-limiting step in the methanogenic degradation of PHAs, however, the overall process occurred quite rapidly, as significant biogradation of PHAs occurred within 3 days at 37°C [35]. As predicted by Galvin [59], PHAs were more rapidly biodegraded by the microbial population in anaerobic sewage sludge than in pond sediment. The lower temperature and most likely lower diversity and numbers of bacteria in sediment probably accounted for the slower biodegradation of PHAs with this inoculum. Nonetheless, cultures in-

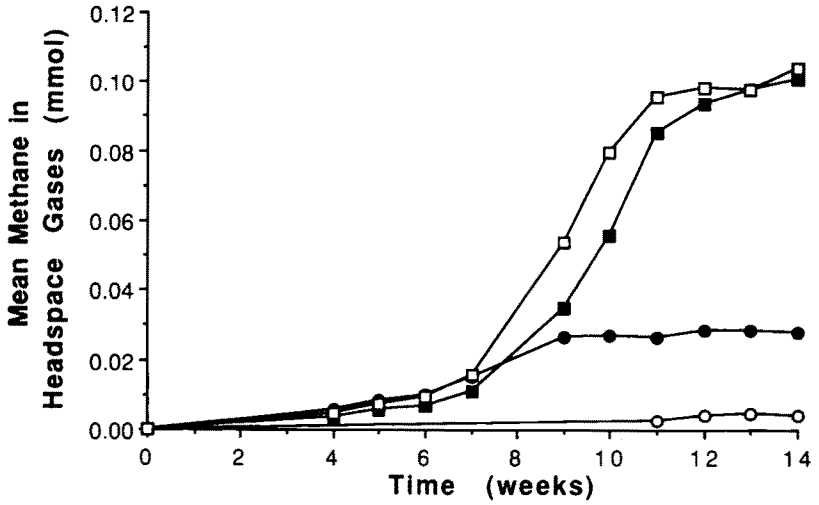

Fig. 4. Mean cumulative CH_4 production from pond sediment-containing cultures incubated at 15°C in the dark. Unamended (○); 3HB (●); P(3HB) (■); P(3HB-co-15%3HV) (□).

oculated with smaller portions of pond sediment, 5 and 2% (v/v), showed CH_4 production from P(3HB) and P(3HB-co-3HV).

Experiments using rumen fluid as inoculum (50%; v/v) showed that there was no enhanced CH_4 production ($P < 0.05$) in the PHA-amended cultures over the unamended cultures. The addition of 5 ml H_2 to one culture set demonstrated that hydrogenotrophic methanogens were present in the rumen fluid, as CH_4 yields were significantly greater ($P < 0.05$) in H_2-amended cultures than in those cultures without H_2. The fact that no enhanced CH_4 production occurred in acetate-amended cultures over the unamended cultures demonstrated that acetoclastic methanogens were not abundant in the rumen fluid.

Despite the fact that CH_4 analyses gave no evidence of PHA biodegradation by the rumen fluid consortium, proof that some rumen fluid microbes are capable of degrading P(3HB) came from plating a dilution series of 10^{-2} to 10^{-7} rumen fluid onto P(3HB) overlay plates in an anaerobic chamber. After a few weeks at 30°C, zones of clearing appeared around several colonies. This indicated that a P(3HB) depolymerase was being secreted by the bacteria. Plates with such colonies were removed from the anaerobic chamber and the colonies tested for aerobic growth at 28°C on PHA overlay plates. One isolate, from the 10^{-6} dilution, was identified as a *Staphylococcus* sp. and it produced a very active PHA depolymerase; clearing around the colonies occurred within 3 days of streaking onto PHA overlay plates.

CONCLUSIONS

This study examined the fate of PHAs under anaerobic conditions with various terminal electron acceptors. Mixed and pure cultures were used as sources of degrading bacteria. Results showed that PHAs were biodegraded under nitrate-reducing conditions with an activated sludge consortium and nitrate was reduced to N_2O and N_2. The amount of PHAs decreased by 20% from the original amount added to cultures within a 40-day period. The copolymer, P(3HB-co-15%3HV), was degraded more quickly than the homopolymer, P(3HB). The biodegradation of PHAs coupled to iron or sulfate reduction could not be demonstrated. Of the methanogenic environments tested, anaerobic sewage sludge yielded the most active PHA-degrading consortia, with lag times of a few days when incubated at 37°C. Pond sediment also contained a microbial population that mineralized PHAs, but only after a lag time of 7 weeks when incubated at 15°C. Although no CH_4 was produced from the PHAs added to rumen fluid, a *Staphylococcus* species capable of degrading and utilizing PHAs was isolated from this source.

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REFERENCES

1. A. Steinbüchel (1991) in D. Byrom (Ed.), *Biomaterials: Novel Materials from Biological Sources*, Stockton Press, Macmillan, and ICI Biological Products, pp. 125-213.
2. H. Brandl, R. A. Gross, R. W. Lenz, and R. C. Fuller (1990) *Adv. Biochem. Eng. Biotechnol.* **41**, 77-93.
3. A. J. Anderson and E. A. Dawes (1990) *Microbiol. Rev.* **54**, 450-472.
4. J. K. Rogers (1992) *Modern Plast.* **69**, 17.
5. F. P. Delafield, M. Doudoroff, N. J. Palleroni, C. J. Lusty, and R. Contopoulos (1965) *J. Bacteriol.* **90**, 1455-1466.
6. K. Mukai, K. Yamada, and Y. Doi (1992) *Int. J. Biol. Macromol.* **14**, 235-239.
7. K. Yamada, K. Mukai, and Y. Doi (1993) *Int. J. Biol. Macromol.* **15**, 215-220.
8. A. Schirmer, D. Jendrossek, and H. G. Schlegel (1993) *Appl. Environ. Microbiol.* **59**, 1220-1227.
9. T. Tanio, T. Fukui, Y. Shirakura, T. Saito, K. Tomita, T. Kaho, and S. Masamune (1982) *Eur. J. Biochem.* **124**, 71-77.
10. Y. Shirakura, T. Fukui, T. Saito, Y. Okamoto, T. Narikawa, K. Koide, K. Tomita, T. Takemasa, and S. Masamune (1986) *Biochim. Biophys. Acta* **880**, 46-53.
11. T. Fukui, T. Narikawa, K. Miwa, Y. Shirakura, T. Saito, and K. Tomita (1988) *Biochim. Biophys. Acta* **952**, 164-171.
12. T. Saito, K. Suzuki, J. Yamamoto, T. Fukui, K. Miwa, K. Tomita, S. Nakanishi, S. Odani, J. I. Suzuki, and K. Ishikawa (1989) *J. Bacteriol.* **171**, 184-189.
13. T. Saito, A. Iwata, and T. Watanabe (1993) *J. Environ. Polym. Degrad.* **1**, 99-105.
14. J. Zhang, T. Saito, A. Ichikawa, and T. Fukui (1992) *Chem. Pharm. Bull.* **40**, 713-717.
15. D. Jendrossek, I. Knoke, R. B. Habibian, A. Steinbüchel, and H. G. Schlegel (1993) *J. Environ. Polym. Degrad.* **1**, 53-63.
16. K. Mukai, K. Yamada, and Y. Doi (1993) *Polym. Degrad. Stabil.* **41**, 85-91.
17. K. Mukai, K. Yamada, and Y. Doi (1993) *Int. J. Biol. Macromol.* **15**, 361-366.
18. Y. Doi, Y. Kanesawa, and M. Kunioka (1990) *Macromolecules* **23**, 26-31.
19. Y. Kumagai, Y. Kanesawa, and Y. Doi (1992) *Makromol. Chem.* **193**, 53-57.
20. H. Nishida and Y. Tokiwa (1993) *J. Environ. Polym. Degrad.* **1**, 65-80.
21. J. Mergaert, A. Webb, C. Anderson, A. Wouters, and J. Swings (1993) *Appl. Environ. Microbiol.* **59**, 3233-3238.
22. L. Lopez-Llorca, M. F. Colom Valiente, and A. Gascon (1993) *Micron* **24**, 23-29.
23. B. H. Briese, D. Jendrossek, and H. G. Schlegel (1994) *FEMS Microbiol. Lett.* **117**, 107-112.
24. D. F. Gilmore, S. Antoun, R. W. Lenz, and R. C. Fuller (1993) *J. Environ. Polym. Degrad.* **1**, 269-274.
25. D. F. Gilmore, S. Antoun, R. W. Lenz, S. Goodwin, R. Austin, and R. C. Fuller (1992) *J. Indust. Microbiol.* **10**, 199-206.
26. M. Matavulj and H. P. Molitoris (1992) *FEMS Microbiol. Rev.* **103**, 323-332.
27. J. Mergaert, C. Anderson, A. Wouters, J. Swings, and K. Kersters (1992) *FEMS Microbiol. Rev.* **103**, 317-322.
28. Y. Doi, Y. Kanesawa, N. Tanahashi, and Y. Kumagai (1992) *Polym. Degrad. Stabil.* **36**, 173-177.
29. L. Lopez-Llorca, M. F. Colom Valiente, and M. J. Carcases (1994) *Micron* **25**, 45-51.
30. H. Brandl and P. Püchner (1990) in E. A. Dawes (Ed.), *Novel Biodegradable Microbial Polymers*, Kluwer Academic, Dordrecht, The Netherlands, pp. 421-422.
31. M. Matavulj, S. T. Moss, and H. P. Molitoris (1993) in H. G. Schlegel and A. Steinbüchel (Eds.), *Proceedings of the International Symposium on Bacterial Polyhydroxyalkanoates*, Goltze-Druck, Göttingen, Germany, pp. 465-466.
32. P. H. Janssen and C. G. Harfoot (1990) *Arch. Microbiol.* **154**, 253-259.
33. J. Urmeneta, J. Mas-Castellá, and R. Guerrero (1995) *Appl. Environ. Microbiol.* **61**, 2046-2048.
34. A. C. Palmisano, D. A. Maruscik, and S. S. Schwab (1993) *J. Gen. Microbiol.* **139**, 387-391.
35. K. Budwill, P. M. Fedorak, and W. J. Page (1992) *Appl. Environ. Microbiol.* **58**, 1398-1401.
36. W. J. Page and O. Knosp (1989) *Appl. Microbiol. Biotechnol.* **55**, 1334-1339.
37. W. J. Page, J. Manchak, and B. Rudy (1992) *Appl. Environ. Microbiol.* **58**, 2866-2873.
38. J. H. Law and R. A. Slepecky (1961) *J. Bacteriol.* **82**, 33-36.
39. I. D. Bossert, M. D. Rivera, and L. Y. Young (1986) *FEMS Microbiol. Ecol.* **38**, 313-319.
40. P. M. Fedorak and D. Grbić-Galić (1991) *Appl. Environ. Microbiol.* **57**, 932-940.
41. M. D. Collins and F. Widdel (1986) *Syst. Appl. Microbiol.* **8**, 8-18.
42. J. R. Postgate (1979) *The Sulphate-Reducing Bacteria*, Cambridge University Press, Cambridge.
43. D. R. Lovley, E. J. P. Phillips, and D. J. Lonergan (1989) *Appl. Environ. Microbiol.* **55**, 700-706.
44. D. R. Lovley and E. J. P. Phillips (1988) *Appl. Environ. Microbiol.* **54**, 1472-1480.
45. D. R. Lovley and E. J. P. Phillips (1986) *Appl. Environ. Microbiol.* **51**, 683-689.
46. E. E. Roden and D. R. Lovley (1993) *Appl. Environ. Microbiol.* **59**, 734-742.
47. C. O. Obuekwe, D. W. S. Westlake, and F. D. Cook (1981) *Can. J. Microbiol.* **27**, 692-697.
48. P. M. Fedorak and S. E. Hrudey (1984) *Water Res.* **18**, 361-367.
49. P. M. Fedorak and S. E. Hrudey (1983) *Environ. Technol. Lett.* **4**, 425-432.
50. B. A. Ramsay, J. A. Ramsay, and D. G. Cooper (1989) *Appl. Environ. Microbiol.* **55**, 584-589.
51. C. W. Dunnett (1955) *Am. Stat. Assoc. J.* **50**, 1096-1121.
52. R. G. D. Steel and J. H. Torrie (1980) *Principles and Procedures of Statistics. A Biometric Approach*, 2nd ed., McGraw-Hill, New York.
53. J. M. Tiedje (1988) in A. J. B. Zehnder (Ed.), *Biology of Anaerobic Microorganisms*, John Wiley & Sons, New York, pp. 179-244.
54. T. J. Dichristina and E. F. Delong (1994) *J. Bacteriol.* **176**, 1468-1474.
55. D. R. Lovley, S. J. Giovannoni, D. C. White, J. E. Champine, E. J. P. Phillips, Y. A. Gorby, and S. Goodwin (1993) *Arch. Microbiol.* **159**, 336-344.
56. E. A. Dawes and P. J. Senior (1973) *Adv. Microb. Physiol.* **10**, 135-266.
57. F. Widdel (1988) in A. J. B. Zehnder (Ed.), *Biology of Anaerobic Microorganisms*, Wiley, New York, pp. 469-586.
58. P. M. Fedorak, K. M. Semple, and D. W. S. Westlake (1987) *J. Microbiol. Methods* **7**, 19-27.
59. T. J. Galvin (1990) in S. A. Barenberg, J. L. Brash, R. Narayan, and A. E. Redpath (Eds.), *Degradable Materials: Perspectives, Issues and Opportunities*, CRC Press, Boca Raton, FL, pp. 39-43.
60. A. M. Buswell and H. F. Mueller (1952) *Ind. Eng. Chem.* **44**, 550-552.