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Isotactic Polypropylene Biodegradation by a Microbial Community: Physicochemical Characterization of Metabolites Produced

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From a selective enrichment culture prepared with different soil samples on starch-containing polyethylene we isolated four microaerophilic microbial communities able to grow on this kind of plastic with no additional carbon source. One consortium, designated community 3S, was tested with pure isotactic polypropylene to determine whether the consortium was able to degrade this polymer. Polypropylene strips were incubated for 5 months in a mineral medium containing sodium lactate and glucose in screw-cap bottles. Dichloromethane crude extracts of the cultures revealed that the weight of extracted materials increased with incubation time, while the polypropylene sample weight decreased. The extracted materials were characterized by performing chromatographic and spectral analyses (thin-layer chromatography, liquid chromatography, gas chromatography-mass spectrometry, infrared spectrometry, nuclear magnetic resonance). Three main fractions were detected and analyzed; a mixture of hydrocarbons at different degrees of functionalization was found together with a mixture of aromatic esters, as the plasticizers usually added to polyolefinic structures.

One serious source of pollution is the huge amount of synthetic polymeric materials (i.e., polyethylene and polypropylene) produced, because these compounds are assumed to be recalcitrant to degradation by bacteria, fungi, and plant or animal cells, although data on the biodegradability of these polymers have been published (1–4, 7, 14, 15, 20, 22, 23).

It is impossible to prevent, even in part, the release of these materials into the environment; consequently, it is important to discover ways to biodegrade these compounds, including the optimal environmental conditions and the possible biological mechanisms involved.

The biodegradability of various plastic films (as well as other substances) has been evaluated by using different analytical approaches and parameters; these include weight loss, tensile strength loss, changes in percentage of elongation, spectroscopic determinations, changes in polymer molecular weight distribution, and the release of $^{14}CO_2$ from ^{14}C-labelled polymers (1–4, 15). The analytical approach used by a worker has often depended on his or her purposes and investigative methods. Some researchers have recently described an extracellular fungal enzyme that is able to degrade polyethylene (20), but little attention has been paid to the metabolic intermediates that are necessarily produced during the first steps of polymer degradation, before these intermediates are converted to CO_2 or CH_4.

The purpose of this research was to test the biodegradative effect of a microbial community (7) on pure isotactic polypropylene by investigating the production of lower-molecular-weight intermediates.

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MATERIALS AND METHODS

Selective enrichment cultures. Soil samples collected from about 15 different sites that were rich in plastic wastes were used as inoculants for enrichment cultures. Portions (1 g) of starch-polyethylene blends (85% low-density polyethylene coextruded with 15% cornstarch [Polybatch Nadeg15; Schulmann]) that were repeatedly washed in sterile water were added as small strips (0.5 by 5 cm; thickness, 0.3 μm) to 50-ml vessels containing 30 ml of sterilized B7 medium (8) supplemented with 0.2% glucose. The vessels were tightly sealed with screw caps after the addition of 1 ml of soil suspension obtained by adding 1 g of soil to 10 ml of tap water. The vessels were incubated for 1 month at 30°C. Three successive subcultures grown in the presence of starch-containing plastic and decreasing glucose concentrations (0.1, 0.05, 0.01%) were then incubated under the same conditions. A final subculture was grown with no addition of glucose.

Isolation and identification of the bacteria. The aerobic strains were isolated on dilution plates containing nutrient agar (Difco) and incubated at 30°C. The strains were studied by using standard morphological and physiological criteria. The rapid API 20B (for the identification of heterotrophic aerobic bacteria), API 20E (for the identification of members of the family *Enterobacteriaceae* and other gram-negative bacilli), and API 20NE (for the identification of gram-negative bacilli that are not members of the *Enterobacteriaceae*) microtube systems were also used (API, La Balme les Grottes, Montalieu Vercieu, France).

The anaerobic strains were isolated by using the rolling tube technique (12). Single colonies were removed in an anaerobic cabinet and were inoculated into serum bottles (16) containing an anaerobic medium (6) under a CO_2-H_2-N_2 (5:10:85) atmosphere. Morphological and physiological analyses were then performed to characterize the strains. Sodium dodecyl sulfate (SDS) electrophoresis of total soluble proteins was carried out as described by Moore et al. (17).

TABLE 1. Characterization and probable identities of the aerobic members of communities 1S, 2S, 3S, and 6

Community	Strain	Gram reaction	Rod-shaped cells	Spores	Starch utilization	Motility	Probable taxon
1S	E	−	−	−	+	+	*Alcaligenes xylophila*
	F	−	+	−	−	−	*Xanthomonas maltophilia*
	G	+	+	−	−	+	NI[a]
2S	A	−	+	−	ND[b]	−	*Pseudomonas* sp.
	B	−	+	−	ND	−	*Pseudomonas* sp.
	C	−	+	−	ND	−	*Pseudomonas vesicularis*
3S	B	−	+	−	+	+	*Pseudomonas chlororaphis*
	C	−	+	−	+	+	*Pseudomonas stutzeri*
	D	−	+	−	−	+	*Vibrio* sp.
6	A	−	+	−	−	+	*Alcaligenes xylophila*
	B	−	+	−	ND	+	NI

[a] NI, not identified.
[b] ND, not detected.

A second technique was used to confirm the presence of sulfate-reducing bacteria with Pankhurst tubes (18) containing a semisolid sulfate API medium (Difco) in the presence of alkaline pyrogallol. Sulfate-reducing bacteria were isolated by using the dilution tube technique described by Postgate (21). Methanogens were recognized by their strong autofluorescence under oxidizing conditions. For fluorescent microscopy a Carl Zeiss Axiophoti microscope was used (420 nm). ATP concentrations in whole cultures were measured by the luciferase assay described by Cole et al. (9).

Growth conditions. The microbial consortia were maintained on B7 medium in screw-cap bottles by using starch-containing polyethylene strips as the sole carbon source. One consortium, designated community 3S, was chosen as the inoculant to study isotactic polypropylene degradation. Portions (1 g) of pure isotactic polypropylene (kindly supplied by E. Martuscelli, Consiglio Nazionale delle Ricerche, Naples, Italy) that had been previously weighed and washed in sterile water were cut as described above and incubated at 30°C in 50-ml vessels containing 30 ml of B7 medium; this medium contained 0.05% glucose and 0.05% sodium lactate and was inoculated with 1 ml of a 10-day-old culture of microbial community 3S (sample 1). Three controls were prepared by using the following conditions: the same amount of polypropylene with no additional carbon source, inoculated with community 3S (sample 2); the same amount of polypropylene in B7 medium containing 0.05% sodium lactate and 0.05% glucose, uninoculated (sample 3); and community 3S in B7 medium supplemented with lactate and glucose, without polypropylene (sample 4). All of the trial preparations were incubated under the same conditions.

Polypropylene extraction. After 0, 40, 150, and 175 days of incubation, the plastic strips from single bottles of the sample 1, 2, and 3 trials were collected by filtration from the cultures, repeatedly washed with distilled water, and subjected to a mild sonication for 3 min at 75 W and 48 kHz. The plastic strips were further washed and then dried for 1 h at 60°C. The removal of bacteria from the plastic surfaces was verified by microscopic examination. The strips were then extracted with methylene chloride (3.3%, wt/vol) overnight at room temperature with stirring, and the solvent was evaporated under reduced pressure. The dry extracts were weighed and analyzed by chromatographic and spectrometric techniques. The residual polypropylene strips were dried under reduced pressure and weighed (residual weight). Both cells and culture medium from sample 4 (without polypropylene) were extracted together three times with methylene chloride, and the organic phase was dried under reduced pressure. Each extract was analyzed by chromatographic and spectrometric techniques.

Chromatographic analysis on silica gel. (i) Thin-layer chromatography. Silica layers on glass plates (type 60 F 254; layer thickness, 0.25 mm; Merck) were eluted with *n*-hexane; the spots were revealed by spraying the plates with formaldehyde-water-sulfuric acid (1:1:2) or by wetting the plates with 10% phosphomolybdic acid in ethanol and heating them at 120°C for 5 min.

(ii) Column chromatography. Dichloromethane extracts dissolved in a minimum volume of *n*-hexane (1 to 2 ml) were loaded on a column (30 by 1 cm; silica gel 60; 230–400 mesh; Merck) and eluted with *n*-hexane and then with chloroform and chloroform-methanol (4:1). Each fraction was analyzed by thin-layer chromatography on silica gel.

Spectral analysis. Infrared spectra in $CHCl_3$ were recorded with a Perkin-Elmer model 298 infrared spectrometer at wave numbers from 4,000 to 600 cm^{-1} for each sample.

^1H nuclear magnetic resonance spectra were recorded in a deuterochloroform solution with a Varian Gemini 200-MHz apparatus and a Varian XL 300-MHz apparatus.

Native polypropylene was dissolved in tetrachlorodeuteroethane. Gas chromatography-mass spectrometry analyses were carried out with a V.G. Analytical Instruments model TS 250 mass spectrometer equipped with a Hewlett-Packard model 5890 gas chromatograph.

For qualitative characterization of the atmosphere composition in the culture bottles, a Poraplot U Chromopack column (27.5 m by 0.32 mm; film thickness, 10 μm) was used with helium as the carrier gas (50 kPa). The temperature program was as follows: 1 min at 30°C, followed by an increase in temperature at a rate of 15°C/min up to a maximum temperature of 120°C and then an additional 8 min at 120°C.

For qualitative characterization of the hydrocarbon mixture, a type OV-1 bonded phase column (25 m by 0.25 mm; film thickness, 0.3 μm; Supelco) was used with helium as the carrier gas (50 kPa). The temperature program was as follows: 1 min at 70°C, followed by an increase in temperature at a rate of 4°C/min up to a maximum temperature of 300°C and then an additional 10 min at 300°C.

RESULTS

Isolation of the microbial communities and partial identification of the strains. Four microbial communities (designated

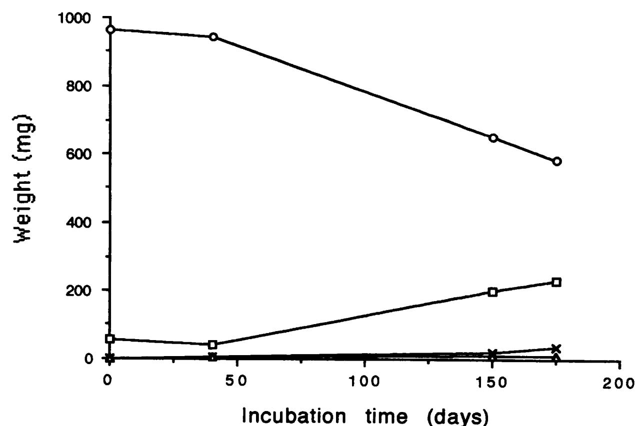

FIG. 1. Time course of degradation of isotactic polypropylene expressed as the insoluble polypropylene residual weight (○) and metabolite formation. Fractions A (□), B (×), and C (△) were obtained by chromatographically separating on a silica gel column the dichloromethane extracts of samples of polypropylene incubated with community 3S. The data shown are typical of the data observed in three different experiments. There was some variation between experiments because of different sampling times during the 175 days of incubation.

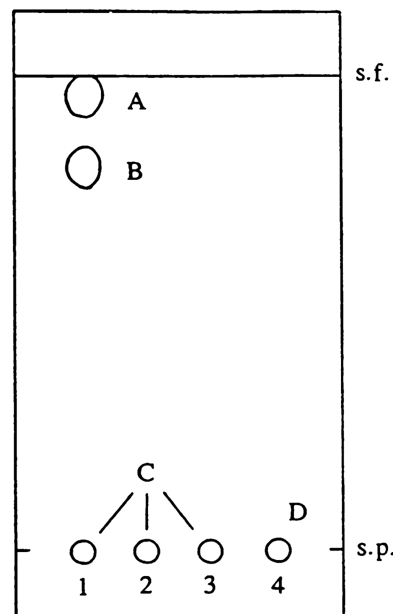

FIG. 2. Thin-layer silica gel chromatography of CH_2Cl_2 extracts of sample 1 (polypropylene incubated in B7 medium supplemented with glucose and lactate and inoculated with community 3S) (lane 1), sample 2 (polypropylene incubated in B7 medium with no additional carbon source and inoculated with community 3S) (lane 2), sample 3 (polypropylene incubated in B7 medium supplemented with lactate and glucose and not inoculated (lane 3), and sample 4 (community 3S [cells and culture medium] grown in B7 medium supplemented with lactate and glucose in the absence of polypropylene) (lane 4). The eluent was *n*-hexane, and the products were detected with 10% phosphomolybdic acid in ethanol (brown spots on a yellow background). s.f., solvent front; s.p., starting point.

1S, 2S, 3S, and 6) adapted to grow on starch-containing plastics as sole carbon sources were obtained from the enrichment cultures. Communities 1S, 2S, and 3S were isolated from samples of different sandy soils containing polyethylene wastes from greenhouses, while community 6 was obtained from a sample of forest soil. The communities appeared to be very similar in their metabolic capabilities and species compositions despite the different sites of sampling. All of the communities were able to grow on lactate and glucose, starch, and paraffin oil and to degrade cellulose when these substrates were added to B7 mineral medium; a blackening of the culture was observed with all of these substrates. A qualitative analysis of the gas phase in all of the trials in the presence of starch-containing plastic revealed that methane and sulfide were formed, indicating that sulfate-reducing bacteria and methanogenic strains were present. Since growth conditions were not strictly anaerobic, traces of oxygen were also detected, but anaerobic conditions may have developed within the microbial communities because aerobic and facultatively anaerobic bacteria were associated with the consortia. Each community consisted of four to seven isolates, some of which were aerobic and some of which were anaerobic. The most important characteristics of the aerobic strains and the tentative identities of these organisms are shown in Table 1. Our results show that the communities contained one or more *Pseudomonas* spp. and *Alcaligenes* spp. and that some of the organisms were amylolytic. A facultative *Vibrio* sp. was isolated from community 3S.

When the communities were subcultured several times under strictly anaerobic conditions, three or four strains were detected. Although these strains have not been fully characterized yet, our preliminary results indicate that a facultatively anaerobic endospore-forming, gram-positive, rod-shaped organism was present in each community. The results of SDS electrophoresis of the total soluble proteins allowed us to identify the strain isolated from community 3S as a *Bacillus* sp. strain. The sulfate-reducing bacteria isolated from each consortium appeared to be morphologically similar, and all of the strains were gram-negative, spore-forming, irregular rod-shaped organisms that were able to utilize acetate as a sole carbon source; they grew in the presence of CO_2, N_2, and H_2 (10:85:5, vol/vol/vol) with no organic substratum and gave negative responses to the desulfoviridin test (21). Methanogenic strains were not isolated from the consortia, but their presence was confirmed by microscopic examination of fluorescence at 420 nm.

Incubation of polypropylene in the presence of community 3S. When community 3S was inoculated onto B7 medium supplemented with glucose and lactate in the presence of pure isotactic polypropylene, growth occurred 2 to 3 days after inoculation. The ATP contents of whole cultures, measured after 72 h, ranged from 8 to 10 μmol/liter. These values did not differ significantly from the values obtained for a culture incubated under the same conditions in the absence of polypropylene, showing, as expected, that plastic did not influence growth over such a short period of time. No attempts were made to detect the contribution of polypropylene to growth over a long period of time compared with the control. When the microbial consortium from the control was plated onto nutrient agar under fully aerobic conditions, about 2×10^8 to 3×10^8 CFU/ml were obtained, while when the bacteria from the same culture were incubated in an anaerobic cabinet, their numbers did not exceed 10^6 CFU/ml. The numbers of sulfate-reducing bacteria counted after 72 h were only 10^3 to 10^4 CFU/ml, but the numbers increased to about 10^6 CFU/ml after 10 to 15 days of incubation.

The community appeared to be stable and viable over the

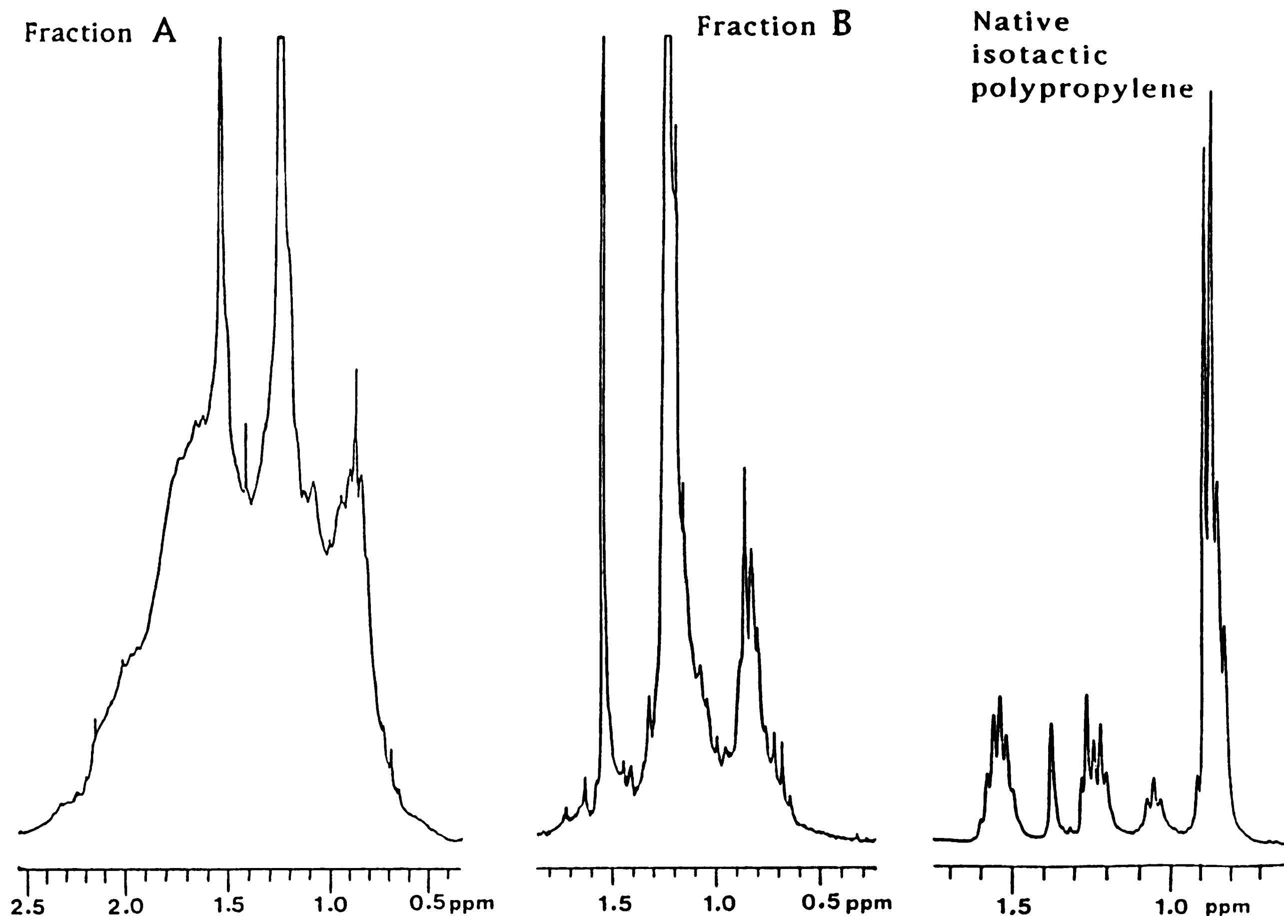

FIG. 3. ^{1}H nuclear magnetic resonance spectra of native isotactic polypropylene and its degradation products (fractions A and B) after 150 days of incubation with community 3S.

whole incubation period. The results of analyses of single colonies obtained from 150-day-old cultures demonstrated that all of the aerobic species present at the beginning of the incubation could be recovered. The *Bacillus* sp. and the sulfate-reducing strains did not lose their viability and were reisolated from rolling tubes inoculated under strictly anaerobic conditions with the microbial consortium at the end of the incubation period.

Weight loss of incubated polypropylene. The formation of metabolites in sample 1 was evident after 40 days of incubation; the amount of material extracted with methylene chloride (a suitable solvent for the extraction of apolar and slightly polar organic materials from polymeric structures) was 40% of the total amount after incubation for 175 days (Fig. 1). The polypropylene films from samples 2 and 3 (controls) contained only 4% extractable products.

Chromatographic analysis and separation of extracted products. The methylene chloride extracts were analyzed by thin-layer chromatography on silica gels to evaluate the nature of the compounds constituting the residues; three main spots (spot A [R_f, 1.0], spot B [R_f, 0.8], and spot C [R_f, 0.0]) obtained by elution with *n*-hexane were observed in sample 1 when community 3S was incubated in B7 medium supplemented with lactate and glucose in the presence of polypropylene (Fig. 2). Product separation, performed by column chromatography, gave three fractions; fractions A and B were obtained by elution with *n*-hexane, and fraction C was eluted with chloroform-methanol (4:1). The weights of fractions A, B, and C increased during the incubation period, as shown in Fig. 1. The extracts of samples 2 and 3 produced only spot C, and the extracts of sample 4 contained polar products that have not been identified yet (spot D [R_f, 0.0]).

Spectral analysis of chromatographed fractions. A comparison of the ^{1}H nuclear magnetic resonance spectra (Fig. 3) revealed that fractions A and B were mixtures of hydrocarbons formed by the degradation of the polypropylene film. We not only detected the signal (doublet at 0.85 ppm) typical of a residual propylene structure, but also observed large proton signals between 0.7 and 2.3 ppm; the latter was evidence that there was a mixture of hydrocarbon chains having different lengths and structures.

In the infrared spectrum of fraction A (1 g/ml) (Fig. 4) strong bands at 2,880 cm^{-1} (CH stretching) and 1,440 cm^{-1} (CH bending) confirmed the hydrocarbon structure. In addition to the hydrocarbon bands at 2,880 and 1,440 cm^{-1}, the infrared spectrum of fraction B also had weak signals both at 1,730 cm^{-1} (ketonic function) and at 3,200 and 1,140 cm^{-1} (alcoholic function); this was evidence that a certain limited oxyfunctionalization of the carbon chain occurred. Fraction C, which was also present in control samples 2 and 3, appeared (as determined by infrared examination) to consist of a mixture of aromatic esters (C=O ester function at 1,730 cm^{-1} and aromatic CH bending at 780 cm^{-1}); the latter are usually added to polyolefinic structures as plasticizers.

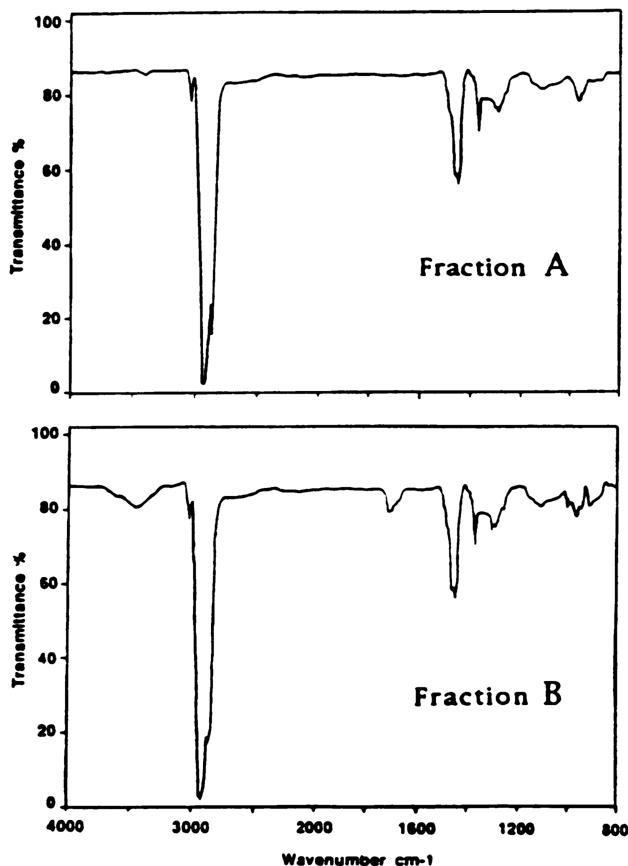

FIG. 4. Infrared spectra of fractions A and B after 150 days of incubation with microbial community 3S.

The hydrocarbon nature of the metabolites obtained from the dichloromethane extracts of the plastic samples was confirmed by a qualitative gas chromatography-mass spectrometry analysis performed at temperatures ranging from 70 to 300°C. An examination of the parent peaks and fragmentation patterns in the mass spectra suggested that hydrocarbon mixtures ($C_{10}H_{22}$ to $C_{31}H_{64}$) having various compositions were formed after incubation (data not shown).

The results of a preliminary chromatographic analysis of methylene chloride extracts in which external standard C_{10} to C_{32} hydrocarbons were used supported these results and indicated that the weight of this hydrocarbon mixture did not exceed 10% of the total weight of the dichloromethane extracts. Attempts to identify hydrocarbons having higher molecular weights by gas chromatography or gas chromatography-mass spectrometry were unsuccessful because of the pyrolysis of the compounds produced that occurred when the temperature was increased from 300 to 450°C.

DISCUSSION

Pure isotactic polypropylene that is incubated with adapted communities of microorganisms under oxygen-limited conditions with no preliminary chemical or physical treatment undergoes biodegradation. Strictly anaerobic and aerobic bacteria appear to coexist in mixed cultures in the presence of low oxygen concentrations, as reported previously by Gerritse and Gottschal (10). From the data reported above we concluded that these aerobic and anaerobic species with different catabolic capabilities can act in close cooperation to degrade polypropylene films. The evidence that biodegradation occurs comes from the increasing concentrations of the methylene chloride extraction products of the incubated polypropylene, together with the contemporary weight loss of the sample. Spectral analysis revealed that the extraction products were mainly hydrocarbons. As such metabolites were absent in the extracts obtained either from the uninoculated controls or from cultures grown without polypropylene, we confirmed that microbial attack of the polymer occurred. The finding that enzymatic attack of polyethylene occurs (20, 23) like enzymatic attack of trypsin on poly(ether urethane) (5) suggests that synthetic polymers may be recognized by natural metabolic machineries and then transformed into lower-molecular-weight compounds.

Since sulfate-reducing strains produce highly reactive oxygen species (19) and they were found in all of the communities tested, we hypothesize that oxidation of polypropylene by these chemical species occurs.

Conventional carbon sources (carbohydrates, starch, organic acids, etc.) have been reported to assist microbial growth and to help in degradation processes, especially under anaerobic conditions (11). In our experience sodium lactate and glucose had to be added to the medium to obtain growth of the consortia, suggesting that these carbon sources had a cometabolic effect. The use of starch as a filler in various plastic manufactured items could be seen as a strategy to drive cometabolic processes (22). Moreover, the filler can enhance the adhesion of bacteria to plastic films and thus increase the susceptibility of the polymer to microbial attack (7, 13).

Hence, we suggest that the well-known metabolic flexibility and adaptability of microorganisms and mycelia can result in the biodegradation of isotactic polypropylene and polyethylene, two macromolecules that supposedly are highly recalcitrant to biological metabolism.

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