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Biodegradation of a Medium-Chain-Length Polyhydroxyalkanoate in Tropical River Water

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Abstract

The medium-chain-length polyhydroxyalkanoate (PHA_{MCL}) produced by *Pseudomonas putida* PGA1 using saponified palm kernel oil as the carbon source could degrade readily in water taken from Kayu Ara River in Selangor, Malaysia. A weight loss of 71.3% of the PHA film occurred in 86 d. The pH of the river water medium fell from 7.5 (at d 0) to 4.7 (at d 86), and there was a net release of CO_2. In sterilized river water, the PHA film also lost weight and the pH of the water fell, but to lesser extents. The C8 monomer of the PHA was completely removed after 6 d of immersion in the river water, while the proportions of the other monomers (C10, C12, and C14) were reversed from that of the undegraded PHA. By contrast, the monomer composition of the PHA immersed in sterilized river water did not change significantly from that of the undegraded PHA. Scanning electron microscopy showed physical signs of degradation on the PHA film immersed in the river water, but the film immersed in sterilized river water was relatively unblemished. The results thus indicate that the PHA_{MCL} was degraded in tropical river water by biologic as well as nonbiologic means. A significant finding is that shorter-chain monomers were selectively removed throughout the entire PHA molecule, and this suggests enzymatic action.

Index Entries: Biodegradation; medium-chain-length polyhydroxyalkanoate; tropical river water; saponified palm kernel oil.

Introduction

With increasing use of plastic materials, the environmental impact that results from the production, application, and disposal of these materials is of great concern to the government, industries, scientists, and consumers. The objective of the ISO 14000 environmental management series is to minimize this impact from all aspects, based on the "cradle to grave" concept. Polyhydroxyalkanoates (PHAs) are polyesters produced naturally

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by a wide range of bacteria *(1)* and can therefore be synthesized from renewable resources. PHA has thermoplastic properties and, being a natural material, is biodegradable in the environment. Consequently, PHA has attracted much research and development as well as commercial interest. Polyhydroxybutyrate (PHB) and its copolymer with hydroxyvalerate (PHBV) have been marketed as packaging material and other products in the agricultural, marine, and medical fields *(1)*. Therefore, bacterial PHA is viewed as a potential candidate in the quest for an environmentally benign plastic material.

The production of a medium-chain-length PHA (PHA_{MCL}) from saponified palm kernel oil (SPKO) by *Pseudomonas putida* PGA1 has already been established in the authors' laboratories *(2)*. Palm kernel oil is a renewable resource abundant in Malaysia. Studies on the degradability of this PHA_{MCL} in different local environments form an integral aspect for the development and application of this material as an industrial product. In the present study, a modified method of the Sturm-Test *(3)* from ASTM standard test method D5209-91 *(4)* was used to determine the inherent biodegradability of PHA and to study the process of PHA degradation in a tropical aquatic environment. The PHA was immersed in water taken from the Kayu Ara River, and the degradability of the PHA was evaluated by monitoring the gross weight loss, changes in film surface morphology, monomer composition, and infrared (IR) spectrum of the PHA film. Carbon dioxide evolution, acid production, and microbial population in the river water medium were also monitored as indications of PHA degradation.

Materials and Methods

PHA Film

PHA_{MCL} was produced by *P. putida* PGA1, which used SPKO as the carbon source *(2)*. The extracted PHA was purified by repeated dissolving in chloroform and precipitating in methanol. It was then cast into a film that was aged for 3 wk at room temperature to allow it to attain equilibrium crystallinity prior to the biodegradability test *(5)*.

Biodegradability Test

The biodegradability test is based on a modified ASTM standard test method for determining the aerobic biodegradation of plastic materials *(4)*. Water collected from the Kayu Ara River in Selangor was first aerated for 4 h, allowed to settle for 30 min, filtered to remove debris, and then stored overnight at 4°C. Two hundred milliliters of this primed river water was placed in a sterile 500-mL conical flask (the test reactor) followed by 0.2 mL of a sterilized mineral salt solution containing the following: 22.5 g/L of $MgSO_4 \cdot 7H_2O$, 40.0 g/L of $(NH_4)_2SO_4$, 27.5 g/L of $CaCl_2$, 0.25 g/L of $FeCl_3 \cdot 6H_2O$, 1.7 g/L of NH_4Cl, 8.5 g/L of KH_2PO_4, 21.75 g/L of K_2HPO_4, and 33.4 g/L of $Na_2HPO_4 \cdot H_2O$. The test reactor was purged free of CO_2 for 24 h

by passing CO_2-scrubbed air through the solution at 1 to 2 bubbles/s. A 15 × 15 mm piece of the PHA film secured within a nylon net bag was submerged in methanol to surface sterilize, allowed to air-dry, and then placed in the solution in the test reactor. The solution was aerated with membrane-filtered CO_2-scrubbed air at 1 to 2 bubbles/s and stirred lightly with a sterilized magnetic stirrer. The gas outlet of the reactor was connected to a flask containing 50 mL of a 5 m*M* barium hydroxide ($Ba[OH]_2$) solution so that CO_2 produced in the reactor would be precipitated as barium carbonate. This CO_2-absorber flask was in turn connected to a second flask containing 50 mL of 5 m*M* $Ba(OH)_2$ to trap any CO_2 that might have "escaped" from the first flask.

The degradation test was carried out at 28°C. Six sets of the test reactors were run to allow monitoring of the PHA film and other parameters at d 0, 6, 20, 41, 60, and 86, respectively. These were the sampling times. Two control reactors were set up: Control 1 contained a PHB film cast from a commercial (Sigma, St. Louis, MO) product and incubated in the river water. This control was used as a positive control because PHB is known to be biodegradable. Control 2 contained an empty net bag suspended in the river water to determine microbial activity in the absence of PHA_{MCL}. Values obtained from these controls, where appropriate, were taken into consideration when evaluating the degradation of the PHA at the end of the study period (i.e., 86 d). An earlier experiment (experiment 1) was run to test the feasibility of the experimental setup. The incubation period was shorter (28 d), and degradation of the PHA_{MCL} was studied in sterilized and unsterilized river water that was not stirred.

PHA Film Analysis

At each sampling time, a test reactor was stopped. The PHA film was removed, rinsed, and sonicated (Bandelin Sonorex RK100, 35kH) twice in distilled water, each time for 15 min. The sonication was to remove debris and particles attached tightly to the PHA film, which could not be removed by rinsing. The PHA film was then dried to constant weight at room temperature. The surface morphology of the PHA film was observed with a scanning electron microscope (Philips SEM 515) after gold coating. The monomer composition of the PHA film was determined as follows: Approximately 8 mg of the PHA film was placed in a solution comprising 0.85 mL of methanol, 0.15 mL of 2 *M* H_2SO_4, and 1 mL of chloroform. The mixture was heated at 100°C for 140 min to break up the PHA into monomers and convert them into methyl esters *(6)*. One milliliter of distilled water was added to the cooled reaction mixture, vortexed, and left to stand for 10 min to induce phase separation. The lower-layer chloroform fraction was analyzed by gas chromatography. This was performed on a Shimadzu GC-14A system equipped with a fused Silica Omegawax™ 250 capillary column (30×0.25 mm) (Supelco) and a flame ionization detector. The temperature of the injector and detector was 170 and 200°C, respectively. The initial

and final temperature of the capillary column was 68 and 200°C, respectively, and the heating rate was 5°C/min.

IR analysis of the PHA was carried out as follows: A portion of the PHA film was cut out and dissolved in 5 mL of chloroform. The solution was filtered through sintered glass (no. 3) to remove insoluble particles. Another 5 mL of chloroform was passed through the sintered glass filter to extract any PHA that might still be attached to it. All the filtrate was collected and 4 mL of methanol was added to precipitate the PHA. After 6 h, the liquid mixture was decanted and the precipitated PHA was dried to constant weight at room temperature. This purified PHA was dissolved in 1 to 2 mL of chloroform, and then a few drops of this solution was put on an NaCl cell. The chloroform was evaporated with a hair dryer. The IR absorption spectra of the PHA were recorded on a Perkin-Elmer FTIR 2000.

CO_2 Analysis

The CO_2 evolved was precipitated in $Ba(OH)_2$ solution and measured by titration of the remaining unreacted $Ba(OH)_2$ with 0.05 *M* HCl *(4)*. Measurement was done every 2 d for the first 10 d and then every 5 to 6 d until the end of the test period.

River Water Analysis

At each sampling time, besides testing the PHA film, the river water in the reactor was analyzed. The pH of the solution was measured. The microbial population in the river water was estimated by viable cell count. An aliquot of the river water was diluted to known levels with sterile distilled water, and 0.1 mL was spread on nutrient agar plates. The plates were incubated at 30°C for 3 to 4 d, when the number of colonies was counted. A series of dilutions were prepared and plated. Only those dilutions that produced between 30 and 300 colonies were used to estimate microbial population.

Results and Discussion

Because of the rather complex assembly of equipment needed to carry out the degradation tests, several batches of the same experiment were run at different times. It was not feasible to test/monitor all parameters of interest within a single run. Therefore, some parameters were measured in one run but not in the another; for example, experiment 1 was set up to compare degradation of the PHA_{MCL} film in nonsterilized and sterilized river water, while experiment 2 was set up to measure CO_2 evolved (Table 1).

Gross Weight Loss

In the first experiment whereby the PHA_{MCL} was incubated in unstirred and sterilized river water, there was 10.6% weight loss in the PHA after 28 d (Table 1). This shows that PHA can be degraded by nonbiologic reactions.

Table 1
Measurements of Gross Weight of PHA_{MCL} Films, pH and Microbial Population of River Water, and Cumulative CO_2 Evolved[a]

Incubation period (d)	Weight loss (%)	pH	Microbial population ($\times 10^5$/mL)	Cumulative CO_2 evolved (mg)
Experiment 1 (unstirred river water)				
0	0	7.47	12.1	ND
28	–5.8	4.33	8.4	ND
28 (sterilized water)	10.6	6.40	0	ND
Experiment 2 (stirred river water)				
0	0	7.48	11.1	0
6	33.6	5.23	4.5	33.88
20	20.9	5.15	26.5	55.44
41	53.0	5.07	18.3	85.36
60	28.6	4.73	1.8	115.72
70	ND	ND	ND	133.76
70 (no PHA)	NA	ND	ND	114.84
86	71.3	4.69	1.1	ND
86 (PHB)	100.0	4.69	>30.0	ND
86 (no PHA)	NA	5.38	2.8	ND

[a]ND, not determined; NA, not applicable.

However, in the same experiment, another PHA film that was incubated in unsterilized river water for 28 d gained 5.8% in weight (Table 1). Since the experiment was conducted in unstirred river water, microbial cells might have been inclined to adhere to the PHA film before active biodegradation set in. This would explain the increase in gross weight of the film (instead of a decrease) and a decrease in microbial population in the water. This weight gain could have been owing to microbial growth on the PHA film being more than the weight loss from PHA degradation.

In the second experiment (stirred river water), by d 86 the PHA_{MCL} and PHB films had disintegrated completely—nothing was left in the net bag. To recover the disintegrated film particles in the solution (if any), the solution in each of the reactors was passed through filter paper (Whatman no. 1), and then the residue was washed, air-dried, and weighed. A weight loss of 71.3% for the PHA_{MCL} film and 100% for the PHB film occurred in 86 d (Table 1).

The results obtained from the two experiments indicate that PHA_{MCL} could be degraded by biologic as well as nonbiologic reactions.

Film Surface Morphology

Under a scanning electron microscope, the undegraded PHA film at d 0 had a smooth surface (Fig. 1A). By d 6 of immersion in stirred river water

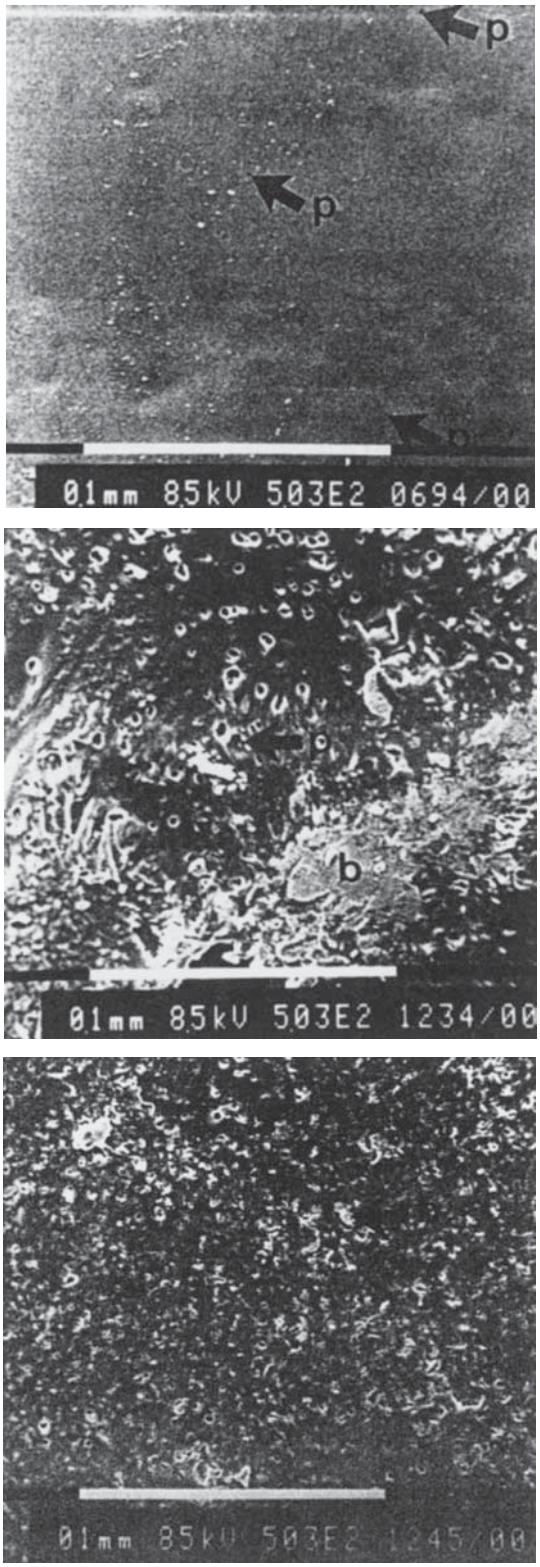

Fig. 1. Scanning electron micrograph (×500 magnification) of **(top)** undegraded PHA_{MCL} film; **(middle)** PHA_{MCL} film after 6 d in stirred river water; and **(bottom)** PHA_{MCL} film after 60 d in stirred river water.

(experiment 2), the film surface appeared rough and had many larger pits (Fig. 1B). By d 60, the film surface appeared uniformly uneven (Fig. 1C). To the naked eye, the PHA film changed from transparent at d 0 to opaque at d 60.

In the first experiment in which the PHA film was incubated in the sterile control for 28 d, the film remained transparent, and under SEM the film surface showed no detectable morphologic changes compared with that of the undegraded film (Fig. 1A). On the other hand, the PHA film that was immersed in the nonsterilized river water for the same 28-d period became opaque and under scanning electron microscope showed a rough, pitted surface. It appears, therefore, that microorganisms and their degradative enzymes caused more extensive physical changes to the PHA film. This might enhance the polymer degradation compared to that caused by abiotic factors alone. This is concurrent with the findings that PHB was not degraded in sterile solutions even after 3 mo *(7)*, nor in sterile seawater *(8)*.

Stirring enhanced the PHA degradation. The PHA film incubated in stirred river water (experiment 2) for 6 d (Fig. 1B) appeared more rough and had more pits compared with a PHA film incubated for a longer period in unstirred river water (experiment 1). Stirring could mechanically erode the PHA film surface and speed up the removal of water-soluble intermediate products known *(9)* to be released from PHA degradation. Another possible effect of stirring was to provide a more regular supply of oxygen and nutrients to the microorganisms around the PHA, thus supporting higher microbial metabolic activity for PHA biodegradation.

Monomer Composition of PHA

The original PHA film at d 0 was composed of 3-hydroxyoctanoate (C8) monomers present in the highest proportion, followed in decreasing proportions by 3-hydroxydecanoate (C10) monomers, 3-hydroxydodecanoate (C12) monomers, and 3-hydroxytetradecanoate (C14) monomers (Fig. 2). By d 6, however, the PHA film did not contain any more C8 monomers, and there was a reversal in the proportions of the other monomers; that is, the C14 monomers were in highest proportion, followed by the C12 monomers, whereas the C10 monomers were in lowest proportion. This same trend was observed in the PHA film analyzed on the other sampling days: d 20, 41, and 60. It appears that the PHA monomers with shorter side chains were more readily removed, and this was probably owing to the selective action of depolymerases present in the water. This observation is concurrent with the finding *(10)* that the rate of PHA hydrolysis depended on the side chain length of the monomers. Whereas C8 monomers were completely removed from the PHA within 6 d of incubation in river water (experiment 2), the C8 monomers were not selectively removed in the PHA incubated for 28 d in the sterilized river water (experiment 1) *(11)*. In the latter case, the C8 monomers comprised 52.7 mol% of the PHA, which was close to the C8 monomer proportion (54.4 mol%) of the original unincubated PHA film.

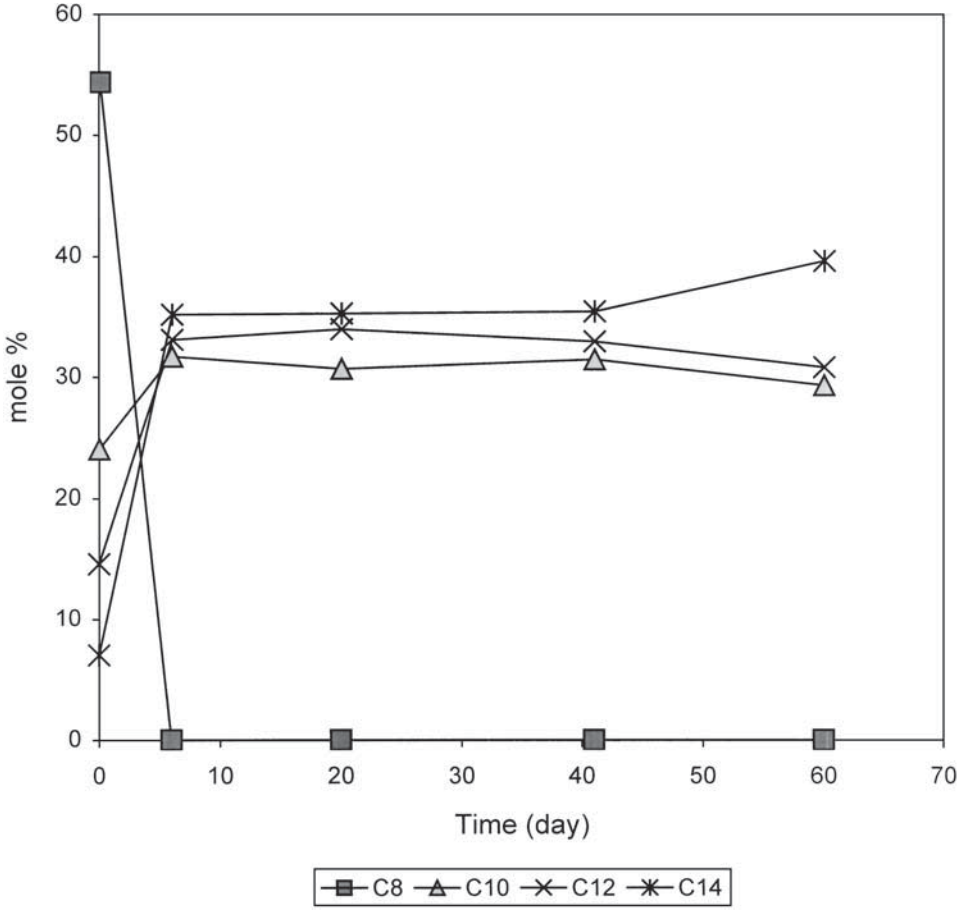

Fig. 2. Monomer composition of PHA_{MCL} material at different incubation periods in stirred river water.

This shows that biologic activity played a major role in removing the C8 monomers from PHA.

Previous findings on the enzymatic degradation of PHA films *(8,12)* showed that biodegradation occurred on the film surface only because the PHA depolymerases did not penetrate the bulk of the polymer. In the present study, biodegradation seemed to have occurred in the entire PHA film because all C8 monomers were completely removed in 6 d. This would only be possible if the PHA film matrix was accessible to PHA depolymerases. It had been postulated *(13)* that PHAs, which have low crystallinity, would allow greater mobility of water within the polymer, thus allowing greater accessibility to the depolymerase enzymes. This means that crystallinity affects the accessibility of PHA to PHA depolymerases. This is further supported by the finding that the rate of enzymatic degradation for P[(*R*)-3HB] chains in an amorphous state was about 20 times higher than the degradation rate of P[(*R*)-3HB] in a crystalline state *(12)*.

The PHA_{MCL} film that we used was completely amorphous *(2)* and, therefore, might be readily accessible to PHA depolymerases compared to a more crystalline material in which the enzymes would act only at the surface and not throughout the entire film. This more rapid biodegradation of PHA_{MCL} would be an advantage in certain applications.

Chemical Composition of PHA

The IR spectra of the PHA films that had been incubated for 6, 20, 41, and 60 d were almost identical to those of the original PHA film. This shows that PHA degradation did not involve removal of the aliphatic side chain, double bond formation, and major changes to the functional groups, i.e., carbonyl (C=O) and hydroxyl (–OH) groups. This observation is supported by a review *(9)* stating that the PHA depolymerases mainly hydrolyze the ester linkages of PHA into monomers, dimers, and/or oligomers. Hence, the aliphatic side chains and carbonyl and hydroxyl groups would still be present in the PHA before and after hydrolysis.

pH

In experiment 1, the pH of the sterilized, unstirred river water dropped from 7.47 to 6.40 after 28 d of incubation with the PHA_{MCL} (Table 1). At the same time, there was a 10.6% weight loss in the PHA. This indicates that nonbiologic degradation of PHA was accompanied by the release of acids. There was a greater decrease in pH (to 4.33) in the unsterilized river water medium, indicating that more acids (probably from PHA biodegradation) were produced when there was biologic activity. In experiment 2, the pH of the nonsterile river water incubated with PHA or PHB dropped significantly, from 7.48 at d 0 to 4.69 at d 86 for both reactors. Microbial activity in the river water in the absence of PHA also caused a drop in pH (to 5.38) but not as much as in the presence of PHA or PHB (Table 1). Oligomers and monomers in the form of hydroxyalkanoic acids were known to be released from PHA degradation *(9)*.

CO_2 Evolved

In experiment 2, the cumulative CO_2 evolved from the test reactor containing PHA (133.76 mg) was similar in amount to the cumulative CO_2 evolved from the control reactor (without PHA) (114.84 mg) at d 70 (Table 1). The CO_2 evolved from the control reactor (without PHA) was an indication of microbial respiration and metabolism on carbon compounds in the river water, while the CO_2 evolved from the reactors containing the PHA would include CO_2 released from PHA degradation as well. While no microbial count was taken on d 70, the microbial population measured on d 86 indicated that the control reactor (without PHA) had double the amount of microbes compared with the test reactor containing PHA. It would be reasonable to believe that since the test reactor produced similar amounts of CO_2 as the control, but had only half the number of microbes, some of the evolved CO_2 would have come from degradation of the PHA.

Microbial Population

In experiment 2, the microbial population in the river water dropped slightly at d 6 (4.5×10^5/mL) from the original count at d 0 (11×10^5/mL), rose to 26.5×10^5/mL at d 20, then dropped steadily to 1×10^5/mL at d 86 (Table 1). This is not entirely surprising considering that the study was conducted in a closed system where deficiency of nutrients, accumulation of metabolites, and PHA degradation products could all contribute to the gradual decrease in microbial numbers over the 86-d period. Interestingly, even though the microbial population in the river water was decreasing from d 20 onward, the trend line of the PHA gross weight loss continued to increase in a polynomial pattern, and CO_2 continued to be evolved at an almost constant rate (Table 1). PHA depolymerases have been reported to function best around pH 7.5–9.8 *(9)*. The continuous and significant reduction in pH with time (Table 1) might have caused the slowing down of the biodegradation process, although nonbiologic reactions might have continued to degrade the PHA. The PHB control, however, showed a high microbial population at d 86 (Table 1) compared to the very low microbial numbers in the PHA reactor in the same period. Perhaps the degradation products of PHB, hydroxybutyric acid, are readily utilized for cell growth, while the degradation products of PHA_{MCL}, longer-chain hydroxyalkanoic acids, might not be readily utilized for cell growth.

Conclusion

The PHA_{MCL} produced by *P. putida* PGA1 using SPKO as the carbon source could degrade readily in tropical river water. The degradation appeared to be attributed to biologic and nonbiologic reactions. In a closed reactor system whereby the PHA film was incubated in stirred river water, a weight loss of 71.3% was achieved in 86 d. Erosion pits and rough spots were formed on the PHA film surface after incubation for 6 d. Such physical signs of degradation were also seen on the PHA film incubated in unstirred river water for 28 d but not on the PHA incubated in the sterile control. This indicates that microorganisms played a major role in degrading the PHA in the river water. Stirring enhanced the rate of PHA degradation, resulting in more erosion pits and rough spots on the surface of PHA incubated in stirred river water compared with the PHA incubated in unstirred river water. The monomers with shorter chain length were removed faster. IR analysis revealed little change in the overall chemical composition of the partially degraded PHA films. These observations could be explained in that hydrolysis of PHA occurs at the ester linkages *(9)* and thus would cause little change in the chemical composition of the polymer. CO_2 was released from PHA degradation. The pH of the water became significantly acidic even after 6 d as a result of microbial metabolism and PHA degradation. Microbial population reflected growth in a batch culture whereby a decrease in some nutrients and accumulation of some metabolic products would combine to reduce cell growth and activity.

There have been reports that microbial degradation occurred on the PHA film surfaces alone *(8,12)* because the enzymes do not penetrate the bulk of the polymer. Our observations with a completely amorphous PHA film *(2)*, however, corresponded to another finding *(13)*—that degradation may occur throughout the polymer bulk depending on the crystallinity of the PHA, which in turn affects the accessibility of PHA depolymerases into the polymer.

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