

Degradation of Medium-Chain-Length Polyhydroxyalkanoates in Tropical Forest and Mangrove Soils

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Abstract

Bacterial polyhydroxyalkanoates (PHAs) are perceived to be a suitable alternative to petrochemical plastics because they have similar material properties, are environmentally degradable, and are produced from renewable resources. In this study, the *in situ* degradation of medium-chain-length PHA (PHA_{MCL}) films in tropical forest and mangrove soils was assessed. The PHA_{MCL} was produced by *Pseudomonas putida* PGA1 using saponified palm kernel oil (SPKO) as the carbon source. After 112 d of burial, there was 16.7% reduction in gross weight of the films buried in acidic forest soil (FS), 3.0% in the ones buried in alkaline forest soil by the side of a stream (FSst) and 4.5% in those buried in mangrove soil (MS). There was a slight decrease in molecular weight for the films buried in FS but not for the films buried in FSst and in MS. However, no changes were observed for the melting temperature, glass transition temperature, monomer compositions, structure, and functional group analyses of the films from any of the burial sites during the test period. This means that the integral properties of the films were maintained during that period and degradation was by surface erosion. Scanning electron microscopy of the films from the three sites revealed holes on the film surfaces which could be attributed to attack by microorganisms and bigger organisms such as detritivores. For comparison purposes, films of polyhydroxybutyrate (PHB), a short-chain-length PHA, and polyethylene (PE) were buried together with the PHA_{MCL} films in all three sites. The PHB films disintegrated completely in MS and lost 73.5% of their initial weight in FSst, but only 4.6% in FS suggesting that water movement played a major role in breaking up the brittle PHB films. The PE films did not register any weight loss in any of the test sites.

Index Entries: Medium-chain-length polyhydroxyalkanoate; degradation; tropical forest soils; mangrove soil.

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Introduction

Petrochemical-derived plastic materials are one of the biggest pollutants of the environment because not only do they persist in the environment, but also they are disposed of in large volumes. This had led to a worldwide quest to develop alternative materials with similar properties but that are environmentally degradable. Such materials should also be made from renewable resources as the amount of fossil fuel is depleting rapidly. Degradable polymers have found applications in a lot of areas, e.g., in medicine such as in sutures, wound dressings, surgical implants, and controlled-release drug delivery systems. Besides that, there is a great demand for degradable plastics for use as garbage bags, food and beverage containers, and for mulching. Biodegradation is defined as that which is the result of enzymes produced by microorganisms, and the process is viewed to be the most complete form of degradation whereby the polymer components are returned to the biogeochemical cycles. Three major requirements need to be established in order to get general acceptance for using biodegradable polymers: (1) justification for their use; (2) a definition which clearly indicates that what they do in the environment is acceptable to everyone; and (3) test protocols which confirm that they do as expected in the environment (1).

Bacterial polyhydroxyalkanoates (PHA) is acknowledged to be a good alternative plastic material because of its wide range of material properties and it could be made from common fermentation substrates such as sugars and plant oils. Two main groups of PHA are recognized and they are synthesized by different groups of bacteria via different biosynthetic pathways. The short-chain-length PHA (PHA_{SCL}) is composed of monomers having 3 to 5 carbon atoms, whereas the medium-chain-length PHA (PHA_{MCL}) is composed of monomers having 6 to 16 carbon atoms. PHA_{SCL} is crystalline and suited for making materials with mechanical strength such as containers. PHA_{MCL} are copolymers and are amorphous. They thus have different applications such as adhesives, impermeable linings, and controlled-release medium for drugs or chemicals. Some of the monomers in PHA_{MCL} may be unsaturated and this allows for chemical modification, e.g., insertion of functional groups or crosslinking to modify the material properties. PHA is a polyester of carboxylic acids, therefore vegetable oils and fatty acids are suitable precursors for PHA biosynthesis especially PHA_{MCL}.

Much studies had been documented about the degradation of PHA_{SCL}, especially poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), in natural environments such as compost (2) and tropical marine waters (3). The *in situ* degradation patterns of PHBV however did not resemble those obtained from *ex situ* studies in controlled environments in laboratories. *Ex situ* studies are nevertheless useful as a means to assess biological degradation such as the analysis of carbon dioxide evolved and determination of PHA-degrading microorganisms and extracellular depolymerases

(4,5). The rate of degradation is influenced by a number of factors which include the properties of the plastic material such as crystallinity, tacticity, morphology, and composition (6), as well as the physical and chemical properties of the environment and the microbial population in it (4). Methods used to determine PHA degradation include visual observation of the material, changes in gross weight, molecular weight and tensile strength, quantitative determination of microbial growth when PHA is used as carbon source, and determination of microbial activities such as oxygen consumption or carbon dioxide production (7).

Compared to the PHA_{SCL}, much less is known about the degradation of the PHA_{MCL}. A PHA_{MCL} produced by fermentation from saponified palm kernel oil had been characterized (8) and the degradation of this material in tropical river water was studied *ex situ* (9). The latter was carried out in a closed bioreactor in order to monitor the evolution of carbon dioxide as a measure of biodegradation (10). As an extension to these studies but with the objectives of assessing the degradability of this PHA_{MCL} in natural environments, the current studies were conducted with the PHA_{MCL} films being buried *in situ* in forest and mangrove soils. Two areas in a tropical rain forest and one in a mangrove swamp were chosen as test sites. Films of PHA_{MCL} made from saponified palm kernel oil were buried in soils in the three locations, and at certain time intervals, film samples were removed for analyses of gross weight, surface morphology, monomer composition, molecular weight, molecular structure, and thermal properties.

Materials and Methods

Polymer Production

The PHA_{MCL} was produced by a two-stage cultivation of *Pseudomonas putida* PGA1. First, the bacteria was grown in a modified rich medium (11) for 24 h, then the cells were harvested, washed with normal saline, and transferred to a nitrogen-limiting E2 medium (12) which contained 0.5% w/v saponified palm kernel oil as the sole carbon source to promote PHA production (8). After 48 h, the cells were harvested by centrifugation, washed with water, resuspended in methanol, and dried to constant weight. The dried cells were refluxed with chloroform for 5 h. The cooled solution was filtered to remove cell debris and the filtrate was concentrated by rotary evaporation. The dissolved PHA in the chloroform was then precipitated in methanol. The methanol–chloroform mixture was decanted and the precipitated polymer was redissolved in chloroform which was allowed to evaporate, leaving behind a polymer film. In order to obtain films of similar thickness, 5 g of PHA film were redissolved in 50 mL of chloroform. After that, every 10 mL of the solution were poured into similar size glass Petri dishes and left to evaporate in the fume cupboard. The PHA films were stored at room temperature for 12 wk to attain equilibrium crystallinity before the degradation studies were performed.

The PHB film was prepared by dissolving 0.46 g PHB granules (Fluka) in 50 mL chloroform which was refluxed at 80°C for 2 h. The solution was poured into a glass petri dish and the chloroform was evaporated. The PE films were cut from commercial plastic sheets.

On-Site Experiments

The on-site degradation studies were conducted at the University of Malaya Field Station, Gombak, Selangor, Malaysia which is a tropical rain forest, and at the mangrove swamp at Matang, Perak, Malaysia. The PHA films (both PHA_{MCL} and PHB) and PE films were encased in nylon nets and buried in the soil, 2 cm deep, at the mangrove swamp (MS) and at two sites in the forest: one was a well-shaded area under fallen leaves termed forest soil (FS), and the other was an unshaded area by the side of a forest stream termed forest soil, stream (FSst). In MS and FSst, the nylon nets holding the films were placed inside grid cages before being immersed in the soil. The grid cages were secured firmly in the soil as a precaution to prevent the nylon nets from being washed away during rain or strong water currents. The PHB and PE films were encased in the same nylon nets and were used as controls in these experiments to compare with the PHA_{MCL} films. At certain time intervals, some of the films were removed from the soils for analysis.

Soil Analyses

Soil temperature and pH were taken at the test sites. Some soil was taken back to the laboratory for measurement of salinity and moisture content. Organic matter content was determined by incinerating the soil at 550°C for 5 h (13) in a Carbolite CSF 1200 furnace. An estimation of microbial biomass in the soil was obtained by measuring the difference in ninhydrin-reactive nitrogen between soil samples which had and had not been fumigated with chloroform vapor (13). The chloroform would kill the microorganisms and a fraction of the cell constituents would solubilize in potassium chloride solution. The amino acid content in this solution would be analyzed by the ninhydrin method. This method was preferred over those which use selective media to analyze bacteria growth because the forest soils are different from the mangrove soils and, therefore, the microbial species might differ and might not be reasonably analyzed by using only one type of selective medium.

Analyses of PHA_{MCL} Films

The weight of the films was measured before and after burial. After the films were removed from the soils, they were rinsed in several changes of sterile distilled water to remove debris, and in 70% ethanol to prevent further biodegradation. The films were then dried at 30°C in a vacuum oven to constant weight. Monomer composition was determined by gas chromatography (14) using a Varian CX 3400 gas chromatograph system

Table 1
Some Properties of FS, FSst, and MS

Parameters	Forest soil (FS)	Forest soil, stream (FSst)	Mangrove soil (MS)
Temperature (°C)	14	25	27
pH	4.2 ± 0.1	8.2 ± 1.2	6.5 ± 0.1
Salinity (ppt)	1	1	3
Moisture content (%)	72.8 ± 1.9	78.1 ± 2.0	77.0 ± 1.1
Organic matter (%)	7.0 ± 0	2.1 ± 0	40.0 ± 5.4
Microbial biomass-N (µg/g dry soil)	118	40	3

with flame ionization detector and a fused silica capillary column (30 m, 0.53 mm internal diameter, 0.50 µm film thickness) (Supelco). Melting temperature, T_m , and glass transition temperature, T_g , were measured by a Perkin-Elmer Differential Scanning Calorimeter-7. Molecular weight, M_w , molecular number, M_n , and polydispersity, M_w/M_n , were determined using a Tosoh Gel Permeation Chromatograph HLC-8020. Functional groups were determined using the Perkin-Elmer FT-IR Spectrometer Spectrum 1000. Nuclear magnetic resonance (NMR) spectrometry was determined with a JEOL JNM-LA 400 FT NMR System, ^1H at 400MHz and ^{13}C at 100MHz. The film surfaces were observed by scanning electron microscopy (Philips SEM 515) after gold-coating (Bio-Rad Microscience Division SEM coating system).

Results and Discussion

Soil Properties

The properties of FS, FSst, and MS are shown in Table 1. The temperature of FS was significantly lower than those of FSst and MS. This is because FS is situated under dense forest canopy and covered with fallen leaves. FSst and MS are more exposed to sunlight and water currents. The pH of the three soils are different: distinctly acidic in FS but alkaline in FSst, whereas the MS is slightly acidic. The acidity of FS is probably owing to its higher organic content (7%) compared to FSst (2%) where soluble and insoluble matters would be washed away by the stream water. The high organic content of MS (40%) is a typical feature of mangrove swamps and the pH is probably regulated by the mixture of seawater and riverwater. As expected, MS is more saline than FS and FSst. The moisture content of FSst (78%) and MS (77%) are similar owing to constant exposure to water currents, whereas that of FS (73%) is only slightly lower, and is probably the result of rain and high humidity in the forest. The microbial biomass nitrogen, as estimated by the soil fumigation method, was highest in FS (118 µg/g dry soil) which was three times the amount in FSst (40 µg/g dry soil) and forty times the amount in MS (3 µg/g dry soil). It is seen therefore that the

Table 2
Percent Gross Weight Loss of the PHA_{MCL}, PHB, and PE Films
After 112 Days of Burial in FS, FSst, and MS

Film	Forest soil (FS)	Forest soil, stream (FSst)	Mangrove soil (MS)
PHA _{MCL}	16.7 ± 1.1	3.0 ± 0.3	4.5 ± 0.2
PHB	4.6 ± 0.1	73.5 ± 18.8	100
PE	0	0	0

three test sites have different soil properties which would be appropriate for comparing the degradability of the PHA_{MCL} films.

Gross Weight of the Films

The greatest weight loss of the PHA_{MCL} films occurred in FS while those buried in FSst and MS registered very little weight loss (Table 2). The outcome was reversed for the PHB films. It appears that PHB, being more brittle, could have been disintegrated by the water currents in FSst and MS and washed away. Where there was no water current as in FS, the PHB films lost less weight than the PHA_{MCL} films. This might be related to the amorphous nature of the PHA_{MCL} films compared to the highly crystalline PHB. Nishida and Tokiwa (15) had observed that the degradability of a polyester decreased as the overall crystallinity increased. The higher temperatures in FSst and MS might also have contributed to the faster rate of PHB degradation in those two sites compared to the much cooler FS as it had been reported (16) that PHB and PHBV hydrolysed at temperatures higher than 28°C. The PHA_{MCL} films, on the other hand, being amorphous and elastic, were not disintegrated by the water currents in FSst and MS as indicated by the low weight loss of the films buried there. The greater weight loss of the PHA_{MCL} films in FS compared to FSst and MS might be owing to the low pH and higher microbial biomass in FS (Table 1). Although not specifically tested, some of the soil microorganisms might have the ability to produce extracellular PHA-depolymerizing enzymes. It is also possible that the PHA_{MCL} films in FS were attacked by larger organisms such as earthworms, ants, and other detritivores that feed on the organic matter in various stages of decomposition on the forest floor. FS has the lowest temperature compared to FSst and MS, but the role of this in enhancing the degradation of the PHA_{MCL} films could not be ascertained under the current studies. The PE films in all three sites did not register any weight loss.

Molecular Weight of the PHA_{MCL}

Table 3 shows that the PHA_{MCL} films that had been buried in FS showed a small reduction in the weight average molecular weight, M_w , and number average molecular weight, M_n , whereas those from FSst and MS showed

Table 3
Molecular Weight and Thermal Properties of the PHA_{MCL} Films Before and After 112 Days of Burial in FS, FSst, and MS

Properties	PHA _{MCL} film before burial	After burial in forest soil (FS)	After burial in forest soil, stream (FSst)	After burial in mangrove soil (MS)
Weight average molecular weight, M _w (×10 ⁴)	10.39	8.32 ± 0.88	9.32 ± 1.21	9.10 ± 1.39
Number average molecular weight, M _n (×10 ⁴)	5.49	4.15 ± 0.66	5.13 ± 0.04	5.23 ± 0.50
Polydispersity, M _w /M _n	1.89	2.02 ± 0.11	1.82 ± 0.22	1.74 ± 0.11
Glass transition temperature, T _g (°C)	-35.7	-36.4 ± 0.1	-37.1 ± 0.3	-36.9 ± 0.7
Melting temperature, T _m (°C)	49.2	50.5 ± 0.3	49.2 ± 0.1	48.9 ± 0.5
Heat enthalpy, ΔH (J/g)	13.9	17.0 ± 6.4	15.1 ± 4.2	17.6 ± 4.5

less reduction. The polydispersity, M_w/M_n of the PHA_{MCL} films from all the sites after 112 d of burial was not significantly different from the films before burial indicating that the PHA_{MCL} was largely intact. The greater reduction in molecular weight of the FS samples correlated with the greater loss of gross weight in the same samples compared with the FSst and MS samples (Table 2). However, as the polydispersity of the FS samples was similar to the films before burial, the degradation of the FS samples was likely to be from surface erosion rather than bond breakage of the bulk polymer. Several reports (17–20) had stated that enzymatic degradation of PHA reduces the gross weight (mass) of the material but does not reduce the molecular weight because the enzymes break the bonds on the surface and not the internal bonds of the molecule. A chemically degraded polymer may however register a reduction in molecular weight before any discernible reduction in mass because the bonds throughout the polymer molecule could be broken if the chemical agent(s) could diffuse into the bulk of the polymer (17).

Thermal Properties of the PHA_{MCL} Films

The thermal properties of the PHA_{MCL} films that had been buried for 112 d in the three test sites did not change much from those of the films before burial (Table 3). This means that the integral properties of the PHA_{MCL} films were maintained, suggesting again that degradation, as indicated by loss of mass (gross weight), was by erosion. In studies whereby PHB films were submerged in seawater in natural and controlled environments (20,21), it was observed that the glass transition temperature, T_g , and the melting temperature, T_m , of the films did not change, concluding that degradation was by surface erosion.

Monomer Composition of the PHA_{MCL}

Before burial, the PHA_{MCL} was composed of 6.9 mole% 3-hydroxyhexanoate (C6) monomers, 58.4 mole% of 3-hydroxyoctanoate (C8) monomers, 26.7 mole% of 3-hydroxydecanoate (C10) monomers, 6.5 mole% of 3-hydroxydodecanoate (C12) monomers, 1.0 mole% 3-hydroxytetradecanoate (C14) monomers, and 0.5 mole% of 3-hydroxyhexadecanoate (C16) monomers. Film samples taken from the three test sites after different burial periods did not show much variation in the proportions of monomers from that of the starting material. This situation is very different from that observed by Ho et al. (9) whereby similarly prepared PHA_{MCL} films were immersed in river water in an enclosed bioreactor. In that study it was observed that the monomer composition of the PHA_{MCL} films was reversed after only 6 d of immersion in river water, i.e., there was no more C8 monomers (which were in highest proportion in the starting material), and only C10, C12, and C14 monomers occurred in ascending proportions (whereas these three monomers occurred in descending proportions in the starting material). Possible explanations for the different outcomes between this

current study and that of Ho et al. (9) are that firstly the PHA_{MCL} films were immersed in different medium: in soils in their natural environments in this study, but in river water under laboratory controlled conditions in Ho et al. (9) where there was no inflow of fresh materials nor outflow of accumulated metabolic by-products. Secondly, the microbial community and density might be different between the soils and the river water although no comparable studies were conducted. It had been postulated (6) that PHA with low crystallinity would allow greater water mobility within the polymer which in turn facilitates the entry of depolymerase enzymes into the polymer. The immersion of the PHA_{MCL} films in river water could have allowed that to happen, but which might not happen when the films were buried in soils. The PHA_{MCL} films are hydrophobic, so in a solid matrix such as soil, the material might not even be accessible to microbial enzymes.

Analyses of Molecular Structure and Functional Groups

There were no differences in the ¹H and ¹³C NMR spectra of the PHA_{MCL} films that had been buried in the three test sites for 112 d compared with the NMR spectra of the films before burial. All the spectra closely resembled those obtained by Gross et al. (22) and Preusting et al. (23), which characterized PHA_{MCL} as a polyester in which the monomeric units are joined to one another at the β-carbon of each unit and to which is also attached the various side arms of the molecule. The IR spectra of the films before and after 112 d of burial were similar, i.e., all the major functional groups and the bending region were similar. These results (NMR and IR) further support the other findings (molecular weight, thermal properties, and monomer composition) that the PHA_{MCL} molecule remained intact during the burial period in the three test sites.

Scanning Electron Microscopy of the PHA_{MCL} Films

The surface of the film before burial was smooth. Films that had been buried in FS showed bigger and deeper holes compared to the tiny pitholes found in the films that had been buried in FSst and MS. This is consistent with the greater loss of gross weight of the films that had been buried in FS compared to those that had been buried in the other two sites. The same factors that could have contributed to the gross weight loss of the PHA_{MCL} films, such as low pH and higher microbial biomass, might also be responsible for the changes in surface morphology of the films.

Conclusion

The PHA_{MCL} films used in this study, which was produced from bacterial conversion of saponified palm kernel oil, registered various degrees of gross weight loss in three types of natural environments in tropical Malaysia: in acidic forest soil under decomposing leaves, in alkaline forest soil along a freshwater stream, and in brackish mangrove soil. The greatest weight loss of the PHA_{MCL} films occurred in the acidic forest soil (FS),

the loss being more than that for the PHB and PE films. Scanning electron microscopy revealed holes in the PHA_{MCL} films after burial compared to the smooth unbroken surface of the films before burial. The chemical properties of the PHA_{MCL} films such as molecular weight and polydispersity, glass transition and melting temperatures, monomer composition, molecular structure, and functional groups of the PHA_{MCL} films, however, remained largely unchanged before and after the burial period. Degradation of the PHA_{MCL} films was therefore likely from surface erosion of the films and did not involve breaking of the molecular structure. The greater weight loss of the PHA_{MCL} films in FS compared to FSst and MS could be attributed to some soil factors such as higher acidity and greater presence of microorganisms in FS. The PHB films also registered gross weight loss in the three test sites with the extent of weight loss being proportional to the water movement at the sites, i.e., the films disappeared completely after 112 d of burial in mangrove soil which experienced tide movements from the sea. There was 73.5% loss in those films buried in FSst which experienced stream water movements and only 4.6% loss in those films buried in FS. This suggests that the PHB films could have disintegrated and the tiny pieces washed away which is plausible because PHB is highly crystalline and brittle. The PE films buried in the three test sites did not register any gross weight loss. A significant conclusion from this study is that the PHA_{MCL} films produced from saponified palm kernel oil degraded very slowly *in situ* in acidic forest soil, alkaline forest soil, and brackish mangrove soil. Surface erosion of the material was indicated with no evidence of molecular disintegration. These data would have important implications in the types of products to be produced from this PHA_{MCL} and its end-use applications.

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