Poly(lactic acid) Degradation in Soil or Under Controlled Conditions

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SYNOPSIS

The fate of a racemic lactic acid polymer (PLA50) was investigated by allowing parallelepiped plates of PLA50 to age in liquid medium containing a mixed culture of *Fusarium moniliforme* and *Pseudomonas putida* at 30°C. Microbial activity was monitored by measuring pH, lactic acid formation by high-performance liquid chromatography, and esterase activity in supernatant. Degradation of the plates was monitored by weighing, size exclusion chromatography, and visual examination. Under the selected conditions, pH increased up to 8, whereas it remained constant in the microorganism-free control. No significant lactic acid formation or esterase activity was detected in the supernatant during the experimental period (32 weeks). For the first 17 weeks, the water absorption rate of the plates was the same in both the microbial and the control media. Subsequently, plates absorbed more water in microbial than in control media. Typical surface/center differential degradation of plates was observed until 17 weeks, but it reduced progressively. At 32 weeks, specimens appeared completely disintegrated in the microbial medium. PLA50 plates were also buried in the soil for 8 weeks. After recovery, plates were allowed to degrade 8 weeks under controlled conditions. Scanning electron microscopy of these plates showed the development of some filamentous fungi at the surface and into the bulk of plates. Five fungal strains were isolated which appeared to be able to assimilate PLA50 oligomers in mixed cultures. It was assumed that PLA50 plates had been first degraded by chemical hydrolysis, followed by the bioassimilation of degradation by-products in both experiments. © 1996 John Wiley & Sons, Inc.

INTRODUCTION

So far, polymeric materials have been developed primarily on the basis of performance, cost effectiveness, and shelf-life. However, their resistance to microbial attack is now considered to be a shortcoming from the viewpoints of environmental protection and solid waste management. Degradable polymers are increasingly considered as an attractive alternative in some cases, because their resistant counterparts require disposal by incineration or landfilling. The degradation by microorganisms of the poly(β-hydroxy alkanoates)-type, such as poly(hydroxy butyrate) (PHB) or higher analogues and their copolymers, has been well documented.[1,2] In contrast, sparse information is available on the mechanism of degradation in the presence of microorganisms of poly(α-hydroxy alkanoates), such as poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) or PLA/PGA copolymers, although these polymers seem to resorb *in vivo* after chemical degradation.[3]

Chemical hydrolysis of PLA-type polymers has been extensively investigated *in vivo* and in aqueous media as well.[3-7] It has been reported that, for large-size devices (>1 mm thick), degradation is faster inside than at the surface because of diffusion-reaction phenomena. When immersed in an aqueous medium such as body fluids, a PLA-based device absorbs water and autocatalytic cleavage of ester bonds starts. Later on, degradation at the surface becomes slower than inside because of the release of water-soluble acidic oligomers and lactic acid, whereas those located well inside remain entrapped.

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At the end, the whole inner part is composed of water-soluble oligomers and a hollow structure is formed, because of their release to the medium, while surface degradation continues slowly.[8]

In this article, the results of investigations on the biodegradation of racemic PLA (PLA50) in the presence of microorganisms are reported. First, parallelepiped plates were allowed to age in liquid culture media containing a mixed culture of a fungus, *Fusarium moniliforme,* and a bacterium, *Pseudomonas putida.* The fate of plates and microbial activity were monitored by weighing, size exclusion chromatography (SEC), chromatography, visual examination, pH, and esterase activity. In a second approach, plates were buried in soil for 8 weeks. Because there was no outer sign of degradation, the plates were then placed on a mineral culture medium and were allowed to age for a further 8 week period under laboratory-controlled conditions.

EXPERIMENTAL

Materials

Racemic PLA

Racemic PLA was obtained by bulk ring-opening polymerization of DL-lactide (Purac Biochem) under a vacuum for 10 days at 140°C. Zinc powder was used as the initiator. Further removal of low-molecular-weight residual compounds was made by the dissolution-precipitation method with acetone/methanol as solvent/precipitant. The resulting polymer was first molded as round plates (75 mm diameter × 2 mm thickness) by compression molding. Parallelepiped specimens (10 × 10 × 2 mm) were then machined from the round plates.

PLA Oligomers

Typically, 1-L commercial LA solutions (racemic form from Sigma and L-pure form from Carlo Erba) were heated under normal pressure to gently distill water. When the temperature reached 130°C, a vacuum was applied and distillation was controlled. The resulting oligomers, the characteristics of which depended on temperature and time conditions, were recovered by dissolution in chloroform and reprecipitation in basal culture medium, in order to eliminate the fraction soluble in this medium. Racemic oligomers appeared as a very sticky transparent material, while L-oligomers were in the form of a white 0.3–0.8 mm powder, after milling and sieving. One fraction of racemic oligomers ($\bar{M}_w$ = 2,000, I = 1.3) and one of L-oligomers ($\bar{M}_w$ = 4,000, I = 1.4) were used.

Degradation Conditions

Microorganisms

A strain of filamentous fungi, *F. moniliforme,* was obtained from the Orstom collection (Fmm) and the bacterium, *P. putida,* was from the Czech Collection of Microorganisms (CCM-3423).

Culture Medium Preparation

The basal culture medium contained (in grams per liter): 0.8 urea, 0.52 $(NH_4)H_2PO_4$, 0.5 glucose, 1.27 NaH_2PO_4, 1.63 $K_2HPO_4.2H_2O$, 0.3 $MgSO_4.7H_2O$, 0.3 KCl, and 7.9 methanol. The initial pH was 6.6. Mineral salts and vitamins were added after sterilization as described elsewhere.[9]

Culture medium (400 mL) was introduced into 1-L Erlenmeyer flasks. After sterilization, parallelepiped plates were added aseptically. Each flask was inoculated with both microorganisms simultaneously. The *F. moniliforme* inoculation was made with a spore suspension recovered with a 0.01% Tween 80 solution from a potato dextrose agar culture (Difco Laboratories, Detroit, MI) after 7 days of incubation at 30°C. The *P. putida* inoculation was made with a 24 h culture cell suspension in a glucose-based medium.[10] The inoculation ratio was 2×10^7 spores or cells per gram of racemic PLA. Culture media were incubated at 30°C under continuous agitation at 150 rpm.

Culture Monitoring

For each degradation time, three specimens were recovered, washed with distilled water, weighed, and vacuum/dried for 1 week at room temperature. As for the standard protocol established by Li et al.,[6] water absorption (WA) was calculated by use of the equation $WA\ (\%) = 100(Ws - Wt)/Wt$, where Ws was the weight of the swollen specimen after wiping the surface with paper, and Wt was the residual dry weight of the specimen. The decrease in average molecular weight ($\bar{M}_n$) was obtained from the ratio $100(M_o - M_t)/M_t$, where M_o and M_t are the initial and the final molecular weights of the degraded material, respectively.

Measurements

SEC Technique

Molecular weights were monitored by size exclusion chromatography (Waters model 600 A gel perme-

ation high-performance liquid chromatography (HPLC); stationary phase, PL Gel Mixed; mobile phase, dioxane; flow rate, 1 mL/min; injection volume, 20 μL). Data were compared with polystyrene standards without any universal calibration.

pH Measurement

The pH values were measured at 25°C, with a Knick digital pH-meter equipped with an Ingold electrode.

Esterase Activity

Esterase activity in the supernatant was assayed by monitoring the fluorescein released from diacetate ester by ultraviolet absorption at 490 nm, as described elsewhere.[11,12]

Determination of LA-Type Compounds

LA-type compounds were measured by HPLC (Waters high-performance liquid chromatograph equipped with a photodiode array detector model 996; stationary phase, Shodex Ionpak KC-811; mobile phase, 0.02% H_3PO_4 solution; flow rate, 0.5 mL/min; injection volume, 20 μL; detection, 210 nm).

Microbial Growth

Microbial growth on the racemic PLA-based culture was evaluated by a colorimetric microplate assay as described by Hernandez et al.,[13] using a glucose-reach medium[10] containing 0.17 g/L of bromocresol purple, as color indicator.

Degradation Test in Soil

Racemic PLA plates were buried in the soil in the forest (north of Montpellier). After 8 weeks, plates were recovered and placed into Petri dishes containing the basal culture media solidified with 15 g/L of agar and without any carbon source. Incubation was at 30°C for 8 weeks in a hydrated environment. After 16 weeks of total degradation time, plates were examined by scanning electron microscopy (SEM).

Microorganisms attached to the surface of the polymer plates, after the two successive 8 week degradation periods, were isolated and cultivated onto a potato dextrose agar culture for 7 days. Spores were recovered with a 0.01% Tween 80 solution. All isolated strains were used in mixed cultures to assay PLA oligomer assimilation. Determination was made by total alkaline hydrolysis (addition of 3 mL of a 10*N* NaOH solution and boiling for 2 min) of the entire content of a 250 mL Erlenmeyer flask for each aged time, followed by HPLC analysis, as described before.[14]

Scanning Electron Microscopy

Pieces of the recovered plates were fixed in a 1.5% glutaraldehyde solution, dehydrated for 10 min each in 10, 20, 40, 60, 80, 95, and 100% ethanol, and mounted on aluminum stubs. Samples were sputter coated with gold and observed under a J.S.M. 35 JEOL scanning electron microscope.

RESULTS

Aging Under Controlled Culture Conditions

The size of the 10 × 10 × 2 mm PLA specimens, which were allowed to age in the presence of *F. moniliforme* and *P. putida,* was selected in order to give rise to heterogeneous degradation, which is characterized by fast central degradation and slow surface degradation.[8] To compare the degradation and biodegradation of the PLA plates, control experiments were performed without microorganisms.

Microbial Activity

Figure 1 shows the variation in the pH of the culture media. During the first week, pH increased up to 8 with time, whereas no pH change was detected in control media. It is suggested that the pH increase reflected microbial activity, probably related to urea assimilation. Microbial activity was also reflected by the turbidity observed in the media during the whole period of investigation. By the use of the colorimetric microplate assay,[13] it was observed that this turbidity corresponded to a ratio of 5×10^7 living cells/mL at 32 weeks postinoculation.

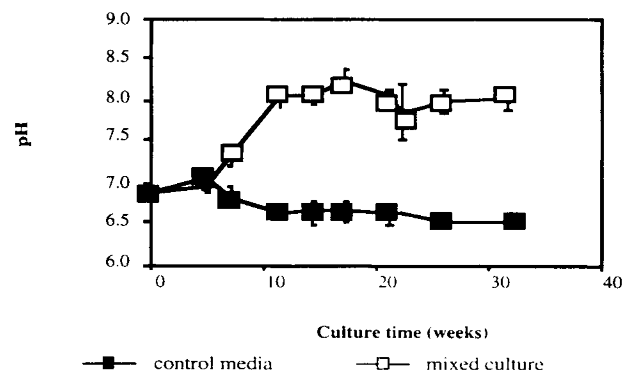

Figure 1 Evolution of pH within microbial and control media containing racemic PLA plates.

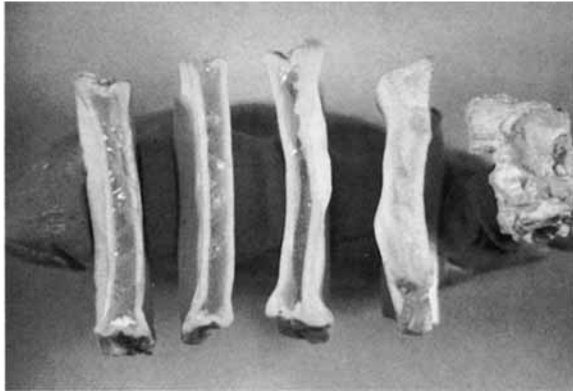

Figure 2 Cross-sections of racemic PLA plates at different times (5, 11, 17, 26, and 32 weeks, from left to right) of aging in microbial liquid medium.

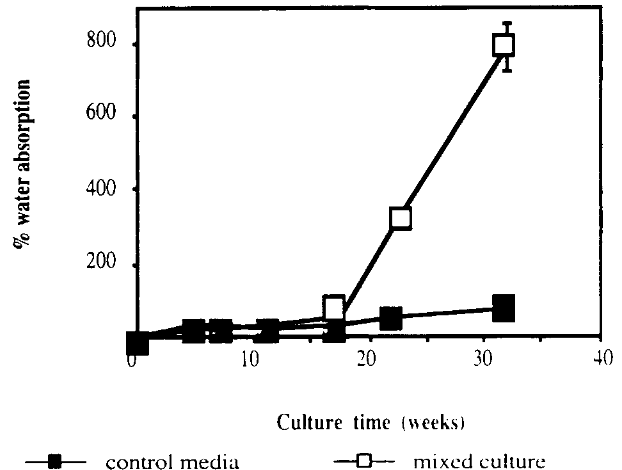

Figure 4 Evolution of WA of racemic PLA plates aged in microbial and in control liquid media.

Physical Modification

Figure 2 shows the pictures of plates at intermediate degradation stages in microbial liquid culture. In agreement with previous findings,[6] at week 5, specimens showed heterogeneous cross-sections that were whitish at the surface and yellow/transparent in the inner part. However, beyond 5 weeks, the evolution of physical parameters differed very much from that of the controls. Differences between inner and outer parts vanished progressively. At week 26, specimens were highly swollen and exhibited homogeneous cross-sections. Specimens were completely disintegrated at 32 weeks. In contrast, plates in controls showed typical heterogeneous cross-sections, with the inner part starting to appear as a very viscous liquid (Fig. 3).

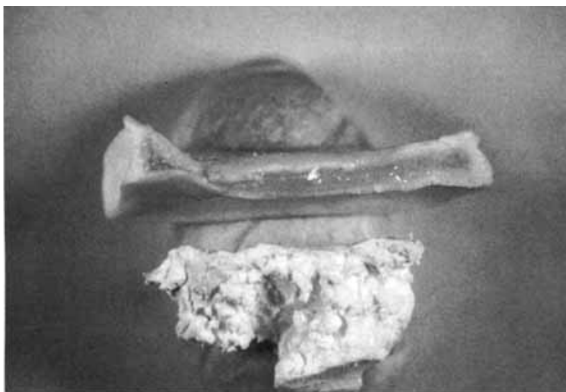

Figure 3 Cross-sections of racemic PLA plates at 32 weeks of aging in control (above) and in microbial (below) liquid media.

Water Absorption

WA was deduced from weight differences between wiped humid specimens, before and after drying. In the case of inoculated media, the profile of WA was biphasic (Fig. 4). In the first phase (0–17 weeks), WA of 2.4% per week was similar for inoculated and control plates. In the second phase (17–32 weeks), WA of the inoculated plates increased very rapidly by 50.5% per week. After 32 weeks, WA increased by 750% for the inoculated plates and only by 75% for control plates.

Molecular Weight Changes

The initial number average molecular weight ($\bar{M}_n$) of plates, taken at the maximum of the SEC peak, was 25,000 (I = 1.6). There was no significant difference in the $\bar{M}_n$ of the plates aged in microbial and control media (Fig. 5). In both cases, $\bar{M}_n$ decreased continuously at a rate of 1.8% per week, to reach 20% of the initial value after 32 weeks for the control. A slight difference was detected between controls and inoculated systems during the last 10 weeks, the degradation of the inoculated samples being lower than that of the controls.

No bimodal SEC chromatograms were observed during the whole experimental time, even in the case of plates degraded chemically, in contrast with all of the observations reported in the literature for thick devices.[6,8] Furthermore, only a very low concentration of LA (0.1 g/L) was detected at the end of the chemical experiment, suggesting a significantly slower degradation at 30 than at 37°C.

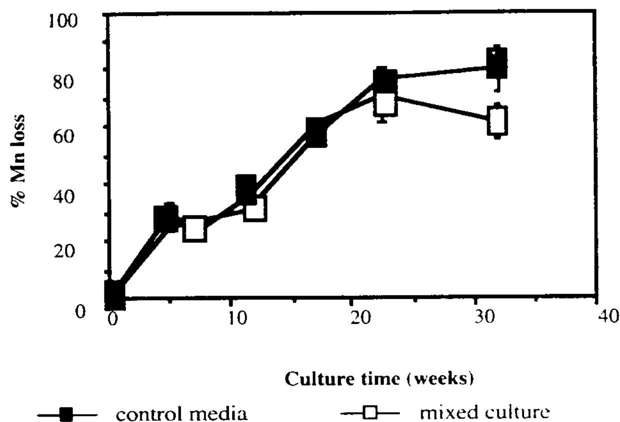

Figure 5 Evolution of number average molecular weight ($\bar{M}_n$) loss of racemic PLA plates aged in microbial and in control liquid media.

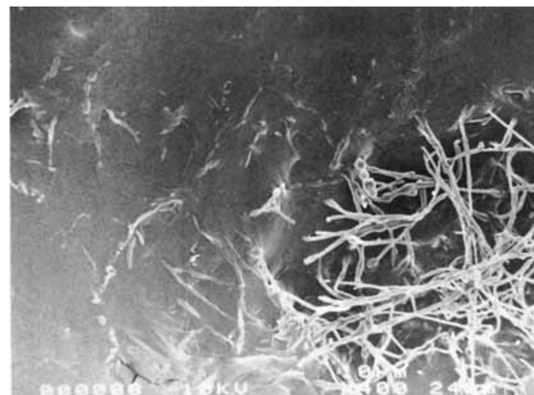

Figure 6 Electron micrograph of racemic PLA plates buried 8 weeks in soil and then incubated 8 weeks in a Petri dish containing mineral agar medium.

Degradation in Soil

Visual and SEM Examination

After the first degradation period in soil, no microbial growth was visible at the surface of the plates. However, the presence of spores was suspected and the plates were allowed to age under *in vitro* culture conditions without any inoculation.

Visual observation of racemic PLA-degraded plates showed that the surface was invaded by filamentous fungi after 8 weeks in culture (16 weeks altogether). Invasion was detected at the periphery of samples, i.e., at the interface between the sample mass and the agar mineral medium. The results of the SEM confirmed surface invasion by mycelia (Fig. 6). It is of significant interest to note that the mycelia were able not only to attach to the surface, but also to penetrate the polymer network. In some locations, filaments penetrated first into the plate, continued penetration through the entire thickness, and got out farther from the bulk (Fig. 7).

Culture of Spores Attached to Soil-Aged Plates

After culture in a conventional medium, five different fungi strains were isolated from soil-aged plates. The ability of these microorganisms to use racemic and L-pure form PLA oligomers as substrate was tested in mixed cultures. The results showed that racemic oligomers were assimilated rapidly by these microorganisms. Actually, 90% of these racemic oligomers were consumed within 8 days of culture, whereas only 30% of L-oligomers were consumed within the same period (Fig. 8).

DISCUSSION

Under the conditions selected for the mixed culture in liquid medium, it was observed that PLA50 plates became whitish and swollen with surface/center differentiation when aged in the control. This behavior agrees with the heterogeneous degradation expected for the rather thick investigated specimens aged in aqueous medium buffered at neutral pH and at 37°C.[6] The lowering of aging temperature from 37 to 30°C caused the degradation to be slower. On the other hand, the initial molecular weight was rather low ($\bar{M}_{peak}$ = 40,000, as referred to polystyrene standards). These two facts are likely to be responsible for the extension to the whole mass of the slow crystallization of stereocomplex oligomers, observed only at the

Figure 7 Electron micrograph showing filamentous fungi developed at the surface and through the bulk of racemic PLA plates buried 8 weeks in soil and then incubated 8 weeks in a Petri dish containing mineral agar medium.

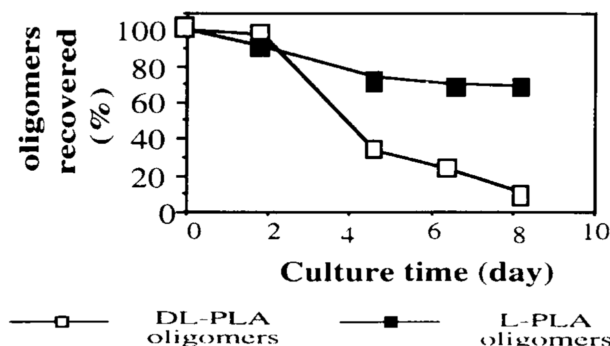

Figure 8 Assimilation of DL- and L-PLA oligomers by mixed cultures of microorganisms isolated from racemic PLA plates buried 8 weeks in soil and then incubated 8 weeks in a Petri dish containing mineral agar medium.

surface so far. This is strongly supported by the fact that late solid residues known to be crystalline are not soluble in normal PLA50 solvents.[15] These characteristics may also explain the finding that the bimodal SEC trace, typical of heterogeneous degradation, was not detected for the control devices, because the SEC static phase was not resolutive enough to reflect the difference between the molecular weights at the surface and inside. Anyhow, there is no doubt from pictures that heterogeneous degradation took place in the case of controls.

The fact that the parallelepiped samples did not show the same evolution in inoculated medium and led to higher swelling and earlier solidification inside beyond 20 weeks (Fig. 2) can have different implications. First, the increase of pH could have affected diffusion-reaction phenomena by making the extraction of acidic degradation by-products easier. Second, the consumption of the released degradation by-products by the microorganisms could have the same effect. Third, the activity of the microorganisms could have led to changes in the osmolarity of the culture medium, a factor which is known to significantly affect the heterogeneous degradation phenomena.[8,16] Last but not least, the release of water-soluble oligomers that normally occurs during the last phase of degradation could have activated the fungal activity to initiate a water transfer from the liquid medium to the solid substrate. The pH did increase because of the microorganism activity under poor substrate utilization conditions; this finding is in agreement with previous results.[14] However, difference in molecular weight variations was detectable, except beyond 23 weeks, when the released water-soluble oligomers were assimilated by microorganisms, thus explaining the observed increase in the molecular weight of the residual solid matrix.

These features, together with the absence of important enzymatic activity in the supernatant of microbial cultures, strongly emphasize the fact that high-molecular-weight chains could not be directly attacked by the microorganisms. In contrast, the ability of microorganisms to attack PLA50 oligomers enzymatically and to assimilate the ultimate degradation products, namely L- and D-lactic acids, well accounts for the late differentiation (molecular weight, swelling, formation of crystalline residues, and deformation) between inoculated specimens and controls.

The behavior of PLA50 plates aged in soil was completely different. There was no change in the visual aspect or size for the 8 week degradation period, although some microorganisms were obviously present on the surface. This phenomenon well agrees with a degradation in a humid but not liquid medium. In fact, homogeneous degradation takes place when temperature is low, water uptake is limited, and water-soluble oligomers cannot be released because of the absence of contact with liquid water. The situation was quite similar when aging was continued on carbon-source free agar, where the specimens were not surrounded by the liquid medium but just put on the surface of the agar. This culture allowed the attached microorganisms coming from the soil to grow, as shown in Figure 6. The penetration of hyphes within the polymer matrix shows that hydrolytic degradation did occur during the 16 weeks of the total aging period and that wild filamentous fungi could invade the partially degraded mass to take advantage of the assimilable oligomers formed and not released. This is well supported by the fact that *F. moniliforme* and *P. putida* mixed cultures and the wild microorganisms isolated from the agar medium were all able to degrade and assimilate PLA50 and PLA100 oligomers, provided that these oligomers were bioavailable, the latter being less degradable because of crystallinity, as previously discussed.[14]

According to these findings, various mechanisms to account for the degradation of PLA polymers can be distinguished, depending on the degradation conditions (Fig. 9). When placed in the presence of microorganisms, such as bacteria or fungi, PLA polymers of high molecular weight will normally resist enzymes, unless those few enzymes which have been shown to be capable of degrading

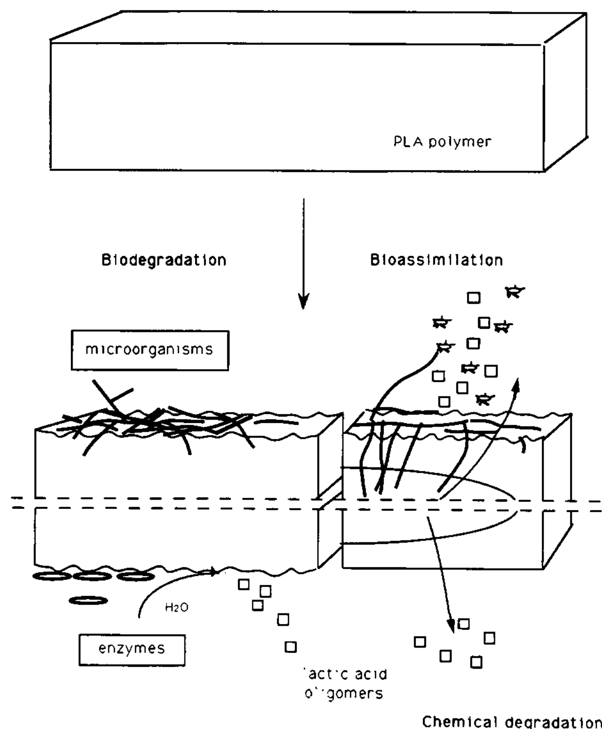

Figure 9 Proposed degradation mechanisms of PLA in the bulk state.

high molecular weight PLA[17,18] are present. So far, the probability is rather low and such degradation was not observed in this investigation. In any case, abiotic hydrolytic degradation will occur. Whether homogeneous or heterogeneous degradation is observed will then depend on the size of devices and on the ability of the surrounding water-containing phase to accommodate (liquid) or not accommodate (vapor) assimilable oligomers. Of course, situations will have to take into account all other factors that are already known to affect the abiotic hydrolytic degradation of aliphatic polyesters of the PLA type.[19] Sooner or later, water-soluble oligomers will be released, which will make the bioassimilation of the predegraded PLA matrix feasible, whenever these oligomers are bioavailable. This will occur earlier for racemic amorphous oligomers than for semicrystalline ones. Under any conditions, PLA polymers will be degraded and assimilated, since the ultimate degradation products (L- and D-lactic acids) have been shown to be metabolized by some common microorganisms. With the inner formation of assimilable compounds and their late release, fungi present in the surrounding aqueous environment can be activated to penetrate the partially degraded mass, to take advantage of the substrates therein, and thus to turn the abiotic degradation mechanisms into a biotic one. This will always occur after a rather long lag time. Whereas *F. moniliforme* managed to reach the substrate materials present within a partially degraded matrix, it seems that *P. putida* was only able to assimilate substrate materials diffused within the surrounding aqueous medium. Let us recall that enzymes such as proteinase K, known as able to directly attack high-molecular-weight PLA, will primarily cause surface erosion, right after being placed in contact with the enzyme-containing aqueous medium.

CONCLUSION

In this study, it is shown that racemic lactic acid polymers in the bulk state are bioassimilable. This means that in the first stages of degradation, only chemical hydrolysis proceeds, resulting in by-product formation. Microorganisms are then able to totally assimilate these products. It is also shown that some microorganisms with the ability to assimilate these kinds of low-molecular-weight PLA exist in nature, thus confirming that PLA polymers are environmentally friendly materials.

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