J Polym Environ (2012) 20:381–387
DOI 10.1007/s10924-011-0387-0

ORIGINAL PAPER

Microbial Degradation of Poly(3-Hydroxybutyrate-co-4-Hydroxybutyrate) in Soil

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Published online: 29 October 2011
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Abstract The microbial degradation of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (P3/4HB) copolymers with different 4HB molar fraction were investigated in soil for 60 days. In the degradation process, changes in weight loss and molecular weight were periodically measured to determine the biodegradability; the surface morphology also was observed using scanning electron microscopy and polarizing optical microscopy. The results showed that the rate of degradation of P3/4HB depends strongly on its crystallinity and surface morphology. Enzymatic degradation, which proceeded via surface erosion mechanisms, was observed mainly during the degradation period in soil. The amorphous interspherulitic regions and crystal center were prone to degrade.

Keywords Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) · Soil degradation · Weight loss · Molecular weight · Surface morphology

Introduction

The growing interest in the development of biodegradable plastics possessing properties similar to those of thermoplastics has focused attention on bacterial polyhydroxyalkanoates (PHA) [1–3]. PHA is one class of biodegradable and biocompatible polyesters. It is accumulated as a carbon and energy storage materials in various microorganisms under conditions of limited nutrition and in the presence of excess carbon [4].

Poly(3-hydroxybutyrate) (PHB) is the most common type of PHA [5] and its material properties such as stiffness, brittleness, high degree of crystallinity, and high melting point (175 °C) are comparable to conventional plastics [4, 6–8]. It can be degraded to water and carbon dioxide (or methane under anaerobic conditions) by microorganisms in various environments [9]. PHB is already produced on an industrial scale but it shows some drawbacks, such as high production costs and brittleness that significantly hinder the widespread application of PHB. Besides, PHB shows poor thermal stability at temperatures above the melting point [10, 11].

Attempts have been made to improve the properties of PHB by copolymerization with other monomers. The copolymerization of 3HB with 3HV (3-hydroxyvalerate; PHBV), 3HP (3-hydroxypropionate; PHBP), 4HB (4-hydroxybutyrate; P3/4HB), and 3HHx (3-hydroxyhexanoate; PHBHHx), has been carried out successfully by microbial fermentation [12]. These PHB-based copolymers show a wide range of physical properties depending on the chemical structure of the comonomer units as well as the comonomer composition [13].

PHBV is the first commercialized bacterial copolyester under the trade name of Biopol [14]. The properties of PHBV vary with the percentage of 3HV. But the 3HB and 3HV components can induce co-crystallization due to the similarity in lattice indexes of the respective unit cells [15–17], which leads to the poor impact toughness of PHBV with 3HV molar fraction ranging from 0 to 95 mol% [18].

Whereas P3/4HB shows substantially reduced crystallinity and melting temperature compared to the PHB homopolymer [19], both the mechanical properties and melt temperature can be adjusted by changing the 4HB

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molar fraction. Consequently, P3/4HB has received considerable attention as an interesting candidate for biomaterials [20].

In contrast to traditional polymers, a significant characteristic of PHB is its biodegradability in natural environments [21]. The biodegradation of PHB and PHBV has been assessed in many ecosystems, such as soil [22, 23], activated sewage sludge [24], sea water [25, 26] and so on. Some PHB depolymerase have been isolated, purified, and characterized [27] and, during the past decade, there have been many intensive studies of the biodegradation of these polymers using purified PHB depolymerase [28].

In addition, many researchers have performed studies on the mechanism of biodegradation [29–31]. Based on scanning electron microscopy (SEM) observations, two modes of degradation were observed on the surface of PHB films: (1) preferential degradation in amorphous regions with crystalline lamellae remaining unchanged and (2) proliferation of microorganisms on the film surface, forming spherical holes in both the amorphous and crystalline parts of the polymer. The spherical holes were due to colonization by the degrading bacterium [32]. It is believed that the surface of the PHB film is an essential growing field for the PHB degrading microorganisms.

Doi [33] studied the hydrolysis and enzymatic degradation of PHB and copolymers with 4HB or 3HV by using depolymerase of the same bacterium. It was concluded that the copolymer containing 4HB was degraded more rapidly than PHB and PHBV. Similar data on the breakdown of these polymers were obtained in soil and activated sludge [34].

The effects of 4HB units on the rate of enzymatic degradation of P3/4HB films were studied in the aqueous solution of an extracellular PHB depolymerase from *Alcaligenes faecalis* [35]. The rate of enzymatic degradation increased significantly with an increase in the 4HB fraction due to a decrease in crystallinity.

The biodegradation behavior of PHB is affected by several factors, such as monomer composition, physical state (i.e. granule suspension, solvent or melt cast films, single crystals), crystallinity and crystal size, morphology and temperature [28, 36, 37].

Though a few research groups have conducted partial studies on the biodegradation behavior of P3/4HB, few of these have considered the effect of surface morphology on polymer degradation and spherulitic morphology in the course of soil degradation.

In this study, the degradation of P3/4HB copolymers with different 4HB molar fractions were studied in soil under laboratory conditions. Degradation was estimated from weight loss, molecular weight change and surface erosion. The influences of time, monomer composition and surface morphology on the rate of degradation were investigated.

Experimental

Materials and Purification

Samples of P3/4HB containing 5 mol% (P3/5%4HB), 7 mol% (P3/7%4HB), 10 mol% (P3/10%4HB), 15 mol% (P3/15%4HB) and 20 mol% (P3/20%4HB) 4HB, were supplied by Green Biological Material Co. Ltd. from Tianjin, China.

Each sample (1 g) was dissolved in 50 mL of chloroform and filtered by vacuum filtration at room temperature to remove any insoluble fraction or impurities to obtain a clear solution. Pure P3/4HB was finally obtained by precipitating the solution into absolute ethanol, filtering and drying under a vacuum at 60 °C for 24 h to remove residual solvent and moisture [38].

Film Preparation

Films were prepared using a solution-casting method. About 1.5 g of pure P(3HB-co-4HB) was dissolved in 50 mL of chloroform. The P3/4HB solution was poured onto a glass plate and allowed to evaporate slowly at room temperature to form a film. The film was dried under vacuum to a constant weight and kept at room temperature for over a week to reach equilibrium crystallinity [28]. The thickness of the resulting films was 0.1–0.3 mm.

Soil Degradation

P3/4HB films with an initial weight of about 10 mg and an initial dimension of approximately 10 mm × 10 mm were buried in soil obtained from a garden. The soil was controlled at room temperature. Water was added periodically to maintain certain water content at about 20% during the soil degradation. The films were periodically removed, washed with distilled water three times and dried in vacuum to a constant weight before analysis.

Analysis and Characterization

Changes in the weight of degraded samples (%) were determined using an electronic balance JJ500. The results obtained for clean and dried samples after biodegradation experiments were compared with those of the respective samples before biodegradation.

Degraded samples were dissolved in chloroform and the intrinsic viscosity of the solution was measured at 30 °C using an Ubbelohde type capillary viscometer. The molecular weight (M_η) was calculated according to the Mark Houwink relationship:

$$[\eta] = KM_\eta^\alpha \quad (1)$$

where [η] is the intrinsic viscosity. For PHB in chloroform at 30 °C, $K = 1.18 \times 10^{-4}$ and $\alpha = 0.78$ [38].

Surface morphology of the P3/4HB films was observed using a JEOL JSM-6380LV SEM with an accelerating voltage of 20 kV. All of the samples were gold coated before observation. Sample morphology was also examined using a XPR-500D polarizing optical microscopy (POM) after crystallizing the samples at 90 °C.

Results and Discussion

In soil, the surfaces of the samples initially became dull and then became progressively rougher the soil exposure time increased, with degradation taking place on all surfaces. Figure 1 illustrates the weight loss of P3/4HB films biodegraded in the soil as a function of biodegradation time. Weight loss started after a lag period and all samples achieved more than 50% weight loss after 60 days in the soil.

There is a biphasic degradation pattern for all the samples: (1) lag period, with samples initially degrading at a slow rate with both the rate of degradation and length of the initial phase varying with 4HB content, which reflects the adherence of microorganisms on the surface of films [4], (2) Once the initial (slow) degradation phase is complete, however, the samples degrade much faster, and the rate of degradation depends upon the copolymeric composition as well. The length of the lag period (weight loss $< 0.5\%$), total weight loss and the degradation rate constants k_e (expressing the rate of weight loss [W] per unit of time [t]), defined by the formula $W = k_e t$, are summarized in Table 1.

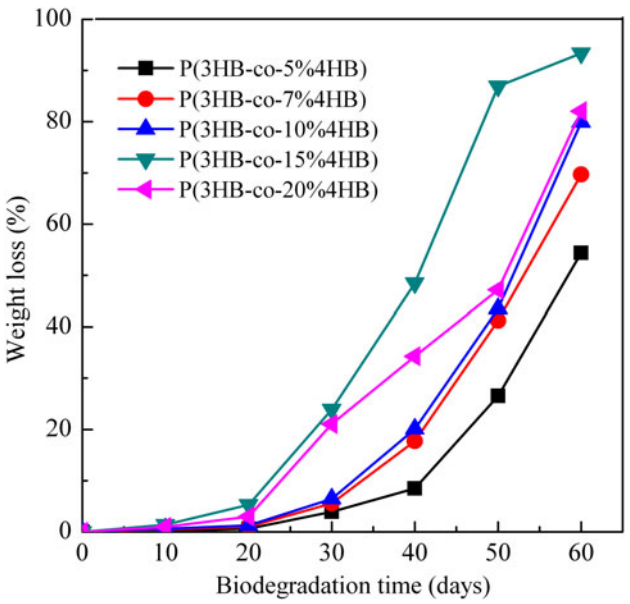

Fig. 1 Weight loss of P3/4HB films biodegraded in soil as a function of biodegradation time

It also can be seen that the rate of soil degradation increases with an increase in the molar fraction of 4HB, and the highest rate is observed at 15 mol% 4HB. Thus, the presence of 4HB units (at 5–15 mol%) in the PHB can accelerate soil degradation. However, increasing the 4HB content to 20 mol% causes a small decrease in the degradation rate relative to P3/15%4HB films.

The degradation rate of P3/4HB has been shown to be dependent on sample crystallinity [35], with crystallinity of the P3/4HB comonomer decreasing with an increase in 4HB content [39]. Thus the accelerated soil degradation of P3/4HB films containing 5–15 mol% 4HB is most likely a result of decreased crystallinity. Conversely, at a 4HB content of 20 mol%, the molecular chain segments are so flexible that they are have a tendency to form uniform and compact structures. This improves the packing density of molecular chains and results in the surfaces of the P3/20%4HB films becoming denser and smoother.

Comparison of SEM photographs of the samples before and after a 60 day test exposure in the soil revealed that surface morphology could be a contributing factor in the biodegradation of the comonomers (Fig. 2). There are large differences between films, both before and after degradation. In general, surface roughness decreased with increasing mol-fraction of 4HB (Fig. 2I), with the surface of the P3/20%4HB film [Fig. 2e(I)] being flatter and smoother than that of the P3/15%4HB film [Fig. 2d(I)]. Wang et al. [40] suggested that a rough surface allows bacteria and water molecules to contact the polymer chains, thus accelerating degradation. Conversely, contact is reduced on a smooth surface, thus retarding degradation.

These results indicate that the rate of soil degradation of P3/4HB films is influenced not only by the degree of crystallinity but also by the surface morphology of the P3/4HB films. The P3/15%4HB film appears to have a optimal combination of low crystallinity and surface roughness that yields enhanced degradation characteristics.

The surface morphologies of P3/15%4HB films before and after soil exposures of 20, 40 and 60 days are shown in Fig. 3a–d, respectively. It can be seen that during the course of soil exposures, surface erosion (degree of roughness) increased as the films became more degraded.

Before soil exposure, the surface of the P3/15%4HB film was relatively smooth. However, after 20 days in the soil the surface of the sample was eroded and covered with a significant number of holes. After another 20 days, the number and size of holes increased dramatically. After 60 days, holes occupied most of the film surface, often aggregating to form large eroded regions bordered by less eroded areas. P3/15%4HB films buried in the soil for periods >60 days were so severely degraded and fragmented that recovery from the soil was not possible.

Table 1 Quantitative data on the weight loss curves of P3/4HB copolymers

Samples	Biodegradation time (days)	Length of the lag period (days)	Total weight loss (%)	Degradation rate constants k_e
P3/5%4HB	60	17	54.38	0.556
P3/7%4HB	60	10	69.69	0.785
P3/10%4HB	60	8	79.91	0.879
P3/15%4HB	60	4	93.39	1.398
P3/20%4HB	60	5	82.03	1.028

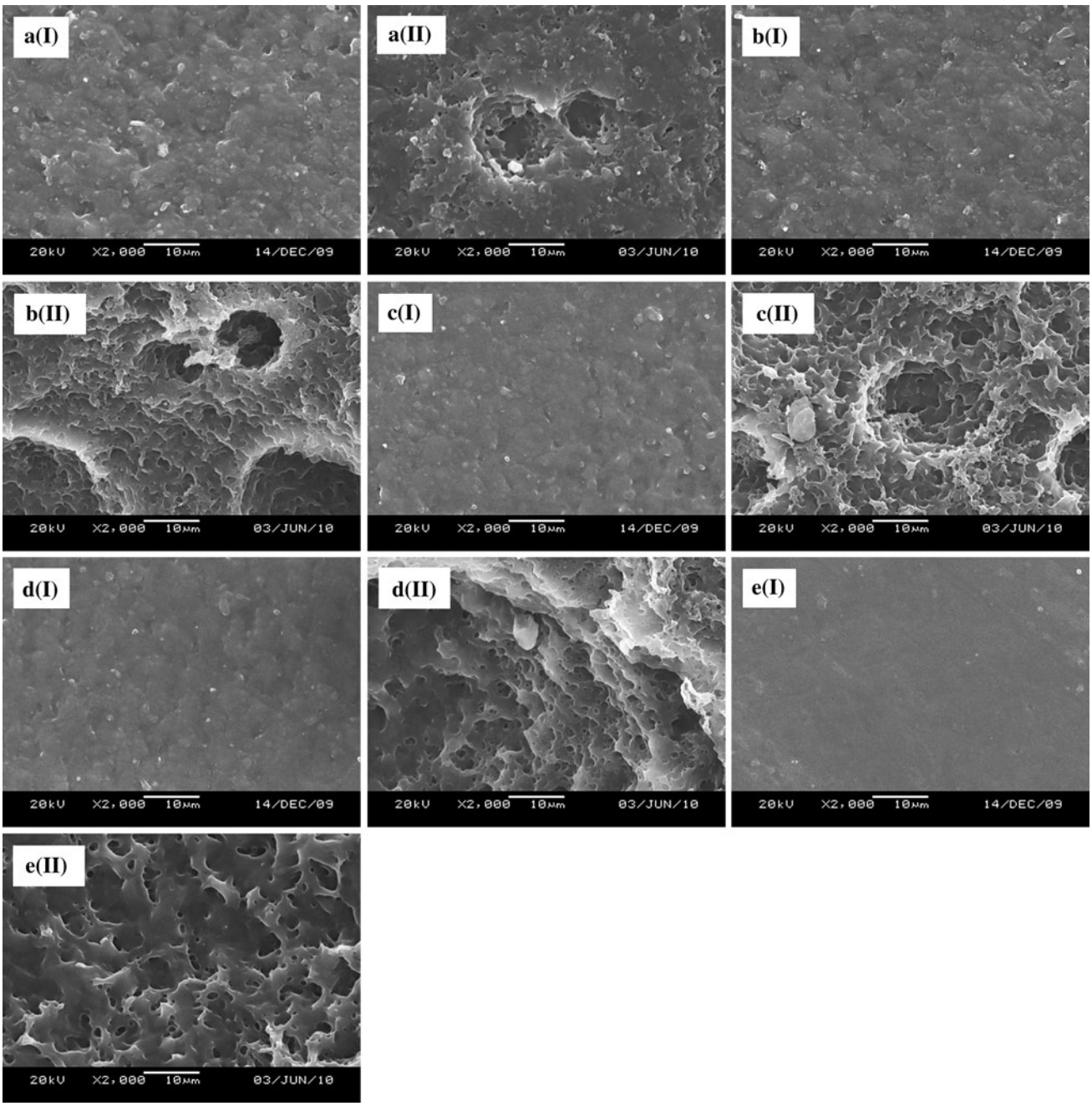

Fig. 2 SEM photographs of P3/4HB films prior to (*I*) and after (*II*) biodegradation in soil: **a** P3/5%4HB, **b** P3/7%4HB, **c** P3/10%4HB, **d** P3/15%4HB, **e** P3/20%4HB

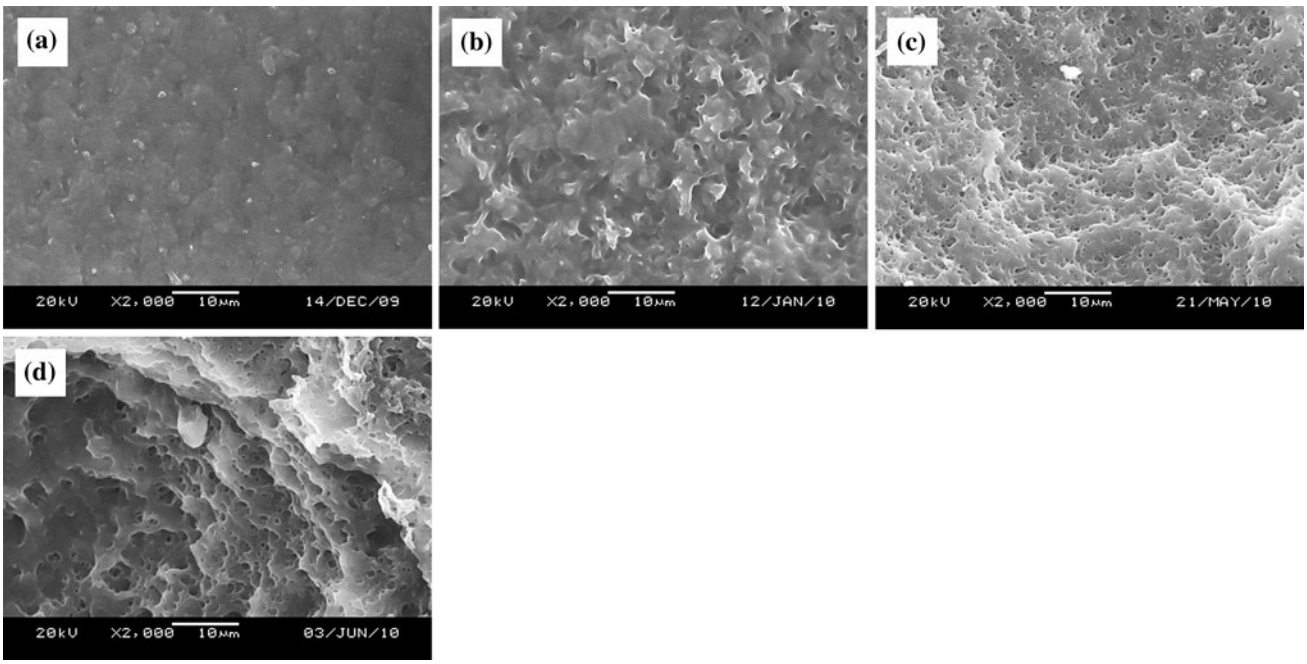

Fig. 3 SEM photographs of the surface of P3/15%4HB films at different biodegradation time: **a** 0 days, **b** 20 days, **c** 40 days, **d** 60 days

Figure 4 shows the change in molecular weight (M_η; calculated using Eq. 1) of P3/4HB, as a function of biodegradation time. It is evident from Fig. 4 that the M_η of the bulk P3/4HB decreases only slightly during degradation in the soil. In general, two types of degradation mechanism are possible for polymers degraded in soil: hydrolytic and enzymatic [28]. Doi et al. [33] found that the molecular weight of PHBV did not change during enzymatic degradation by PHB depolymerases, but did decrease during hydrolytic degradation at 55 °C in a phosphate buffer (pH 7.4). Our result indicates that polymer chain cleavage occurred only at the point of microbial attack. The cleaved molecules were then either mineralized to produce new cells and respired CO_2, leached into the soil, or lost during washing. As a result, the remaining bulk polymer shows only a small (9–16%) decrease in molecular weight. These data suggest that biodegradation of P3/4HB in soil takes place mainly via an enzymatic process with only a minor influence of hydrolysis [41].

To further investigate the biodegradation behavior of P3/4HB films, polarization photomicrographs of P3/10%4HB were taken before and after biodegradation in soil (Fig. 5). The samples were crystallized at 90 °C before being buried in the soil; the samples were supported on glass slide, thus only one side was exposed to the soil.

Before the soil exposure, spherulites with a maximum radius of approximately 100 μm were observed for all P3/10%4HB samples (Fig. 5a). After 10 days of biodegradation, circular holes were observed at the impinging fronts of neighboring spherulites (Fig. 5b). In contrast, the spherulite

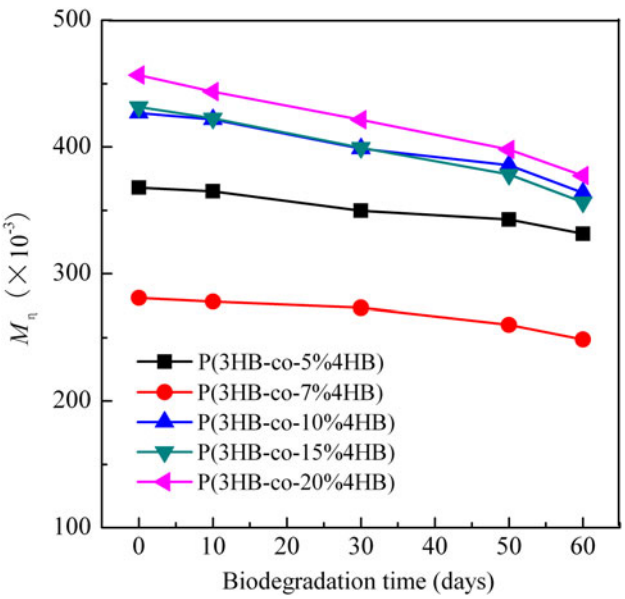

Fig. 4 Molecular weight change of P3/4HB biodegraded in soil as a function of biodegradation time

center exhibits less degradation, keeping the spherulitic morphology almost intact. This indicates that the amorphous interspherulitic regions are prone to microbial degradation. This phenomenon can be attributed to preferential degradation of highly disordered material accumulated at the growing fronts of impinging spherulites. That is, during film solidification from the melt, it is quite reasonable to assume that polymer chain segments that are unable to crystallize on the lamellar fronts of either of two spherulites

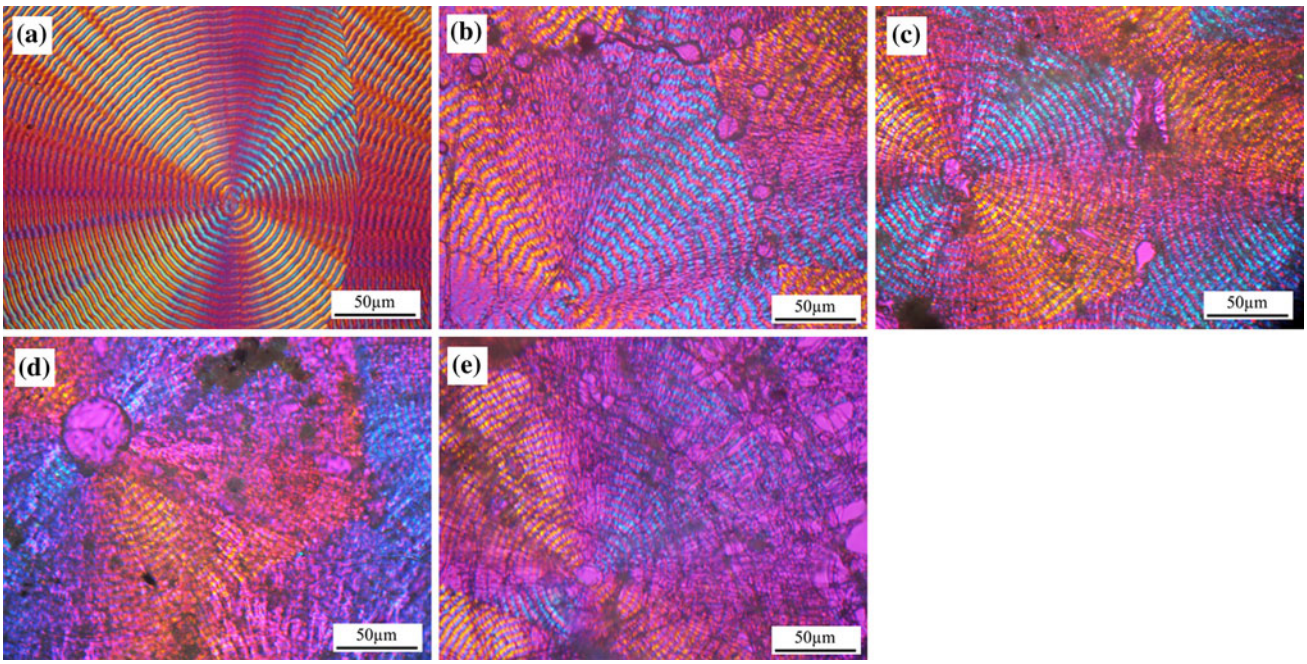

Fig. 5 POM photographs of P3/10%4HB films at different biodegradation time: **a** 0 days, **b** 10 days, **c** 20 days, **d** 30 days, **e** 40 days

growing in opposite directions remain trapped in disordered conformations and constitute an amorphous fraction liable to selective enzymatic attack [27].

After a total of 20 days in the soil, circular holes also were observed at the spherulite center (Fig. 5c). Circular cavities at the center of spherulites in PHB films subjected to microbial degradation by *Alcaligenes paradoxus* and *Comamonas testosteroni* previously were reported by Nishida and Tokiwa [42]. However, whereas the center of melt-crystallized PHB spherulites often show cracks that form and concentrate at the spherulite core during crystallization [43], we observed no cracks in the P3/4HB spherulites (Fig. 5c).

Using real-time, small-angle light scattering, Takashi [44, 45] found that at the early stages of spherulitic crystallization, an isotropic embryo (i.e., a highly disordered crystalline domain with low crystallinity) formed. The spherulite then developed from this central nucleus by increasing the size and the degree of ordering. Extrapolation of such a model to melt-crystallized P3/4HB suggests that at the initial stages of spherulitic growth, the degree of order in the center is lower than in the rest of the polycrystalline aggregate. If such a situation does not change appreciably with time, the spherulite core should be particularly liable to enzymatic attack as it becomes exposed at the sample surface during the course of biodegradation. Thus, our observation that the holes tend to be formed on a boundary line between spherulites and at a crystal center, can be explained by the assumption that chain segments in a disordered conformation are more susceptible to enzymatic attack than segments in an ordered crystalline structure.

After 40 days in the soil, the P3/10%4HB film shows significant biodegradation with part of the spherulitic morphology destroyed by the microorganisms.

Conclusions

The degradation in soil of P3/4HB copolymers with different 4HB molar fractions was investigated using capillary viscometry, SEM and POM to monitor weight loss and changes in molecular weight and surface morphology.

Both crystallinity and surface morphology play important roles in the degradation of P3/4HB. The biodegradation of P3/4HB films generally increased as the mol-fraction of 4HB increased from 5 to 15%, further increasing the mol-fraction of 4HB to 20%, however, resulted in a decrease in biodegradability. It appears that the advantages of low crystallinity and surface roughness are optimized in P3/15%4HB, yielding films that exhibit the best biodegradation characteristics.

Overall weight loss by the P3/4HB films was more significant than the change in molecular weight, indicating that biodegradation of solution-cast films in soil occurs mainly via an enzymatic process. The SEM and POM photographs show the development of holes in the films during the soil exposure; these holes tend to be formed at the boundary between spherulites and at crystal centers.

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