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Nanocomposites of poly(3-hydroxybutyrate)/organomodified montmorillonite: Effect of the nanofiller on the polymer's biodegradation

Elpiniki Panayotidou,[1,2] Apostolos Baklavaridis,[1] Ioannis Zuburtikudis,[1,3] Dimitris S. Achilias[2]

[1]Department of Mechanical and Industrial Design Engineering, TEI of Western Macedonia, 50100 Kozani, Greece

[2]Department of Chemistry, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

[3]Department of Chemical and Petroleum Engineering, United Arab Emirates University, Al Ain, UAE

Correspondence to: D. Achilias (E-mail: axilias@chem.auth.gr) and I. Zuburtikudis (E-mail: ioannis.z@uaeu.ac.ae or izub@teikoz.gr)

ABSTRACT: Poly(3-hydroxybutyrate) (PHB) is a biopolymer that can be degraded by extracellular PHB depolymerase. This enzyme is secreted by various microorganisms, but bacterial PHB depolymerases are the most widely studied. The biodegradability rate depends on various factors. By controlling them, the biodegradability rate can change and be customized, and thus, the applications of the polymer can increase and become more diverse. In this work, the role of organomodified montmorillonite (OMMT) on PHB biodegradation was investigated. Using the melt-mixing method, nanocomposites of PHB and OMMT as the nanofiller were prepared. The enzyme was isolated from the fungus *Penicillium pinophilum* and the enzymatic degradation was studied for both pure polymer and its nanocomposites. It was found that, after 25 days of enzymatic degradation, the mass loss was very low, while the polymer's average molecular weight as measured by gel permeation chromatography was significantly reduced (more than 50%). Additional peaks corresponding to PHB oligomers (from pentamers to nonamers) appeared after biodegradation. This behavior was observed for pure PHB and the hybrid materials. Scanning electron microscopy imaging of the biodegraded surfaces and analysis of these images showed that the higher amount of nanoclay (10 wt %) resulted in larger biodegraded area of the specimens. The results presented here demonstrate that the presence of the nanoclays enhances the biodegradation rate of pure PHB polymer and provide quantitative data for the biodegradation of PHB/organoclay hybrid materials. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41656.

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INTRODUCTION

Biodegradable polymers are environmental friendly and with properties comparable to those of the conventional polymers. They qualify for many diverse applications, such as in food packaging and biomedicine,[1–20] and their use is constantly increasing. They constitute a good answer to the plastics-waste disposal problem and its subsequent environmental impact.

Polyhydroxyalkanoates (PHAs) are a class of biodegradable polymers that are being produced from renewable resources. The most widely studied member of PHAs is polyhydroxybutyrate (PHB). PHB is an aliphatic polyester with an *R* configuration and physical properties similar to those of polypropylene (PP), a characteristic that makes it a very promising candidate for substituting PP, an oil-derived synthetic polymer. In addition of being biodegradable, PHB is biocompatible.[21,22] However, it is brittle and has low thermal stability at processing temperatures slightly higher than its melting point. Bionanocomposites, consisting of a biopolymer reinforced with fillers having at least one dimension in the nanoscale, improve the mechanical and thermal properties of the pristine biopolymer. For that reason, nanocomposites with PHB as the polymer matrix are considered to be a promising solution to the drawbacks of neat PHB.[1,23–28]

PHB is biodegraded in all environments and the final products are water and carbon dioxide.[29,30] PHB can be degraded either intracellular or extracellular by PHB depolymerase that is secreted by various microorganisms.[31]

Extracellular PHB depolymerase has been studied more than intracellular PHB depolymerase and plays an important role in the metabolism of PHB in the environment. There are several extracellular PHB depolymerases that are secreted by various fungi and bacteria,[29,32] but all of them have some common properties:

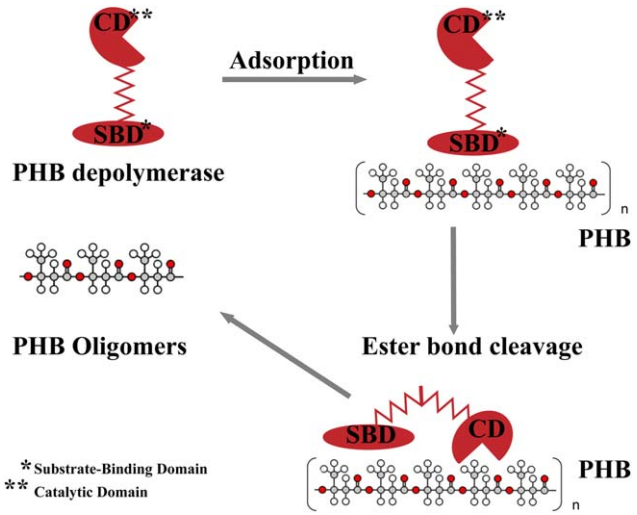

Figure 1. The two steps of PHB biodegradation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

1. small molecular weight, below 100 kDa,
2. high stability under various conditions such as pH, temperature, and ionic strength,
3. optimal pH in the alkaline range (7.5–9.8), only the depolymerases of *Pseudomonas pickettii* and of *Penicillium funiculosum* have pH optima at 5.5 or 6.0, respectively,
4. pronounced affinity to hydrophobic materials,
5. most of them are inhibited by serine hydrolase inhibitors such as diisopropylfluorophosphate or acylsulfonyl compounds.[33–35]

Extracellular PHB depolymerases are composed of a C-terminal substrate binding domain, a N-terminal catalytic domain, and a linker region connecting the two domains.

The enzyme binds to the polymer surface with the binding domain and the catalytic domain hydrolyzes the ester bonds. The catalytic domain contains the triad Serine-Histidine-Aspartate, which are the catalytic sites of the domain.[12,35,36] As it is shown in Figure 1, the enzymatic degradation of polymers is a two-step heterogeneous process: Adsorption of the enzyme onto the surface of the polymer and catalytic ester-bonds cleavage by the active site of the enzyme.[36,37]

Extracellular PHB depolymerase from *Alcaligenes faecallis* has been studied extensively and it has been found that the enzyme mainly hydrolyzes the second and third ester bonds from the hydroxyl terminus. The ester linkage is attacked by the oxygen atom of the serine side-chain, which is the nucleophile and is supported by the imidazole ring of histidine. The carboxyl group of aspartate stabilizes the positive charge of histidine.[34]

The enzyme is specialized in the PHB enzymatic hydrolysis. However, its biodegrading action depends on various factors related to the polymer itself. The most important of these are:

(i) the stereoregularity of the polymer (the enzyme hydrolyzes polymer with only *R*-configuration), (ii) the crystallinity (the rate of the biodegradation decreases as the degree of crystallinity increases), (iii) the molecular mass (high molecular mass polymers have small rate of biodegradation, in general), and (iv) the monomeric composition of PHB.[34]

The polymer's biodegradation can be controlled by manipulating all or some of these factors. However, a customized control of the polymer biodegradation may broaden its application field, and therefore, it is a very interesting problem that is drawing the attention of many researchers.

Kasuya *et al.* studied the adsorption kinetics of extracellular PHB depolymerase from *A. faecallis* on the surface of poly(3-hydroxybutyrate) (P3HB), poly(3-hydroxypropionate) (P3HP), poly(4-hydroxybutyrate) (P4HB), poly[(*S*)-2-hydroxypropionate] (P2HP), and poly(6-hydroxyhexanoate) (P6HH) films. PHB depolymerase was bound on the surface of all the films, but it hydrolyzed the surface of P3HB, P3HP, and P4HB, only.[38] Abe and Doi investigated the rate of degradation of pure PHB and its copolymers by PHB depolymerase from *A. faecallis* and the results showed that the copolymers are biodegraded more rapidly than pure PHB.[39] Wada *et al.*[40] investigated the biodegradability of PHB using the radiation graft copolymerization method. They concluded that the biodegradation of PHB was increased by the introduction of hydrophilic 2-hydroxyethyl methacrylate (HEMA). The disadvantage of this method is that the graft chains remained without being degraded. Ryou *et al.* studied the effects of plasma treatment by CF_3H and O_2 on the biodegradability of PHB and P(3HB-*co*−3 HV). The CF_3H plasma exposure decreases the rate of biodegradation of the materials, but the O_2 plasma exposure has no significant change in the biodegradation rate.[41]

Yoshie *et al.*[42] investigated the enzymatic degradation of PHB/low-molecular-weight-additives mixtures by extracellular PHB depolymerase isolated from *A. faecalis* T1. They found the presence of the additives at low concentrations enhances the rate of biodegradability of the pure polymer.[42]

In the present work, nanocomposites of PHB with organomodified montmorillonite (OMMT) have been prepared by the melt-mixing method. Extracellular PHB depolymerase was isolated from the fungus *Penicillium pinophilum* (the process is reported in another work that has been submitted elsewhere) and the effect of the nanofiller in the polymer's biodegradation was investigated and is reported here.

EXPERIMENTAL

Materials

Poly[(*R*)-3-hydroxybutyric acid] (PHB) was supplied by Sigma–Aldrich Chemical Co. and was used as received. Sodium montmorillonite (NaMMT) with a cation exchange capacity CEC = 92.6 meq/100 g was purchased from Southern Clay Products (TX). Octadecylamine ($C_{18}H_{37}NH_2$) (purity> 99% and MW = 269.52), HCl (37% wt/vol), ethanol absolute, chloroform, and tris(hydroxymethyl)aminomethane (Tris) were supplied by Sigma–Aldrich Chemical Co.

Modification of Sodium Montmorillonite

The natural montmorillonite was dried for 24 h at 80°C before use. Its modification took place via a cation exchange reaction between sodium montmorillonite and octadecylammonium salt.

In a 1 : 1 solution of ethanol and water, at 75°C, the suitable quantity of octadecylamine equivalent to 1.50 times the CEC of inorganic material, and an equivalent amount of HCl were dissolved. The inorganic material was dispersed in water at 75°C and then, this aqueous suspension (3 wt %) was added to the alkylamine solution. The mixture was stirred vigorously for 24 h at 75°C. Applying vacuum filtration, the precipitate was collected and then, it was rinsed with a 1 : 3 solution of ethanol and water that had been heated at 45°C until a $AgNO_3$ test verified the absence of chloride. The final product (organomodified montmorillonite or C_{18}MMT) was placed in a vacuum drying oven, at 60°C, until all the absorbed water was evaporated. Consequently, the organo-modified clay was stored in a desiccator for use.

Production of the PHB Nanocomposites

The hybrid materials were produced by the melt mixing method in a co-rotating twin-screw micro-extruder/compounder (Haake MiniLab^{TM}). The isothermal drum temperature was kept at 175°C, while constant nitrogen flow was employed to avoid thermal degradation of the polymer. The mixture was mixed for 3 min at a screw speed of 130 rpm until the torque and the mixture viscosity became constant. Hybrids with loadings of 1, 3, 5, and 10 wt % were prepared.

Study of Crystallinity

The degree of crystallinity was determined by differential scanning calorimetry (DSC). The DSC measurements were done using a Diamond Perkin Elmer instrument and under nitrogen flow. Thermographs were obtained by heating the samples from −30 to 190°C at a heating rate of 20°C/min, and keeping the sample material at the temperature of 190°C for 1 min. The degree of crystallinity was determined by the following equation[43]

$$X_c(\%) = \frac{\Delta H\left(m_c/m_p\right)100}{\Delta H_0} \quad (1)$$

where:

X_c is the degree of crystallinity (%),

ΔH is the melting enthalpy measured by the heating experiments,

ΔH_0 is the theoretically melting enthalpy of PHB 100% crystalline, $\Delta H_0 = 146$ J/g,43

m_c is the mass of the nanocomposite material,

m_p is the mass of PHB in the nanocomposite.

Biodegradation Study

Extracellular PHB depolymerase was purified and isolated from *P. pinophilum* (ATCC 9644). The enzyme has molecular weight of about 35 kDa and disintegrates PHB with (*R*) configuration only.

Films of PHB and its nanocomposites were prepared by thermal pressing the materials at 175°C under 200 atm for 3 min. Before using the thermo-press, the quantity of the materials, about 0.05 g, had been heated in a vacuum drying oven at 175°C for 3 min. The produced circular films had a width of 0.1 cm and a diameter of 1 cm.

Enzymatic solutions with different enzyme concentrations (mg enzyme/mL buffer solution) such as 1 mg enzyme/mL, 2 mg enzyme/mL, 4 mg enzyme/mL, and 8 mg enzyme/mL, were tested for the determination of the optimal biodegradation results. The best results were given by the 4 mg enzyme/mL concentration.

The films of pure PHB and its nanocomposites were incubated into 1 mL buffer solution, Tris-HCl (pH = 7.4), and with 4 mg of enzyme, for 2 days in an oven at 37°C. After 2 days, the solution was replaced by a new one and the system (film and solution) was placed in the oven for 2 more days. After that, the films were rinsed with de-ionized water and placed in a vacuum drying oven at 37°C for 24 h. The mass of the films before and after the interaction of the enzyme with the films was measured, and the differences in weight were used to deduce the biodegradation rate of the film.

Simultaneously, the molecular weight distribution (MWD) and the average molecular weights of PHB and its nanocomposites were determined by gel permeation chromatography (GPC). A Polymer Laboratories model PL-GPC 50 Plus instrument was used; it included an isocratic pump, a differential refractive index detector, and three PLgel 5 μm MIXED-C columns in series. The samples were dissolved in chloroform and solutions with concentration 0.1% wt/vol were produced. Before injecting into the GPC instrument, the samples were filtered using Whatman Nylon Membrane Filters with 0.45 μm pores to remove any insoluble fractions or clay additives. The sample volume injection was 100 μL. Tetrahydrofuran (THF) was used as an eluent. The flow rate was 1 mL/min and the analysis temperature 30°C. Narrow-molecular weight polystyrene standards, ranging from 200 to 2,000,000, were applied for the calibration. The MWD and average molecular weights were determined before the samples were emerged into the enzyme solution and the measurements were repeated after 10, 20, and 25 days from the first immersion into the enzymatic solution.

The universal calibration procedure was used to transform the elution volume to polymer molecular weight using the equity of the hydrodynamic volumes:

$$K_X M_X^{1+\alpha_x} = K_{PS} M_{PS}^{1+\alpha_{PS}} \quad (2)$$

where K_{PS} and α_{PS} are the Mark–Houwink constants for polystyrene in tetrahydrofuran ($K_{PS} = 14.1 \times 10^{-5}$, $\alpha_{PS} = 0.7$) and K_X and α_X are the Mark–Houwink constants for PHB in chloroform ($K_X = 1.18 \times 10^{-4}$, $\alpha_X = 0.78$).[21]

In addition, the morphology of surface of the PHB films before and after their exposure to the enzymatic solution was investigated by using scanning electron microscopy (SEM). All images were recorded using a JEOL 6610 LV SEM. Prior to the SEM observations, all samples were coated with gold (Au) using a Au sputtering device (Quorum 150R S) in order to eliminate charging under the electron beam. The percentage of the surface that has been disintegrated by the enzyme was determined by image analysis of the SEM micrographs using the appropriate software (Image J).

RESULTS AND DISCUSSION

Degree of Crystallinity

The melting enthalpy and degree of crystallinity of pure PHB and its nanocomposites were determined using DSC and the

Table I. Melting Enthalpy, ΔH, and Degree of Crystallinity, X_c, of Pure PHB and its Nanocomposites

Sample	Degree of crystallinity (X_c) (%)	ΔH (J/g)
PHB	64.5	94.25
PHB+1% C18MMT	59.1	85.39
PHB+3% C18MMT	55.3	78.30
PHB+5% C18MMT	54.2	75.23
PHB+10% C18MMT	51.1	67.17

results are illustrated in Table I. As shown, the degree of crystallinity of the neat polymer is high enough, but it is reduced by the presence of the nanofiller. The nanocomposite with concentration 10 wt % C_{18}MMT has the lowest degree of crystallinity.

Study of Biodegradation

By measuring the mass loss that took place during immersion of the samples into the enzymatic solution every five days, conclusions about the rate of biodegradation can be obtained. There are several reports in literature on PHB biodegradation by bacterial extracellular depolymerases.[36,44] Canetti *et al.* studied the biodegradation of PHB by PHB depolymerase from *Aureobacterium saperdae* and observed a decrease in biodegradation rate by increasing the degree of crystallinity.[45] Hsieh *et al.* investigated the effect of hydrophilic and hydrophobic monomers grafted onto PHB powder and concluded that the monomer with the hydrophilic chain improved PHB's biodegradability.[46] The biodegradation of PHB and its nanocomposites with OMMT in compost media has been studied by Maiti *et al.*, who found that the presence of the nanoclay enhances the biodegradation rate of the pure polymer.[25]

In this work, the enzyme was isolated from the fungus *P. pinophilum*, which has not been extensively studied.[47] Figure 2 presents the results from the biodegradation studies.

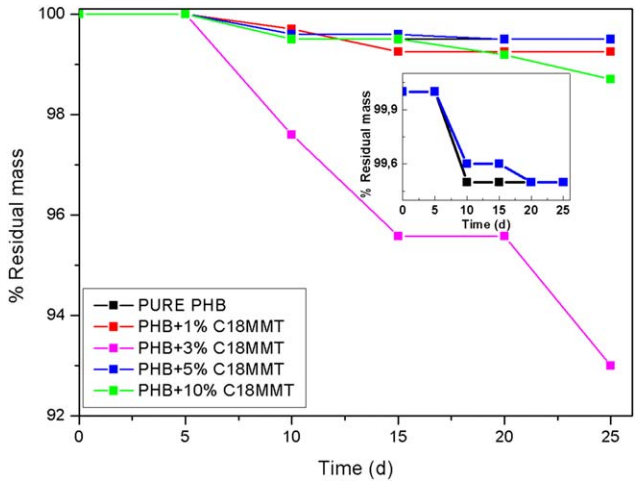

Figure 2. Residual mass (%) of pristine PHB and its nanocomposites after 25 days of exposure to the enzymatic solution. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

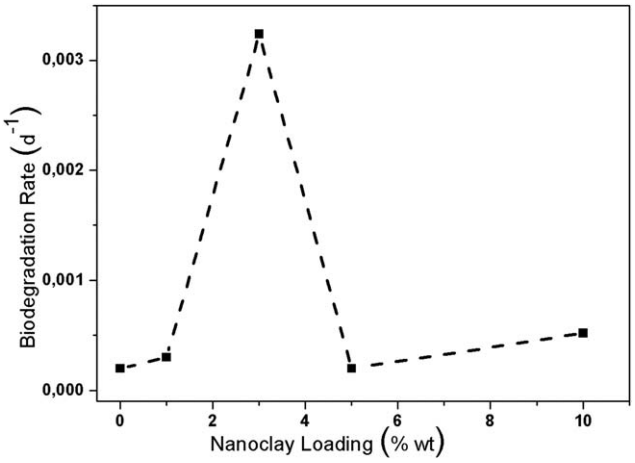

Figure 3. The biodegradation rate for pure PHB and its nanocomposites. (The dashed line connecting the experimental points is a guide to the eye).

As shown in Figure 2, the rate of biodegradation of pristine PHB is low. This is due to its high degree of crystallinity, since the enzyme prefers the amorphous areas at first. This experimental finding is in agreement with the literature.[8,48–50] It is concluded from Figure 2 that the presence of the nanofiller increases the rate of biodegradation. As presented in Table I, the nanocomposites have a lower degree of crystallinity than the neat polymer, thus indicating that the rate of biodegradation increases by the decrease in crystallinity. However, this increase is greater for the nanocomposite with 3 wt % loading and not for the 10 wt % nanocomposite. The results show that the biodegradation rate of the PHB nanocomposites goes through a maximum that corresponds to a loading of 3 wt % (Figure 3).

The X-ray diffraction (XRD) results of these nanocomposite[51] indicate that the nanocomposites with loadings 3, 5, and 10 wt % have an intercalated structure, with the intensity of the characteristic $d_{(001)}$-spacing peak to increase from the 3 to the 10 wt % loading. These results suggest that, as the loading increases, more polymer is inserted in the galleries of the swollen clay platelets. In addition, as the clay loading increases, the number density of the clay platelets per unit volume of the hybrid material increases. These observations, in turn, propose that less polymer is available and accessible for the PHB depolymerase to bind and, subsequently, to hydrolyze. Therefore, as the loading of the clay increases in the PHB nanocomposite materials, two antagonistic effects with respect to the biodegradation rate are in action: (1) The decrease in crystallinity of the hybrid material that increases its biodegradation rate and (2) the decrease in the availability and accessibility of the polymer chains by PHB depolymerase that lowers the biodegradation rate of the hybrid material. These two antagonistic effects result in a maximum biodegradation rate that seems to be at a loading of 3 wt %.

Furthermore, the MWD for both the neat polymer and its nanocomposites was measured with GPC, before the exposure to the enzymatic solution and after several days of exposure to it. The results of the GPC chromatograms obtained before the immersion into the enzymatic solution are presented in Figure

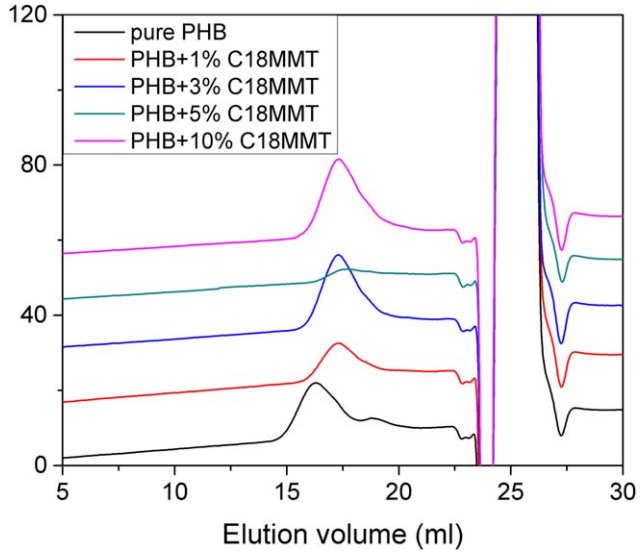

Figure 4. GPC chromatograms of pure PHB and all the nanocomposites before their immersion into the enzymatic solution. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

4. As it can be seen, a strong peak appears for all samples at ~25 mL corresponding to the elution solvent. Another small negative peak at 27 mL, which corresponds to THF, was present for all samples at exactly the same elution volume. This means that results derived from the chromatograms correspond to differences in the polymer MWD and they are not instrument artifacts due to unstable solvent flow rate, temperature variations, etc. From the curves in Figure 4, it is clear that the MWD of all nanocomposites is shifted to higher elution volumes compared to pure PHB, which means that all nanocomposites have slightly lower molecular weights compared to pure PHB. Moreover, the amount of the nanofiller does not seem to influence much the MWD, which is always unimodal.

Using the universal calibration procedure, the average molecular weights of all samples were estimated and are illustrated in Table II. As can be seen, the number average molecular weight (M_n) of pure PHB is nearly 37,000, while that of the nanocomposites starts at 34,300 and goes to 31,700, as the amount of the nano-clay increase from 1 to 10%. The polydispersity of the MWD ranges from 1.46 to 1.58, which means that the weight average molecular weight of all samples is in the vicinity of 50,200. These results indicate that the melt mixing method of producing PHB nanocomposites results in a small-scale reduction of the polymer chain length.

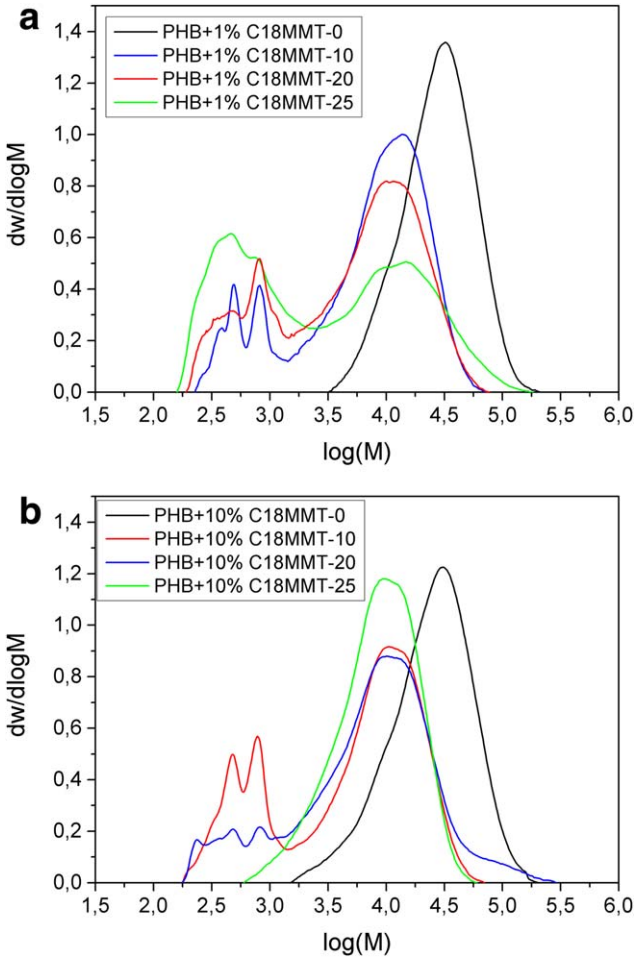

Figure 5. Effect of the biodegradation time on the MWD of the nanocomposites with 1% (a) or 10% (b) organomodified nanomontmorillonite. Different curves correspond to 0, 10, 20, and 25 days. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table II also presents results for the number average molecular weight of the polymer after its immersion in the enzymatic solution for 10, 20, and 25 days, respectively. A clear decrease in M_n with time was observed for all different hybrid materials. The overall average molecular weight reduction ΔM after 25 days ranges from

Table II. Number Average Molecular Weight (M_n) of Pure PHB and Nanocomposites at Different Biodegradation Time Periods

	Biodegradation time (days)				
Sample	Initial	10	20	25	Overall ΔM (%)
Pure PHB	36,940	23,240	17,120	16,840	54.4
PHB+1% C18MMT	34,290	17,690	16,550	15,230	55.5
PHB+3% C18MMT	32,760	16,710	14,690	14,510	55.7
PHB+5% C18MMT	32,320	16,050	14,660	14,220	56.0
PHB+10% C18MMT	31,730	15,770	14,590	13,760	56.6

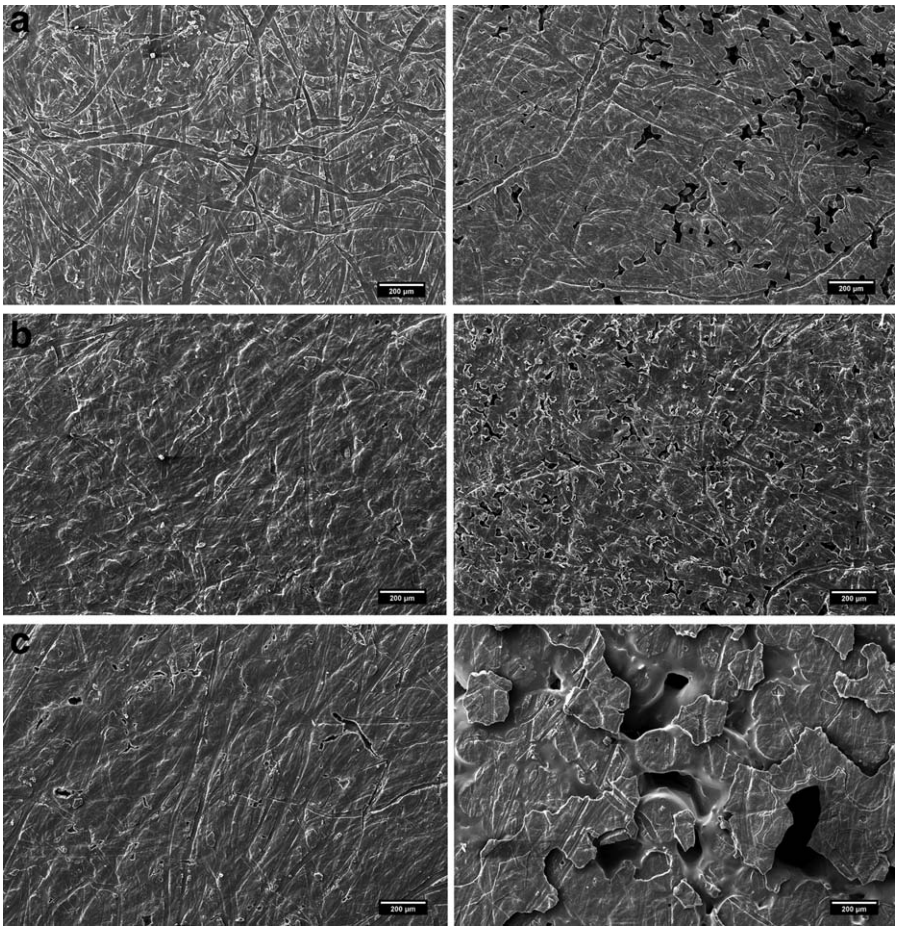

Figure 6. The surface morphology of pure PHB (a) and its nanocomposites with 3 wt % (b) and 10 wt % (c) clay before the exposure to the enzyme (left side) and after 25 days exposure (right side).

54.4% for the pure polymer to 56.6% for the nanocomposite with 10% OMMT.

The variation of the full MWD with biodegradation time for the nanocomposites with 1 or 10% nanoclay is displayed in Figure 5(a,b), respectively.

Figure 5 also shows that a clear, second peak, (which is double in some cases), appears in molecular weights around 447–794. Though a precise determination of these molecular weights is not feasible, a first prediction is that these peaks correspond to PHB oligomers with 5–9 structural units, i.e., to pentamers to nonamers. These findings are in agreement with literature data, where it was reported that the multiphase degradation of PHAs can be described as follows: In the first period (a few weeks), the amorphous phase is eroded, thus somewhat increasing the crystallinity of the polymer. Then, the polymer chains are disrupted, tetramers, dimmers, and monomers are formed, and molecular mass decreases. Later on, erosion processes develop and the polymer losses its mass. This process can last for months, up to 2–3 years, depending on environmental conditions and physicochemical properties of the specific PHA.[50]

This reduction in the MW of the neat polymer and the hybrid materials after their exposure to the enzymatic solution in conjunction with the small weight reduction of the exposed sample films indicate that the enzyme, due possibly to its small size, gets into the volume of the sample film, it cleavages the polymer chains, but most of the resulting oligomers cannot come out to the surface of the film due to steric hindrances induced by the size of the oligomers and their insolubility to water. Similar findings and observations have been made by Boskhomdzhiev *et al.*, who studied the long-term biodegradation kinetics of pure PHB and its blends with copolymer poly(3-hydroxybutyrate-*co*−3-hydroxyvalerate) and with polylactic acid.[48]

The surface morphology of PHB and its nanocomposites films was investigated by SEM. Figure 6 presents the surface morphology for the pure polymer [Figure 6(a)], the 3 wt % [Figure 6(b)], and 10 wt % [Figure 6(c)] nanocomposites, before their exposure to the enzyme and after 25 days exposure. As it can be seen, the films before the immersion into the enzymatic solution are continuous. Their surface is micron-scale rough due to the surface topography of the hydraulic press plates used for the formation of the film. After 25 days of exposure to the enzyme, holes and traces of erosion appear on the surface of the films. These traces of erosion are most pronounced in the nanocomposite with 10 wt % loading. The 10 wt %

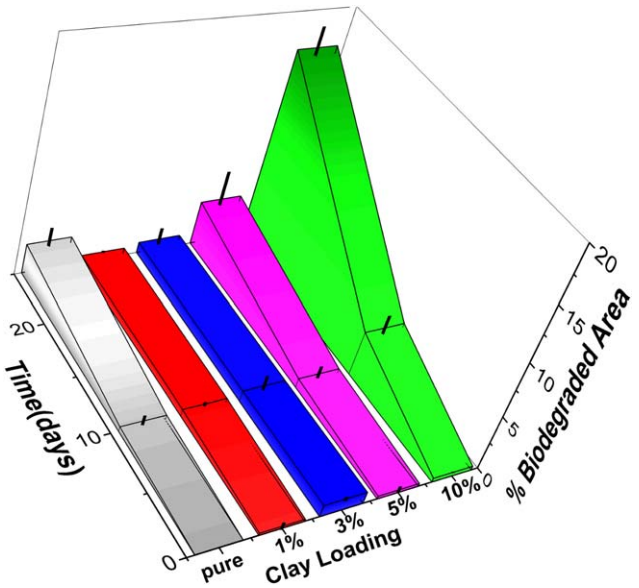

Figure 7. The percentage of the biodegraded area of the samples after their immersion into the enzymatic solution for 25 days. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

nanocomposite has the lowest degree of crystallinity (Table I). This result is reasonable, knowing that PHB-depolymerase prefers the amorphous state.

The percentage of the biodegraded area of all the samples during the exposure to the enzyme is shown in Figure 7. All the calculations were made from the ×65 magnification images. The lowest magnification images are preferable in order to obtain results that correspond to the highest possible fraction of the area of the sample. The calculation was made in an automated andconsistent way using a well-established methodology by Impoco *et al.* and Image J Software.[52,53] As it can be seen, the samples' surface is disintegrated during the exposure to the enzyme. The presence of the nanoclays, in loadings greater than 3 wt %, increases the biodegraded area of the sample surface.

CONCLUSIONS

In the present work, nanocomposites of PHB with OMMT were prepared by the melt-mixing method. Extracellular PHB depolymerase was isolated from the fungus *P. pinophilum* and the effect of the nanofiller in polymer biodegradation was investigated. The biodegradation rate goes through a maximum that corresponds to an organoclay loading of 3 wt %. This is rather due to the antagonistic effects caused by the increase in the clay loading: The lowering of the crystallinity of the hybrid material (increase in biodegradation rate) and the smaller availability and accessibility of the polymer chains by the enzyme (decrease in biodegradation rate). However, the mass loss after 25 days of enzymatic degradation was very low, while the polymer's average molecular weight was significantly reduced (more than 50%). Additional peaks corresponding to PHB oligomers appeared after biodegradation. This was observed for both pure PHB and the nanocomposites. These observations indicate that the biodegradation process takes place in the bulk mass of the sample films, as well. The SEM imaging of the biodegraded surfaces and analysis of these images showed that the higher amount of nanoclay (10 wt %) resulted in larger biodegraded area. In conclusion, this research demonstrates that the presence of the nanoclays enhances the biodegradation rate of pure PHB polymer and provides quantitative data for the biodegradation of PHB/organoclay hybrid materials.

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