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Environmental Degradation of Blends of Atactic Poly[(R,S)-3-hydroxybutyrate] with Natural PHBV in Baltic Sea Water and Compost with Activated Sludge

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Abstract Degradation of atactic poly[(R,S)-3-hydroxy-butyrate] (a-PHB) binary blends with natural poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV, 12 mol% of 3HV units), has been investigated and compared with plain PHBV in the compost containing activated sludge and under marine exposure conditions in the dynamic water of the Baltic Sea. Characteristic parameters of compost and the Baltic Sea water were monitored during the incubation period (6 weeks) and their influence on the degree of biodegradation is discussed. After specified degradation times of the experiments the weight loss of the samples, surface changes, changes in molecular weight and polydispersity as well as changes of the composition and thermo-mechanical properties of the blends have been evaluated. Macroscopic observations of the samples were accompanied by investigations using optical microscopy, size-exclusion chromatography (SEC), nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC) and tensile testing. The degree of degradation of blends of a-PHB with PHBV depends on the blend composition and environmental conditions. In both environments studied the weight loss of plain PHBV was more significant than changes the molecular weight. In both environments only enzymatic degradation of the blends, which proceeds via surface erosion mechanisms, was observed during the incubation period.

Keywords Atactic poly[(R,S)-3-hydroxybutyrate] ·
Natural poly(3-hydroxybutyrate-co-3-hydroxyvalerate) ·
Biodegradation · Compost with activated sludge ·
Baltic Sea water

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Introduction

The development of new technologies for the production of biodegradable polymers could improve market opportunities and decrease plastic waste [1]. Among biodegradable polymers, polyhydroxyalkonoates (PHA) are of special academic as well as technological and commercial interest. Bacterial PHA, produced from renewable resources, offers wide ecological advantages [2]. Natural PHAs are stereoregular thermoplastics containing a stereogenic site in each molecular repeat unit. Their physical properties may be tuned from crystalline–brittle to soft–sticky materials depending on the length of the side aliphatic chain at the chiral carbon [3]. On the other hand, the properties of chemically prepared PHA analogues may be additionally controlled by the stereochemical configuration of the repeating units along the polymer chain [4]. Synthetic analogues of PHA are obtainable by direct copolymerization of epoxides with carbon monoxide [5] and via ring-opening polymerization (ROP) of respective β-lactones [6].

The atactic poly[(R,S)-3-hydroxybutyrate] (a-PHB), a synthetic amorphous analogue of natural PHB (n-PHB), can be prepared by anionic polymerization of β-butyro-lactone [7, 8]—the monomer that could be obtained using synthetic gas derived from coal or waste biomass

gasification [9]. The a-PHB attracted special attention as an amorphous polymer suitable for modifying mechanical and biodegradation properties of natural PHA [8, 10]. Enzymatic hydrolysis of a-PHB was observed when this amorphous polymer was blended or copolymerized with a variety of crystalline polyesters [10–14]. Enzymatic attack by PHB depolymerase from *Ralstonia pickettii* T1 can be induced by blending a-PHB with other amorphous polymers of a higher glass transition temperature than a-PHB, e.g., poly(methyl methacrylate) (PMMA) or atactic polylactide (PLA) [15–18]. Moreover, extracellular PHB depolymerase able to degrade pure synthetic a-PHB were described [19–21]. The water-soluble a-PHB oligomeric products of enzymatic hydrolysis are bioassimilated by selected bacterial strains, thus showing total biodegradability of this synthetic polymer [22, 23]. It was also found that a-PHB oligomers are non-toxic and biocompatible and can be used as potential carriers for drug delivery [24].

However, little is known about the environmental degradation of materials containing a-PHB. Recently, the degradation in soil of a-PHB containing blends, with n-PHB was reported. It has been found, that in soil degradation of investigated blends proceeded faster than respective reference plain n-PHB film [25].

The development of polymeric materials which undergo biodegradation under natural conditions would be the key to solving pollution problems caused in seawater by marine plastic waste discarded into marine environments every year and accumulate in this region. The same problem was observed in public waste-fields. The degradation of natural polyesters in a biological environment such as seawater or activated sludge may involve a simple hydrolytic degradation process in addition to a microbial (enzymatic) degradation. Thus the biodegradation of microbial polyesters may occur through the cleavage of the ester linkages by specific enzymes under natural conditions or by hydrolysis.

The aim of the present study is to examine the biodegradation of blends of atactic poly[(R,S)-3-hydroxybutyrate] with a selected biopolyester i.e. PHBV in two environments: compost containing activated sludge and under marine exposure conditions in seawater, respectively. Degradation experiments of these blends under laboratory conditions, both hydrolytic (pH = 7.4, T = 70 °C) and enzymatic (in the presence of *P. lemoignei* PHB-depolymeraze, pH = 8.0, T = 37 °C) were reported previously [10].

Experimental

Materials

- Natural PHBV (3HV content 12%, M_n = 231,800; M_w/M_n = 1.5) was supplied by Aldrich. Solvent casted

films with the thickness of 0.115 mm were used in degradation experiments.

- Atactic PHB (a-PHB; M_n = 13,000; M_w/M_n = 1.3) was synthesized via anionic ring opening polymerization of racemic β-butyrolactone initiated with the 18-crown-6 supramolecular complexes of potassium hydroxide [26].

- The binary a-PHB/PHBV blends containing 30, 40, 50 and 60 wt% of a-PHB were prepared by casting the chloroform solution on glass plates at room temperature. Films with the thickness of 0.015–0.200 mm were used in degradation experiments.

Environments

The incubation of polymer samples was conducted in compost with activated sludge and dynamic seawater.

- *Biodegradation in compost with active sludge* The compost pile 1.5 × 2 × 1 m (width, length and height) was prepared under natural conditions of a sewage farm. It consisted of the activated sludge, from a municipal waste treatment plant in Gdynia, burnt lime and straw. Burnt lime (0.45 kg CaO/1 kg dry mass of compost) was added to ravage phatogenic bacteria and eggs of parasites, deacidificate activated sludge and convert the dehydrated activated sludge to compost. Straw was added to maintain a higher temperature and to loosen the structure of the compost pile. The compost pile was not adequately aerated, so it was expected that a combination of conditions from aerobic at the upper part, micro-aerophilic in the middle part and facultative anaerobic at the bottom of the pile could occur [27, 28]. Characteristic parameters of the compost with activated sludge used in the experiment are presented in Table 1.
The samples of PHBV and its blends with a-PHB were put into a special perforated basket and buried in the compost pile at the depth of 1 m. The incubation lasted 6 weeks and for some samples it was prolonged.

- *Biodegradation in seawater* The samples of PHBV and its blends with a-PHB were located in a special

Table 1 The characteristic parameters of compost with activated sludge

Parameter	Month	
	July	August
Temperature (°C)	19.5	22.6
pH	5.6	5.1
Moisture content (%)	48.8	46.8
Activity of dehydrogenases (mol/mg d.m.)	0.0398	0.0331

Table 2 The characteristic parameters of Baltic Sea water[a]

Parameter	Month	
	July	August
Temperature (°C)	16.9	20.3
PH	8.1	8.1
Cl content (g/kg)	3.66	3.40
Oxygen content (cm^3/dm^3)	6.65	5.82
Salinity (ppt)	6.61	6.14

[a] Parameters received from the Institute of Meteorology and Water Management in Gdynia, Poland

stainless steel basket at a depth of 2 m under the sea surface, near the ship of the Polish Ship Salvage Company, in Gdynia Harbour. The incubation lasted 6 weeks and for some samples it was prolonged. Characteristic parameters of seawater environment according to The Institute of Meteorology and Water Management in Gdynia, Poland are presented in Table 2.

Methods

Characterization of Compost

The moisture content of compost was determined by drying a sample at 105 °C until constant weight was obtained.

The activity of the dehydrogenases was measured by a spectrophotometric method using triphenyltetrazolium chloride (TTC), which is a method for the estimation of biochemical activity of microorganisms in active sludge by the oxidation process. The method is based on the dehydrogenation of glucose added to the compost with a subsequent transfer of the hydrogen to the colourless biologically active compound of TTC, which undergoes a reduction to $TTCH_2$ (red compound) [29, 30]. Glucose (1.5 g) and TTC (0.2 g) were added to distilled water (100 mL) to form a solution. One millilitre of that solution was added to 5 mL of a compost solution (50 g compost homogenized with 100 mL of distilled water). The final mixture was incubated at 37 °C for 15 min. The dehydrogenation reaction was stopped by adding ethanol and the intensity of the colour in the filtered liquid was measured using a Specol colorimeter at 490 nm.

The pH of the compost was determined with a Teleko N 5172 pH-meter. The determinations were done on 50 g samples in 100 mL distilled water after homogenization for 30 min and incubation for 1 h at room temperature.

Investigation of Polymeric Materials

The films of natural PHBV and its blends with synthetic a-PHB were cut into 15 × 100 mm rectangles. After incubation in compost and seawater, the samples were taken from the natural environments, washed thoroughly with distilled water and dried at room temperature to a constant weight.

The weight changes of degraded samples (%) were estimated using an electronic balance Gibertini E 42s. The results obtained for clean and dried samples of blends after biodegradation experiments were compared with those of the respective samples before biodegradation.

Microscopic observation of polymer surface changes during degradation was done using optical microscope Axioplan—Opton equipped with phase contrast and linked to a computer through a photocamera Nikon Coolpix 4500 Digital. Microscopic observations were performed at a magnification of 120×.

Size-exclusion chromatography SEC

- SEC of a-PHB: SEC measurements were performed in chloroform solution at 35 °C and a flow rate of 1 mL/min using a Spectra-Physics 8800 solvent delivery system with two Mixed C Styragel columns in series and a Shodex SE 61 refractive index detector. A volume of 10 μL of sample solutions in THF (concentration 2%, w/v) was injected. Polystyrene standards with low polydispersity were used to generate a calibration curve. Number-average molecular weight, weight-average molecular weight, and polydispersity index M_w/M_n (PDI) of a-PHB were obtained.

- SEC of a-PHB/PHBV blends: The measurements were performed at 25 °C on a Perkin-Elmer liquid chromatograph equipped with an LC-30 differential refractometer (DRI). Separations have been carried out using a 5 μm PSS SDV linear XL column (300 mm length and 8 mm ID) with a precolumn (Polymer Standards Service GmbH, Mainz). The column was calibrated with polystyrene (PS) standards of low polydispersity. Acid free chloroform was used as an eluent with a nominal flow rate of 1.0 mL/min. The samples of investigated blends were dissolved at 60 °C (5 h) in acid free chloroform at the concentration of 0.4%. The mass of the samples injected onto the column was on the average 1.0×10^{-4} g.

- SEC of PHBV: Measurements were performed as described in Ref. 3(b) at 25 °C using a Hewlett Packard 1100 series pump coupled to a Dawn-DSP laser photometer (LS detector) equipped with a He–Ne laser, ($\lambda = 633$ nm) and an Optilab-DSP interferometric refractometer (DRI detector, both instruments from Wyatt Technology Corp., USA). The mass of PHBV

samples injected onto the column was on the average 2.5×10^{-4} g. Data acquisition and evaluation were done using Astra 4.73.04 software and Corona 1.40 software (Wyatt Technology Corp.).

Nuclear magnetic resonance (NMR) The NMR spectra were recorded using a Varian VCR-300 multinuclear magnetic resonance spectrometer. Samples were prepared as solutions in $CDCl_3$ using tetramethylsilane (TMS) as an internal standard.

Mechanical properties of the blends in form of films were measured using an Instron Model 4204 tensile tester at 20 mm/min tensile speed.

Differential scanning calorimetry (DSC) measurements were performed using a Du Pont 9900 thermal analyzer in the temperature range from −80 to +200 °C at a heating rate of 20 °C/min. The instrument was calibrated with high purity indium and gallium standards.

Results and Discussion

Degradation Environments

It is known that the abiotic parameters of compost (temperature, pH, and moisture content) and seawater (temperature, pH, salinity and oxygen content) have a significant influence on the development of living microorganisms in natural environments.

Characteristic parameters measured for the compost with activated sludge and water in the Baltic Sea according to the Institute of Meteorology and Water Management, Maritime Branch are presented in Tables 1 and 2, respectively.

Looking at the characteristic parameters of compost we can state, that the average temperature of compost (∼21.0 °C) during incubation was on the level preferred for enzymatic degradation [31]. The pH values in both months were similar and the average pH in compost with activated sludge was acidic (5.3). Taking the conditions of compost into consideration we can expect that the microaerophilic conditions resulting from an activity of living organisms occurred by the end of composting process. The temperature about 20 °C and acidic pH of compost in our experiment caused that psychrotrophic acidofilic microorganisms could play the main role in the experiment [32].

It is known that a wide population of micro- and macro-organisms can exist in the compost. According to the literature the most abundant are bacteria, actinomycetes and fungi [33]. The activity of dehydrogenases depends on the degree of growth microorganisms of populations, which are producing enzymes involved in biodegradation process.

During the experiment the activity of dehydrogenases in compost had been changing. With decreasing of moisture content the lower absolute value of activity of dehydrogenases was observed. It could have an influence on development of living microorganisms and the process of environmental degradation of the biopolyesters and blends with atactic PHB.

The moisture content of the natural (outdoor) compost depends on the weather conditions, but condensation of water connected with respiration of microorganisms also has an influence on the humidity.

The characteristic parameters of the Baltic Sea water according to the Institute of Meteorology and Water Management, Maritime Branch presented in Table 2 indicate that the average temperature (∼18.6 °C) of the Baltic Sea water was slightly lower than that preferred for enzymatic degradation which is in the range of 20–60 °C [31]. The value of pH in seawater was alkaline (8.1) and was the same in both months.

The low temperature of the Baltic Sea and the slight alkalinity of water can be expected to have had an influence on the development psychrotrophic bacteria. In the first period of incubation (July) we could observe lower temperatures and the highest oxygen content. These conditions have an influence on the activity of oxidising enzymes. The situation was also favourable for development of aerobic epilithic bacteria. The metabolism of these microorganisms probably caused the decrease of oxygen content at the end (August—5.82 cm^3/dm^3) and changing concentration of carbon dioxide in the seawater. On the other hand, the pH of the seawater remained unchanged throughout the entire period of sample incubation [34].

The comparison of characteristic parameters of compost and seawater, presented in Tables 1 and 2, indicates very different conditions in both environments which could have a significant influence on the rate of environmental degradation of the biopolyester and its blends with a-PHB. Generally, the conditions in seawater favoured the growth of bacteria, whereas the conditions in compost with activated sludge favoured the growth of fungi.

Evaluation of the Changes in Polymer Samples During Environmental Degradation

Weight loss, changes in surface morphology, composition and molecular weight as well as thermal and mechanical properties of polymer samples before and after specified incubation times in compost and seawater, were determined in order to evaluate the influence of a-PHB content on the degradation rate of the polymeric blends studied in the environments described above.

Weight Loss

Weight loss after 2, 4 and 6 weeks of natural PHBV and its blends with 30, 40, 50 and 60 wt% of a-PHB exposure in compost (presented in Fig. 1) indicates that all blends degrade, but with different rates. The weight loss of the investigated binary blends was dependent on the content of a-PHB in the blends. However, the weight loss of binary blends was lower than that of the plain PHBV.

Almost all blends studied disintegrated after 6 or 7 weeks and this disintegration made it impossible to carry out reliable measurements of the weight loss after a given incubation time. Obviously, disintegradability does not indicate total sample degradation. Therefore, remaining small particles of the samples should still be degraded and bioassimilated by microorganisms present in the compost, until the samples would be completely converted to carbon dioxide and water [22, 35, 36]. In the case of our blends that this should occur is beyond doubt as it has been proven that both components are biodegradable [19].

In seawater the degree of weight loss after the same incubation time was lower for all samples. For example, the PHBV sample weight lost was about 60% after 6 weeks in seawater, whereas it disintegrated after the same incubation time in the compost (Fig. 2). The higher weight loss
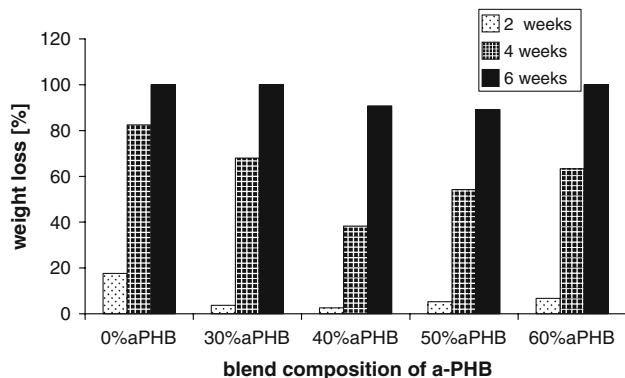

Fig. 1 The weight loss of natural PHBV and its blends with a-PHB after specific biodegradation periods in compost with activated sludge
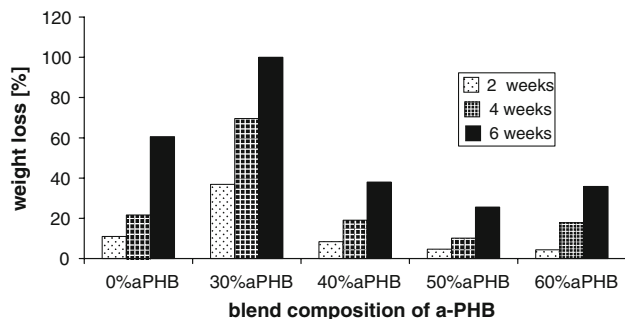

Fig. 2 The weight loss of natural PHBV and its blends with a-PHB after specific biodegradation periods in Baltic Sea water

observed for the sample of the blend with 30 wt% a-PHB may be caused by its lower thickness (0.15 mm) as compared with other blends (∼0.20 mm).

The weight losses of samples in the dynamic seawater may also be influenced by mechanical stress due to the natural movement of water. However, similar biodegradation behaviour was reported earlier for PHBV degraded under laboratory conditions, and the weight loss of PHBV was found to decrease in the following order of environments: anaerobic sludge > aerobic sludge > river sediment > seawater [37, 38].

Changes of Polymer Surface

The morphology of the sample surface can influence the degradation process. Tsuji and coworkers reported that high surface hydrophilicity and the presence of pores on the surface significantly enhance the biodegradability of polyesters in seawater [39]. Moreover, films of blend prepared by solvent evaporation may be heterogeneous with the surface enriched in one component. In our work all samples were prepared following the same procedure.

Macroscopic observations suggest the vulnerability of the investigated samples to microbiological attack. The changes of samples surface were more visible for biodegradation in compost than in seawater, which is in agreement with weight loss results described above. The formation of holes and pinholes on the surfaces of degraded samples, especially on those degraded in compost (where degradation proceeds faster: compare Figs. 1–3) was observed.

Presented as an example, Fig. 4 shows the photomicrographs of the surface of the blend of PHBV with 40 wt% a-PHB after specified times of biodegradation in both environments. Before degradation the surface of the blend was flat and smooth. After 14 days of the degradation process in compost, deep pinholes are visible on the photomicrographs, and after another 14 days, the holes of about 0.5 µm in diameter appear. The remaining part of the surface did not undergo any significant changes. After 28 days of incubation in compost, this binary blend became prone to fragmentation and on its surface agglomeration of microorganisms appeared (Fig. 4). These results reveal that degradation of samples in the compost with activated sludge proceeds via formation of holes (surface erosion) formed on specific parts of surface. After this initial stage, the degradation proceeded to the inner part of samples (Fig. 3a). On the contrary, the degradation in seawater of samples with the same a-PHB content occurs on the entire surface through the formation of a large amount of small pinholes (about 0.1 µm in diameter), but without formation of large holes. This is clearly evident after longer degradation period (42 days, Fig. 4).

Fig. 3 Macroscopic photographs of PHBV and its blend containing 30 and 40 wt% of a-PHB before and after specific biodegradation periods in (**a**) compost with activated sludge and (**b**) Baltic Sea water
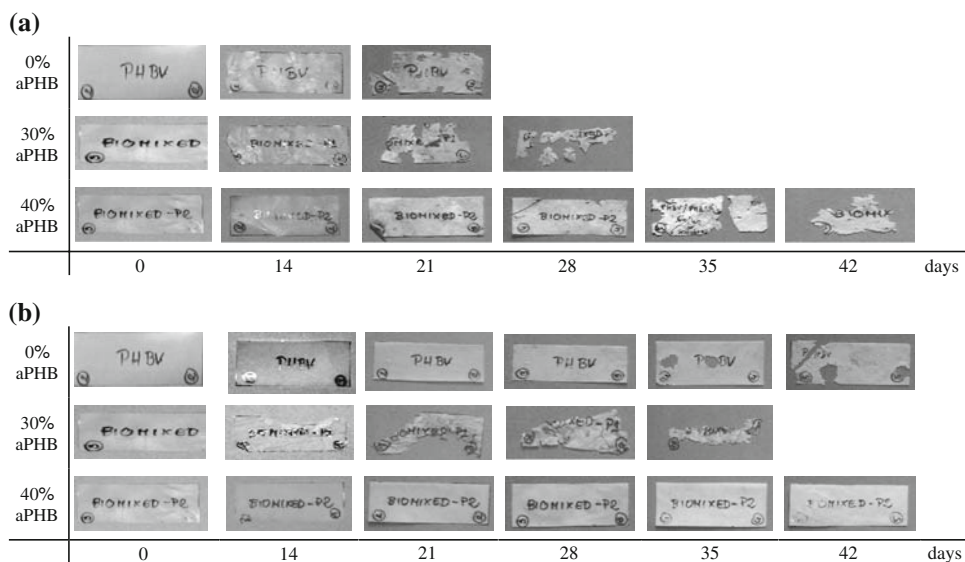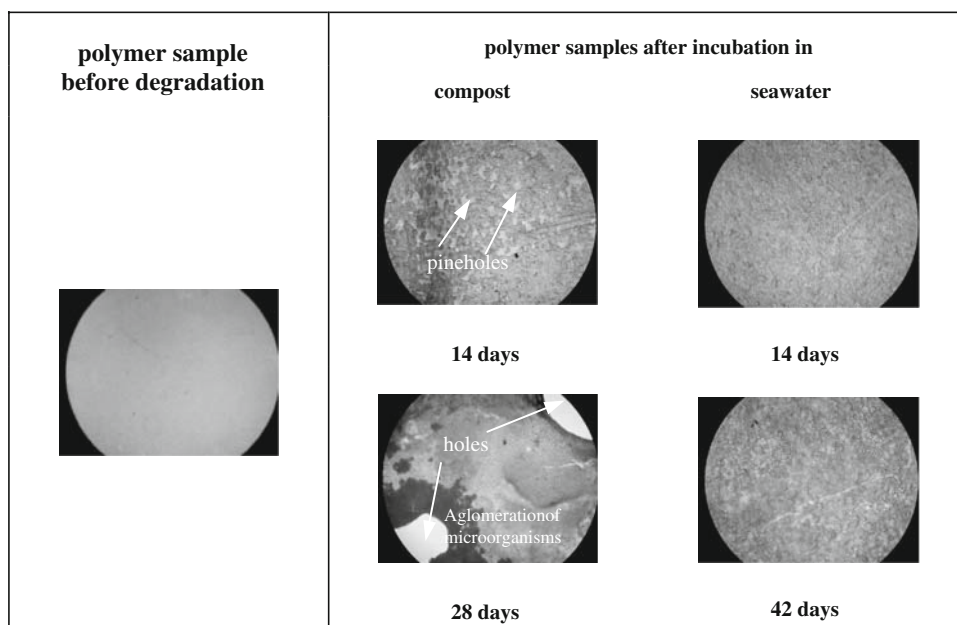

Fig. 4 The photomicrographs of the surfaces of the blend PHBV containing 40 wt% of a-PHB before and after specific biodegradation periods in natural environments (observations were done at a magnification of 120×)

In addition, microscopic observation also indicated, that the amorphous phase of the blends degraded faster and as a consequence the crystallinity of samples should gradually increases.

Changes of Composition and Molecular Weight

The composition of binary blends was determined by ^{1}H NMR spectroscopy [8]. As presented in Table 3 the a-PHB content in the blends decreased during the degradation process. These results reveal that degradation occurred primarily in the amorphous phase, which is in agreement with the results of microscopic observation.

The monitoring of changes in molecular weights was carried out by SEC and SEC-MALS analysis. The obtained results for plain PHBV before and after degradation in compost and seawater are presented in Table 4.

The molecular weight of pure PHBV decreased slightly during the time of incubation in both environments, suggesting that PHBV degraded via the enzymatic process (connected with weight loss of the sample comp. Figs. 1 and 2) with a minor influence of the hydrolytic process. On the contrary, the binary blends of PHBV and a-PHB show no measurable changes in the molecular weight, indicating that biodegradation of blends in both environments takes place only by the enzymatic process via a surface erosion mechanism [40].

Table 3 The changes of blend composition after specific biodegradation periods in compost with activated sludge and seawater, respectively

Time (weeks)	a-PHB Content (%) from ^{1}H NMR	
	Compost	Seawater
0	40	40.0
2	30	31.4
6	[–]	24.1
0	50.0	50
2	44.8	43.4
6	[–]	40.5
0	60.0	60
2	57.0	59.8
6	[–]	59.5

[–] Disintegrated sample

Thermal and Mechanical Properties

The blends of natural PHBV and synthetic a-PHB are miscible in the melt in the wide range of compositions (10–50% a-PHB) and solidify with spherulitic morphology. The influence of a-PHB content on the thermal and mechanical properties of the blends was already reported [10]. The degree of crystallinity decreased with increasing content of a-PHB in the film samples. Thus, the tensile properties of the brittle PHBV biopolymer may be remarkably improved through blending with synthetic, amorphous a-PHB. Moreover, the blends containing less than 50 wt% of a-PHB possess mechanical and thermal characteristics satisfactory for processing [41].

The mechanical and thermal properties of investigated blends before and after specific time of exposure in compost and seawater are presented in Tables 5 and 6, respectively.

As reported previously, the crystallinity of samples decreases with increasing a-PHB content in the blends [10]. During the degradation process the enthalpy (ΔH) of the endothermic transition of blends increases with incubation time. Enthalpies between approximately 12 and 30 J/g were determined before and after the degradation, respectively. Such results indicate an increase of blend crystallinity as a consequence of degradation of the amorphous phase, which is in agreement with microscopy observations.

The mechanical properties such as tensile strength, elongation at break and tensile modulus change during the degradation process. This is connected with the changes in the blend composition and morphology after the specific time of degradation. Since the degradation process

Table 4 The changes of molecular weight and polydispersity of PHBV after specific biodegradation periods in compost with activated sludge and seawater, respectively

[–] Disintegrated sample

nm, not measured

Degradation environment	Time (weeks)	Change of M_w (%)	Change of M_n (%)	Change of M_w/M_n
Compost	2	7.95	10.44	1.54
	4	[–]	[–]	[–]
Seawater	2	1.58	3.36	1.53
	4	nm	nm	nm
	6	7.4	11.78	1.58

Table 5 Thermal and mechanical properties of investigated polymer blends before (0) and after 2 weeks of biodegradation test in compost with activated sludge

Sample	Time (weeks)	T_m (°C)	Tensile strength (MPa)	Elongation at break (%)	Tensile modulus (MPa)
a-PHB/PHBV 40/60	0	138.8	8.5	15	400
	2	140.6	13.4	11.2	604
a-PHB/PHBV 60/40	0	138.0	1.4	39.2	180
	2	138.7	4.6	14.0	115

Table 6 Thermal and mechanical properties of investigated polymer blends before (0) and after 2 and 6 weeks of biodegradation test in seawater

Sample	Time (weeks)	T_m (°C)	Tensile strength (MPa)	Elongation at break (%)	Tensile modulus (MPa)
a-PHB/PHBV 40/60	0	138.8	8.5	15	400
	2	nm	12.0	10.7	471
	6	148.8	7.8	8.1	342
a-PHB/PHBV 60/40	0	138.0	1.4	39.2	180
	2	nm	5.8	20.1	184
	6	140.6	3.7	9.0	130

nm, not measured

proceeds mainly in the amorphous phase leading to the decrease of a-PHB content, consequently the sample crystallinity increases, which is connected with the increase in tensile strength and decrease of elongation at break (comp. Tables 5 and 6). The observed decrease of elongation at break with incubation time is consistent with the suggested preference of degradation of the amorphous phase. Nevertheless, mechanical tests are limited, because the samples gradually lost integrity and some started disintegrating after 6 weeks (compare Figs. 1 and 2).

Conclusions

The results of degradation studies of blends of natural PHBV with synthetic a-PHB indicate enzymatic degradation in both environments i.e. in compost with activated sludge and in the dynamic Baltic Sea water. The degrees of degradation of samples incubated in compost are higher than those of the samples incubated in seawater. In both environments the natural PHBV degrades together with its binary blends containing a-PHB, for that the degree of degradation is dependent on the a-PHB content in the blend. The amorphous phase of the binary blend is degraded first, resulting in the increased sample crystallinity, which in turn causes higher values of tensile strength and tensile modulus. The increase of blend crystallinity during the first stage of degradation is observed in both environments, but it is more pronounced for the blends degraded in compost than for samples degraded in seawater.

Based on the results of this study it may be concluded, that amorphous a-PHB (which is not able to form self supporting films) is degradable in binary blends with PHBV in compost with activated sludge as well as in dynamic seawater.

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