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Biodegradation of polyhydroxyalkanoates (PHAs) in tropical coastal waters and identification of PHA-degrading bacteria

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ABSTRACT

Biodegradability patterns of two PHAs: a polymer of 3-hydroxybutyric acid (3-PHB) and a copolymer of 3-hydroxybutyric and 3-hydroxyvaleric acids (3-PHB/3-PHV) containing 11 mol% of hydroxyvalerate, were studied in the tropical marine environment, in the South China Sea (Nha Trang, Vietnam). No significant differences have been observed between degradation rates of 3-PHB and 3-PHB/3-PHV specimens; it has been found that under study conditions, biodegradation is rather influenced by the shape of the polymer item and the preparation technique than by the chemical composition of the polymer. Biodegradation rates of polymer films in seawater have been found to be higher than those of compacted pellets. As 3-PHB and 3-PHB/3-PHV are degraded and the specimens lose their mass, molecular weight of both polymers is decreased, i.e. polymer chains get destroyed. The polydispersity index of the PHAs grows significantly. However, the degree of crystallinity of both PHAs remains unchanged, i.e. the amorphous phase and the crystalline one are equally disintegrated. PHA-degrading microorganisms were isolated using the clear-zone technique, by inoculating the isolates onto mineral agar that contained PHA as sole carbon source. Based on the 16S rRNA analysis, the PHA-degrading strains were identified as *Enterobacter* sp. (four strains), *Bacillus* sp. and *Gracilibacillus* sp.

1. Introduction

As the human population of the world grows and economic activity intensifies, increasing amounts of chemical substances are produced and consumed and large quantities of chemicals accumulate in the biosphere. This is a global environmental problem, which poses a threat to biotic and abiotic components of the environment [1]. In spite of the increasing efforts to bring down accumulation of wastes and recycle them, more and more damage is done to the environment. In the “Agenda 21” adopted at the special United Nations Conference on Environment and Development in Rio de Janeiro (1992), stress is laid on the need for the development and application of new, environmentally friendly technologies and materials [2]. Development of materials that can be involved in the biospheric cycling is in line with the conception of environmentally safe sustainable industrial development. The greatest concern is accumulation of synthetic plastics in natural ecosystems. The worldwide annual production of synthetic polymers has reached about 180 million tons, and they largely accumulate in landfills and are disposed underground, polluting the land and natural waters, including vast areas of the Global Ocean. This is detrimental to aquatic ecosystems and can cause increased mortality of freshwater and marine biota. An escape from this ecological “dead-end” is gradual replacement of synthetic polymers with new, biodegradable, materials [3]. Most of the research in this field is focused on developing biodegradable synthetic polymers and so-called “green polymers” – polymeric materials of natural origin [4,5]. Polyhydroxyalkanoates (PHAs) – polymers synthesized by microorganisms under specific growth conditions – currently occupy a special position among biodegradable natural polyesters as materials with numerous useful properties and a wide range of applications [6–8]. These polymers are degraded in biological media to form products innocuous to the environment: carbon

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dioxide and water under aerobic conditions or methane and water under anaerobic conditions. They are degraded by soil and aquatic microorganisms that have specific enzymes – PHA depolymerases [9,10]. Six hundred PHA depolymerases from various microorganisms have been identified by now; comparison of their amino-acid sequences provided a basis for uniting them in 8 superfamilies including 38 families [11]. Among PHA biodegraders are various microorganisms that degrade PHAs in soil [12–14], compost [15], fresh waters [16,17], and marine environments [18–21]. There are a few marine microorganisms that have been identified as PHA degraders: the bacteria *Pseudoalteromonas* sp. NRRL B-30083 [22], *Marinobacter* sp. NK-1 [23], *Alcaligenes faecalis* AE122 [24] and the actinomycetes *Nocardiopsis aegyptia* [25] and *Streptomyces* sp. SNG9 [26]. Their ability to secrete exodepolymerases and utilize both 3-PHB and 3-PHB/3-PHV has been proven experimentally.

Laboratory experiments showed that PHAs are effectively degraded in model systems with seawater and marine bacterial sediment and that degradation rate can vary depending upon the chemical composition and initial molecular mass of the polymer, the technique used to prepare samples and their mass, maintenance conditions, including pH and temperature of the medium, and specificity of PHA-degrading microorganisms as well as substrate specificity and catalytic activity of PHA-depolymerising enzymes excreted by these microorganisms [27–30].

As the range of potential applications of PHAs grows wider, studies examining degradation of these polymers in natural environments acquire increasing significance. Results obtained in model systems cannot be used to predict PHA biodegradation in multicomponent and diverse natural ecosystems. We have extensively studied PHA degradation in natural environments, including water bodies with various ecosystems, under aerobic and anaerobic conditions, etc. [17,31,32].

The purpose of this study was to examine degradation of PHAs with different chemical structures in the tropical marine environment and identify polymer-degrading bacteria.

2. Material and method

2.1. Preparation of PHA samples

The tested material was the PHA samples synthesized by the bacterium *Ralstonia eutropha*. The strain of *R.eutropha* B5786 is registered in the Russian National Collection of Industrial Microorganisms [33]. A polymer of 3-hydroxybutyric acid (3-PHB) and a copolymer of 3-hydroxybutyric and 3-hydroxyvaleric acids (3-PHB/3-PHV) containing 11 mol% of hydroxyvalerate, synthesized in the Institute of Biophysics SB RAS, Russia, were used in experiments [34]. The PHAs were extracted from bacterial biomass with chloroform and precipitated with ethanol. The chemical purity of the resulting specimens was estimated by conventional biochemical methods. The presence of protein impurities was determined by the Kjeldal micro-method [35] and carbohydrates by the anthrone method [36]. The specimens were subjected to methanolysis [37] and their PHA composition was analyzed with a gas chromatograph-mass spectrometer (GC/MS, model GCD Plus, Hewlett Packard, USA).

Films were prepared by casting chloroform solution (from 3 % w/v) on degreased glass and subsequent drying at room temperature for 2–3 days in a dust-free box. Film thickness was measured with an MKO-25 micrometer (Russia) at sensitivity 0.01 mm. The film thickness (average of 10 measurements) was 5.507 μm (the error was 0.026). Segments of equal thickness were selected and disks of diameter 30 mm, thickness 0.1 mm, and mass 73 ± 5 mg were cut out to be further used in the experiments. Three-dimensional specimens were prepared by cold compaction of finely powdered polymer, using an AutoPellet 3887 laboratory press (Carver, USA) at 120 kg-f/cm^2 (diameter 10 mm, height 0.5 cm, mass 330 ± 25 mg).

2.2. Experimental designs and sampling gears

PHA specimens were weighed and placed in 20 × 25 cm close-meshed gauze jackets with pouches. Twelve specimens, three of each type, were placed separately in the pouches. Four types were examined: 3-PHB film, 3-PHB/3-PHV film, 3-PHB pellet, and 3-PHB/3-PHV pellet (Fig. 1a). The jackets were fixed on standard stainless-steel stationary frames (Fig. 1b). The frames were placed in the sluice chamber of the floating platform (5 × 8 m, 40 m^2) (Fig. 2); the specimens were submerged to a depth of 120 cm.

PHA degradability was examined in the tropics, at the Marine Research and Testing Station of the Vietnam-Russia Tropical Centre, in the South China Sea (Vietnam). The station is situated on the island of Hon Tre, in the Nha Trang Bay (the Socialist Republic of Vietnam), 12°11′ N, 109°17′ E. The experiment was carried out from 11 March to 27 July 2009. Changes in the mass of polymers were monitored taking into account the properties of the seawater. Every 20 days, 3 specimens of each type were taken out of the water,

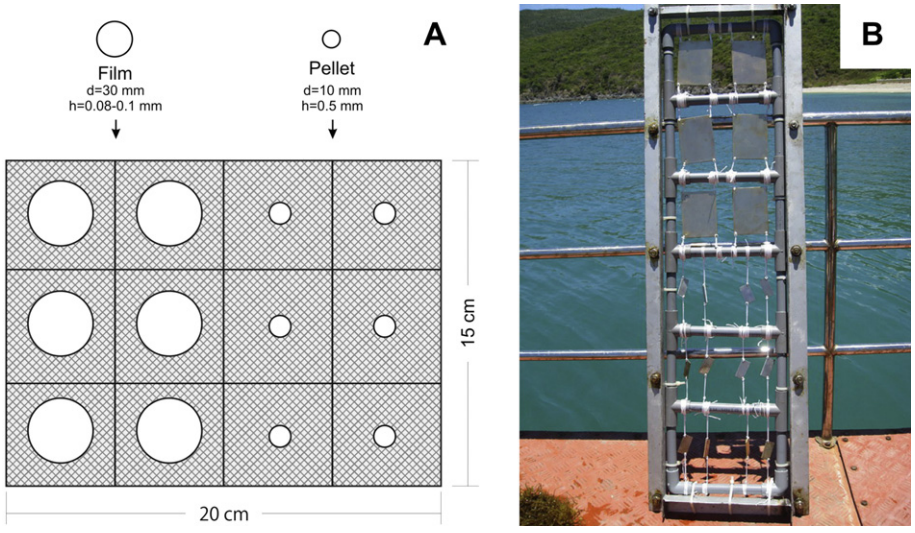

Fig. 1. The placement of PHA specimens (A); the specimen holding frame (B).

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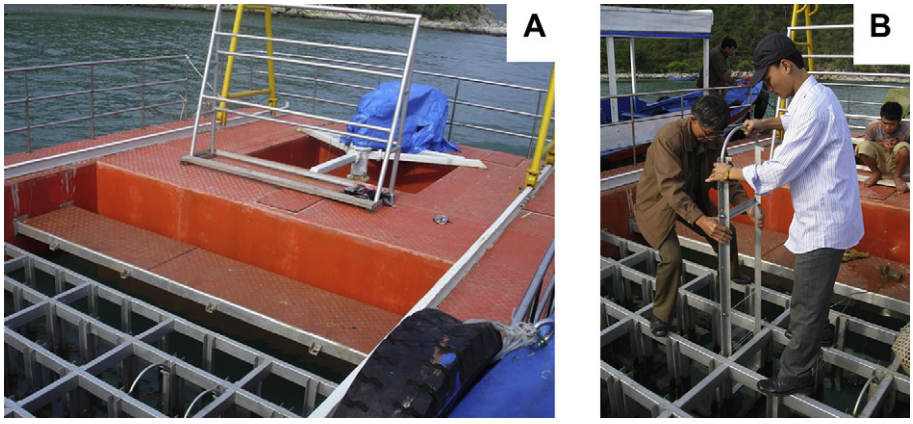

Fig. 2. The floating platform of Vietnam–Russia Tropical Centre (Nha Trang, Vietnam) (A); submerging the specimens in the sea (B).

carefully cleaned to remove biofilm, rinsed in distilled water, and dried for 24 h at the temperature 40 °C.

Seawater temperature and pH and dissolved oxygen concentration were measured at the same time points, when PHA specimens were retrieved. Dissolved oxygen was measured with an HI 9142 portable meter (Hanna Instruments, Italy); water pH – with an HI 98127 – HI 98128 portable pH-meter (Hanna Instruments, Italy); salinity – using an S/Mill-E 2442-W10 handheld refractometer (Japan).

2.3. Determination of PHA biodegradation

Biological degradation of PHAs was evaluated based on the following parameters: a decrease in the mass of the specimens, changes in the molecular mass and molecular-mass distribution of polymers, a change in fine structure properties such as the ratio between the ordered (crystalline) phase and the disordered (amorphous) phase.

Polymer specimens were weighed prior to submersion and in the course of experiment, using a Mettler balance (USA) of precision class 4. The mass loss was calculated as a ratio of the final mass to the initial mass of the specimen (X, %):

$$X = \frac{X_2}{X_1} \times 100,$$

where X_1 and X_2 are masses of the specimen before and after the experiment, respectively, mg.

2.4. Physicochemical examination of PHAs

3-PHB and 3-PHB/3-PHV films and pellets were subjected to X-ray structure analysis and crystallinity determination prior to the experiment and after 160 days of exposure to seawater. Measurements were conducted using a D8 ADANCE X-ray spectrometer ("Bruker", Germany) (graphite monochromator on a reflected beam) in a scan-step mode, with a 0.04° step and exposure time 2 s, to measure intensity at point. The instrument was operating at 40 kV × 40 μA.

Molecular mass and molecular-mass distribution of PHAs were examined using a gel permeation chromatograph (Waters Breeze System, USA) relative to reference polystyrenes from Sigma (USA). The average molecular mass was calculated as follows:

$$M_n = \sum(N_i \cdot M_i/N),$$

where N_i is the number of molecules of mass I; N is the total number of molecules; M_i is the mass of molecules of length I.

The weight average molecular mass of the PHA was determined as

$$M_w = \sum(w_i \cdot M_i),$$

where w_i is the portion of the mass ($w_i = N_i M_i/\Sigma(N_i \cdot M_i)$.

Polydispersity, which provides an estimate of the proportions of fragments with different polymerization abilities in the polymer, was calculated from the formula

$$PD = M_B/M_H.$$

Surface free energy (γ_S) (erg/cm^2), as a surface property of the specimens, was calculated by measuring contact angles for water in air (θ, degrees), using known equations [38]. To determine contact angles for water in air, film samples were placed on the microscopic stage, and distilled water was dropped on them with an automatic macropipette – 100, 200, and 300 μl, 10 drops of each volume. A computer image of the drop was obtained with a digital camera, and the angular value was determined (the average angular value was calculated from the measurement of 10 drops of each volume). Free surface energy of polymer films (erg/cm^2) was found from the equation $\gamma_s = \gamma_L(1 + \cos\theta)^2/4$, where γ_L is free water surface tension, 72.8 erg/cm^2.

Electron microscopy was performed using an FEI Company Quanta 200 scanning electron microscope coupled to an EDAX attachment for X-ray microanalysis with nitrogen-free cooling – GENESIS XM 2 60 – Imaging SEM with APOLLO 10 (USA).

2.5. Microbiological study

Microbiological examination aimed at identifying PHA-degrading microorganisms was performed in the period of active polymer degradation, on 27 August 2009 (after 140 days of exposure of the specimens to seawater). PHA specimens were aseptically removed from the jackets. The specimens were rinsed with sterile seawater to remove unattached microorganisms. Microbial biofilm samples were scraped off the surface of the polymer. On the same day, seawater samples were collected and plated on media.

Bacterial counts in the seawater and on the surface of PHA specimens were performed as follows. Samples were serially diluted and inoculated onto the Yoshimizu–Kimura (Y–K) nutrient medium: 5 g of peptone; 2 g of yeast extract; 1 g of glucose; 0.2 g of K_2HPO_4; 0.1 g of $MgSO_4 \times 7H_2O$; 18 g of agar; 500 ml of tap water and 500 ml of seawater; pH – 7.8–8. Fungi were counted on Sabouraud dextrose agar and acidic Czapek medium: 20 g of sucrose; 2 g of $NaNO_3$; 1.0 g of K_2HPO_4; 0.5 g of $MgSO_4 \times 7H_2O$; 0.5 g of KCl; 3.0 g of $CaCO_3$; 20 g of agar; 500 ml of tap water and 500 ml of

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seawater. Before the melted Czapek medium was dispensed, it was supplemented with sterile lactic acid, 4 ml/L. Inoculated plates were maintained at a temperature of 28–30 °C. Bacteria were counted at days 1–3 of incubation and fungi – at days 4–5. Axenic cultures of microorganisms were prepared by selecting 8–10 morphotypes of each isolate and re-inoculating them onto plates with the corresponding medium. Morphology of bacterial cells was examined in Gram-stained smears. Phenotypic properties of PHA-degrading microorganisms were examined using conventional microbiological methods [39,40]. Identification of bacterial isolates involved comparative analysis of their morphological, cultural, and biochemical properties. Morphology of vegetative cells was determined using a Meiji ML2000 light microscope (Meiji Techno, Japan). An Infiniti I camera (Luminera, Canada) was used to make micrographs. The examined parameters were as follows: spore formation, motility, Gram staining, growth factor requirements, capacity for anaerobic growth, the presence of nitrate reductase, catalase, oxidase, amylase, and proteinase activity, acid formation from glucose, lactose, sucrose, maltose, and mannite. Taxonomic identification was done in accordance with the handbook on the biology of bacteria [41].

PHA-degrading microorganisms were detected using the clear-zone technique [12], by inoculating the samples onto mineral agar that contained PHA as sole carbon source and KH_2PO_4–Na_2HPO_4 buffer (1:1) (0.033 M); NH_4Cl (1 g); $MgSO_4 \times 7H_2O$ (0.5 g); iron citrate (0.05 g); $CaCl_2 \times 2H_2O$ (0.005); yeast extract (0.05 g); casein hydrolysate (0.1 g); agar (20 g); tap water (500 ml); seawater (500 ml). Five millilitres of the medium containing 0.25% of finely powdered PHB (milled and subsequently treated with ultrasound) was added to the Petri dish with the solidified mineral agar of the same composition. On the surface of the agar medium, clear zones were formed around colonies of microorganisms with PHA-depolymerase activity.

2.6. DNA manipulations

Extraction of DNA was carried out using a commercial AquaPure Genomic DNA Isolation reagent kit (Bio-Rad, USA), following the manufacturer's protocol. The 16S rRNA gene was amplified using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), corresponding to *Escherichia coli* positions 8–27 and 1510–1492, respectively.

PCR was performed using a Mastercycler Gradient amplifier (Eppendorf, Germany) in the final volume 50 μl, containing 65 mM Tris–HCl (pH 8.9), 16 mM ammonium sulfate, 3.5 mM $MgCl_2$; 0.05% Tween 20; 0.2 mM dNTP; 0.2 μM solutions of oligonucleotide primers, 20–100 ng of DNA, and 1 unit of active Taq DNA polymerase (Bio-San, Novosibirsk, Russia). The PCR was run as follows: primary denaturation at 95 °C for 3 min was followed by 28 cycles with denaturation at 95 °C for 10 s, primer annealing at 60 °C for 10 s, and extension at 72 °C for 20 s. Final extension was performed at 72 °C for 5 min.

The sizes, quantity and purity of PCR products were tested by electrophoresis in a 1.2% agarose gel, using 0.5% TAE buffer. Visualization was performed by ethidium bromide staining, followed by documenting with a Doc Print transilluminator (Vilber Lourmat, France).

The DNA nucleotide sequence was determined in the Inter-Institute Sequencing Centre SB RAS (Novosibirsk) using Sanger sequencing methods, with a BigDye Terminator Cycle Sequencing Kit v 3.1 (Applied Biosystems, USA), on an ABI PRISM 3100 genetic analyzer (Applied Biosystems, USA), following the manufacturer's protocol.

The obtained nucleotide sequences of the 16S rRNA gene were compared with the sequences in the GenBank, EMBL and DDBJ databases, using the BLAST tool for the search for sequences with high homology, of the NCBI Web site (http://www.ncbi.nlm.nih.gov/BLAST/). The obtained nucleotide sequences were deposited in the database of GenBank NSBI under the following numbers: HM587328, HM587329, HM587330, HM587331, HM587332, and HM587333.

2.7. Statistical analysis

Statistical analysis of the results was performed using the standard software package of Microsoft Excel. Arithmetic means and standard deviations were found. Significant differences between mean values were tested using Student's t test (significance level: $p = 0.05$) by standard methods.

3. Results

3.1. Degradation of different PHAs

As the PHA biodegradation rate is known to be influenced not only by different factors in a given environment such as weather conditions and microbial population but also by the chemical composition of the PHA, the technique used to process it, and geometry of the polymer [9], in this study we compared the rates of biodegradation of two PHA types (homogenous PHB and a PHB/PHV copolymer) in the form of films and compacted pellets, prepared using different techniques, in seawater.

Hydrochemical parameters of seawater remained practically unchanged throughout the experiment (Table 1). The average temperature of the water was 28.75 ± 1.65 °C, with the minimum 27.1 °C and the maximum 30.4 °C. Water pH values were close to neutral, varying insignificantly, between 7.0 and 7.5. The salinity of the water varied within the range 32–35‰; the average salinity in the study period was 34%. Dissolved oxygen concentration varied from 5.4 to 8.3 mg/ml.

Recorded mass loss parameters of different PHA specimens are shown in Fig. 3: specimens of different shapes prepared by different techniques biodegraded at different rates. The most rapid degradation was recorded in films, which had a large surface area and had been prepared by the technique that affected the polymer in the least degree. In 160 days after the specimens had been submerged in seawater, the residual mass of 3-PHB and 3-PHB/PHV films was 58% and 54%, respectively, i.e. the mass loss of the specimens was almost equal. Over the first 120 days of exposure, the mass loss of the specimens occurred gradually, i.e. their degradation rate remained the same; a more significant weight loss was recorded for the following 20 days, suggesting more rapid degradation (Fig. 1; % 1b and 2b).

The degradation pattern of 3D compacted PHA pellets was somewhat different (Fig. 3, 1a and 2a). Over the first 80 days, the mass of the specimens remained almost unchanged; degradation occurred between days 80 and 160. The mass loss of 3-PHB and

Table 1
Hydrochemical parameters of seawater in the South China Sea.

Sampling date	Temperature of the water, °C	pH	Salinity (ppm)	Oxygen (mg/ml)
11.03.2009	27.3	7.4	32	8.3
30.03.2009	28.6	7.5	34	7.4
20.04.2009	29.0	7.0	34	6.5
07.05.2009	27.1	7.1	33	6.1
28.05.2009	29.5	7.1	33	6.9
17.06.2009	28.7	7.2	35	5.4
07.07.2009	27.7	7.2	35	6.1
27.07.2009	30.4	7.4	34	5.7

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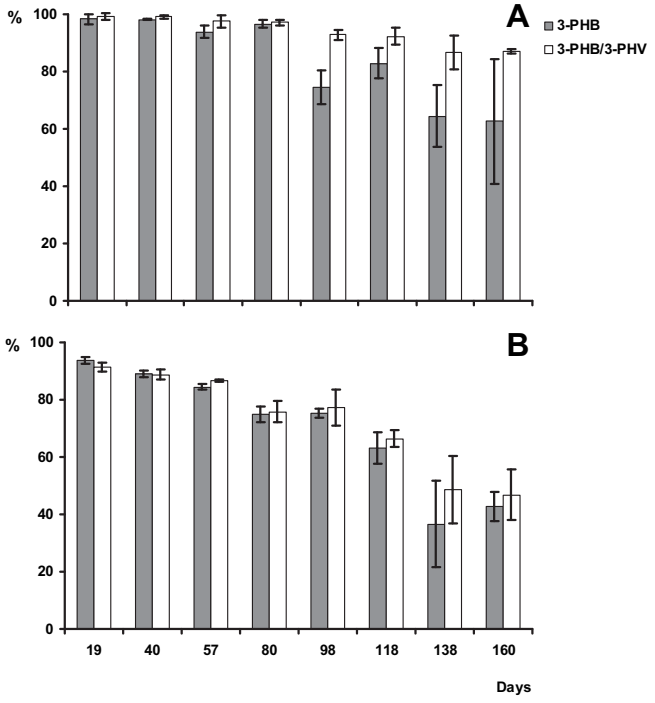

Fig. 3. Dynamics of the mass loss of PHA pellet (A) and film (B) specimens incubated in the marine environment (March–August 2009).

3-PHB/PHV pellets was 38% and 13%, respectively, but these differences are unreliable. These results cannot provide a basis for the conclusion that the processes of degradation of the two PHA types in the seawater are different or that the copolymer degrades at a higher rate than the 3-PHB homopolymer. Compacted pellets have a smaller surface area and, hence, a smaller polymer/water interface area than polymer films, and it may take a longer time for microorganisms to get attached to the surface and a longer time for the microorganisms to get adapted to the PHA as the substrate that has been subjected to compacting. There could be two phases of biodegradation of the polymer: in the first phase PHAs could be depolymerised and their polymer chains broken under the impact of depolymerising enzymes of PHA-degrading microorganisms; in the second phase polymer biodegradation products (tetra-, di-, and monomers of hydroxy acids) could be utilized, resulting in a decrease in the molecular weight of the polymer itself and a total mass loss of the specimens.

3.2. Changes in structure and properties of degrading PHAs

Differences that we recorded in biodegradation patterns of PHA specimens of different shapes, prepared using different techniques, could, in our opinion, be accounted for by different structures and surface areas of specimens, which must have influenced the adhesion of microorganisms and their enzymatic activity. Thus, it seemed reasonable to answer the question what processes occurred in the degrading polymer. Specimens were examined using X-ray structure analysis to determine whether disintegration of the polymer matrix changed the ratio of the ordered phase to the disordered one, i.e. the degree of crystallinity of the polymer. Depolymerising enzymes preferentially degrade the amorphous (disordered) part of a polymer rather than its crystalline region [27]. If PHA specimens submerged in the water of the South China Sea were degraded in this way, the decrease in the amorphous proportion must have enhanced the crystallinity of the polymer. Initially, the crystallinities of the 3-PHB and 3-PHB/3-PHV films were similar (71% and 69%, respectively); the crystallinities of the pellets of different composition were also very similar – 70% and 71% (Table 2). Analysis of X-ray spectra of PHA specimens after 160 days of marine exposure did not reveal any change in their crystallinity. Fig. 4 shows X-ray spectra of 3-PHB/3-PHV films before submersion and at day 160, when they were retrieved from the water. Thus, no changes were found in the proportions of the amorphous phase and the crystalline one in PHAs of both types as they degraded, suggesting a conclusion that in the studied marine environment, the amorphous phase of the PHA and the crystalline one were equally degraded, and, hence, the integrated indicator for the state of the polymer structure (crystallinity) remained unchanged.

Table 2
Comparative characterization of PHA specimens before and after marine exposure.

Parameter	Initial	At day 160	Initial	At day 160
	3-PHB	3-PHB	3-PHB/3-PHV	3-PHB/3-PHV
Crystallinity (films), Cx, %	71	70	69	69
M_w (films), kDa	1400 ± 106	1040 ± 33	1320 ± 85	1110 ± 80
Polydispersity (films)	2.00 ± 0.37	2.26 ± 0.20	2.01 ± 0.23	2.00 ± 0.22
Crystallinity (pellets), Cx, %	70	71	71	69
M_w (pellets), kDa	1360 ± 63	1090 ± 87	1430 ± 46	610 ± 25
Polydispersity (pellets)	1.83 ± 0.11	2.57 ± 0.51	2.06 ± 0.12	2.41 ± 0.11

Properties of the polymer are determined to a large extent by its molar mass, M_w (weight average molecular weight) in particular. Changes in molecular mass of PHA specimens after marine exposure were analyzed using gel-permeating chromatography (Table 2). Molecular mass of the specimens of all types was found to decrease during degradation; a decrease in M_w was recorded for films and pellets, prepared from both the 3-PHB homopolymer and the 3-PHB/3-PHV copolymer. The most dramatic, 57%, decrease in this parameter was obtained for copolymer pellets; in the other variants the decrease ranged between 16% and 26%. Polydispersity increased in specimens of all types, suggesting that the number of fragments having different degrees of polymerization was growing.

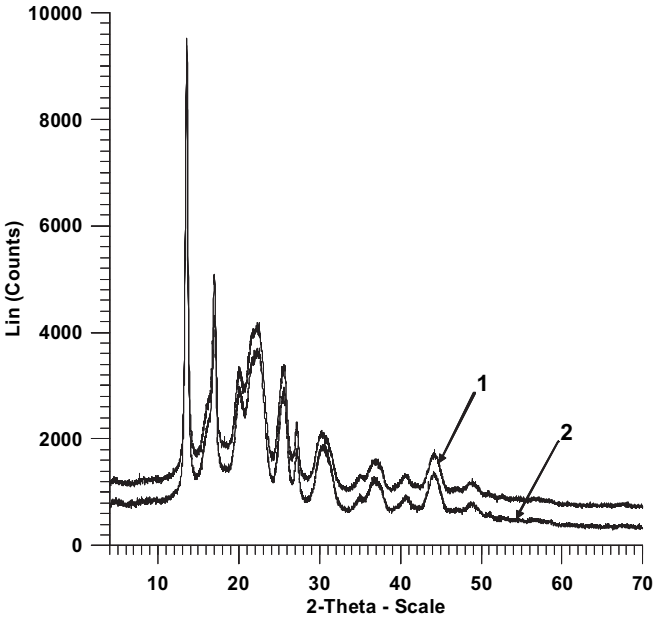

Fig. 4. X-ray spectra of 3-PHB/3-PHV films: 1 – the spectrum of the initial specimen; 2 – the spectrum of the specimen after marine exposure for 160 days.

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Table 3
Comparative characterization of the surface of PHA specimens before and after marine exposure.

Parameter	Initial	At day 160	Initial	At day 160
	3-PHB	3-PHB	3-PHB/3-PHV	3-PHB/3-PHV
Contact angle for water in air (θ, degrees) (films)	67°	67°	67°	69°
Surface tension (γ, erg/cm^2) (films)	35.1	35.2	35.1	33.5
Contact angle for water in air (θ, degrees) (pellets)	72°	73°	72°	71°
Surface tension (γ, erg/cm^2) (pellets)	31.2	30.4	31.2	31.8

Thus, during the course of PHA biodegradation in the marine environment, as the mass of the specimens was reduced, their M_w decreased too, which is in agreement with the data reported by other authors [10,13,27]. However, the crystallinity of both PHAs remained unchanged, i.e. the amorphous phase of the polymer and the crystalline one were equally disintegrated.

The surface properties of the specimens are characterized in Table 3, which shows that in all degraded specimens, the contact angle for water in air and the calculated surface tension values practically did not change after marine exposure, i.e. adhesive properties of the surface were preserved as the specimens were degraded. Before and after marine exposure, the contact angle for water in air was significantly greater in pellets than in films (prepared from either PHA), i.e. the adhesive properties evaluated based on this parameter were better in films than in compacted pellets, which were degraded at a much lower rate than films. To a certain extent, this can serve as evidence for greater "attractiveness" of film surface for biofilm-forming microorganisms.

Degradation and mass loss of the specimens altered morphology of their surface, particularly that of films, whose degradation was more pronounced. As the number of pinholes increased, the films became prone to fragmentation (Fig. 5A). The surface of compacted pellets changed less significantly. First, the surface of the pellets became rough and then pores and holes appeared (Fig. 5B). Electron micrographs show these changes more clearly: as the specimens degraded, the number and sizes of pores and pinholes, which were absent on the initial specimens, increased (Figs. 6 and 7).

3.3. Isolation of PHA-degrading microorganisms

Microbiological investigations showed that in the seawater of the South China Sea, the total number of heterotrophic bacteria on the Y–K medium was 1.6×10^3 CFU per ml and the number of microscopic fungi 1×10^2 CFU per ml. Similar data are reported by other authors, who have been conducting microbiological investigations in the Sea of Japan and in coastal waters of Vietnam [42–44].

Polymer specimens incubated in seawater for 160 days became covered with microbial biofilms. A total of 58 heterotrophic bacteria were isolated in pure cultures. Biofilm-forming microorganisms were represented by diverse morphological types. Gram-negative rods prevailed, amounting to between 60% and 65% on different specimens. Isolated bacteria were representatives of the genera *Pseudomonas*, *Pseudoalteromonas*, *Corynebacterium*, *Staphylococcus*, *Planococcus*, *Micrococcus*, *Arthrobacter*, *Cellulomonas*, *Enterobacter*, *Bacillus*, and *Gracilibacillus*. Microscopic fungi were represented by *Aspergillus*, *Penicillium*, *Trichoderma*, *Verticillium*, *Mucor*, and *Malbranchea*.

PHA-degrading microorganisms were isolated by inoculating biofilm samples onto mineral agar that contained finely powdered PHA as sole carbon and energy source. Most of the isolated bacteria were unable to degrade the polymer. The occurrence of clear zones on the polymer-containing diagnostic medium (depolymerase activity) (Fig. 8) was recorded for six isolates of biofilm bacteria. Based on similarities of morphological types, three strains capable of polymer biodegradation were selected from these isolates.

Based on the 16S rRNA analysis, we identified the isolated PHA-degrading strains as *Enterobacter* sp. IBP-VN1, *Bacillus* sp. IBP-VN2, and *Gracilibacillus* sp. IBP-VN3, *Enterobacter* sp. IBP-VN4, *Enterobacter* sp. IBP-VN5 and *Enterobacter* sp. IBP-VN6. The obtained nucleotide sequences of the 16S rRNA gene were deposited in the database of GenBank NSBI.

PHA-depolymerase activity of representatives of *Bacillus megaterium* was reported in the literature, and genes of intercellular PHB depolymerase *phaZ* were cloned and characterized for two strains of this taxon (ATCC 11561 and N-18-25-9) [15,45]. There are no data on PHA-depolymerase activity in representatives of the genera *Gracilibacillus* and *Enterobacter* in the available literature.

4. Discussion

Gradual replacement of synthetic materials, which persist in the environment, with polymers that can be degraded to end products, without releasing compounds harmful to nature, is an environmental and social issue of great importance. Synthetic plastics have accumulated in the biosphere in great quantities and just an insignificant portion is recycled. Thus, designing biodegradable polymers and studying their properties, including biodegradability patterns under varying and complex natural conditions, can help overcome pollution problems. Among the most promising

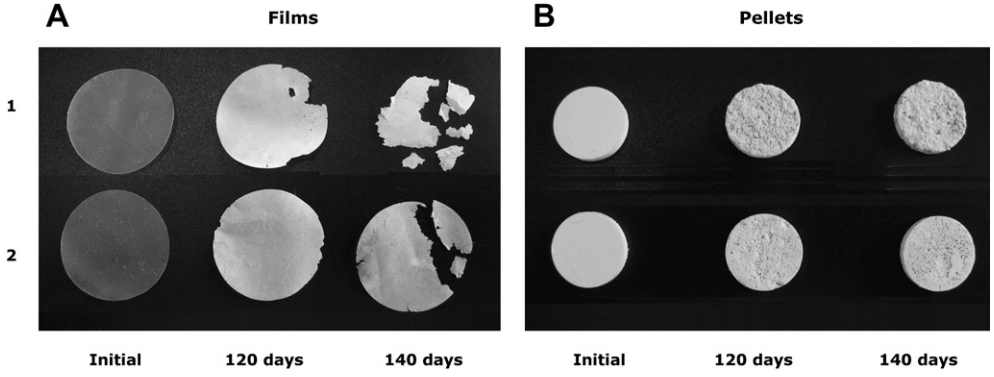

Fig. 5. PHA specimens (films (A) and pellets (B)) after different periods (days) of marine exposure: 1 – 3-PHB; B – 2-PHB/3-PHV.

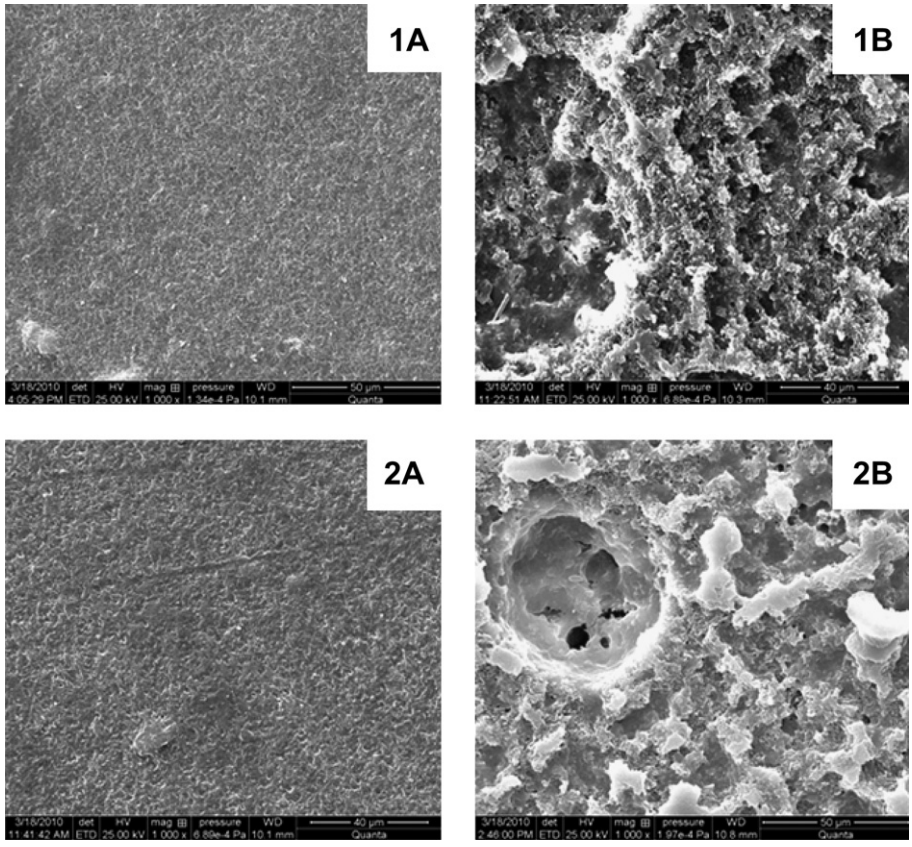

Fig. 6. Electron micrographs of PHA films: 1 – 3-PHB and 2 – 3-PHB/3-PHV; A – initial specimens, B – specimens after marine exposure for 160 days.

materials are lactic acid polymers and PHAs, whose production has been started all over the world [7]. As outputs of PHAs are growing and potential applications widening, there is an increasing need for the knowledge of mechanisms and kinetics of their biodegradation in different biological environments. Laboratory experiments and occasional natural observations cannot provide a sufficient basis for predicting the behaviour of polymer products in complex and dynamic natural environments.

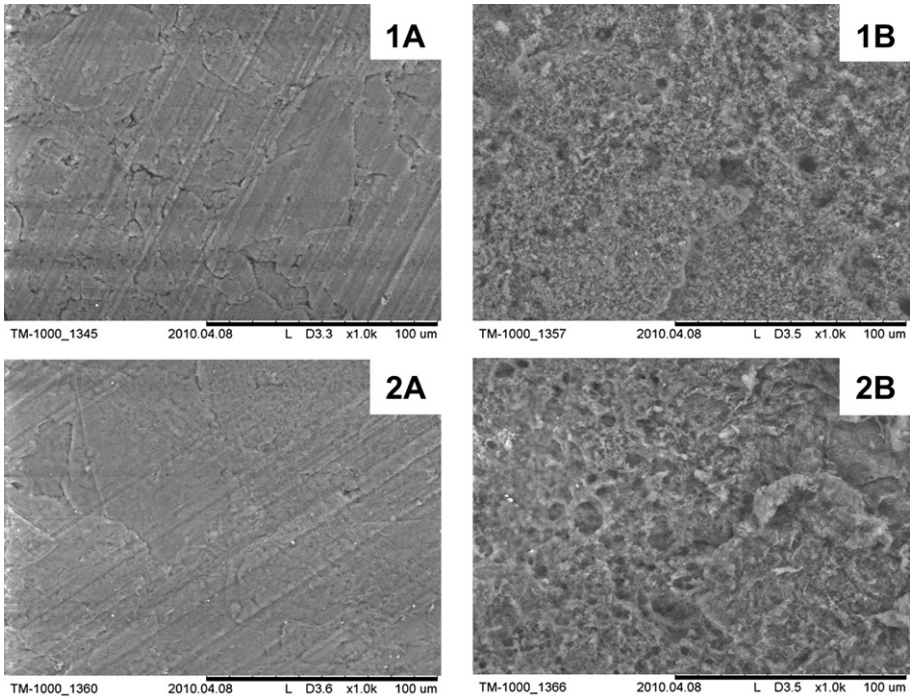

Fig. 7. Electron micrographs of PHA pellets: 1 – 3-PHB and 2 – 3-PHB/3-PHV; A – initial specimens, B – specimens after marine exposure for 160 days.

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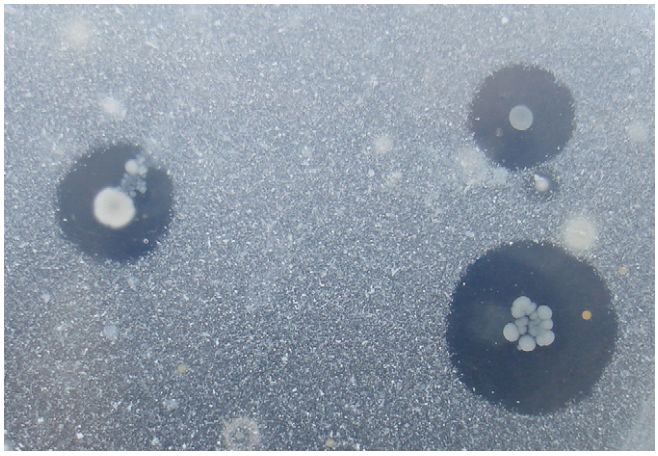

Fig. 8. Manifestation of depolymerase activity of PHA-degrading bacteria (clear-zone formation on diagnostic medium).

For a number of years we have been studying PHA degradation in laboratory and field experiments, proving that this process is influenced by the chemical structure of the polymer, the temperature of the environment, hydrochemical and hydrobiological parameters of water bodies, including the structure of the aquatic ecosystem [17,32]. We have isolated PHA-degrading microorganisms from natural sources and identified them [31]. Field studies were performed in Lake Shira – a salt-water lake with therapeutic properties, situated in Khakassia (south of the Krasnoyarskii Krai, Russia), with stratification of temperature, oxygen, chemical composition, and biota. Our observations showed that PHA was degraded not only in the best heated and aerated part of the lake, epilimnoion, at a depth of 3 m, but also in the oxic–anoxic chemocline, at a depth of 13 m, and at a depth of 20 m, in the monimolimnion, where there is no oxygen, but there is hydrogen sulfide, and where the temperature is low (1–2 °C) (unpublished data).

This study addressed degradation patterns of polymer specimens, prepared using different techniques (films and compacted pellets) from PHAs of different chemical composition (3-PHB and 3-PHB/3-PHV) in the tropics, at the Marine Research and Testing Station of the Vietnam–Russia Tropical Centre, in the South China Sea (Vietnam).

There have been rather few reports regarding PHA degradation patterns under natural conditions, in marine environments, in particular. Authors of one of the first papers in this field monitored degradation of 3-PHB/3-PHV and 3-PHB/4-PHB specimens in the Sea of Japan for one year [19]. They recorded a decrease in polymer molecular mass and erosion of the surface of the specimens, which were related to the season and water temperature. Imam and co-authors [18] studied degradation of 3-PHB/3-PHV compared to cornstarch and blends of cornstarch and 3-PHB/3-PHV in tropical coastal waters of Puerto Rico. The specimens were placed at the edge of a mangrove stand, within a mangrove stand, among the coastal reefs, and in open water, at a depth of 1 meter. Specimens exposed to seawater were degraded slower than those at the other stations. Degradation of 100% 3-PHB/3-PHV started after a pronounced lag period, at day 50 of the exposure; the degradation rate was very low, the rates of weight loss were not higher than 0.1%/day; the polymer had a half-life of 158 days. Microbial counts were much lower in the sea than in the mangrove stand, leading to different polymer degradation rates. Microbial biofilm on the surface of the specimens placed in the sea was less pronounced than on the specimens placed in other stations, and it was only formed after a certain lag period. Rutkowska and co-authors [21] monitored degradation of 3-PHB/3-PHV films in Gdynia Harbor of the Baltic Sea for 6 weeks and recorded 60% degradation. Blending of biological polymer and atactic poly [(R,S)-3-hydroxybutyrate) was found to slow down degradation rates. Professor Mergaert and colleagues [46] monitored PHA degradation in small freshwater ponds and in seawater, in the Zeebrugge Harbor (Belgium). The recorded degradation rate of 3-PHB/3-PHV in the sea was higher than in freshwater ponds. In freshwater ponds, the mass of 3-PHB specimens had decreased to 31% of their pre-exposure mass and the mass of the copolymer to 77% over one year, while in seawater the mass of 3-PHB specimens had decreased to 31% of their pre-exposure mass over 270 days and the mass of the copolymer – to 49–52%. Although the mass loss was considerable, no changes in the molecular weight of the PHA itself were recorded. Sridewi and co-authors studied degradation of 3-PHB, 3-PHB/3-PHV (5 mol% of 3-hydroxyvalerate) and 3-PHB/3-PHHx (5 mol% of hydroxyhexanoate) films in tropical mangrove ecosystems of Sungai Pinang, Malaysia, in different zones (in the surface water layer, deep in the sediment, in open water, and in mangrove stand). Copolymer specimens, which were less crystalline and whose surface was more porous, degraded faster than 3-PHB; the highest degradation rate was recorded for 3-PHB/3-PHHx films. The authors ascribed the differences in degradation rates to different structural and physico-chemical properties of the surfaces of the films prepared from various PHAs, causing stronger or weaker attachment of microorganisms onto the surface, and to the specificity of PHA exodepolymerases produced by these microorganisms. These data, obtained in various marine ecosystems of different parts of the world, cannot provide a universal biodegradation pattern under natural conditions for the two most studied PHAs (3-PHB and 3-PHB/3-PHV).

Our study of PHA degradation in the tropical marine environment did not reveal any significant differences between degradation rates of homopolymer and copolymer specimens. The weight loss of compacted pellets for 160 days was not greater than 20% of their initial weight; degradation rates of 3-PHB and 3-PHB/3-PHV films were similar and they lost half of their weight. Conversely, in our earlier studies, when PHAs were degraded in laboratory soil microcosms and natural water bodies, 3-PHB/3-PHV specimens degraded faster than 3-PHB ones, and their degradation rates increased with the growing percentage of 3-hydroxyvalerate in the copolymer [17,31,32]. That was in agreement with the results reported by other authors [18,19], who also showed that 3-PHB/3-PHV specimens degraded faster than 3-PHB ones. However, Kita and co-authors [24] reported that in the pure culture of the bacterium *Alcaligenes faecalis*, degradation rates of PHB were higher than those of 3-PHB/3-PHV specimens. Thellen and co-authors [28] studied degradation patterns of melt-extruded PHA films in the laboratory media containing slurry of marine sediment. PHA films were biodegraded in the marine environment; all samples achieved a minimum of 70% mineralization in 40 days; under dynamic conditions (in tanks with marine sediment and continuously flowing seawater) the mass loss of 3-PHB specimens was greater than that of 3-PHB/3-PHV ones.

In our study, during PHA degradation in seawater, as the mass of the specimens was reduced, M_w decreased and polydispersity increased, which was in agreement with the results reported by other authors [9,13,27]. However, while polymer chains were depolymerised and M_w decreased, crystallinity of both PHAs remained unchanged, suggesting a conclusion that in the studied marine environment, the amorphous phase of the PHA and the crystalline one were equally disintegrated, which is in disagreement with the current opinion that depolymerising enzymes preferentially degrade the amorphous part of a polymer rather than its crystalline region [9,27].

What role do PHA depolymerases play in PHA biodegradation process? As shown quite convincingly in a number of studies, PHA biodegradation pattern is determined to a large extent by the type of microorganisms that take part in polymer biodegradation and

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specificity and catalytic activity of extracellular PHA depolymerases. Numata and co-authors [30] showed that the rate of PHA biodegradation by depolymerase isolated from *Ralstonia pickettii* is determined by polymer composition, which is responsible for the basic properties of the polymer (temperature characteristics, crystallinity, the structure of the crystalline region, spherulite morphology and size, in particular), and the composition of monomeric units. The authors found that extracellular PHB depolymerase from *R. pickettii* had a higher affinity for the 3-PHB/3-PHV copolymer, which was degraded *in vitro* by the enzyme at a higher rate than 3-PHB; this difference became more pronounced with increasing 3-hydroxyvalerate content. The authors suggest that the higher erosion rate of 3-PHB/3-PHV is due to the isodimorphic crystals of 3-PHB/3-PHV, which can be more readily degraded by the enzyme. Feng and co-authors [29] examined *in vitro* biodegradation of sterile films prepared from PHAs of various chemical structures by extracellular PHA depolymerases isolated from *R. pickettii* T1 and *Acidovarans* sp. TP4 and showed that degradation rates were determined to a large extent by the type of the enzyme. Depolymerase from *R. pickettii* degrades 3-PHB/3-PHV specimens at different rates depending on the content of 3-hydroxyvalerate; depolymerase from *Acidovarans*, on the other hand, degrades the copolymer if the content of 3-hydroxyvalerate in it is below 80 mol%. The rate of copolymer degradation by either depolymerase accelerates with increasing 3-hydroxyvalerate content, reaching the highest values at 40 mol% of 3HV; at 3HV content higher than that the activity of the enzymes subsides.

Analysis of microorganisms that formed biofilms on PHA specimens incubated in seawater in the South China Sea revealed a great variety of both heterotrophic bacteria and fungal microflora. However, just a few species were found to have PHA depolymerase activity and to be able to grow using PHA as sole carbon and energy source. Using 16S rRNA analysis, we identified the isolated PHA-degrading strains as *Enterobacter* sp. IBP-VN1, *Bacillus* sp. IBP-VN2, *Gracilibacillus* sp. IBP-VN3, *Enterobacter* sp. IBP-VN4, *Enterobacter* sp. IBP-VN5 and *Enterobacter* sp. IBP-VN6. So far, there has been no mention of PHA depolymerase activity in representatives of the genera *Gracilibacillus* and *Enterobacter* in the available literature.

5. Conclusion

The study of the biodegradation patterns of PHAs in the tropical marine environment showed that they are rather influenced by the shape of the polymer item and the preparation technique than by the chemical composition of the polymer. Biodegradation rates of polymer films in seawater were found to be higher than those of compacted pellets. As 3-PHB and 3-PHB/3-PHV are degraded and the specimens lose their mass, molecular weight is decreased, i.e. polymer chains get destroyed. The polydispersity index of the PHAs grows significantly. However, the degree of crystallinity of both PHAs remains almost unchanged, i.e. the amorphous phase of the PHA and the crystalline one are equally disintegrated. Based on the 16S rRNA analysis, the PHA-degrading strains isolated from seawater were identified as *Enterobacter* sp. IBP-VN1, *Bacillus* sp. IBP-VN2, *Gracilibacillus* sp. IBP-VN3, *Enterobacter* sp. IBP-VN4, *Enterobacter* sp. IBP-VN5 and *Enterobacter* sp. IBP-VN6. Until now, there has been no data regarding PHA depolymerase activity in representatives of the genera *Gracilibacillus* and *Enterobacter*.

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References

[1] Bombach P, Richnow HH, Kästner M, Fischer A. Current approaches for the assessment of in situ biodegradation. Applied Microbiology and Biotechnology 2010;86:839–52.
[2] Agenda 21: earth summit – The United Nations Programme of Action from Rio. United Nations; 1993. 294 pp.
[3] Cornell DD. Biopolymers in the existing postconsumer plastics recycling stream. Journal of Polymers and the Environment 2007;15:295–9.
[4] Greene J. Biodegradation of compostable plastics in green yard-waste compost environment. Journal of Polymers and the Environment 2007;15:269–73.
[5] Tokiwa Y, Calabia BP. Biodergadability and biodegradation of polyesters. Journal of Polymers and the Environment 2007;15:259–67.
[6] Philip S, Keshavarz T, Roy I. Polyhydroxyalkanoates: biodegradable polymers with a range of applications. Journal of Chemical Technology and Biotechnology 2007;82:233–47.
[7] Chen G- Q. A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chemical Society Reviews 2009;38:2434–46.
[8] Tokiwa Y, Calabia BP. Degradation of microbial polyesters. Biotechnology Letters 2004;26:1181–9.
[9] Jendrossek D, Handrick R. Microbial degradation of polyhydroxyalkanoates. Annual Review of Microbiology 2002;56:403–32.
[10] Kim DY, Rhee YH. Biodergadation of microbial and synthetic polyesters by fungi. Applied Microbiology and Biothechnology 2003;61:300–8.
[11] Knoll M, Hamm TM, Wagner F, Martinez V, Pleiss J. The PHA depolymerase engineering database: a systematic analysis tool for the diverse family of polyhydroxyalkanoate (PHA) depolymerases. BMC Bioinformatics 2009; 10:89–97.
[12] Mergaert J, Webb A, Anderson C, Wouters A, Swings J. Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in soils. Applied and Environmental Microbiology 1993;59:3233–8.
[13] Mergaert J, Swings J. Biodiversity of microorganisms that degrade bacterial and synthetic polyesters. Journal of Industrial Microbiology 1996;17:463–9.
[14] Suyama T, Tokiwa Y, Ouichanpagdee P, Kanagawa T, Kamagata Y. Phylogenetic affiliation of soil bacteria that degrade aliphatic polyesters available commercially as biodegradable plastics. Applied and Environmental Microbiology 1998;64:5008–11.
[15] Takaku H, Kimoto A, Kodaira S, Nashimoto M, Takagi M. Isolation of a Gram-positive poly(3-hydroxybutyrate) (PHB)-degrading bacterium from compost, and cloning and characterization of a gene encoding PHB depolymerase of *Bacillus megaterium* N-18-25-9. FEMS Microbiology Letters 2006;264:152–9.
[16] Kasuya KI, Inoue Y, Tanaka T, Akehata T, Iwata T, Fukui T, et al. Biochemical and molecular characterization of the polyhydroxybutyrate depolymerase of *Comamonas acidovorans* YM1609, isolated from freshwater. Applied and Environmental Microbiology 1997;63:4844–52.
[17] Volova TG, Gladyshev MI, Trusova MY, Zhila NO. Degradation of polyhydroxyalkanoates in eutrophic reservoir. Polymer Degradation and Stability 2007;92:580–6.
[18] Imam SH, Gordon SH, Shogren RL, Tosteson TR, Govind NS, Greene RV. Degradation of starch-poly(beta-hydroxybutyrate-co-beta-hydroxyvalerate) bioplastic in tropical coastal waters. Applied and Environmental Microbiology 1999;65:431–7.
[19] Doi Y, Kanesawa Y, Tanahashi N, Kumagai Y. Biodegradation of microbial polyesters in the marine environment. Polymer Degradation and Stability 1992;36:173–7.
[20] Sridewi N, Bhubalan K, Sudesh K. Degradation of commercially important polyhydroxyalkanoates in tropical mangrove ecosystem. Polymer Degradation and Stability 2006;91:2931–40.
[21] Rutkowska M, Krasowska K, Heimowska A, Adamus G, Sobota M, Musioł et M, et al. Environmental degradation of blends of atactic poly[(*R, S*)-3-hydroxybutyrate] with natural PHBV in Baltic sea water and compost with activated sludge. Journal of Polymers and the Environment 2008;6:183–91.
[22] Leathers TD, Govind NS, Greene RV. Biodegradation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by a tropical marine bacterium, *Pseudoalteromonas* sp. NRRL B-30083. Journal of Polymers and the Environment 2000;8:119–24.
[23] Kasuya K, Mitomo H, Nakahara M, Akiba A, Kudo T, Doi Y. Identification of a marine benthic P(3HB)-degrading bacterium isolate and characterization of its P(3HB) depolymerase. Biomacromolecules 2000;1:194–201.
[24] Kita K, Mashiba S, Nagita M, Ishimaru K, Okamoto K, Yanase H, et al. Cloning of poly(3-hydroxybutyrate) depolymerase from a marine bacterium, *Alcaligenes faecalis* AE122, and characterization of its gene product. Biochimica et Biophysica Acta 1997;1352:113–22.
[25] Ghanem NB, Mabrouk ME, Sabry SA, El-Badan DE. Degradation of polyesters by a novel marine *Nocardiopsis aegyptia* sp. nov.: application of Plackett–Burman experimental design for the improvement of PHB depolymerase activity. The Journal of General and Applied Microbiology 2005;51:151–8.
[26] Mabrouk MM, Sabry SA. Degradation of poly (3-hydroxybutyrate) and its copolymer poly (3-hydroxybutyrate-co-3-hydroxyvalerate) by a marine *Streptomyces* sp. SNG9. Microbiological Research 2001;156:323–35.
[27] Abe H, Marsubara I, Doi Y. Physical properties and enzymatic degradability of polymer blends of bacterial poly[(*R*)-3-hydroxybutyrate] and poly[(*R,S*)-3-hydroxybutyrate] stereoisomers. Macromolecules 1995;28:844–53.
[28] Thellen C, Coyne M, Froio D, Auerbach M, Wirsen C, Ratto JA. A processing, characterization and marine biodegradation study of melt-extruded

polyhydroxyalkanoate (PHA) films. Journal of Polymers and the Environment 2008;16:1–11.
[29] Feng L, Wang Y, Inagawa Y, Kasuya K, Saito T, Doi Y, et al. Enzymatic degradation behavior of comonomer compositionally fractionated bacterial poly (3-hydroxybutyrate-co-3-hydroxyvalerate)s by poly(3-hydroxyalkanoate) depolymerases isolated from Ralstonia pickettii T1 and Acidovorax sp. TP4. Polymer Degradation and Stability 2004;84:95–104.
[30] Numata K, Kikkawa Y, Tsuge T, Iwata T, Doi Y, Abe H. Enzymatic degradation processes of poly[(R)-3-hydroxybutyric acid] and poly[(R)-3-hydroxybutyric-co-(R)-3-hydroxyvaleric acid] single crystals revealed by atomic force microscopy: effects of molecular weight and second-monomer composition on erosion rates. Biomacromolecules 2005;6:2008–16.
[31] Volova TG, Gladyshev MI, Trusova MY, Zhila NO. Degradation of polyhydroxyalkanoates and the composition of microbial destructors under natural conditions. Microbiology 2006;75(5):593–8.
[32] Voinova ON, Gladyshev MI, Volova TG. Comparative study of PHA degradation in natural reservoirs having various types of ecosystems. Macromolecular Symposia 2008;269:34–7.
[33] Stasishina GN, Volova TG. Patent of RF No. 2053292: Shtamm bakterii *Alcaligenes eutrophus* – produtsent (The strain of the bacterium *Alcaligenes eutrophus* – a producer). Bjulleten' izobretenij (Invention bulletin); 1996:1 (in Russian).
[34] Volova TG, Kalacheva GS, Plotnikov VF. Biosynthesis of heteropolymeric polyhydroxyalkanoates by chemolithoautotrophic bacteria. Microbiology 1998;67:420–4.
[35] McKenzie HA, Wallace HS. The Kjeldahl determination of nitrogen: a critical study of digestion conditions – temperature, catalyst, and oxidizing agent. Australian Journal of Chemistry 1954;7:55–70.
[36] Seifter S, Dayton S, Novic B, Muntwyler E. The estimation of glycogen with the anthrone reagent. Archives of Biochemistry and Biophysics 1950;25 (1):191–200.
[37] Brandl H, Knee EJ, Fuller RC, Gross RA, Lenz RW. Ability of the phototrophic bacterium *Rhodospirillum rubrum* to produce various poly (β-hydroxyalkanoates): potential sources for biodegradable polyesters. International Journal of Biological Macromolecules 1989;11:49–55.
[38] De Gennes P-G. Wetting: statics and dynamics. Uspekhi phizicheskikh nauk [Advances in Physical Sciences] 1987;151:619–81 (in Russian).
[39] Netrusov AI, Egorova MA, Zakharchuk LM. Praktikum po mikrobiologii [Practical course in microbiology]. Moscow: Akademiya; 2005. 608 pp. (in Russian).
[40] Garrity GM, editor. Bergey's manual of systematic bacteriology. The proteobacteria. 2nd ed., vol. 2. Springer; 2005. 2816 pp.
[41] Dworkin M, editor. The prokaryotes – a handbook on the biology of bacteria. 3rd ed., vols. 1–7. Springer; 2006.
[42] Beleneva IA, Zhukova NV, Le Lan H, Nguyen Tran DH. Taxonomic composition of bacteria associated with cultivated mollusks *Crassostrea lugubris* and *Perna viridis* and with the water of the Gulf of Nha Trang Lagoon, Vietnam. Microbiology 2007;76(2):220–8.
[43] Beleneva IA. Distribution and characteristics of *Bacillus* bacteria associated with hydrobionts and the waters of the Peter the Great Bay, Sea of Japan. Microbiology 2008;77(4):497–503.
[44] Beleneva IA, Zhukova NV. Seasonal dynamics of cell numbers and biodiversity of marine heterotrophic bacteria inhabiting invertebrates and water ecosystems of the Peter the Great Bay, Sea of Japan. Microbiology 2009;78(3): 369–75.
[45] Chen HJ, Pan SC, Shaw GC. Identification and characterization of a novel intracellular poly(3-hydroxybutyrate) depolymerase from *Bacillus megaterium*. Applied and Environmental Microbiology 2009;75(16):5290–9.
[46] Mergaert J, Wouters A, Anderson C, Swings J. In situ biodegradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in natural waters. Canadian Journal of Microbiology 1995;41(13):154–9.

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