 DOI: 10.1002/masy.201251004

Biodegradation of Polyhydroxyalkanoate Films in Natural Environments

Anatoly N. Boyandin,[*1,2] *Valentin P. Rudnev,*[3] *Vladimir N. Ivonin,*[3] *Svetlana V. Prudnikova,*[2] *Ksenia I. Korobikhina,*[2] *Maxim L. Filipenko,*[4] *Tatiana G. Volova,*[1,2] *Anthony J. Sinskey*[5]

Summary: Biodegradation of film specimens from polyhydroxyalkanoates (PHAs) of two types – poly-3-hydroxybutyrate (PHB) and poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate (PHBV) – was analysed in different environments: tropical sea waters of the South China Sea (Nha Trang, Vietnam) and soils in the environs of Hanoi (Vietnam), Nha Trang (Vietnam) and Krasnoyarsk (Siberia, Russia). In seawater, the mass loss of the specimens of both types was almost equal. However, in tropical soils, PHB degraded quicker than PHBV. In the Siberian soil, the degradation rate of the PHBV was generally higher than that of PHBV. Analysis of molecular mass of PHA specimens showed its decreasing during biodegradation. In the tropical sea conditions, PHA degrading microorganisms were represented by bacteria of *Enterobacter*, *Bacillus* and *Gracilibacillus* genera. Among PHA degrading bacteria, *Burkholderia*, *Alcaligenes*, *Bacillus*, *Mycobacterium* and *Streptomyces* genera were identified in Vietnamese soils, and *Variovorax*, *Stenotrophomonas*, *Acinetobacter*, *Pseudomonas*, *Bacillus* and *Xanthomonas* genera in Siberian soils. Micromycetes of *Gongronella*, *Paecilomyces*, *Penicillium* and *Trichoderma* genera exhibited PHA degrading activity in Vietnamese soils, and *Paecilomyces*, *Penicillium*, *Acremonium*, *Verticillium* and *Zygosporium* genera – in Siberian soils.

Keywords: biodegradation; biopolymers; microbial degradation; polyhydroxyalkanoates

Introduction

The use of chemically synthesized polymers leads to accumulation of large quantities of chemicals in the biosphere. Polyhydroxyalkanoates (PHAs) – polyesters of bacterial origin – currently occupy a special position among biodegradable natural polymers as materials with numerous useful properties and a wide range of applications.[1–3] Due to the decline in the cost, PHA application is more and more directed toward the production of degradable containers, packages, and disposable dishware and household goods. Therefore, PHAs are ecologically important alternative materials that can replace synthetic plastics.

In the natural environment PHAs are decomposed by PHA degrading microorganisms to carbon dioxide and water (aerobic conditions) or methane and water (anaerobic conditions). Such microorganisms were isolated more than 40 years ago.[4] Later, different groups of these microorganisms were identified and characterized.[5–8] However, it is still discussed which PHAs are degraded more actively in different environmental conditions.

[1] Institute of Biophysics SB RAS. 50/50 Akademgorodok, Krasnoyarsk, 660036, Russia
Fax: (3912) 43 34 00;
E-mail: araneus@mail.ru
[2] Siberian Federal University. 79 Svobodnyi Av., Krasnoyarsk, 660041, Russia
[3] The Joint Vietnam-Russian Tropical Research and Technological Centre, Nguyen Van Huyen, Nghia Do, Cau Giay, Hanoi, Vietnam
[4] Institute of Chemical Biology and Fundamental Medicine SB RAS, 8 Lavrentiev Ave., Novosibirsk, 630090, Russia
[5] Massachusetts Institute of Technology, Cambridge, Massachusetts, 02139, USA

Poly-3-hydroxybutyrate (PHB) and poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) are among the most commonly occurring bacterial PHAs. PHB is a highly crystalline and brittle thermoplastic. PHBV has much better properties, including reduced brittleness. This copolymer is more useful commercially because its melting point can be lowered, and its mechanical properties and thermoplastic characteristics can be greatly improved by increasing the ratio of 3-hydroxyvalerate to 3-hydroxybutyrate repeating units.[9]

As the volume of production increases and the fields of PHA application are extended, it becomes relevant to study the mechanisms of their destruction under various natural conditions. The purpose of this study was to investigate degradation of polymers with different chemical structure – poly-3-hydroxybutyrate and 3-hydroxybutyrate-*co*-3-hydroxyvalerate – in natural environments and to identify PHA degrading microorganisms.

Experimental Part

Preparation of PHA Samples

Poly-3-hydroxybutyrate (PHB) and copolymer poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) containing 10 mol% of hydroxyvalerate, synthesized in the Institute of Biophysics SB RAS, Russia, by cultivating bacterium *Ralstonia eutropha*, strain B5786 (Russian National Collection of Industrial Microorganisms), were used in experiments.[10] Films were prepared by casting chloroform solution (3% w/v) on degreased glass and subsequent drying at room temperature for 2–3 days in a dust-free box. Segments of equal thickness were selected and disks of diameter 30 mm were cut out to be further used in the experiments.

Experimental Designs and Sampling Gears

PHA specimens were weighed and placed in close-meshed gauze jackets with pouches. Experiments were conducted in most warm period for every region (summer and nearest times). PHA degradability in seawater was examined in the Nha Trang Bay (the South China Sea, Vietnam). The experiments were carried out from March to August 2009. Experiments in Vietnamese soils were conducted at Hanoi and Nha Trang stations of the Joint Russian-Vietnamese Tropical Research and Technological Centre from May to November 2010. The study of biodegradation of PHAs in Siberian soils was performed at Krasnoyarsk (Siberia, Russia), in the Arboretum of the Institute of Forest SB RAS. PHA specimens were maintained in root zones of larch (*Larix sibirica L.*) and birch (*Betula pendula L.*) from June to September 2010. Periodically one of the jackets was withdrawn, specimens were washed in distilled water, dried at 40 °C for 48 h and weighed (on a Metler balance, USA). Molecular mass and molecular mass distribution of PHAs were analysed using GPC (Waters, USA). Polymer crystallinity was measured using X-Ray spectrometer (D8 ADVANCE, Bruker, Germany).

Microbiological Study

Analysis of microorganisms was conducted at or near the end of experiments (after about 180 days of exposure in Vietnamese soil experiments, about 140 days in Vietnamese marine experiments and about 100 days in Siberian soil experiments, due to a shorter summer period in Siberia). Soil debris were washed off the polymer surface, and different dilutions of the resulting suspension were inoculated onto nutrient agar plates (Yoshimizu-Kimura (Y-K) agar medium for seawater samples and peptone agar medium for soil samples). True PHA degraders were detected by inoculation of the samples removed from the surface of polymer specimens onto the diagnostic media (mineral agar for bacteria and Czapek agar media for micromycetes) that contained 0.25% powdered β-polyhydroxybutyrate as sole carbon source.[11] Growth of microorganisms with PHA-depolymerase activity was accompanied by the formation of clear zones around colonies of microorganisms.

In addition to conventional morphological and biochemical examination, PHA degrading bacteria were identified by 16S rRNA gene sequence analysis. 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 universal primers 27F (5′-AGAGTT TGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTT GTTACGACTT-3′), corresponding to *Escherichia coli* positions 8-27 and 1510-1492, respectively. PCR was performed using a Mastercycler Gradient amplifier (Eppendorf, Germany). The DNA nucleotide sequence was determined using Sanger sequencing methods, with a BigDye Terminator Cycle Sequencing Kit v 3.1 (Applied Biosystems, USA), on an ABI PRISM 3100 genetic analyser (Applied Biosystems, USA), following the manufacturer's protocol.

Soil micromycetes were identified based on their micro- and macro-morphological traits (structure and colour of colonies, structure of mycelium and spore-bearing organs).[12,13]

Results and Discussion

In 160 days after the specimens had been submerged in seawater, the residual masses of PHB and PHBV films were 58% and 54%, respectively, i.e. the mass losses of the specimens of both types were almost equal (Figure 1A). However, there were differences in PHB and PHBV degradation in soil experiments (Figure 1B). In tropical soils, PHB was degraded quicker than PHBV. In this experiment, the site of exposure was the most important factor defining the degradation rate, which was significantly higher in Hanoi than in Nha Trang. After 184 days of Hanoi soil exposure, PHB specimens lost 97% of their initial mass whereas PHBV specimens lost 33%. In Nha Trang soil experiments, PHA specimens were degraded much slower: the mass loss was about 16% for PHB and 7% for PHBV specimens. It was apparently due to low precipitation in Nha Trang and, therefore, very low soil moisture content during the experimental period.

Siberian soil experiments were conducted in the rhizosphere of different tree species: the Siberian larch (*Larix sibirica L.*) and the silver birch (*Betula pendula L.*). In the soil under the larch, which was moister and housed more microorganisms, PHA degradation rates were higher than those recorded under the birch (Figure 1C). By the end of the experiment, the residual mass of PHB specimens in the larch soil had decreased to 45% of their initial mass, and the residual mass of PHBV specimens – to 22%. In the soil of the birch rhizosphere, the degradation rates of both PHA types were lower, in spite of the great variety of the fungi present in this soil. At day 109 of the exposure, the residual masses of PHB and PHBV specimens amounted to 84% and 74% of their initial masses, respectively. Therefore, in contrast to Vietnam soil experiments, the degradation rate of the PHBV was generally higher than that of PHBV. The differences in degradation rates of two PHA types may be determined by substrate specificity of PHA depolymerases of major PHA degrading microorganisms in different ecosystems.

Degradation and mass loss of the films altered morphology of their surface. As the number of pinholes increased, the films became prone to fragmentation (an example is shown on Figure 2). Electron micrographs also show these changes: as the specimens degraded, the number and sizes of pores and pinholes, which were absent on the initial specimens, increased. Analysis of X-ray spectra of PHA specimens after marine exposure did not reveal any change in their crystallinity. On the contrary, specimens in Siberian soil experiments showed increases in the degrees of crystallinity of both PHAs, suggesting preferential disintegration of the amorphous phase in the soil. This effect may also depend on substrate specificity of PHA depolymerases of dominant PHA degrading microorganisms. Analysis of molecular

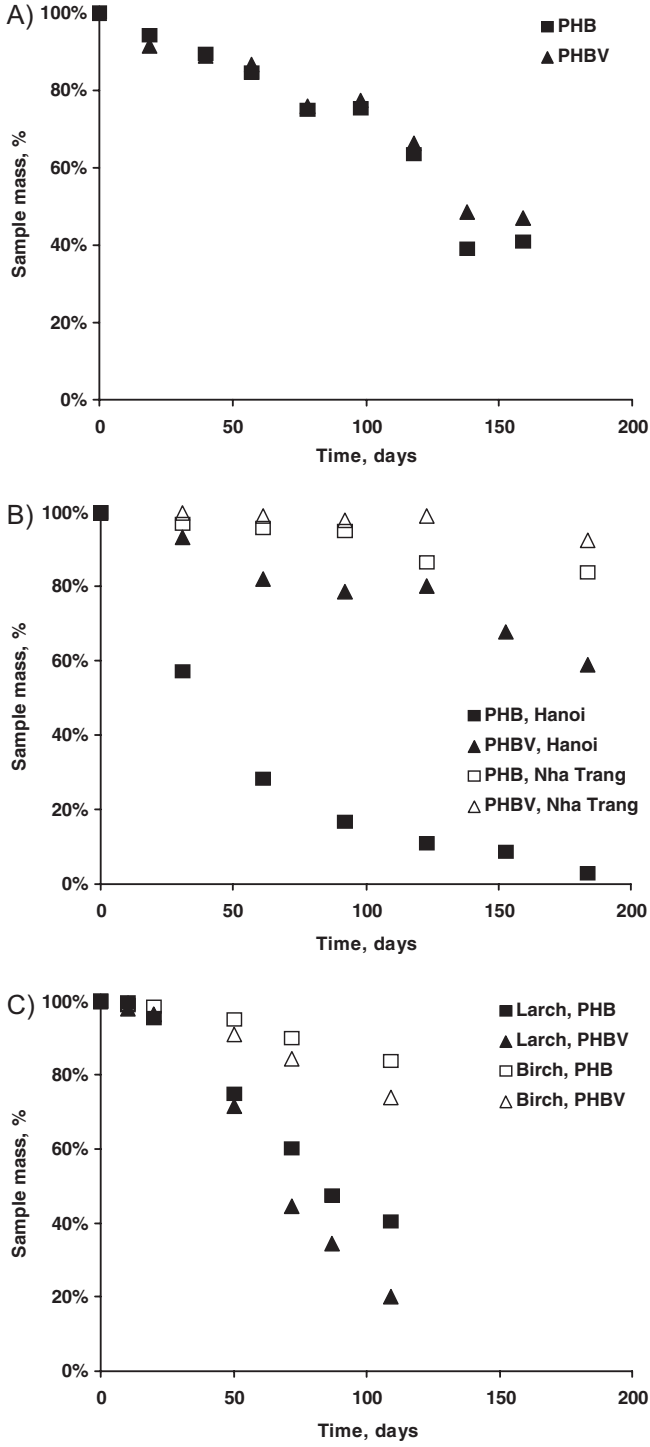

Figure 1.
Dynamics of the mass of polymer specimens: A – seawater; B – Vietnamese soil; C – Siberian soil.

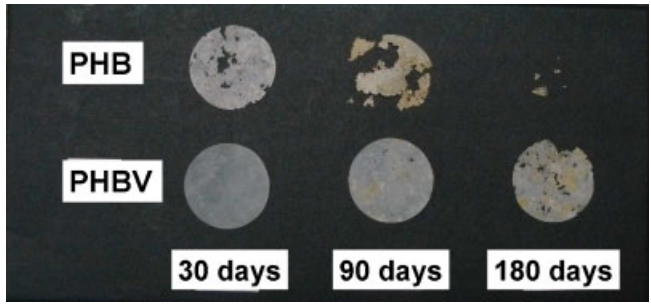

Figure 2.
Biodegradation of PHA specimens in the soil of Hanoi.

mass of PHA specimens showed that it decreased during biodegradation in all measurements, suggesting that PHA chains were destroyed.

The specimens retrieved at the end of field experiments were subjected to microbiological study. In all experiments, total bacterial counts showed a stimulating effect of PHAs on bacterial growth: concentration of bacteria on polymer surface was significantly higher than in the environment. Soil micromycetes behaved in a similar way, which was indicative of their significant contribution to PHA degradation.

Microbiological analyses were conducted to identify PHA degrading microorganisms. In the tropical sea, PHA degrading microorganisms were represented by bacteria of *Enterobacter*, *Bacillus* and *Gracilibacillus* genera. In the soils, micromycetes also contributed much to PHA degradation. Among PHA degrading bacteria, *Burkholderia*, *Alcaligenes*, *Bacillus*, *Mycobacterium* and *Streptomyces* genera were identified in Vietnamese soils, and *Variovorax*, *Stenotrophomonas*, *Acinetobacter*, *Pseudomonas*, *Bacillus* and *Xanthomonas* genera in Siberian soils. Micromycetes of *Gongronella*, *Paecilomyces*, *Penicillium* and *Trichoderma* genera exhibited PHA degrading activity in Vietnamese soils, and *Paecilomyces*, *Penicillium*, *Acremonium*, *Verticillium* and *Zygosporium* genera – in Siberian soils. Thus, *Bacillus* species contributed to PHA degradation in all ecosystems studied. Similarly to this, micromycetes of *Paecilomyces* and *Penicillium* were characteristic PHA degraders in both Vietnamese and Siberian soils.

Acknowledgements: The study was supported by the project initiated by the Government of the Russian Federation for governmental support of scientific research conducted under the guidance of leading scientists at Russian institutions of higher learning (Agreement No. 11.G34.31.0013) and the Program of Integrated Research of the Presidium of SB RAS (Project No. 96).

[1] S. Philip, T. Keshavarz, I. Roy, *J. Chem. Tech. Biotechnol.* **2007**, *82*, 233.
[2] G.-Q. Chen, *Chem. Soc. Rev.* **2009**, *38*, 2434.
[3] Y. Tokiwa, B. P. Calabia, *Biotechnol. Lett.* **2004**, *26*, 1181.
[4] A. A. Chowdhury, *Arch. Mikrobiol.* **1963**, *47*, 167.
[5] J. Mergaert, A. Webb, C. Anderson, A. Wouters, J. Swings, *Appl. Environ. Microbiol.* **1993**, *59*, 3233.
[6] H. Brandl, P. Puchner, *Biodegradation.* **1991**, *2*, 237.
[7] B. H. Briese, D. Jendrossek, H. G. Schlegel, *FEMS Microbiol. Letts.* **1994**, *117*, 107.
[8] J. Mergaert, C. Anderson, A. Wouters, J. Swings, *J. Environ. Polym. Degrad.* **1994**, *2*, 177.
[9] A. Dufresne, D. Dupeyre, M. Paillet, *Journal of Applied Polymer Science.* **2003**, *87*, 1302.
[10] T. G. Volova, G. S. Kalacheva, V. F. Plotnikov, *Mikrobiologiya.* **1998**, *67*, 512 (in Russian).
[11] F. P. Delafield, M. Doudoroff, N. J. Palleroni, C. J. Lusty, R. Contopoulos, *J. Bacteriol.* **1965**, *90*, 1455.
[12] L. N. Egorova, "*Pochvennyye griby Dalnego Vostoka. Gifomitsety (Soil fungi of the Far East. Hyphomycetes)*", Nauka, Leningrad **1986**, 192 p. (in Russian).
[13] D. A. Sutton, A. W. Fothergill, M. G. Rinaldi, "*Guide to Clinically Significant Fungi*", Williams & Wilkins, Baltimore (MD) **1998**.

