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EXPERIMENTAL ARTICLES

The Biodegradation of Poly-β-Hydroxybutyrate (PHB) by a Model Soil Community: The Effect of Cultivation Conditions on the Degradation Rate and the Physicochemical Characteristics of PHB

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Abstract—The biodegradation of films made of poly-β-hydroxybutyrate (PHB) with a molecular mass of 1500 kDa was studied using a model soil community in the presence and absence of nitrate and at different concentrations of oxygen in the gas phase. The biodegradation of PHB was investigated with respect to changes in its molecular mass, crystallinity, and some mechanical properties.

Key words: *Azotobacter chroococcum*, biodegradation, poly-β-hydroxybutyrate, molecular mass, crystallinity.

Poly-β-hydroxybutyrate (PHB) is a storage polymer in some microorganisms, which is synthesized under the conditions of unbalanced growth. Generally, polymers occurring in the environment are degraded through their hydrolysis, mechanical, thermal, oxidative, and photochemical destruction, and biodegradation. One of the valuable properties of PHB is its biodegradability, which can be evaluated using various field and laboratory tests. The ASTM test for the aerobic and anaerobic degradation of PHB includes the evaluation of changes in the chemical composition and physicochemical characteristics of PHB and its degradation products [1]. Requirements for the biodegradability of PHB may vary in accordance with its applications. The most attractive property of PHB with respect to ecology is that it can be completely degraded to CO_2 and H_2O by microorganisms. The degradation of PHB and its composites in natural ecosystems, such as soil, compost, and bodies of water, was described in a number of publications [2–6]. Maergaert *et al*. [2] isolated from soil more than 300 microbial strains capable of degrading PHB in vitro, of which denitrifying bacteria are of particular practical interest due to their potential ability to be used in the immobilized state on PHB films for the purification of water from nitrates [7]. Accordingly, the investigation of the reduction of nitrates by denitrifying microbial communities in the presence of PHB as a carbon source is by no means of great interest.

The aim of the present work was to study the biodegradation of PHB in a model soil community in the presence and absence of nitrate and at different concentrations of oxygen in the gas phase and to investigate changes in its molecular mass, crystallinity, and some mechanical properties.

MATERIALS AND METHODS

Experiments were carried out with the high-molecular-weight poly-β-hydroxybutyrate (molecular mass, 1500 kDa; the molecular weight distribution index, 2.7) synthesized by the nitrogen-fixing bacterium *Azotobacter chroococcum* 32B, which was isolated from the rhizosphere of wheat grown in soddy podzolic soil. Strain 32B was maintained on Ashby medium containing (g/l) $K_2HPO_4 \cdot 3H_2O$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.2; $Na_2MoO_4 \cdot 2H_2O$, 0.006; $CaCO_3$, 5.0; sucrose, 20; and agar, 20. To enhance the synthesis of PHB up to 80% by the dry weight of cells, the strain was grown at an excess of carbon sources in a medium containing (g/l) $K_2HPO_4 \cdot 3H_2O$, 1.05; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.4; $FeSO_4 \cdot 7H_2O$, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.006; $CaCl_2$, 0.1; sodium citrate, 0.5; and glucose, 40.

PHB was isolated from the biomass of *A. chroococcum* 32B by a procedure consisting of the following stages: PHB extraction with chloroform on a shaker at 37°C for 12 h; the removal of cell debris by filtration; PHB purification by means of thrice precipitation with isopropanol and dissolution in chloroform; and PHB drying at 60°C. PHB films 0.03–0.04 mm in thickness

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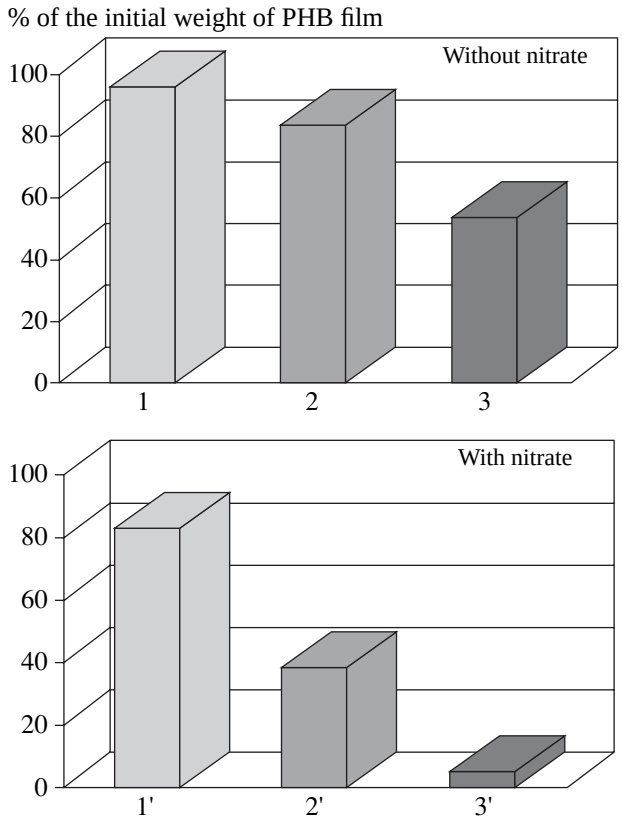

Fig. 1. The effect of oxygen concentration in the gas phase and the presence of nitrate in the soil suspension on the degree of degradation of PHB films after 2 months of incubation under (1) anaerobic, (2) microaerobic, and (3) aerobic conditions.

were prepared by pouring a chloroform solution of PHB onto the bottom of petri dishes.

The molecular mass of PHB was determined viscosimetrically. The intrinsic viscosity η of PHB was measured in a chloroform solution at 30°C, and the molecular mass *M* was calculated according to the equation $[\eta] = 7.7 \times 10^{-5} M^{0.82}$ [8].

The mechanical properties of PHB were tested using an Instron 1121 universal dynamometer and test specimens in the form of a blade 10 mm long and 0.03–0.04 mm thick. Testing was carried out at 20°C at a traverse velocity of 5 mm/min.

The content of volatile fatty acids in the liquid phase of soil suspensions was determined using a Chrom-5 gas chromatograph equipped with a flame ionization detector and a glass column (3 mm × 1.2 m) packed with Chromosorb 101. The column was kept at 170°C, the carrier gas was argon, and the sample volume was 2 μl.

The content of N_2O in the gas phase was determined using the same chromatograph equipped with a thermal-conductivity detector and a glass column (3 mm × 1.2 m) packed with Porapak Q. The detector was kept at 20°C; the carrier gas was argon.

The degree of PHB crystallinity was evaluated by X-ray analysis using a Bruker AXS device equipped with a rotating copper anode and a two-dimensional detector.

The degradation of PHB was studied by placing polymer film pieces weighing about 40 mg into 50-ml flasks with 20 ml of a suspension containing 10% soil in 0.1 M potassium phosphate buffer. Potassium nitrate was added at a concentration of 5 g/l. To create anaerobic conditions in the flasks, they were sealed with rubber stoppers, evacuated, and filled with argon containing 5% acetylene to inhibit N_2O reductase. In experimental variants with microaerobic conditions, the sealed unevacuated flasks were filled with air containing 5% acetylene. In experimental variants with aerobic conditions, the flasks were merely closed with cotton-wool plugs. The flasks were incubated at 28°C. At regular intervals, the film pieces were withdrawn from the flasks, thoroughly washed with 0.1 M K phosphate buffer, dried at 60°C for 2 h, and weighed to determine the loss of their weight because of PHB degradation.

By the end of the experiments, residual film pieces weighing about 10 mg were transferred to sterile water and shaken for 1 h. Then the water with desorbed bacterial cells was diluted tenfold to a millionfold, and the dilutions were plated onto Czapek and nutrient agars to determine the total bacterial population. The number of denitrifying bacteria was determined by plating the dilutions onto Gil'tai medium.

All the experiments were performed in triplicate. The data presented in the Tables are average values.

RESULTS AND DISCUSSION

Figure 1 shows how the presence of nitrate in the soil suspension and different aeration conditions affect the degree of PHB degradation. Two months after the onset of the experiment, when the PHB film was almost completely degraded in one of the experimental variants (namely, variant 3', characterized by aerobic conditions and the presence of nitrate), the experiment was stopped, and the residual film pieces were withdrawn from the flasks and weighed. It can be seen that the rate of PHB degradation was maximum under aerobic conditions and that the addition of nitrate enhanced the degradation rate under all of the aeration conditions studied. Nitrate, as an alternative electron acceptor, presumably stimulated the development of microaerophilic and anaerobic denitrifying microflora. Of interest is the fact that the rate of PHB degradation under aerobic conditions in the absence of nitrate (experimental variant 3) was lower than under microaerobic conditions in the presence of nitrate (experimental variant 2').

On the 20th day of incubation, N_2O (the gas product of denitrification) in the gas phase of experimental flask 2' amounted to 178.7 μmol/flask, while its content was close to zero (specifically, 8.0 μmol/flask) in the gas phase of the control flask containing soil and nitrate but

Table 1. Reduction of NO_3^- to N_2O during the biodegradation of PHB films

Experimental variant	Degradation conditions	N_2O in the gas phase, μmol/flask		
		20 days	30 days	60 days
1'	0% O_2 and 5 g/l NO_3^-	82.3	114.4	128.5
	Control to 1' (no PHB film)	28.1	40.2	56.2
2'	10% O_2 and 5 g/l NO_3^-	178.7	271.0	259.0
	Control to 2' (no PHB film)	8.0	16.1	12.0
3'	20% O_2 and 5 g/l NO_3^-	ND	ND	ND
	Control to 3' (no PHB film)	ND	ND	ND

Note: ND stands for “not detected.”

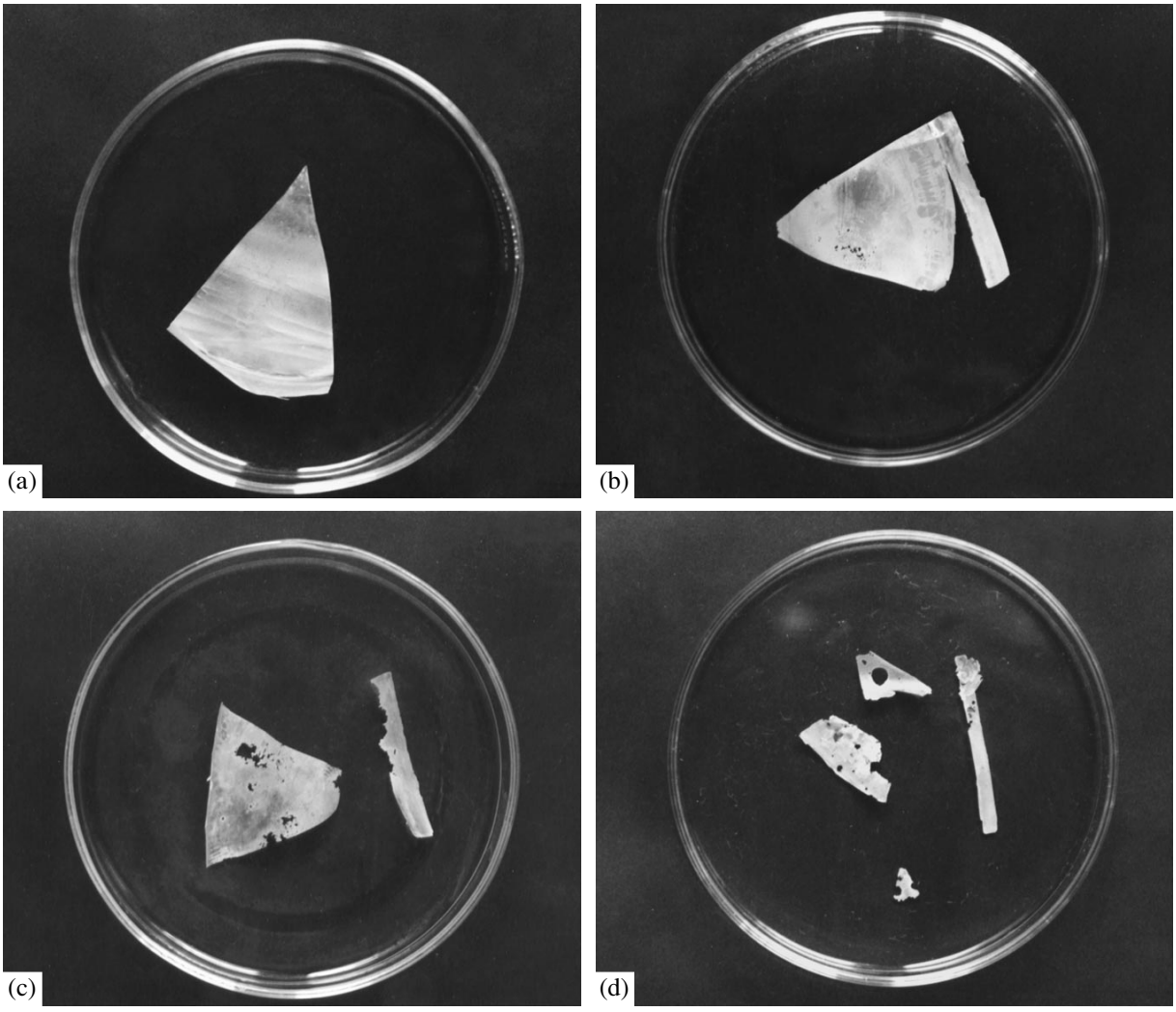

Fig. 2. (a) Undegraded PHB film and PHB films with different degrees of degradation after 2 months of incubation in experimental flasks (b) 1, (c) 2, and (d) 2'.

Table 2. Changes in the physicochemical characteristics of PHB films during their biodegradation

Experimental variant	Degradation conditions	PHB degradation, % of the initial weight	Intrinsic viscosity, dl/g	Average molecular mass, kDa	Degree of crystallinity, %
	Undegraded film	100	8..9	1490	72.9
1	0% O_2 and 0 g/l NO_3^-	96	8.0	1310	73.9
1'	0% O_2 and 5 g/l NO_3^-	83	8.1	1330	74.1
2	10 % O_2 and 0 g/l NO_3^-	83	6.8	1080	74.2
2'	10% O_2 and 5 g/l NO_3^-	38	6.3	980	75.2
3	20% O_2 and 0 g/l NO_3^-	53	7.0	1110	73.9
3'	20% O_2 and 5 g/l NO_3^-	5	5.2	780	76.3

Note: The characteristics of PHB films were determined after 60 days of incubation.

Table 3. Changes in the mechanical properties of PHB films during their biodegradation

Experimental variant	Degradation conditions	PHB degradation, % of the initial weight	Breaking strength, MPa	Ultimate elongation, %	Modulus of elasticity, MPa
	Undegraded film	100	31.3	4.0	1660
1	0% O_2 and 0 g/l NO_3^-	96	36.6	3.2	2010
1'	0% O_2 and 5 g/l NO_3^-	83	31.2	3.4	1965
2	10% O_2 and 0 g/l NO_3^-	83	26.0	3.7	1695
2'	10% O_2 and 5 g/l NO_3^-	38	12.1	3.0	840
3	20% O_2 and 0 g/l NO_3^-	53	13.2	3.8	825
3'	20% O_2 and 5 g/l NO_3^-	5	ND*	ND*	ND*

* The mechanical properties of PHB films were determined after 60 days of incubation. ND stands for "not determined" (PHB film pieces were almost completely degraded).

no PHB film (Table 1). In the presence of nitrate, microaerobic conditions were more favorable to PHB degradation than anaerobic conditions (the amount of N_2O accumulated under anaerobic conditions was twofold smaller than under microaerobic conditions, where the content of oxygen in the gas phase was about 10%). Denitrifying activity was observed throughout the experiment. The addition of nitrate obviously stimulated the development of denitrifying bacteria, which are essential in PHB degradation. Figure 2 illustrates the degradation of PHB films in different experimental variants.

On the 60th day of incubation, soil suspensions were analyzed by gas chromatography for the content of volatile fatty acids. The content of acetic and butyric acids was found to be, respectively, 596 and 325 μmol/flask under anaerobic conditions, 158 and 53 μmol/flask under microaerobic conditions, and zero under aerobic conditions. These data suggest that PHB is completely degraded to CO_2 and H_2O under aerobic conditions.

The total number of microorganisms on the surface of the PHB films degraded under aerobic and microaerobic conditions was 4×10^4 to 5×10^6 (fungi) and 10^5 to 2×10^7 (bacteria). The PHB film degraded under anaerobic conditions contained no microorganisms. The bacteria detected on the degraded PHB films were dominated by the genera *Pseudomonas* (pseudomonads were represented by both fluorescent and nonfluorescent forms), *Bacillus*, *Azospirillum*, *Mycobacterium*, and *Streptomyces*. The fungi were dominated by the genus *Penicillium*. The number of denitrifying bacteria was about 10^2 cells per flask in almost all experimental variants, except that their number in flask 2' (PHB degradation under microaerobic conditions in the presence of nitrate) was higher (10^3 cells per flask).

The molecular mass of PHB tended to decrease in the course of its degradation (Table 2). In experimental variant 3' (PHB degradation under aerobic conditions in the presence of nitrate), where the degree of polymer degradation was visually maximum, the evaluated molecular mass of PHB on the 60th day of incubation was 780 kDa, i.e., nearly twofold lower than the molecular mass of an undegraded PHB (1490 kDa). In contrast to our findings, Mergaert *et al.* observed no decline in the molecular mass of polyhydroxyalkanoates in the course of their degradation [2]. The investigation of the biodegradation of the bulk pieces of polyhydroxyal-

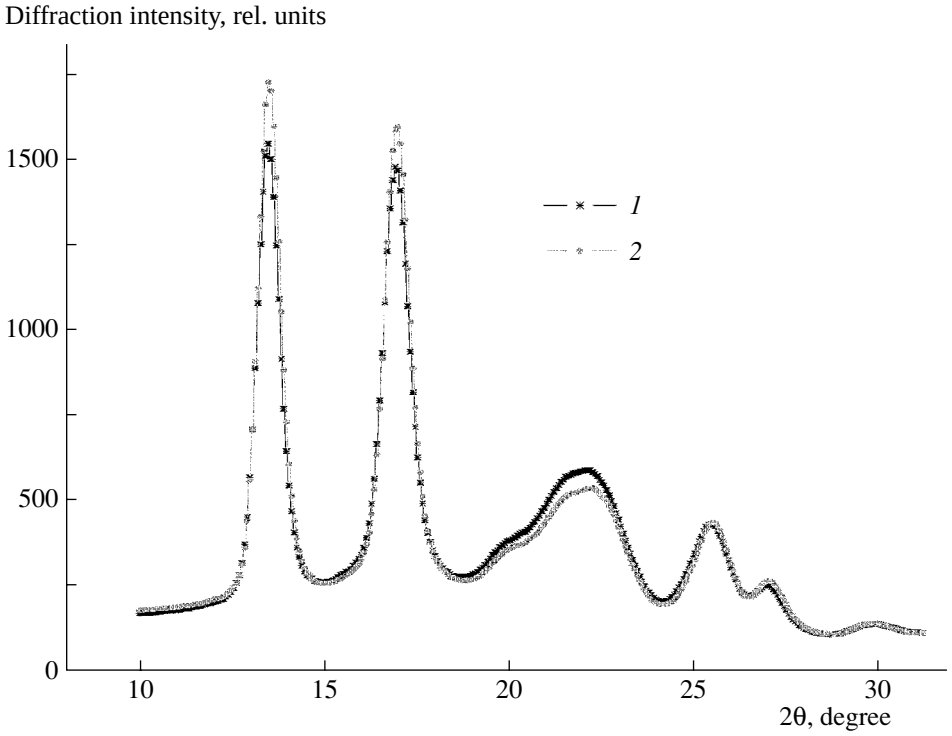

Fig. 3. Diffractograms of (*1*) an undegraded PHB film and (*2*) the PHB film degraded in experimental flask 3'.

kanoates in soil and compost performed by these researchers showed that the molecular mass of PHB did not decrease when this polymer was degraded by 20 wt % and the molecular mass of the copolymer of 80% β-hydroxybutyrate and 20% β-hydroxyvalerate did not decrease when the copolymer was degraded by 60 wt % over a period of 150 days [2].

Our experiments revealed a correlation between the degree of PHB degradation and the molecular mass of degraded PHB (Fig. 1 and Table 2). In experimental flasks with equal aeration levels (variant 2 vs. 2' and variant 3 vs. 3'), the presence of nitrate promoted the degradation of PHB films and enhanced the decline in the molecular mass of PHB. It should be noted that the most degraded PHB films from experimental flasks 2' and 3' exhibited the highest values of the crystallinity index (75.2 and 76.3%, respectively, as compared with 72.9% typical of an undegraded PHB film). The crystallinity index was calculated from diffractograms (Fig. 3 exemplifies the diffractograms of an undegraded PHB film and that degraded in experimental flask 3'). As was shown by Spyros *et al.*, polyhydroxyalkanoates contain amorphous and crystalline regions, of which the former are much more susceptible to microbial attack [9]. If so, the microbial degradation of PHB must be associated with a decrease in its molecular mass and an increase in its crystallinity, which was actually observed in the experiments. The degradability of polymers considerably depends on the degree of their crystallinity. For instance, Volova *et al.* showed that films manufactured of poly-3-hydroxybutyrate-co-3-hydroxyvalerate are more amorphous and more degradable than PHB films [10].

Mechanical testing showed (Table 3) that the moduli of elasticity of PHB films degraded in experimental variants 1, 1', and 2 increased and those of PHB films degraded in variants 2', 3, and 3' decreased as compared with the modulus of elasticity of an undegraded PHB film. These observations can be accounted for by the fact that the microbial degradation of the amorphous regions of PHB films makes them more rigid. However, further degradation of the amorphous regions makes the polymer structure looser.

Thus, the high-molecular-weight poly-β-hydroxybutyrate can easily be degraded under aerobic and microaerobic conditions in the presence of nitrates with the involvement of denitrifying bacteria. This process is of great practical and theoretical interest. Changes in the molecular mass, crystallinity, and mechanical properties of PHB films observed in the course of the experiments provide insight into the mechanism of PHB degradation in soil and give an idea of its rate in nature.

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