BIODEGRADABLE PLASTIC POLY-β-HYDROXYBUTYRATE PRODUCED BY *Azotobacter chroococcum* ON FOOD INDUSTRY WASTES

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Abstract

The food industry wastewater may serve as raw material for PHB synthesis by *Az. croococcum*. The content of polymer in bacterial cells grown on that raw materials may reach 75%. Biodegradation of films made of poly-β-hydroxybutyrate (PHB) was studied in a model soil community in the presence and absence of nitrate and at different concentrations of oxygen in the gas phase. PHB biodegradation was investigated with respect to changes in its molecular mass, crystallinity, and mechanical properties.

The enrichment culture of bacteria degrading PHB films during a short time (3 -7 days) was obtained from soil suspension. In that culture, the dominant species were *Pseudomonas fluorescens* and *Pseudomonas stutzeri*. The rate of PHB degradation in the enrichment culture depended on polymer molecular weight which reduced with time during biodegradation.

Introduction

Polyhydroxyalkanoates (PHAs), are polyesters formed by many prokaryotic microorganisms when unbalanced nutritional conditions are chosen for the producing cells. PHAs can be biodegradable substitutes to fossil fuel plastics that can be produced from renewable raw materials such as saccharides, alcohols and low-molecular weight fatty acids. They are completely degradable to carbon dioxide and water through natural microbiological mineralization. Consequently, neither their production nor their use or degradation have a negative ecological impact. PHB derived plastics have considerable potential application as biodegradable bulk plastics, replacing non-biodegradable products from polypropylene or polyethylene. These products can be used as packing materials, coating for seeds and agrochemicals. PHB can serve as matrix for denitrifying bacteria in drinking water purification. An interest market for PHB will be in high value products (e.g.) biodegradable pins, plates, pegs, sutures and implants for drug delivery [1, 2].

A microorganism - producer should be characterized by the high content of the polymer in cells (at least 60% of cell dry weight) and relatively high degree of polymerization (molecular weight of at least 300 kDa). In addition, such properties of a producer as capacity to utilize inexpensive carbon source and synthesize, primarily, a single reserve compound are important [3, 4].

Azotobacter is an nitrogen-fixing organism growing and synthesizing PHB at a wide range of C and N concentration in the medium. However, high carbon concentration and C/N ratio ranging from 20/1 to 100/1 has the most beneficial effect on PHB biosynthesis. These parameters are usually characteristic of sugar industry wastewaters (40g COD/l), potato and maize wastewaters (up to 20g COD/l) and winery wastewaters (10-20 g COD/l). Different wastes from agricultural products can successfully be used for PHB production. Thus, the cultivation of Azotobacter on food industry waste aims at two purposes: PHB synthesis and cleaning of food industry wastewater until the ecologically permissible concentration of organic substances [5, 6].

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 [7-12]. Maergaert *et al.* [8] 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 [13]. 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.

In this study, we aimed to obtain a biosynthetic plastic by growing the *Azotobacter chroococcum* on the food-industry wastes, as well as to evaluate biodegradability of the material in soil suspension and on test-media, and to isolate a community of microorganisms capable of utilizing the PHB.

Materials and methods

The PHB producer nitrogen-fixing bacterium *Azotobacter chroococcum* 32B, isolated from the rhizosphere of wheat grown in soddy podzolic soil was used. In our preliminary work the semi-industrial technology of PHB production by *A. chroococcum* 32B which ensure the biomass yield 50 g dry biomass/ liter with high content of polymer (up to 85% of the cell dry weight, coefficient of conversion Yx - 0.35) was developed [14, 15]. In the present work experiments were carried out in batch culture. The basic fermentation medium contained (per liter): K_2 HPO_4 • 3H,0. 1.05; KH_2PO_4 ,0.2; $MgSO_4$ • $7H_2O$, 0,4; FeS04 • $7H_2O$, 0.01; Na_2MoO_4 • $2H_2O$ 0.006; $CaCl_2$, 0.1; sodium citrate, 0.5. Flask experiments were carried out in the 750 ml flasks containing 200 ml culture solution at 30^0 C and 190 rpm on a rotatory shaker for 48 hours. *A. chroococcum* 32B was cultivated in the fermentation medium supplemented with one of seven different carbon sources (3% w/v of carbohydrates): (1) glucose; (2) sucrose; (3) raw sugar; (4) raw sugar with 3g/l sodium acetate; (5) sugar-beet molasses (containing (w/w) 81-84% dry matter comprised 52-54% sucrose, 10-30% nitrogenous compounds, 9-10% nitrogen-free compounds, and 11-12% ash); (6) vinasse (winery waste containing 75 g/l sugars, 3.3 g/l ethanol, 1.3 g/l volatile fatty acids, 1.2 g/l polyphenols); (7) soluble starch.

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

Biodegradation experiments were carried out with the poly-β-hydroxybutyrate films (PHB molecular mass 1540 kDa and 890 kDa; the molecular weight distribution index 2.2- 2.7).

The molecular mass of PHB was determined viscosimetrically. The intrinsic viscosity [η] of the chloroform solution of PHB was measured at 30°C, and the molecular mass *M* was calculated according to the equation $[\eta] = 7.7 \times 10^{-5} M^{082}$ [16].

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 N_2O in the gas phase was determined .using a Chrom-5 gas chromatograph equipped with a thermal-conductivity detector and a glass column (3 mm x 1.2 m) packed with Porapak Q. The detector was kept at 20°C, and 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 [17].

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/1. 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 the 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.

To obtain the enrichment culture of denitrifying bacteria capable of degrading PHB the residual film pieces from soil suspension supplemented with nitrate were transferred to sterile water and shaken for 1 h. The resultant suspension was inoculated into liquid medium used to cultivate the denitrifying bacteria (Gil'tai medium). Further plating from a mixed culture of bacteria displaying heavy growth was made on the following media: a) liquid Gil'tai medium + PHB film; b) liquid Gil'tai medium without carbon source + PHB film; c) on solid Gil'tai medium without carbon source containing powdered PHB.

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

Results and discussion

The A. chroococcum culture growth was highest on raw sugar and raw sugar with addition 3g/l sodium acetate (10.2 and 10.8 g/l respectively) (Table 1) The addition of 3g/l sodium acetate does not reduce PHB content in cells (78% then grown on raw sugar and 75% on raw sugar with acetate). Then grown on glucose, sucrose and raw sugar A. chroococcum synthesized high amount of PHB (at least 70%), which somewhat decreased on molasses and starch (63% and 67% respectively).

Table 1. A. chroococcum growth and PHB production in media containing different carbon sources

Carbon source	Dry cell weight, g/l	PHB content, %	PHB molecular mass, kDa
Glucose	9,8	76	1660
Sucrose	10,1	81	1540
Raw sugar	10,2	78	1490
Raw sugar + 3g/l sodium acetate	10,8	75	640
Sugar-beet molasses	8,9	67	590
Vinasse	9,5	76	890
Soluble starch	9,1	63	1310

Starch is the cheap carbon source available in large quantities. Azotobacter chroococcum is the only species that can hydrolyze starch among the Azotobacter [18]. The culture growth and PHB content in cells were 9.1 g/l and 63 % ,respectively, when starch was used as the only carbon source in the medium. These values are comparable to those obtained on sugars.

Carbon sources affect not only PHB production but also molecular weight of the polymer. Upon acetate addition to the fermentation medium with raw sugar PHB content was at least twice reduced, from 1490 to 640 kDa. PHB with lowest molecular mass (560 kDa) was obtained on

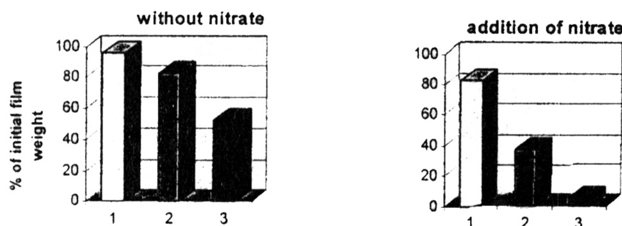
molasses. Then grown on the starch containing media the cells synthesized PHB with molecular mass quite similar to that of PHB synthesized in cells grown on glucose and sucrose.

Therefore, our results show unrefined carbon sources, namely molasses, vinasse and starch, to be suitable raw materials for PHB production by A. chroococcum 32B.

Biodegradation of PHB films in soil suspension

Biodegradation of PHB films in soil suspension was studied under different oxygen conditions and additional nitrogen source (nitrate) was added which may also serve as electron acceptor. Figure 1 shows how the presence of nitrate in the soil suspension and different aeration conditions affect the degree of PHB degradation.

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.



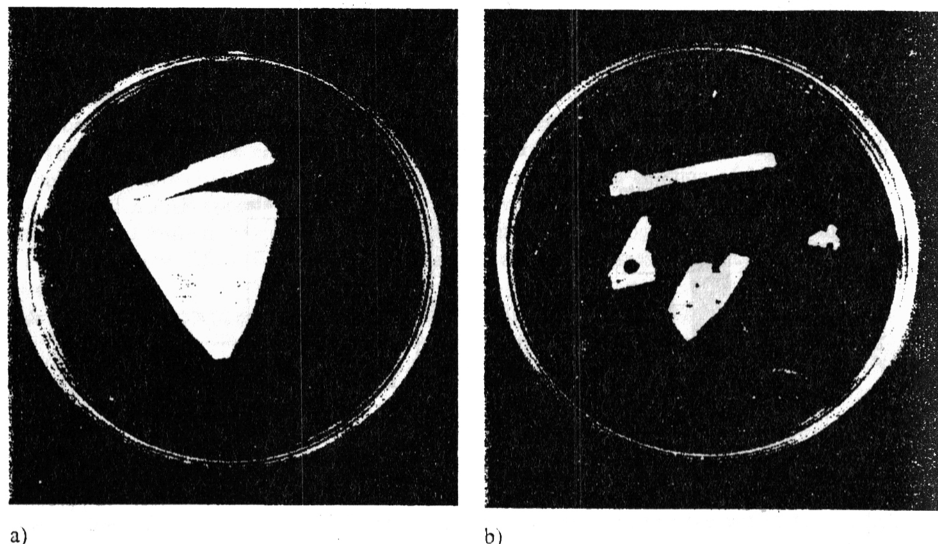
Two months after the onset of the experiment, when the PHB film was almost completely degraded in one of the experimental variants (namely, variant 3', characterized by aerobic conditions and the presence of nitrate), the experiment was stopped, and the residual film pieces were withdrawn from the flasks and weighted. 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').

As soon as on the 20th day of incubation, N_20 (the gas product of denitrification) in the gas phase of experimental flask 2' amounted to 178.7 μmol/flask, while was close to zero (specifically, 8.0 μmol/flask) in the gas phase of the control flask containing soil and nitrate but no PHB film (Table 1). N_20 content in aerobic flasks (variant 3') was not determined, as they were closed by cotton -wool plugs.

Table 1. Reduction of NO_3^-; to N_20 during the biodegradation of PHB films

Experimental variant	Degradation conditions	N_20 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

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.



a) b)

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

The total number of microorganisms on the surface of the PHB films degraded under microaerobic and aerobic 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).

Changes in the physicochemical characteristics of PHB films during their biodegradation

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 ia the molecular mass of polyhydroxyalkanoates in the course of their degradation [8]. The investigation of the biodegradation of the bulk pieces of polyhydroxyalkanoates 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 [8].

Table 2. Changes in molecular mass and cristallinity of PHB during 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.

Our experiments revealed a correlation between the degree 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). As was shown by Spyros *et al.*, polyhydroxyalkanoates contain amorphous and crystalline regions, of which the former are much more susceptible to microbial attack [19]. 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 really observed in the experiments.

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).

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.

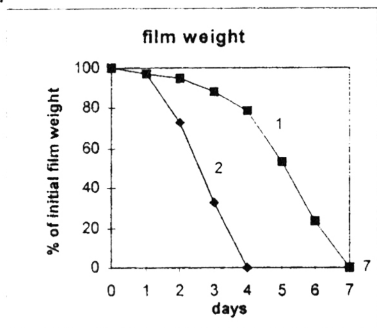
PHB film biodegradation in the soil -derived enrichment culture

In addition to experiments on PHB film biodegradation in soil suspension, PHB biodegradation in the enrichment culture obtained on the medium used to cultivate denitrifying bacteria (Gil'tai medium) was also studied.

The PHB film degraded in both the conventional Gil'tai medium including citrate 5 g/l and in the same medium containing PHB as the only carbon source. However, the rate of biodegradation in the conventional Gil'tai medium was much higher than in the Gil'tai medium deprived of the carbon source (3-7 days and 15-20 days respectively). To identify bacteria, contributing into a PHB-degrading enrichment the latter was plated on the following solid media: MPA, KB and potato agar.

The similar dominant bacterial species, *Ps. fluorescens* and *Ps. stutzeri*, were identified upon plating from conventional Gil'tai medium + PHB film and from the same medium containing PHB as the only carbon source.

Under denitrifying conditions, biodegradation of the PHB films was observed during seven days. Figures 3 and 4 show that both the film weight and PHB molecular mass decreased with time.



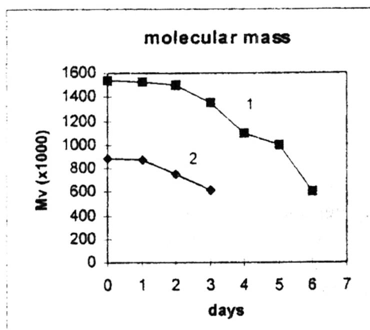


Fig.3. PHB film biodegradation by mass 1540 kDa; 2- initial molecular mass 890 kDa

Fig.4. Changes in molecular mass of PHB during its biodegradation by enrichment culture; 1- initial molecular mass 1540 kDa; 2- initial molecular mass 890 kDa

The PHB film with molecular mass of 1540 kDa and weighting 6 mg completely degraded for seven days. The same-weight film of PHB with molecular mass of 890 kDa degraded during four days. In contrast to the data of Doi et al. [20] who found that PHB molecular mass remained unchanged upon enzymatic biodegradation in an aquatic solution of PHB-depolymerase from *Alcaligenes faecalis,* in our experiments, the average viscosity molecular mass of the higher- and lower-molecular polymers decreased gradually from 1540 to 580 kDa and from 890 to 612 kDa, respectively (Fig. 4). Biodegradation of the lower-molecular polymer was observed as soon as a day from the very beginning of the experiment. (After 48 h, the film weight was 27% reduced and the average viscosity molecular mass decreased from 890 to 727 kDa). Biodegradation of the higher-molecular polymer was initiated as late as three days after beginning of the experiment. After 48 h, a loss in the film weight comprised as little as 5%, whereas the molecular mass remained almost unchanged.

The "exo"-type cleavage of the polymer chain, i. e. a successive removal of the terminal groups, is known to occur at a higher rate than the "endo"-type cleavage, i. e., a random breakage of the polymer chain at the enzyme-binding sites. Thus, the former type of polymer degradation is primarily responsible for changes in its average molecular mass. However, the "endo"-type attack plays the important role at the initiation of biodegradation, because at the beginning, a few polymer chains are oriented so that their ends are accessible to the effect of the enzyme [21]. Biodegradation of the lower-molecular polymer, which contains a higher number of terminal groups, is more active, probably, because the "exo"-type degradation is more active in lower than in higher-molecular polymer.

To test the capacity of a denitrifying enrichment to utilize PHB on solid media, the bacterial suspension was plated on solid test-media containing polyhydroxybutirate. On the fifth day, a heavy growth of bacteria was observed on dishes; after two weeks, polyhydroxybutirate was utilized completely.

Conclusions

The food industry wastewaters, vinassa, molasses and soluble starch, may serve as raw material for PHB synthesis by *Az. croococcum.* The content of polymer in the bacterial cells may reach 75%. PHB degradation observed in soil suspension was stimulated upon addition of nitrates. The enrichment culture degrading PHB films for a short time (3--7 days) was obtained from soil suspension. In that culture *Ps. fluorescens* and *Ps. stutzeri* were dominant species. The rate of PHB degradation in the enrichment culture depended on polymer molecular weight which decreased with time during biodegradation.

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