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Aerobic and Anaerobic Microbial Degradation of Poly-β-Hydroxybutyrate Produced by *Azotobacter chroococcum*

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Abstract

Food industry wastewater served as a carbon source for the synthesis of poly-β-hydroxybutyrate (PHB) by *Azotobacter chroococcum*. The content of polymer in bacterial cells grown on the raw materials reached 75%. PHB films were degraded under aerobic, microaerobic, and anaerobic conditions in the presence and absence of nitrate by microbial populations of soil, sludges from anaerobic and nitrifying/denitrifying reactors, and sediment from a sludge deposit site. Changes in molecular mass, crystallinity, and mechanical properties of PHB were studied. Anaerobic degradation was accompanied by acetate formation, which was the main intermediate utilized by denitrifying bacteria or methanogenic archaea. On a decrease in temperature from 20 to 5°C in the presence of nitrate, the rate of PHB degradation was 7.3 times lower. Under anaerobic conditions and in the absence of nitrate, no PHB degradation was observed, even at 11°C. The enrichment cultures of denitrifying bacteria obtained from soil and anaerobic sludge degraded PHB films for a short time (3–7 d). The dominant species in the enrichment culture from soil were *Pseudomonas fluorescens* and *Pseudomonas stutzeri*. The rate of PHB degradation by the enrichment cultures depended on the polymer molecular weight, which reduced with time during biodegradation.

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Index Entries: Aerobic, microaerobic, and anaerobic conditions; *Azotobacter chroococcum*; denitrification; denitrifying microorganisms; methanogenesis; microbial degradation; poly-β-hydroxybutyrate.

Introduction

Polyhydroxyalkanoates (PHAs) are polyesters produced by many prokaryotic microorganisms usually under unbalanced growth conditions. Their physical and technical properties suggest their use as a substitute for conventional nonbiodegradable plastic materials. In addition to the applicability of poly-β-hydroxybutyrate (PHB)-derived plastics as packages, coatings, and so forth, high-value medicinal products, such as biodegradable pins, plates, pegs, sutures, and implants for drug delivery, could be based on PHB as well. Neither PHB production nor degradation have a negative ecologic impact *(1,2)*.

The microorganism producer of PHB should be characterized by the high content of the polymer in cells (above 60% of cell dry weight) and relatively high degree of polymerization (mol wt of at least 300 kDa). In addition, a capacity to utilize inexpensive carbon source and to synthesize, primarily, a single reserve compound is important *(3,4)*. Azotobacter species are nitrogen-fixing organisms growing and synthesizing PHB at a wide range of carbon and nitrogen concentrations in the medium. However, a high carbon concentration and C/N ratio ranging from 20:1 to 100:1 has the most beneficial effect on PHB biosynthesis. PHAs can be produced from renewable raw materials such as saccharides, alcohols, and low molecular weight fatty acids that are contained in food and agriculture industry wastewater. The cultivation of Azotobacter on food industry waste has two purposes: PHB synthesis and cleaning of food industry wastewater until reaching the ecologically permissible concentration of organic substances *(5–7)*.

The most attractive property of PHB with respect to ecology is that it can be completely degraded by microorganisms. The degradation of PHB and its composites in various ecosystems, such as soil, compost, and water bodies, has been described in a number of publications *(8–13)*. Maergaert et al. *(9)* isolated from soil more than 300 PHB-degrading microbial strains, of which denitrifying bacteria are of particular practical interest owing to their potential ability to be used in the immobilized state on PHB films for the purification of water from nitrates *(14)*.

In the present study, we aimed to obtain a biosynthetic plastic by growing *Azotobacter chroococcum* on the raw materials, to evaluate biodegradability of the material under aerobic, microaerobic, and anaerobic conditions in the presence and absence of nitrate by microbial populations of soil, sludges from anaerobic and nitrifying/denitrifying reactors, and sediment of a sludge deposit site, as well as to obtain active denitrifying enrichment culture degrading PHB.

Materials and Methods

Production of PHB

The PHB producer nitrogen-fixing bacterium *A. chroococcum* 32B was isolated from the rhizosphere of wheat grown in soddy podzolic soil. The basic fermentation medium contained 1.05 g/L of $K_2HPO_4 \cdot 3H_2O$, 0.2 g/L of KH_2PO_4, 0.4 g/L of $MgSO_4 \cdot 7H_2O$, 0.01 g/L of $FeSO_4 \cdot 7H_2O$, 0.006 g/L of $Na_2MoO_4 \cdot 2H_2O$, 0.1 g/L of $CaCl_2$, and 0.5 g/L of sodium citrate. Batch experiments were carried out in 750-mL flasks containing 200 mL of culture solution at 30°C and 190 rpm on a rotatory shaker for 48 h. Strain 32B was cultivated in the fermentation medium supplemented with one of seven different carbon sources (3% [w/v] of carbohydrates): glucose; sucrose; raw sugar; raw sugar with 3 g/L of sodium acetate; sugar beet molasses (containing [w/w] 81–84% dry matter comprising 52–54% sucrose, 10–30% nitrogenous compounds, 9–10% nitrogen-free carboxylic acids, and 11–12% ash); vinasse (winery waste containing 75 g/L of sugars, 3.3 g/L of ethanol, 1.3 g/L of volatile fatty acids (VFA), 1.2 g/L of polyphenols); soluble starch.

The procedure for PHB isolation from the biomass of strain 32B consisted 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 triple precipitation with isopropanol and dissolution in chloroform, and PHB drying at 60°C. PHB films 0.03–0.04 mm thick were prepared by pouring a chloroform solution of PHB into the bottom of Petri dishes.

Degradation of PHB

Biodegradation experiments were carried out with the PHB films (PHB molecular mass of 1540 and 890 kDa; molecular weight distribution index of 2.2–2.7). The degradation of PHB was studied under aerobic, microaerobic, and anaerobic conditions in the presence and absence of nitrate by microbial populations of soil, sludges from anaerobic upflow anaerobic sludge bed (UASB) and nitrifying/denitrifying reactors, and sediment of a sludge deposit site.

Degradation of PHB by soil microorganisms was studied by placing polymer film pieces weighing about 40 mg into 50-mL flasks with 20 mL of a suspension containing 10% of an air-dried soil in 0.1 *M* potassium phosphate buffer. Potassium nitrate was added at a concentration of 5 g/L. Anaerobic incubation was carried out in rubber-sealed flasks with an atmosphere of 95% argon and 5% acetylene to inhibit N_2O reductase. In variants with microaerobic conditions, the gas phase consisted of 10% oxygen, 5% acetylene, and 85% argon. 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* potassium phosphate buffer, dried at 60°C for 2 h, and weighed to determine weight loss.

Anaerobic degradation of PHB by sludges in the presence and absence of nitrate was investigated with three types of an activated sludge received

Table 1
Growth of *A. chroococcum* 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 + 3 g/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

from different sources: microbial biomass from a laboratory anaerobic UASB reactor treating pig manure wastewater *(15)*, activated sludge from a nitrifying/denitrifying reactor of a municipal wastewater treatment plant (Moscow), sediment of a sludge deposit site (Moscow region) *(16)*.

To investigate degradation of PHB under denitrifying conditions, a modified medium was used *(17)*. It contained 2.6 g/L of KH_2PO_4, 5.3 g/L of K_2HPO_4, 0.1 g/L of $MgCl_2$, 0.08 g/L of $CaCl_2$, 3.0 g/L of KNO_3, 0.1 g/L of $Na_2S{\cdot}9H_2O$, 0.01 g/L of yeast extract, 0.002 g/L of resazurin, 2.5–3.0 g/L of PHB, 2 mL of Lippert's solution of trace elements *(18)*, and 10 mL of Wolin's vitamin solution *(19)* at pH 7.0–7.2 at 20°C. The gas phase contained 6% acetylene and 94% argon. Nitrate was added fractionally, when exhausted.

To investigate anaerobic PHB degradation in the absence of nitrate Pfennig's fermentation medium was used *(20)*. It contained 0.33 g/L of NH_4Cl, 0.5 g/L of $MgCl_2{\cdot}6H_2O$, 0.33 g/L of $CaCl_2{\cdot}6H_2O$, 0.33 g/L of KCl, 0.33 g/L of KH_2PO_4, 0.5 g/L of yeast extract, 2.5 g/L of $NaHCO_3$, 0.6 g/L of $Na_2S{\cdot}9H_2O$, 0.002 g/L of resazurin, 2.5–3.0 L of PHB, 2 mL of Lippert's solution of trace elements *(18)*, and 10 mL of Wolin's vitamin solution *(19)* at pH 7.0–7.2 at 20°C. The gas phase contained 100% N_2.

Anaerobic degradation of PHB by sediment from sludge deposit sites (Moscow region) was investigated at 20, 11, and 5°C, respectively.

Each experiment had four replicates. The data presented in Tables 1–4 are average values.

Analytical Methods

The nitrate concentration was measured using analytical test kits (Merck).

Gaseous products (N_2O, CH_4, CO_2) were determined by gas chromatography (GC) (Chrom-5, Czech Republic) with a thermal conductivity detector. Porapak Q was used as sorbent for definition of N_2O and activated coal AG-3 for CH_4 and CO_2. Oven temperature was 25°C. VFA were determined by GC (Chrom-5) with a flame ionization detector, at an oven

Table 2
Changes in Molecular Mass, Crystallinity, and Mechanical Properties of PHB After 60 d of Biodegradation

Variant	Degradation conditions		PHB degradation, % of the initial weight	Intrinsic viscosity (dL/g)	Average molecular mass (kDa)	Degree of crystallinity (%)	Breaking strength (MPa)	Ultimate elongation (%)	Modulus of elasticity (MPa)
	O_2 (%)	5 g/L NO_3^-							
Undegraded film			100	8.9	1490	72.9	31.3	4.0	1660
1	0	–	96	8.0	1310	73.9	36.6	3.2	2010
1'	0	+	83	8.1	1330	74.1	31.2	3.4	1965
2	10	–	83	6.8	1080	74.2	26.0	3.7	1695
2'	10	+	38	6.3	980	75.2	12.1	3.0	840
3	20	–	53	7.0	1110	73.9	13.2	3.8	825
3'	20	+	5	5.2	780	76.3	ND[a]	ND[a]	ND[a]

[a]ND, not determined.

Table 3
Basic Parameters of Anaerobic Degradation of PHB at 20°C by Activated Sludges in Presence and Absence of Nitrate

Source of active biomass	Initial TSS (g/L)	Initial Cell number (cells/mL)	Conditions of incubation	Amount of used PHB (%)	Amount of used NO_3^- (g/L)	Maximum amount of formed acetate (m*M*)	Average rate of acetate formation (acetate/d)	Average rate of butyrate formation (butyrate/d)	Average rate of denitrification (m*M* NO_3^-/d)
Anaerobic UASB reactor	0.68	3.0×10^8	$+NO_3^-$	100	8.9	35.3	—	—	10.6
			$-NO_3^-$	92	—	91	5.6	0.94	—
Nitrifying/ denitrifying reactor	0.91	3.7×10^8	$+NO_3^-$	100	8.7	5.6	—	—	9.5
			$-NO_3^-$	92	—	92	4.6	1.3	—

Table 4
Anaerobic Degradation of PHB at Different Temperatures
by Microbial Population of Sediment Sludge Deposit Site (Moscow Region) in Presence and Absence of Nitrate

Temperature of incubation (°C)	Conditions of incubation	Time of incubation (d)	Amount used		Maximum rate of denitrification (m*M* NO_3^-/d)	Average rate of PHB degradation (g PHB/[L·d])
			PHB (%)	NO_3^- (g/L)		
20	$+NO_3^-$	13	100	5.5	7.1	0.19
	$-NO_3^-$	18	84	—	—	0.12
11	$+NO_3^-$	20	100	4.72	5.1	0.11
	$-NO_3^-$	50	0	—	—	0
5	$+NO_3^-$	50	50	2.3	1.0	0.026
	$-NO_3^-$	50	0	—	—	0

temperature of 170–180°C and injector and detector temperature of 200°C. Chromosorb-101 was used as sorbent. In both cases a 1.2-m glass column and argon as carrier gas (40 mL/min) were used.

To determine total suspended solids (TSS), the experimental sample at a volume of 5 mL was centrifuged, twice washed out in 0.85% NaCl solution, and dried at 105°C for 12 h.

Physicochemical and Mechanical Properties of PHB

The mean 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 $[\eta] = 7.7 \times 10^{-5} M^{0.82}$ *(21)*. 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 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 *(22)*.

Enrichments from Soil Suspension

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 containing 2.1 L of KNO_3, 1.0 L of asparagine, 5.0 L of sodium citrate, 2.0 L of KH_2PO_4, 2.0 L of $MgSO_4$, and 0.2 L of $CaCl_2$. Further plating from a mixed culture of bacteria was made on the Gil'tai medium with PHB film.

Enrichments from Activated Sludge

The enrichment cultures of microorganisms from activated sludge of the laboratory anaerobic UASB reactor treating pig manure wastewater *(15)*, and activated sludge from the nitrifying/denitrifying reactor of the municipal wastewater treatment plant (Moscow), were obtained by the method of serial dilution. Activated sludges from these reactors were inoculated in the modified liquid medium for denitrifying microorganisms *(17)*. PHB films or VFA were used as a carbon source.

Total Cell Count

To count the cell number on the surface of PHB films degraded in soil suspension, residual film pieces were placed in 10 mL of sterile water and shaken for 1 h. The cell number on the surface of the films was determined by serial dilution of the resultant suspension and plating on solid media (beef extract agar and wort agar).

Total cell count from sludge was obtained by 4',6'-diamidino-2-phenylindol (DAPI) staining. A cell suspension in a volume of 0.01 mL was

spread over a 1-cm^2 area on a slide, air-dried, and fixed in 96% ethanol for 20 min. The slides were stained for 10 min in a solution of dye containing 0.01 mg/mL of DAPI and washed off with water.

Microscopy

Cells were counted on a LUMAM I1 UF-microscope (LOMO, Russia) with UFS 6-3 filters (360 nm), a SZS-24-4 filter, and a blue plate (300–380 nm, 420–500 nm) for DAPI staining.

Cell morphology was studied with an Amplival light microscope (Carl Zeiss, Germany) with phase contrast.

Results and Discussion

Production of PHB

Growth of *A. chroococcum* was highest on raw sugar and raw sugar with the addition of 3 g/L of sodium acetate (10.2 and 10.8 g/L, respectively) (Table 1). When grown on glucose, sucrose, and raw sugar, *A. chroococcum* synthesized a high amount of PHB as well (at least 70%), which somewhat decreased on molasses and starch (63 and 67%, respectively).

Starch is a cheap carbon source available in large quantities. *A. chroococcum* is the only species that can hydrolyze starch among the Azotobacter *(23)*. Carbon sources affect not only PHB production but also molecular weight of the polymer. When grown on the starch containing media, the cells synthesized PHB with a 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 by Microbial Population of Soil Suspension

Biodegradation of PHB films in soil suspension was studied for 2 mo under different aeration conditions, and nitrate was added as an additional nitrogen source (and an electron acceptor) (Fig. 1, Table 2). Degradation of PHB was highest under aerobic conditions, and practically did not proceed under anaerobic conditions. The addition of nitrate enhanced degradation under all of the aeration conditions studied and stimulated the development of denitrifying microbial populations.

Changes in the physicochemical and mechanical characteristics of PHB films during their biodegradation are presented in Table 2. The molecular mass of PHB tended to decrease in the course of its degradation. When PHB was degraded under aerobic conditions in the presence of nitrate, the degree of polymer degradation was visually maximum. The evaluated molecular mass of PHB on d 60 of incubation was 780 kDa, i.e., nearly twofold lower than the mass of an undegraded PHB (1490 kDa). Maergaert et al. *(9)* observed no decline in the molecular mass of PHAs in the course of their

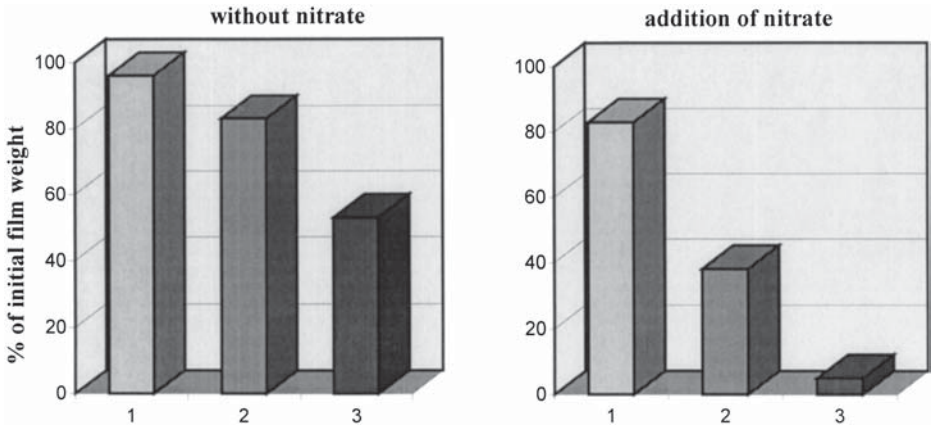

Fig. 1. Effect of oxygen concentration in gas phase and presence of nitrate in soil suspension on degree of degradation of PHB films after 2 mo of incubation under anaerobic (1), microaerobic (2), and aerobic conditions (3).

degradation. Our experiments revealed a correlation between the degree of PHB degradation and the molecular mass of degraded PHB (Fig. 1, Table 2). In experimental flasks with equal aeration levels, the presence of nitrate promoted the degradation of PHB films and enhanced the decline in the molecular mass of PHB. The most degraded PHB exhibited the highest values of the crystallinity index. As shown by Spyros et al. *(24)*, PHAs contain amorphous and crystalline regions, of which the former are much more susceptible to microbial attack. Thus, the microbial degradation of PHB must be associated with a decrease in its molecular mass and an increase in its crystallinity, which was observed in the experiments. Mechanical testing showed (Table 2) that the moduli of elasticity of PHB films degraded under anaerobic and microaerobic conditions (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 structure of the polymer looser.

Anaerobic Degradation of PHB Film by Activated Sludge from UASB and Nitrifying/Denitrifying Reactors in Presence and Absence of Nitrate at 20°C

Degradation of PHB by activated sludge from a UASB reactor treating pig manure wastewater and from a nitrifying/denitrifying reactor of the plant treating municipal wastewater (Moscow) in the presence and absence of nitrate at 20°C was studied. The activated sludge from both reactors had similar characteristics of degradation of PHB and average rates of denitrification (Table 3). In both the presence and absence of nitrate, the intensity

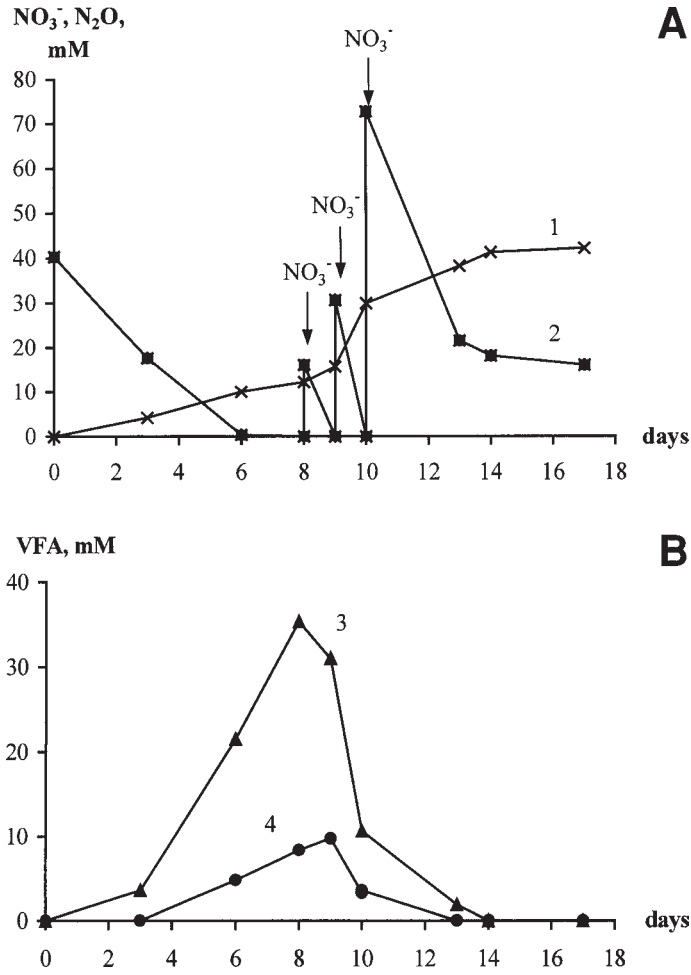

Fig. 2. Anaerobic degradation of PHB in presence of nitrate by activated sludge from anaerobic UASB reactor treating pig manure wastewater at 20°C: **(A)** nitrate consumption and N_2O formation (1, N_2O, m*M*; 2, NO_3^-, m*M*); **(B)** formation and consumption of VFA (3, acetate, m*M*; 4, butyrate, m*M*).

and character of degradation of PHB was also similar. The rate of degradation of PHB in the presence of nitrate was only 1.11 times higher than in the absence of nitrate. The activated sludge from the anaerobic UASB reactor showed slightly higher average rates of denitrification and acetate accumulation.

The results on anaerobic degradation of PHB by activated sludge from the UASB reactor in the presence and absence of nitrate are provided in Figs. 2 and 3. In the presence of nitrate, degradation of PHB was accompanied by consumption of nitrate and N_2O formation (Fig. 2). PHB was degraded through stages of formation of butyrate and acetate, as the intermediate products, and CO_2 as the end product. Exhaustion of nitrate from the medium resulted in a sharp increase in VFA (mainly acetate) accumu-

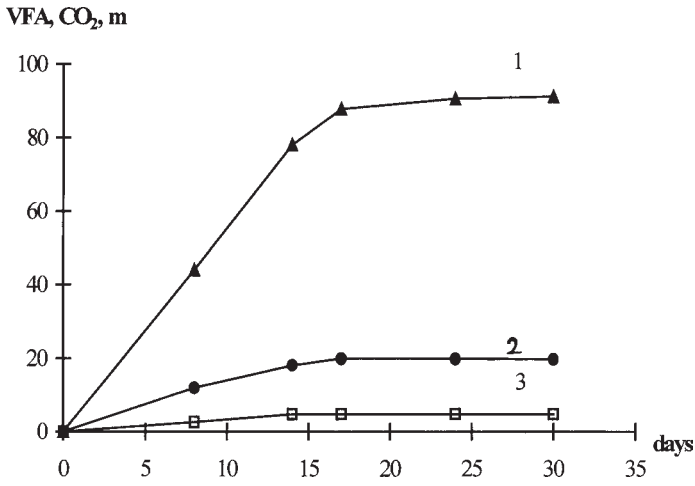

Fig. 3. Products of anaerobic degradation of PHB in absence of nitrate by activated sludge from anaerobic UASB reactor treating pig manure wastewater at 20°C (1, acetate, m*M*; 2, butyrate, m*M*; 3, propionate, m*M*).

lation. An addition of nitrate in the medium resulted in VFA consumption. Obviously, denitrifying microorganisms serve as the final consumer of VFA when nitrate is available.

In the absence of nitrate, PHB also was effectively degraded with formation of acetate, butyrate, and a small amount of propionate (Fig. 3). Unlike the denitrifying community, in the anoxic one the final consumers of acetate are acetoclastic methanogenes. However, rapid hydrolysis of PHB led to an accumulation of high amounts of acetate (up to 90 m*M*), which decreased the pH to 5.0 and caused a complete suppression of methanogenesis. Acetoclastic methanogens are sensitive to low pH as well as to high concentration of acetate. When the experimental samples were diluted by the fresh mineral medium up to an acetate concentration of 7–10 m*M*, methane production was initiated. After 1 mo, 0.5 m*M* CH_4 was detected in these diluted samples. This experiment showed the presence of viable methanogens in community, as well as the possibility of degradation of PHB with methane formation.

The results obtained indicate that anaerobic hydrolysis of PHB at 20°C could be provided by hydrolytic anaerobic nondenitrifying microorganisms. However, the presence of PHB-degrading denitrifiers could not be excluded.

Anaerobic Degradation of PHB by Microbial Population of Sediment from Sludge Deposit Site (Moscow Region) in Presence and Absence of Nitrate at Low Temperatures

The low-temperature influence on the rate of anaerobic PHB degradation was studied with sediment of a sludge deposit site (Moscow region) that was known to be active at low temperature *(16)*. The rates of degradation

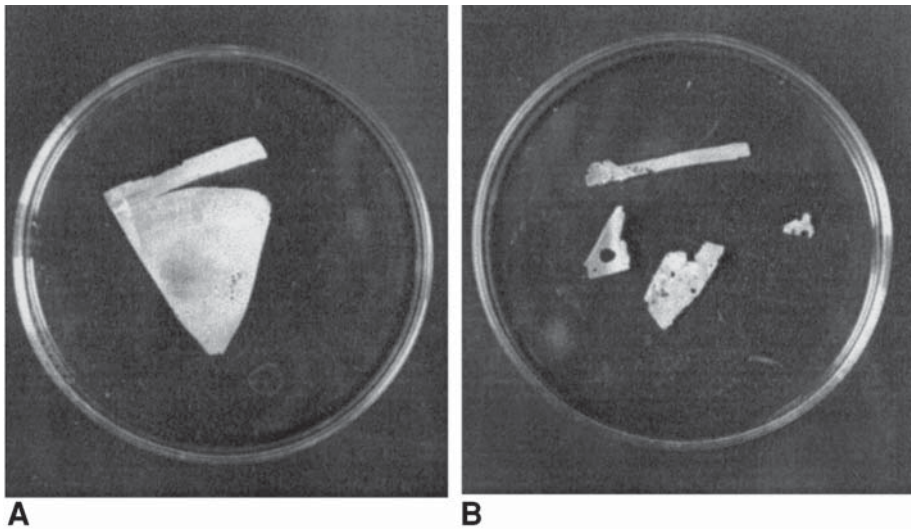

Fig. 4. PHB films with different degrees of degradation after 2 mo of incubation in soil suspension **(A)** anaerobic conditions without nitrate and **(B)** microaerobic conditions with nitrate.

of PHB by a microbial population of sediment depended on temperature (Table 4). In the absence of nitrate, PHB was sufficiently degraded at 20°C, but it was not degraded at all at 5°C but was degraded at 11°C. No acetate and other VFA were produced from PHB. Thus, a decrease in temperature drastically suppressed the growth of PHB-degrading microorganisms under anoxic conditions.

In the presence of nitrate, PHB continued to be degraded even at 5°C through the rates of PHB degradation, and denitrification decreased. The average rate of PHB degradation was 7.3 times lower and the maximal rate of denitrification was 7.1 times lower at 5°C compared with the same values at 20°C. These results suggest a possible importance of a group of hydrolytic denitrifiers.

Microorganisms Participating in Biodegradation of PHB

Figure 4 illustrates the degradation of PHB films by a microbial population of soil suspension under anaerobic conditions without nitrate and microaerobic conditions with the addition of nitrate after 2 mo of incubation in soil suspension. Under aerobic conditions with nitrate, PHB film was degraded completely. The total number of microorganisms on the surface of the PHB films degraded under microaerobic and aerobic conditions was 1.1×10^4 to $1.3 \times 10^6/cm^2$ of film surface (fungi) and 2.5×10^4 to $5 \times 10^6/cm^2$ of film surface (bacteria). Among the bacteria detected on the degraded PHB films, those of genera *Pseudomonas*, *Bacillus*, *Azospirillum*, *Mycobacterium*, and *Streptomyces* dominated. The fungi were dominated by

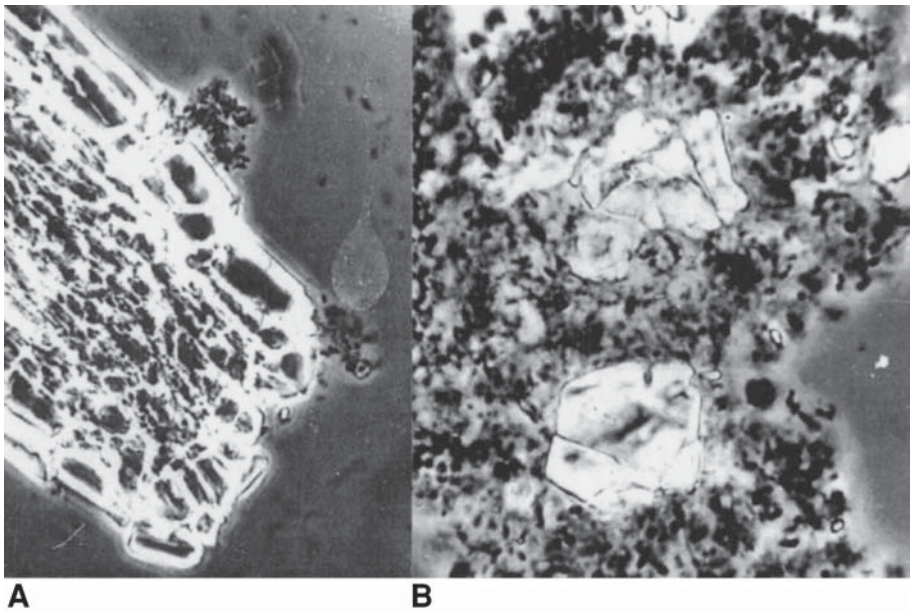

Fig. 5. Steps of degradation of PHB by enrichment culture from activated sludge of anaerobic UASB reactor treating pig manure wastewater. Magnification: **(A)** ×1140; **(B)** ×1570.

the genus *Penicillium*. In the absence of nitrate, the number of denitrifying bacteria was about 1.5×10^2 cells/cm^2 of film surface. Under microaerobic conditions in the presence of nitrate it was higher (1.7×10^3 cells/cm^2 of film surface).

The enrichment denitrifying cultures grown on media with VFA or PHB films as a single carbon source were obtained. The cultures were derived from the activated sludges of anaerobic UASB and nitrifying/denitrifying reactors. PHB hydrolysis in the presence and absence of nitrate was caused by rods of different length and thickness. Degradation of PHB film by the enrichment denitrifying culture derived from the activated sludge of the anaerobic UASB reactor was also studied (Fig. 5). Degradation started with cell adsorption on a surface of polymer film and its consequent decomposition. Colonization of polymer films by microbial community was apparent usually in 3–5 d. In a couple of days, polymer was completely covered by a cell layer and the film surface began to degrade (Fig. 5A). The film became thin and broke up into large pieces. Large pieces of polymer were decomposed to fine ones during the next 2 to 3 d (Fig. 5B).

Biodegradation of PHB in the enrichment culture obtained from soil on the medium used to cultivate denitrifying bacteria (Gil'tai medium) was also studied. The dominant bacterial species, *Pseudomonas fluorescens* and *Pseudomonas stutzeri*, were identified in this enrichment culture. Under denitrifying conditions, PHB films were completely degraded for 7 d. Figures 6 and 7 show that both the film weight and molecular mass of PHB

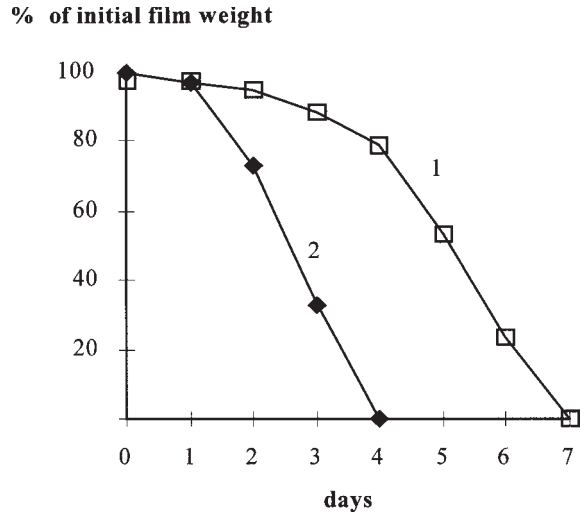

Fig. 6. Biodegradation of PHB film by enrichment culture (1, initial molecular mass of 1540 kDa; 2, initial molecular mass of 890 kDa).

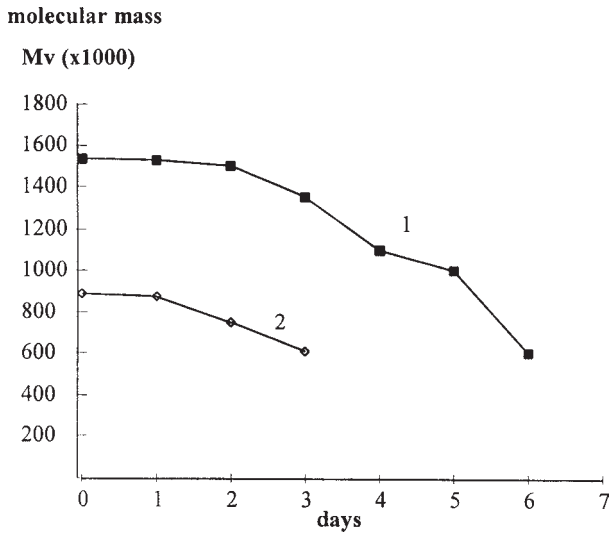

Fig. 7. Changes in molecular mass of PHB during its biodegradation by enrichment culture (1, initial molecular mass of 1540 kDa; 2, initial molecular mass of 890 kDa).

decreased with time. In contrast to the data of Doi et al. *(25)*, who found that the molecular mass of PHB remained unchanged on 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. 7). 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 an 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 *(26)*. 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.

Conclusion

Food industry wastewater, vinasse, molasses, and soluble starch may serve as raw material for PHB synthesis by *A. chroococcum*. The content of polymer in the bacterial cells may reach 75%.

Degradation of PHB observed in soil suspension was stimulated on the addition of nitrates. Microbial degradation of PHB was associated with a decrease in its molecular mass and an increase in its degree of crystallinity.

Anaerobic degradation of PHB in the presence and absence of nitrate occurred in all sludges examined. The presence of nitrate in the medium slightly influenced the rate of PHB degradation at 20°C.

PHB was consistently degraded to VFA and CO_2 with participation of different microbial groups. Acetate was a primary product of anaerobic PHB degradation and in the presence of nitrate provided denitrification. Under anaerobic conditions in the absence of nitrate, PHB could be degraded to H_2O and gaseous products, CO_2 and CH_4.

At a temperature decrease from 20 to 5°C, the rate of anaerobic degradation of PHB and the rate of denitrification decreased 7.3 and 7.1 times, respectively. Under the same conditions without nitrate, degradation of PHB was suppressed completely at 5 and 11°C.

The enrichment cultures degrading PHB films for a short time (3–7 d) were obtained from anaerobic sludge and from soil suspension. In the culture obtained from soil, *P. fluorescens* and *P. stutzeri* were the 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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