MICROBIAL DEGRADATION OF POLY-3-HYDROXYBUTYRATE UNDER DENITRIFYING AND STRICT ANAEROBIC CONDITIONS AT LOW TEMPRATURE

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Abstract

The food industry wastewater served as a carbon source for PHB synthesis by *Azotobacter croococcum*. The content of polymer in bacterial cells grown on the raw materials reached 75 %. The films of poly-β-hydroxybutyrate (PHB) were degraded under anaerobic conditions in the presence and absence of nitrate and at low temperatures by microbial populations of sludges from anaerobic and nitrifying/denitrifying reactors, and of sediment from a sludge check. In the presence of nitrate, PHB was degraded to CO_2 with formation VFA (acetate, propionate and butyrate) as intermediate products. Then VFA were consumed by denitrifying microorganisms and nitrate was reduced to N_2O. Under anaerobic conditions in the absence of nitrate, VFA were formed from PHB rapidly. High concentrations of acetate (to 90 mM) accumulated in medium and suppressed acetoclastic methanogenesis. The rate of PHB degradation at 20° C on all investigated sludges were similar in both the presence and absence of nitrate. PHB degradation was temperature-dependent. In the presence of nitrate, the average rate of PHB degradation and the maximal rate of denitrification decreased 7.3 and 7.1 times, respectively, with a temperature decrease from 20° C to 5°. Under anaerobic conditions without nitrate PHB was not degraded even at 11° C. PHB-degrading microorganisms belong to different morphological types.

Introduction

In recent years, PHB attracts much attention as a biodegradable material which can be used in treating systems for nitrogen removal (nitrification/ denitrification process) as material for microbial immobilisation and an additional carbon source for microbial growth [1, 2].

PHB is a biodegradable microbial reserve polyester. It is one of the general class of optically active microbial polyesters termed polyhydroxyalkanoates. It consists of 3-hydroxybutyrate as a monomer. Microbial decomposition of PHB is mediated by a specific enzyme – PHB-depolymerase synthesised by microbial cells, which are adsorbed on a surface of a polymer film and degrade it to oligo- and monomers [1, 3- 5].

The main goal of this paper was an investigation of anaerobic PHB degradation by microbial communities in the presence and absence of nitrate and the effect of a temperature decrease on PHB degradation.

Materials and methods

Production of PHB

In this study samples PHB with high-molecular weight (1400 kDa) were used. The PHB producer nitrogen-fixing bacterium *Azotobacter chroococcum* 32B was isolated from the rhizosphere of wheat grown in soddy podzolic soil. To induce superproduction of polymer by *A. chroococcum* 32B (up to 85 % of the cell dry weight), the following fermentation medium was used. It contained (per liter): $K_2HPO_4 \cdot 3H_20$, 1.05; KH_2PO_4 , 0.2; $MgS0_4 \cdot 7H_2O$, 0.4; $FeS04 \cdot 7H_20$, 0.01; $Na_2MoO_4 \cdot 2H_2O$. 0.006; $CaCI_2$, 0.1; sodium citrate, 0.5; glucose, 40.

The procedure of PHB isolation from the biomass of *A. chroococcum* 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 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 into the bottom of Petri dishes.

Degradation of PHB

Three types of an active sludge derived from different sources were used: microbial biomass from laboratory anaerobic UASB- reactor, treating pig manure wastewater [6]; active sludge from nitrifying/denitrifying reactor of Luberetskaya station treating municipal wastewater (Moscow); sediment of sludge deposit sites (Moscow region) [7].

Anaerobic PHB degradation in the presence of nitrate was studied on the modified liquid medium [8]. It contained (per liter): KH_2PO_4, 2.6; K_2HPO_4, 5.3; $MgCl_2$, 0.1; $CaCl_2$, 0.08; KNO_3, 3.0; $Na_2S \cdot 9H_2O$, 0.1; yeast extract, 0.01; resazurin, 0.002; PHB, 2.5-3.0; 2 ml of microelements on Lippert [9]; 10 ml of vitamins on Wolin [10]; pH 7.0-7.2 at 20°C. The gas phase contained 6 % acetylene and 94 % argon. Nitrate was added fractionally, when exhausted.

Anaerobic degradation of PHB in the absence of nitrate was studied on the Pfennig's fermentation medium [11]. It contained (per liter): NH_4Cl, 0.33; $MgCl_2 \cdot 6H_2O$, 0.5; $CaCl_2 \cdot 6H_2O$, 0.33; KCl, 0.33; KH_2PO_4, 0.33; yeast extract, 0.5; $NaHCO_3$, 2.5; $Na_2S \cdot 9H_2O$, 0.6; resazurin, 0.002; PHB, 2.5-3.0; 2 ml of microelements on Lippert [9]; 10 ml of vitamins on Wolin [10]; 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 studied at the temperatures of 20, 11 and 5°C, respectively.

For each samples four replicates were used. The data presented in the Tables are average values. The control samples contaned nitrate without PHB and PHB without nitrate.

Analytical methods

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

Gaseous products (N_2O, CH_4, CO_2) were determined by gas chromatography (Chrom-5, Prague, Czechia) 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. The oven temperature was 25°C. VFA were determined by gas chromatography (Chrom-5, Prague, Czechia) with a flame-ionisation detector, temperature oven- 170- 180°C, injector and detector- 200°C. Chromosorb-101 was used as sorbent. In both cases 1.2 m glass column and argon as carrier gas (40 ml/min) were used.

To determined total suspended solids (TSS) the experimental sample in volume of 5 ml was centrifuged, twice washed out in 0.85 % NaCl solution and dried at 105°C during 12 hours.

Enrichment cultures

The enrichment cultures of microorganisms from active sludge of laboratory anaerobic UASB- reactor, treating pig manure wastewater [6] and active sludge from nitrifying/denitrifying reactor of Luberetskaya station treating municipal wastewater (Moscow) were obtained by the method of tenfold dilution. Active sludges from these reactors were inoculated in the modified liquid medium for denitrifying microorganisms (see above)[8]. PHB films or VFA were used as a carbon source.

Total cell count

Total cell count from sludges was obtained by DAPI (4 ', 6 '-diamidino-2-phenilindol) staining. Cell suspension in volume of 0.01 ml was spread over a 1 cm^2 area on a slide, air- dried,

fixed in 96 % ethanol during 20 minutes. The slides were stained for 10 minutes in a solution of dye containing 0.01 mg/ ml DAPI and washed off by water.

Microscopy

Microbial cells were counted with UF- microscope " LUMAM I1 " with filters UFS 6- 3 (360 nm), filter SZS-24- 4, a blue plate (300- 380 nm, 420- 500 nm) for DAPI staining.

Cell morphology was examined on a "Amplival" phase contrast light microscope (Carl Zeiss, Germany).

Results and Discussion

1. Anaerobic degradation of PHB film by active sludge from UASB and nitrifying/denitrifying reactors in the presence and absence of nitrate at 20 °C.

PHB degradation by active sludge from UASB reactor treating pig manure wastewater and from nitrifying/denitrifying reactor treating municipal wastewater (Moscow) in the presence and absence of nitrate at 20° C was investigated. The active sludge from both reactors had similar characters of PHB degradation and average rates of denitrification (Table 1). In both presence and absence of nitrate, the intensity and character of PHB degradation was similar. The degradation rate was only 1.11 times higher in the presence than in the absence of nitrate. The active sludge from anaerobic UASB- reactor showed a bit higher average rates of denitrification and acetate accumulation.

Figures 1 and 2 show the results of anaerobic PHB degradation by active sludge from UASB-reactor in the presence and absence of nitrate.

In the presence of nitrate, PHB degradation was accompanied by consumption of nitrate and N_2O formation (Fig. 1). Nitrate was added fractionally, when it was exhausted from the medium. High value of the ionic force of the solution could lead to suppression of microbial growth. The amount of N_2O formed in experiment was lower then that theoretically expected. It can be explained by the fact that a part of nitrate was consumed by microbial cells of the community including microorganisms which were not involved in denitrification.

PHB was degraded to CO_2 with formation butyrate and acetate, as the intermediate products. Exhaustion of nitrate from the medium resulted in a sharp increase of VFA (mainly acetate) accumulation. An addition of nitrate 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 small amounts of propionate (Fig. 2). Unlike the denitrifying community, in an anoxic one the consumers of acetate are acetoclastic methanogenes. However fast hydrolysis of PHB led to an accumulation of high amounts of acetate (up to 90 mM), which decreased pH value down to 5 and caused a complete suppression of methanogenesis. Acetoclastic methanogens are sensitive to low pH as well as to high concentration of acetate. In control samples without PHB, slow methanogenesis proceeded the presence of viable methanogens in microbial community. Our results differ from those Budwill and co-authors, who studied PHB degradation in a wet sediments. These authors showed that methane formation comprised 87 % of that theoretical expected and was more intense than in control sample without polymer [2].

When experimental sample, in which PHB degraded to VFA, was diluted by fresh mineral medium to reduce acetate content to 7- 10 mM, methane started to produce slowly. One month after that, 0.5 mM CH_4 was produced in this diluted sample. This experiment showed the presence of viable methanogens in the community, as well as the possibility of PHB degradation with methane formation.

Results obtained indicate that anaerobic hydrolysis of PHB at 20°C could be provided by hydrolytic anaerobic non-denitrifying microorganisms. However a presence of denitrifyers degrading PHB could not be either excluded.

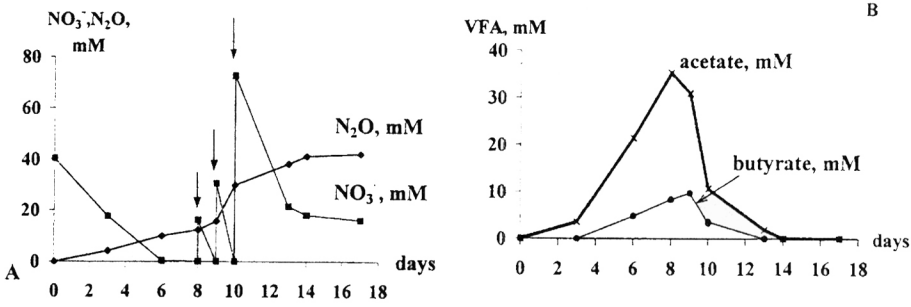

Fig. 1. Anaerobic PHB degradation at 20° C by active sludge from anaerobic UASB- reactor treating pig manure wastewater in the presence of nitrate; (A) nitrate consumption and N_2O formation, (B) formation and consumption of VFA (⟶ - addition of nitrate).

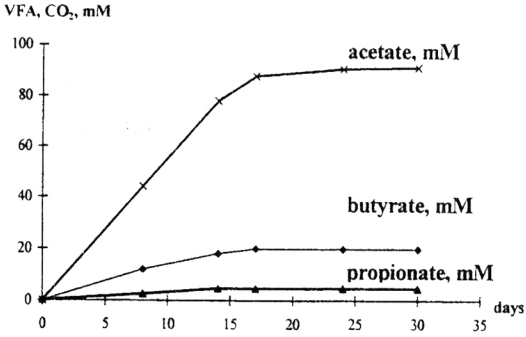

Fig. 2. Products of anaerobic PHB degradation in the absence of nitrate by active sludge from anaerobic UASB- reactor treating pig manure wastewater at 20° C.

Table 1. The basic parameters of anaerobic PHB degradation at 20°C by active sludges in the presence and absence of nitrate

Source of active biomass	Conditions of incubation	Initial TSS, g/l	Initial cell number, cells/l	Amount of used PHB, %	Amount of used NO_3^-, g/l	Max. amount of formed acetate, mM	Average rate of acetate formation, acetate/ day	Average rate of butyrate formation, butyrate/ day	Average rate of denitrification, mM NO_3^-/ day
anaerobic UASB-reactor	+ NO_3^-	0.68	3.0×10^{11}	100	8.9	35.3	-	-	10.6
	- NO_3^-			92	-	91	5.6	0.94	-
Nitrifying /denitrifying reactor	+ NO_3^-	0.91	3.7×10^{11}	100	8.7	5.6	-	-	9.5
	- NO_3^-			92	-	92	4.6	1.3	-

2. Anaerobic PHB degradation at low temperatures by microbial population of sediment from sludge deposit site (Moscow region) in the presence and absence of nitrate.

The low temperature effect on the rate of anaerobic PHB degradation in sediment of sludge deposit site (Moscow region), that was known to be active at low temperature [7], was investigated. It was shown that the rate of PHB degradation by microbial population of sediment depended of temperature (Table 2). In the absence of nitrate PHB was sufficiently degraded at 20°C but it was not degraded at all even at 11°C, not to mention at 5°C. No acetate and other VFA were produced from PHB. Thus, under anoxic conditions, a temperature decrease sharply suppressed the growth of PHB- degrading microorganisms.

Low temperature did not so dramatically effect on PHB degradation in the presence of nitrate. Therefore PHB continued to be degraded even at 5°C, through the rates of PHB degradation and denitrification decreased with decreasing temperature. The average rate of PHB degradation and maximal rate of denitrification were 7.3 and 7.1 times lower, respectively, at 5°C than at 20°C These results indicated a possible importance of the hydrolitic denitrifyers group.

High rate and sufficient efficiency of denitrification even at low temperatures allows to draw a conclusion on an opportunity of use PHB as an additional carbon source for microbial growth of denitrifying biomass during treating wastewater from nitrate. Its use is attractive in systems treating wastewater where nitrification/ denitrification occurs at last polishing step. In this step the majority part of organic pollution has been already utilized. So there is a lack of an organic substrates for microbial growth in medium. Methanol and sucrose are usually used as a carbon source addition [12]. PHB also can use as a material for microbial biomass immobilisation. It has been coordinated with other researchers which also offer use PHB in treating tap water. Biedermann with co-authors [13] showed that the reactor with anaerobic and the aerobic columns loaded by PHB-co-PHV granules ensured denitrification of tap water with simultaneous polymer hydrolysis. Wurmthfler and Muller [14] also used PHB granules stimulate the process of the tap water denitrification.

Table 2. Anaerobic PHB degradation at different temperatures by microbial population of sediment of sludge deposit site (Moscow region) in the presence and absence of nitrate.

Temperature of incubation, °C	Conditions of incubation	Time of incubation, days	Amount of used		Max. rate of denitrification, mM NO_3^-/ day	Average rate of PHB degradation, g PHB/l· day
			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	4.2	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

Anaerobic PHB degradation at 20°C by the enrichment cultures.

The enrichment denitrifying cultures grown on media with VFA or PHB films as a single carbon source were obtained. This cultures were derived from the active sludges of anaerobic UASB- reactor and nitrifying/denitrifying reactor. Hydrolysis of PHB in the presence of nitrate was carried out by different length and thickness rods (Figure 3 (A)). On the medium with VFA, the rod- shaped thick denitrifyers domineted (Figure 3 (B)). They were also detected in the enrichment culture grown on the PHB- containing medium. The final definition of PHB- degrading microorganisms is only possible in pure cultures.

The PHB film degradation by the enrichment denitrifying culture from the active sludge of anaerobic UASB-reactor was investigated (Fig. 4 (A, B)). Degradation started with a cells adsorption on a surface of polymer film and its consequent decomposition. Colonization of polymer films by microbial community was apparent usually on 3-5 days. In a couple of days polymer film proved completely covered by a cell layer and the film surface began to degrade (Figure 4, A). The film became thin and broke up to large pieces. Large pieces of polymer were decomposed to fine during the next 2- 3 days (Figure 4, B).

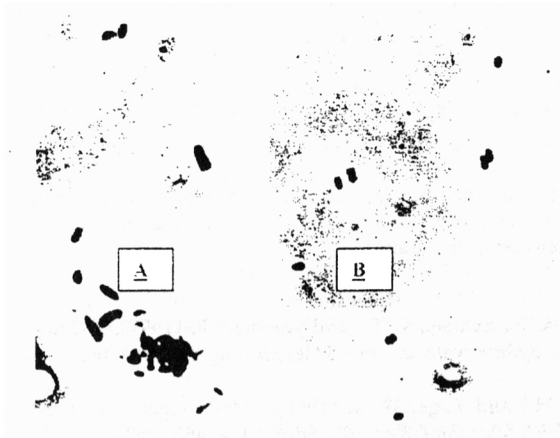

Fig.3. Cells morphology in denitrifying enrichment cultures from nitrifying/denitrifying reactor (A)- with PHB as substrate, (B)- with VFA as substrate. (× 1850).

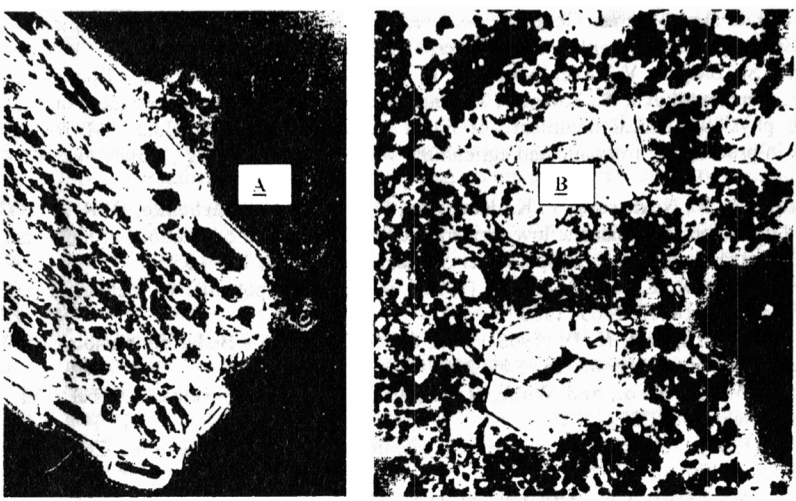

Fig. 4. Steps of PHB degradation by enrichment culture from active sludge of anaerobic UASB-reactor treating pig manure wastewater; (A)× 1140, (B) × 1570.

Conclusions

All investigated active sludges degraded PHB under anaerobic conditions in both presence and absence of nitrate. Nitrate had a little effect on the rate of PHB degradation at 20°C. PHB was gradually degraded to VFA and CO_2 by different groups of microorganisms. 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. PHB-degrading microorganisms belong to different morphological types.

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

High rate and sufficient efficiency of denitrification allows to draw a conclusion on an opportunity of use PHB as an additional carbon source for microbial growth of denitrifying biomass during treating wastewater from nitrate.

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