Biosynthesis, biodegradation, and application of poly(3-hydroxybutyrate) and its copolymers - natural polyesters produced by diazotrophic bacteria

A.P. Bonartsev[1,3], **V.L. Myshkina**[1], **D.A. Nikolaeva**[1], **E.K. Furina**[1], **T.A. Makhina**[1], **V.A. Livshits**[1], **A.P. Boskhomdzhiev**[1], **E.A. Ivanov**[1], **A.L. Iordanskii**[1,2], and **G.A. Bonartseva**[*,1]

[1]Laboratory of biochemistry of nitrogen fixation, A.N.Bach's Institute of Biochemistry, Russian Academy of Sciences, Leninskii prosp. 33, 119071 Moscow, Russia
[2]Laboratory of transport phenomena in polymers, Joint Institute of Chemical Physics, Russian Academy of Sciences, Kosygin str. 4, 119991 Moscow, Russia
[3]Laboratory of enzyme chemistry, Faculty of Biology, Moscow State University, Leninskie gory 1-12, 119992 Moscow, Russia

Polyhydroxyalkanoates (PHAs) are bacterial polymers that are formed as naturally occurring storage polyesters by a wide range of microorganisms. Biodegradable and biocompatible poly(3-hydroxybutyrate) (PHB) and its copolymers with 3-hydroxyvalerate (PHBV) are the best known representatives of PHA family. For more than 20 years biosynthesis, biodegradation and applications of PHB and its copolymers have been studied in the Bach Institute of Biochemistry RAS. An effective technology for production of PHB and PHBV of different molecular weight (from 200 to 1500 kDa) by diazotrophic bacteria of Azotobacter and Rhizobium genus has been developed. In order to clarify mechanism of PHB biodegradation degradation of PHB at different conditions in vitro and in vivo have been studied. A number of medical devices on basis of PHB: surgical meshes, screws and plates for bone fixation, periodontal membranes, and wound dressing are developed. High biocompatibility of PHB films and medical devices implanted in animal tissues has been demonstrated. Nowadays, development of systems of sustained drug delivery on the base of PHAs microspheres and microcapsules as a new and promising trend in the modern pharmacology is intensively in progress.

Keywords: polyhydroxyalkanoates; poly(3-hydroxybutyrate); diazotrophic bacteria; Azotobacter; Rhizobium; biosynthesis; biodegradation; biocompatibility; medical devices; sustained drug delivery; controlled release; microspheres; microcapsules

1. Introduction

Polyhydroxyalkanoates (PHAs) are bacterial polymers that are formed as naturally occurring storage polyesters by a wide range of microorganisms usually under unbalanced growth conditions [35]. Mechanical properties of PHAs make them suitable replacements for petrochemically produced bulk plastics (polyethylene, polypropylene etc.), but in contrast to these commodity plastics PHA are completely degradable to carbon dioxide and water through natural microbiological mineralization. PHAs can be produced by biotechnological processes under controlled conditions. Microorganisms are able to incorporate up to 60 different types monomer into their storage polymer and a series of PHAs with different monomeric composition (i.e. different physical and chemical properties) can be produced [45]. Novel polymer materials have revolutionized the polymer applications. In the immediate future, one of the dominant factors to be expected from human endeavor will become the environmental friendliness. Along this line, serious efforts are mounted to the developments of biopolymers with appropriate properties and processability, so-called the ‘‘green’’ polymers, contrast to the conventional petrochemically originated polymers [26, 29]. Poly-3-hydroxybutirate (PHB) and its copolymers with 3-hydroxyvalerate (3HV), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), are the best known representatives of PHA family. These polyesters have attracted widespread attention, as environmentally friendly polymers which can be used in a wide range of agricultural, industrial, and medical applications. Biodegradability and biocompatibility are main characteristics that allow PHAs

[*] Corresponding author: e-mail: bonar@inbi.ras.ru, Phone: +7 4959544008

to be competitive in special market sectors. Both PHB homopolymer and PHBV copolyester have been receiving commercial interest as a promising candidate for the large-scale production of biodegradable and biocompatible thermoplastics, since this polymer exhibits a considerable range of transport and thermo-mechanical properties which depend on its 3HV content. Perspective areas of PHAs applications are following: the use of PHA as filler for non-biodegradable plastics (production of biodegradable plastics); disposable packages; in agriculture - systems for prolonged release of fertilizers and agrochemicals; in medicine – medical devices and systems of sustained drug delivery [45].

In the last decades, potential application of bacterial PHB and related PHAs as biodegradable and biocompatible materials in medicine attracts much attention. A challenging combination of biomedical and biodegradable properties of PHB is a perspective tool in design of novel medical devices and tissue engineering. Over the past years, PHAs, particularly PHB, have been used to develop devices including sutures, repair devices, repair patches, slings, cardiovascular patches, orthopedic pins, adhesion barriers, stents, guided tissue repair/regeneration devices, articular cartilage repair devices, nerve guides, tendon repair devices, bone marrow scaffolds, and wound dressings [20]. Moreover, the capability of PHB for encapsulation and controlled release of different drugs allows applying PHB for development of therapeutic systems of sustained drug delivery [3]. A high biocompatibility of PHB was demonstrated both in vitro and in vivo [20].

In spite of investigation of PHB biosynthesis, degradation, biocompatibility, application etc. by many researches these data are generally fragmentary and often contradictory because each of these basic problems is studied by researches focused on special scientific fields. For example, mechanical and physical-chemical properties of PHB are studied in physical and chemical laboratories whereas biocompatibility of PHB is studied in physiological and biomedical laboratories.

In the Bach Institute of Biochemistry (Russian Academy of Sciences) a group of experts from different scientific areas: microbiology, polymeric chemistry, physiology, and medicine works in close cooperation. For more than 20 years our team has been focused on a comprehensive study of biosynthesis, biodegradation and applications of PHB and its copolymers.

2. A fluorescent method of PHB vital staining in bacterial cells

To start an extensive search of PHB producers among diazotrophic microorganisms it was necessary to develop simple qualitative and quantitative methods of PHB content estimation in living bacterial cells. A simple fluorescent technique of PHB vital staining in bacterial cells (colonies) grown on a solid medium in Petri dishes in the presence of phosphine 3R, a vital lipophylic due, has been developed. The fluorescence of colonies in UV light shows the presence of the polymer. The method has been used for a primary qualitative selection of the bacterial strains with high PHB content. Colonies of strains containing PHB in small quantities had no fluorescence. Colonies of the strains with the high content of the polymer produce bright-green fluorescence in UV light. The method is very simple and is applied for serial analysis [5].

The content of PHB in the bacterial cells was assayed quantitatively by measuring the fluorescence intensity of cells grown in the presence of phosphine 3R. The florescence intensity of microbial suspension was determined using a spectrofluorimeter at the maximal excitation wavelength (380 nm) and the maximal fluorescence wave length (470 nm). This quantitative method has been used for differentiation of diazotrophic bacterial strains with various accumulation of PHB when the bacteria were grown in pure solid and liquid cultures. Strains with rich content of PHB had a high fluorescence peak at 470 nm [6].

This method has allowed selecting the strains of diazotrophic bacteria of *Azotobacter* and *Rhizobium* genus that are able to synthesize PHB intensely.

3. Biosynthesis of PHB and PHBV

3.1. Biosynthesis of PHB and nitrogen fixation in *Rhizobium*

It is well known that PHB synthesis and expenditure are closely connected with the energy requirements of the cell. Many nitrogen-fixing microorganisms can synthesize PHB in considerable quantity, because its synthesis and consumption are closely connected with such energy - intensive process as the biological fixation of molecular nitrogen. PHB synthesis depends on a number of conditions including the nature of carbon and nitrogen sources utilized, on their concentration ratio in the medium, on partial oxygen pressure and so on. In considering PHB synthesis by root nodule bacteria, it should be noted that this property is fully inherent in all *Rhizobium* species.

Nitrogen deficiency or the excess of organic substrates results in PHB accumulation. The highest PHB level in *Rhizobium* cells is also reached by oxygen limitation.

The amount of synthesized PHB may be stabilized by cultivating bacteria under strictly definite conditions, and in this case may be used as quantitative characteristics of individual *Rhizobium* strains. The presence of storage biopolymers enhances the survival of *Rhizobium*, serving as a carbon and energy source during periods of starvation outside the nodule [2].

In our laboratory the relationship between PHB content and nitrogenase and hydrogenase activity in some strains of *Rhizobium* has been studied. A strict inverse correlation was revealed between nitrogenase activity and the PHB content during anaerobic growth in the presence of nitrates. This dependence was apparently due to the PHB expenditure on nitrogen fixation under such conditions. A direct correlation between hydrogenase activity and the PHB content has been found [7, 8].

It may be assumed that the presence of hydrogen-assimilating hydrogenase gives the strains studied an advantage, in terms of energy, that is due to the secondary involvement of molecular hydrogen in metabolism and thereby enables surplus energy accumulating in the form of PHB to be used more effectively.

3.2. PHB content in cells of various *Rhizobium* species during grows with different carbon and nitrogen sources

The capacity for PHB synthesis has been tested in active and less active collection strains of *Rhizobium phaseoli*, *R. meliloti,* and *R. trifolii* during growth on media with different carbon and nitrogen sources.

It has been shown that the nature of the carbon and nitrogen sources utilized during growth of root nodule bacteria determines both their growth yield and PHB synthesis [9].

The maximum PHB content depends on the type of culture. In *R. trifolii*, maximum PHB content has been found in cells grown with sucrose as the carbon source and glutamine as the nitrogen source. PHB content was higher in the active strain than in the strain with low activity, and reached 45% of dry cell weight. The PHB content was much lower when organic acids (succinate, fumarate or acetate) were used. Testing of various combinations of carbon and nitrogen sources for cultivation of *R. trifolii* has shown that with combined use of glucose and nitrate, the PHB content was many times higher in the less active strain than in the active strain.

In the group of lucerne root nodule bacteria, the PHB content was highest in the less active strain *R. meliloti* grown on sucrose and glycine (up to 59% of dry cell weight). Mainly, the PHB synthesis in *R. meliloti* cells was higher in the lowly active strain than in the active strain for most of the tested carbon and nitrogen sources. However when fumarate was the carbon source and ammonium sulfate was the nitrogen source the active strain accumulated more PHB than the strain with low activity. Just like *R. trifolii, R. meliloti* showed low PHB accumulation during growth on media containing organic acids.

Among all *Rhizobium* strains cultivated on different sources of carbon and nitrogen the highest level of PHB accumulation (up to 65% of dry cell weight) has been observed in the less active *R. phaseoli* strain grown on sucrose and nitrate. With other nitrogen sources the polymer synthesis was also high and reached 40-45% of dry cell weight that was considerably higher than in other groups of nodule bacteria (Fig. 1).

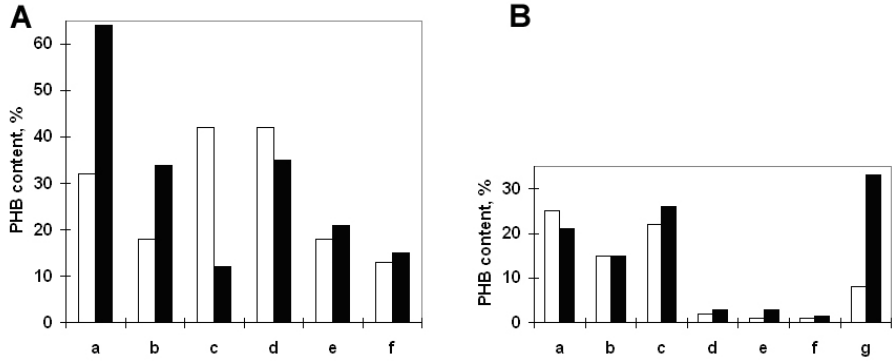

Fig.1. Content of PHB in cells of active A_3 (■) and low active A_1 (□) *R. phaseoli* strains grown on different nitrogen and carbon sources. (A) Cells grown on sucrose with different nitrogen sources: a) KNO_3; b) $(NH_4)_2SO_4$; c) glutamine; d) urea; e) asparagine; f) glycine; (B) Cells grown on different carbon sources in the presence of NO^{3-} or NH^{4+}: a) glucose + NO^{3-}; b) arabinose + NO^{3-}; c) mannitol + NO^{3-}; d) succinate + NO^{3-}; e) fumarate + NO^{3-}; f) acetate + NO^{3-}; g) fumarate + NH^{4+}.

3.3. Influence of various oxygen, sucrose and nitrate concentrations on PHB synthesis in *Rhizobium phaseoli*

Optimization of conditions for PHB overproduction in strains A_1 (low active) and A_3 (active) of *R. phaseoli* has been developed. The degree of PHB content in the cells of nodule bacteria is determined not only by chemical nature of carbon and nitrogen source used for growth but also by their concentration ratio in the medium. The method of mathematical design of experiment that we have used is highly illustrative of it. Sucrose and KNO_3 served as carbon and nitrogen sources are the best ones for the synthesis of the polymer for this group of nodule bacteria. It has been used a complete factor experiment - CFE 2^3 with the application of 3 variables (O_2, sucrose, KNO_3) on two concentration levels (- lower and + upper ones). Maximum PHB content of the studied strains corresponded to the concentration ratio of sucrose and KNO_3 in the medium, which is equal to 20:1. A minimal PHB content in both strains was observed at a C : N ratio of 3 : 1, and oxygen did not play any substantial role in this case. Carbon and nitrate concentration used in CFE were low; their low level was necessary for a theoretical elucidation of the C : N ratio required for a maximal PHB synthesis. Therefore, we have employed the increased sucrose concentration gradient in the medium to achieve a maximal intracellular PHB accumulation.

On the base of CFE 2^3 data obtained, we have used the gradient of increased sucrose concentration in the medium at fixed potassium nitrate and oxygen levels, thus setting various C : N ratios in the medium and using high concentrations of the carbon source to achieve a maximal biomass yield and PHB content. A high PHB level was achieved at a C : N ratio of 10 : 1 and was stabilized up to a C : N ratio of 20 : 1 and higher (Fig 2). At high concentrations of carbon substrate in the medium, cellular PHB content in poorly active strains was significantly higher than in active strains. A maximal PHB content in the poorly active strain of *R. phaseoli* A_1 was as much as 80% of the dry cell weight. The biomass yield in the poorly active strain was much greater than the biomass yield in the active strains under these conditions [11, 12].

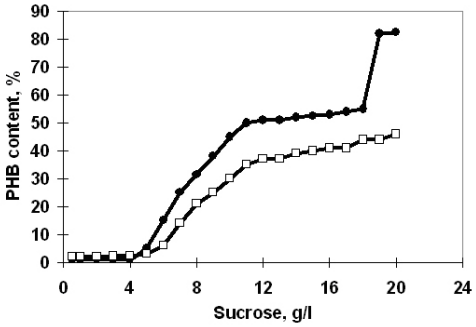

Fig. 2 PHB content (% of dry cell weight) in cells of R . phaseoli versus the gradient of sucrose concentration in the medium: (●) low active strain A_1, (□) active strain A_3.

Thus, a design for selection of potential biotechnological producer of PHB has been developed. The optimal conditions have been selected for polymer overproduction and PHB accumulation in cells.

3.4. Biosynthesis of PHB and PHBV by *Azotobacter* strains

Nutrientregulated hyperaccumulation of PHB and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) has been reported in *Azotobacter vinelandii* strain UWD, which produced PHAs during growth on a variety of unrefined sugar sources including sucrose, molasses, cane molasses and corn syrup [39]. Less is known about PHA production by *Azotobacter chroococcum* strains, although PHB homopolymer production by *A. chroococcum* strain 7B in various mediums has been reported [13, 16]. We have shown that various strains of a single *Azotobacter* species varied widely in the level of reserve polymers. Under similar growth conditions the content PHB may differ from 0 to 85% of biomass dry weight [10, 42, 46].

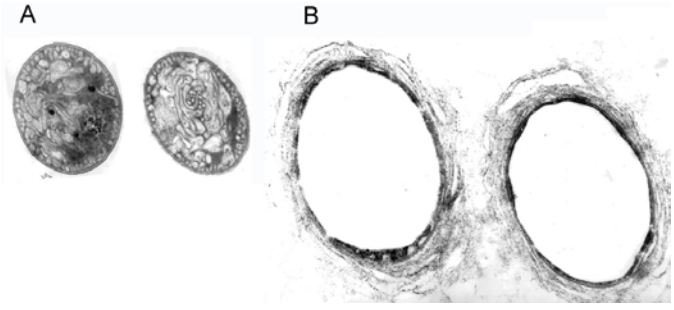

Fig. 3. Transmission electron microscopy observation of *Azotobacter chroococcum* cells.
(A) PHB content - 0%;
(B) PBH content - 85%.
The magnification is 50 000 x.

We have selected some strains capable to accumulate PHB up to 85% (fig. 3). Cultivation of the producer strain was divided into two stages due to directed regulation of aeration and carbon source concentration. At the first stage active cell division, as well as extensive conversion of a carbon source into biomass containing no polymer occurred. At the second stage of a fermentation process PHB was actively synthesized in the producer cells because culture growth was limited by oxygen at an excess of carbon source. *A. chroococcum* 7B has been found to utilize glucose, fructose, sucrose, galactose, maltose, mannitol, as major carbon source. On melassa-containing medium, the strain grows well and accumulates PHB. Among the carbon sources studied, glucose, sucrose, and melassa are the best carbon sources to provide the highest yield of PHB with the molecular weight (Mw) as high as 1300 - 1500 kDa, which is an important advantage of this producer. Citrate, succinate, propionate, malate, acetate, ethanol, butanol, isobutanol, and aminobutyrate can be used as additional carbon sources.

We have developed the technology of PHB production by *Azotobacter* strains that ensues the yield (50 g dry biomass /liter) with content of polymer up to 85% of the cell dry weight. An advantage of the selected PHB producing microorganisms is that they utilize cheap unrefined raw material as a carbon sources. High carbon concentration and C/N ratio ranging from 20/1 to 100/1 has the most beneficial effect on PHB biosynthesis. Different wastes from agricultural production can be successfully used for PHB synthesis [16, 18].

The method of polymer obtaining is highly competitive. The PHB-producing strain and the method of it biosynthesis have been patented [14]. A technology of the large-scale PHB production has been tested on the industrial fermenters. Advantage of the developed technology is the high molecular weight of the polymer (1500 kDa) and the lack of side products of biosynthesis. The method is readily reproducible and ensures isolation of a polymer with standard molecular weight, physicochemical properties, technological parameters, the degree of purity and toxicity.

Unlike other PHB producers (*Alcaligenes, Methylobacterium* etc.), bacteria of the genus *Azotobacter* can synthesize polymer at a wide range of molecular weight. The molecular weight of the synthesized polymer has been found to depend on cultivation conditions of the PHB producer. Like other physico-chemical properties of PHB, Mw is known to depend on such factors of producer cultivation as carbon and nitrogen sources and their concentration ratio in the medium, temperature, pH, oxygen concentration etc. We have managed to synthesize polymer of different molecular weights with definite physico-chemical properties using different concentration ratios of the major and additional carbon sources in the cultivation medium of *A. chroococcum* 7B, and by varying medium pH [38]. The method of production of PHB of different molecular weight from 300 kDa to 1500 kDa has been patented [14].

The biosynthesis of PHBV by *A. chroococcum* 7B grown under different carbon nutrition conditions has been studied in order to develop novel culture methods for the production of biodegradable microbial polyesters. We have demonstrated the production of large amounts of PHBV copolymers with different 3HV content by *A. chroococcum* stain 7B in culture media containing sucrose (3% w/v) as the primary carbon source and organic acids at different concentration: propionate (20 mM) and valerate (10 and 20 mM) as additional carbon sources. The parameters to be analyzed included the yield of biomass, the yield, synthesis rate, and composition of copolymers. The yield of biomass of *A. chroococcum* 7B grown under 10 mM, 20 mM valerate and 20 mM propionate was 4.8, 3.2 and 3.1 g/l, respectively in comparison with 5.6 g/l under pure sucrose. Thus, addition of valerate and propionate caused inhibition of cell growth. PHAs were accumulated to 78, 76 and 76% of the cellular dry weight, respectively, in comparison with 82% in control. Gas chromatographic analysis of PHA isolated from bacterial cells grown under 10 mM, 20 mM valerate and 20 mM propionate showed that the polyester (Mw 800-1300 kDa) was mainly composed of 3-hydroxybutyrate (3HB) and of 17, 32 and 3 mol% 3-hydroxyvalerate (3HV), respectively [unpublished data]. The method of PHB copolymers obtaining with different percentage of 3HV in PHBV chain has been patented [19].

These results indicate that it is possible to synthesize a range of PHBV copolymers with different 3HV content. Biosynthesis of PBHV with different composition of copolymers allows producing and applying these biopolymers for development of medical devices with various mechanical, physical-chemical and biological properties.

4. Biodegradation of PHB.

4.1. Degradation of PHB in vitro

In vitro degradation of PHAs in the aqueous media proceeds via a random bulk hydrolysis of ester bonds in the polymer chain [32]. To understand mechanisms of PHB biodegradation it is necessary to examine hydrolysis of PHB in vitro. Degradation of films from PHB of different Mw (300, 450, 1000 kDa) in vitro has been demonstrated under three different conditions: at 37°C in phosphate buffer (pH = 7.4) and at 70°C in phosphate buffer (pH = 7.4), and at 37°C in human blood serum. At 37°C hydrolysis of PHB (for example, films with thickness of 50 mkm, Mw of PHB

= 1000 kDa) was very slow: only 5% weight loss of PHB films for 6 months, the complete degradation of PHB we could not examined at these conditions (more than 1 year). Degradation of PHB films in human blood serum was similar to degradation in phosphate buffer at 37°C with high correlation rate. At 70°C degradation of PHB was much more rapid: 5% weight loss of PHB films (50 mkm, 1000 kDa) was for 2.5 months and the complete degradation of PHB films was occurred for 3.5 months. But the feature of PHB degradation at both conditions was similar. At initial long-term phase (6 months for 37°C incubation and 2.5 months for 70°C incubation) weight loss only of approximately 5% was observed. But simultaneously the increase of crystallinity and the decrease of Mw were occurred. As consequence, mechanical properties became worse: PHB films became more rigid and fragile; the surface of PHB films became distended, microcracks appeared. At second relatively short-term phase rapid fragmentation and following complete degradation of small fragments of PHB films were observed. We have showed that the rate of hydrolysis of PHB films depends on Mw of PHB (fig. 4). The films from PHB of high molecular weight (450 and 1000 kDa) degraded as it was described above whereas films from PHB of low molecular weight (150 and 300 kDa) lost weight relatively gradually [unpublished data]. Our results are confirmed by data of other investigators [21, 32].

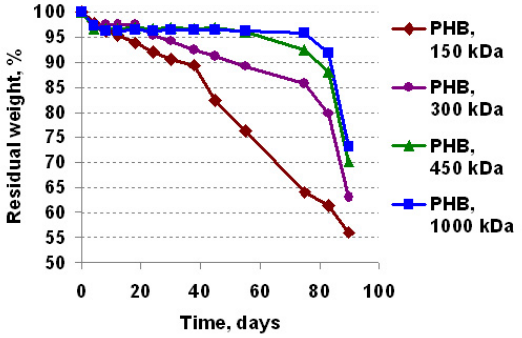

Fig. 4. In vitro degradation of films from PHB of different molecular weight (150, 300, 450 and 1000 kDa). The films were submerged in phosphate buffer (pH=7.4) at 70ºC for 90 days.

4.2. Degradation of PHB by soil microorganisms

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. 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. Maergaert et al. 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 [36]. 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".

We have also studied biodegradation of films prepared from the synthesized PHB under model conditions [17]. We have studied biodegradability of PHB films under aerobic, microaerobic and anaerobic condition 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. Changes in molecular mass, crystallinity, and mechanical properties of PHB have been studied.

The biodegradation of PHB films in soil suspension (fig. 5) has been studied during 2 months under different aeration conditions and nitrate was added as an additional nitrogen source (and as an electron acceptor). Degradation of PHB was highest under aerobic condition, and practically did not proceed under anaerobic condition. The addition of nitrate enhanced the degradation under all of the aeration conditions studied and stimulated the development of denitrifying microbial populations.

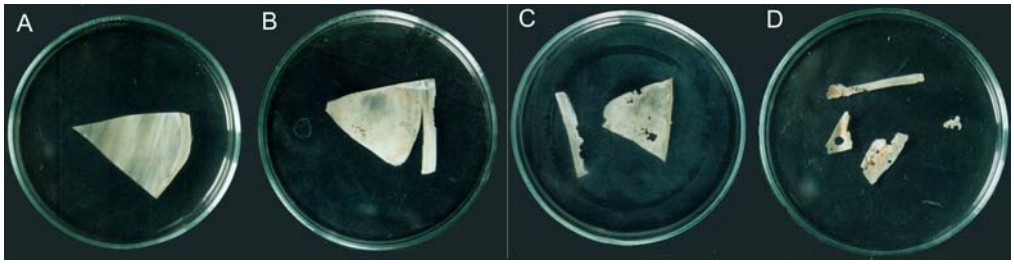

Fig. 5. Undegraded PHB film (A) and PHB films with different degrees of degradation after 2 months incubation in soil suspension: anaerobic conditions without nitrate (B), microaerobic conditions without nitrate (C), and microaerobic conditions with nitrate (D).

Our experiments have revealed a correlation between the degree PHB degradation and the molecular weight of degraded PHB. In experimental flasks with equal aeration levels, the presence of nitrate promoted the degradation of PHB films and enhanced the decline in the Mw of PHB. The most degraded PHB exhibited the highest values of the crystallinity index. As it has been shown by Spyros *et al.,* PHAs contain amorphous and crystalline regions, of which the former are much more susceptible to microbial attack [44]. If so, the microbial degradation of PHB must be associated with a decrease in its molecular weight and an increase in its crystallinity, which was really observed in the experiments. Moreover, microbial degradation of the amorphous regions of PHB films made them more rigid. However, further degradation of the amorphous regions made the structure of the polymer much looser [17].

The total number of microorganisms on the surface of the PHB films degraded under microaerobic and aerobic conditions in soil was 4 x 10^4 to 5 x 10^6 (fungi) and 1 x 10^5 to 2 x 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 [15].

PHB biodegradation in the enriched culture obtained from soil on the medium used to cultivate denitrifying bacteria (Gil'tai medium) has been also studied. The dominant bacterial species, *Pseudomonas fluorescens* and *Pseudomonas stutzeri*, have been identified in this enrichment culture. Under denitrifying conditions, PHB films were completely degraded for seven days. Both the film weight and Mw of PHB decreased with time. In contrast to the data of Doi et al. [22] who found that Mw of PHB remained unchanged upon enzymatic biodegradation in an aquatic solution of PHB- depolymerase from *Alcaligenes faecalis,* in our experiments, the average viscosity molecular weight of the higher- and lower-molecular polymers decreased gradually from 1540 to 580 kDa and from 890 to 612 kDa, respectively. 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 weight. 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 [25]. 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 [15, 17].

The samples of PHB have been tested for fungicidity and resistance to fungi by estimating the growth rate of test fungi from the genera *Aspergillus, Aureobasidium, Chaetomium, Paecilomyces, Penicillum, Trichoderma* under optimal growth conditions. PHB film did not exhibit neither fungicide properties, nor the resistance to fungal damage, and served as a good substrate for fungal growth [37].

PHB degradation by activated sludge from reactor treating pig manure wastewater and from nitrifying/denitrifying reactor of the plant treating municipal wastewater (Moscow) in the presence and absence of nitrate at 20° C has been studied [30]. The activated sludge from both reactors had similar characters of PHB degradation and average rates of denitrification. In both presence and absence of nitrate, the intensity and character of PHB degradation were also similar. The rate of PHB degradation in the presence of nitrate was only 1.11 times higher, than in absence of nitrate. The activated sludge from anaerobic reactor showed a bit higher average rates of denitrification and acetate accumulation.

In the presence of nitrate, PHB degradation was accompanied by consumption of nitrate and N_2O formation. 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 of volatile fatty acids (mainly acetate) accumulation. An addition of nitrate in the medium resulted in volatile fatty acids consumption. Obviously denitrifying microorganisms serve as the final consumer of volatile fatty acids when nitrate is available.

In the absence of nitrate, PHB was also effectively degraded with formation of acetate, butyrate and small amount of propionate. Unlike the denitrifying community, in anoxic one the finally consumers of acetate are acetoclastic methanogenes. However rapid 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. When the experimental samples were diluted by the fresh mineral medium up to acetate concentration of 7- 10 mM, the methane production was initiated. After a month 0.5 mM CH_4 was detected in these diluted samples. This experiment has shown the presence of viable methanogens in community, as well as the possibility of PHB degradation with methane formation.

The obtained results have indicated that anaerobic hydrolysis of PHB at 20^{o}C could be provided by hydrolytic anaerobic non-denitrifying microorganisms. However the presence of PHB-degrading denitrifyers could not be also excluded. Under anaerobic conditions and in the absence of nitrate, no PHB degradation was observed even at 11º C [17].

4.3. Biodegradation of PHB in vivo

There is increasing interest regarding the degradation of PHAs in recent years, due to the biomedical industry's need for biodegradable polymer implants and controlled drug release systems [20]. Even though PHAs are considered to be quite resistant to degradation in the animal body, both enzymatic and non-enzymatic processes can occur simultaneously under normal conditions. In animal tissues degradation in vivo of PHAs is due to hydrolysis and biodegradation by tissue enzymes (nonspecific esterases) that are involved in degradation, especially, at the late stage of degradation process. It is generally accepted that the rates of degradation are influenced by the characteristics of the polymer, such as chemical composition, structure, crystallinity, and molecular weight [1, 24].

The biodegradation of PHB has been observed by many investigators [20]. But these researches have been carried out mainly in connection with application of devices based on PHB. Therefore, at each paper Mw of PHB, thickness of films or devices, site of implantation, species of laboratory animal, and etc. were appropriate for purpose of PHB device application.

We have studied fundamentally the biodegradation of PHB in vivo. Films of PHB of different Mw (300, 450, 1000 and 1500 kDa) and different thickness (10, 20 and 40 mkm) have been implanted subcutaneously to Wistar rats. At 0.5, 1, 2, 3 and 6 months after implantation PHB films have been isolated and the weight of PHB films, the crystallinity degree and Mw of PHB have been examined. PHB films have degraded relatively slowly. Our results indicate that the rate of biodegradation of PHB films depends on film thickness as well as on Mw of PHB. For example, PHB film with thickness of 50 mkm and PHB Mw of 1000 kDa completely degraded for 3 months (fig. 6). The process of PHB biodegradation consists of several phases. At initial phase PHB films was covered by fibrous capsule. At second phase capsulated PHB films very slowly lost weight with simultaneous increase of crystallinity and decrease of Mw and mechanical properties of PHB. At third phase PHB films were rapidly disintegrated and then completely degraded. At 4th phase empty fibrous capsule resolved [unpublished data]. Our results are confirmed by data of other investigators [24, 40]. It has been suggested that the degradation of resorbable polymers proceeds in two stages. Initially, the chain scission takes place in the amorphorous regions of a polymer and overall crystallinity increases. Crystallinity may then decrease as hydrolysis leads to degradation of chains in the crystalline regions, although no strict demarcation between these two stages can be made [24].

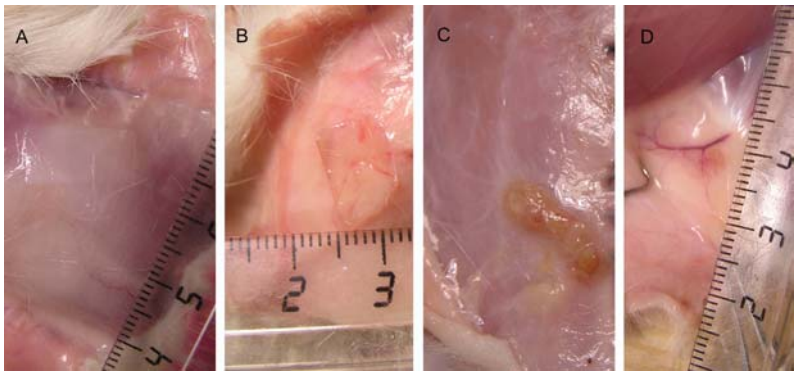

Fig. 6. Biodegradation of PHB films in vivo. PHB films (film thickness = 50 mkm; molecular weight of PHB = 1000 kDa) were implanted subcutaneously in Wistar rats for 0.5 month, 98% residual weight of the film (A), 2 months, 94% residual weight (B), 2.5 months, 28% residual weight (C), and 3 months, 0% residual weight (D).

5. Biocompatibility of PHB

5.1. Biocompatibility of PHB in vitro

Biocompatability of PHA in vitro has been demonstrated at different cell cultures: fibroblasts, mesenchymal stem cells, osteoblasts, bone marrow cells, articular cartilage chondrocytes, endothelial cells, smooth muscle cells etc. [20]. We have examined biocompatibility of PHB at cell cultures isolated from monkey kidney AGMK and FRLK4 grown on PHB films as on scaffolds for 3-60 days. PHB didn't influence on growth and vitality of cells for whole period of cell cultures incubation. The cells were not exposed to any polymer-depending factors: delivering toxic substances (products of polymer destruction), incompatible polymer surface etc. We have examined also the cytotoxicity of extract from PHB films at the same cell cultures. The addition of the extract to cell cultures grown on laboratory plastic Petri dishes didn't influence on growth, development and migration of the cells. The index of cytotoxicity was evaluated as 0.84 that indicates that PHB is not toxic [unpublished data]. Thus, PHB is biologically inert and highly biocompatible for isolated cell cultures in vitro.

5.2. Biocompatibility of PHB in vivo

Biocompatibility of PHB has been demonstrated in vivo under subcutaneous implantation of PHB films. Tissue reaction to films from PHB of different Mw (300, 450, 1000, 1500 kDa) implanted subcutaneously was relatively low and didn't change from tissue reaction to control glass plate. The low tissue reaction to implanted PHB films indicates the high biocompatibility of PHB in vivo that was observed by many investigators [20, 24, 40].

The possible reason of high biocompatibility of PHB is presence of natural PHB oligomers and 3-hydroxybutyrate, the intermediate product of PHB degradation, in animal tissues at normal conditions [34, 43] in comparison with chemically synthesized biodegradable polymers, for example, polylactides and polyglycolides.

We have obtained a toxicological certificate of Institute of Medical Technique (Ministry of Health, Russia) (no. 388-99, 24.12.1999) that approves of PHB for application in medicine as nontoxic and biocompatible material suitable for implantation in human tissues.

6. Blending of PHA

Blending of friendly environmental biopolymers with industrial synthetic polymers is a perspective tool to produce novel materials with combined characteristics in having both improved application properties and low cost advantages in material performance. We have designed and studied the blends on the base of bacterial poly-3-hydroxybutyrate (PHB) and universally known industrial polymers with different content of hydrophilic groups (diverse hydrophilicity) such as typical hydrophobic low-density polyethylene (LDPE), hydrophilic polyvinylalcohol (PVA) as well as moderately hydrophilic polyamide (Nylon 6,6) and segmented polyetherurethanes (SPEU) [27].

Theory and practice of polymer blends' applications are based on studies of transport phenomena because these phenomena control mainly efficiency of polymer exploitation. Water permeability and sorption as well as diffusivity in polymer materials are regulated by chemical structure of macromolecules and the polymer morphology [28].

Preliminary mixed compositions with different ratio were loaded in the single-screw extruder (ARP-20). Each of the origin components was also identically processed. The impact of three structural levels (molecular, crystalline and supermolecular) in above polymer blends upon water transport has been studied. From DSC and x-ray data the partial compatibility of blends for the polyester (PHB) and PVA is observed until 20 wt % of PVA. In this concentration interval (0-20%) the water permeability monotonically decreased with PHB concentration due to immobilization of hydroxyl groups by carbonyl groups of PHB and, hence, due to water solubility depression.

A detailed study of PHB/LDPE blends including mechanical properties and morphologies enables to propose an immiscible blend formation with decreasing of crystallinity degree for each component and formation of PHB fibrils with band-like and cylinder-like architecture. For both PHB/LDPE and PHB/SPEU blends the specific inflection points on permeability curves are shown. The initial stage of permeability reveals the relaxation of elements of blend structure on the molecular and crystalline levels. The variation of PHB concentration in the blended films permits to regulate their special morphology and as result the water barrier properties [28].

The influence of polymer hydrophilicity on the immiscibility degree has been studied. On the above data the permeability model of water transport in the heterogeneous matrices is presented. Because of the special features of morphology, the novel films are superior to the origin polymer films (PHB, LDPE, PVA, and SPEU) in mechanical

characteristics and water flux resistance. Besides, the formation of such heterogeneous compositions allow to regulate the rate of (bio)degradation in living systems or under wet climatic environment conditions [29].

7. Application of PHB

7.1. Medical devices

The perspective area of PHB application is development of implanted medical devices for dental, cranio-maxillofacial, orthopaedic, hernioplastic and skin surgery. A number of potential medical devices on the base of PHB: bioresorbable surgical sutures (fig. 7A) [23, 41], biodegradable screws and plates for cartilage and bone fixation (fig. 7B), biodegradable membranes for periodontal treatment (fig. 7C), surgical meshes with PHB coating for hernioplastic surgery (fig. 7D) [3], wound coverings [31] have been developed. We have obtained a toxicological certificates of Institute of Medical Technique (Ministry of Health, Russia) for application of surgical meshes with PHB coating (no. 371-06 and no. 372-06, 02.10.2006) and periodontal membranes (no. 377-06 and no. 378-06, 03.10.2006) in medicine. Investigation of biocompatibility, biodegradation and therapeutic effectiveness of these devices is intensively in progress [unpublished data].

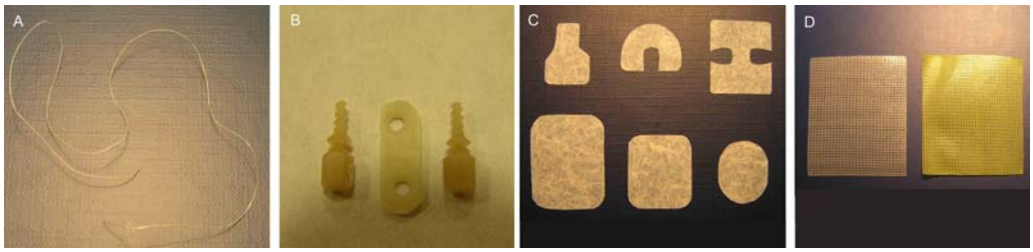

Fig. 7. Medical devices on the base of PHB. (A) bioresorbable surgical suture; (B) biodegradable screws and plate for cartilage and bone fixation; (C) biodegradable membranes for periodontal treatment; (D) surgical meshes with PHB coating for hernioplastic surgery, pure (left) and loaded with antiplatelet drug, dipyridamole (right).

7.2. Systems of sustained drug delivery on the base of PHB films

An improvement of medical devices on the base of biopolymers by encapsulating of different drugs opens up the wide prospects in applications of these new devices with pharmacological activity in medicine. We have designed the novel systems for sustained delivery of antiproliferative drug – dipyridamole (DP) and anti-inflammatory drug - indomethacin. The kinetics of drug release from PHB films has been studied. The release occurs via two mechanisms, diffusion and degradation, operating simultaneously. Dipyridamole and indomethacin diffusion processes determine the rate of the release at the early stages of the contact of the system with the environment (the first 6-8 days). The coefficient of the release diffusion of a drug depends on its nature, the thickness of the PHB films containing the drug, the weight ratio of dipyridamole and indomethacin in polymer, and the molecular weight of PHB. Thus, it is possible to regulate the rate of drug release by changing of molecular weight of PHB, for example [3]. A number of other drugs have been also used for development polymeric systems of sustained drug delivery: antibiotics (rifampicin, metronidazole, ciprofloxacin, levofloxacin), anti-inflammatory drugs (flurbiprofen, dexamethasone, prednisolone), and antitumor drugs (paclitaxel).

7.3. Systems of sustained drug delivery on the base microspheres and microcapsules

Development of therapeutic systems of sustained drug delivery on the base of microspheres and microcapsules from biodegradable polymers is a new and promising trend in the modern pharmacology. We have developed systems of controlled dipyridamole release on the base of PHB microspheres. The coefficient of the release diffusion of DP extensively depends on diameter of microspheres. But it is possible to produce a system with prolonged uniform drug release that is important for producing therapeutic systems with adjusted drug dosing. For example, the sustained release of DP from PHB microspheres (diameter – 60 mkm) occurred with almost constant rate for more that 1 month (fig. 8).

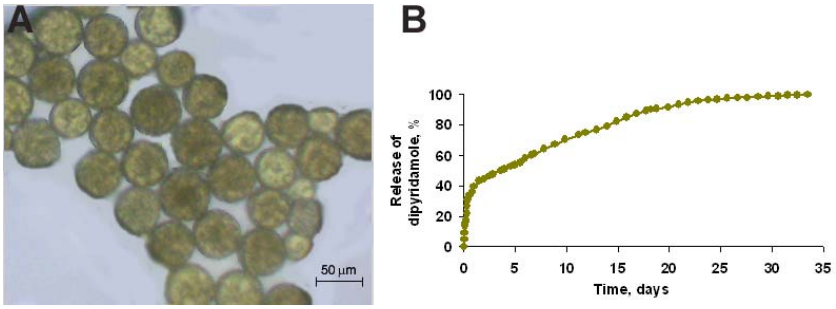

Fig. 8. PHB microspheres for sustained delivery of drugs. (A) PHB microspheres (average diameter = 60 mkm, PHB Mw = 1000 kDa) loaded with dipyridamole (10% w/w); (B) Sustained delivery of dipyridamole from PHB microspheres for more than 1 month.

For developing systems of controlled release of water-soluble drugs, proteins and peptides (hormones, tissue factors, enzymes etc), and nucleic acids (DNA and RNA plasmids encoding various proteins and peptides) is necessary to produce polymer systems on the base of biodegradable microcapsules. We have produced PHB microcapsules with encapsulated model drug – methylene green (fig. 9). The prospects for development and investigation of systems with biological activity based on microcapsules from polyhydroxyalkanoates look bright.

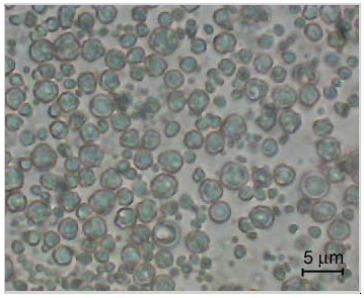

Fig. 9. PHB microcapsules (average diameter = 3 mkm, PHB Mw = 450 kDa) with encapsulated water solution of model drug, methylene green.

7.4. A physiological model on the base of PHB devices loaded with chemical substances

Besides application of PHB for producing of medical devices and systems of sustained drug delivery, PHB can be used for producing systems of sustained enzyme activators or inhibitors release for development physiological models. PHB with its minimal adverse inflammatory tissue reaction under implantation is a perspective tool in design of novel physiological models of prolonged local enzyme activation or inhibition in vivo. We have developed a system of sustained nitric oxide (NO) donor delivery on basis of PHB. This system can be used for investigation of prolonged NO action on normal tissues of blood vessels in vivo. The development of in vivo model of prolonged NO local action on vascular tissues is a difficult problem, because NO donors deliver NO only for a few minutes. We have developed a model of prolonged local NO action on appropriate artery on basis of PHB loaded with a new effective NO donor, FPTO [33]. It has been shown that FPTO-loaded PHB cylinders can release FPTO (and consequently NO) for up to 1 month with relatively constant rate. FPTO-loaded PHB cylinders with sustained FPTO delivery were implanted around left carotid artery of Wistar rats (fig. 10), pure PHB cylinders were implanted around right carotid artery as control. At 1st, 4th and 10th days after implantation arteries and cylinders have been isolated. Nowadays, the investigation of enzymes expression and activity in isolated arterial tissues is carried out [4].

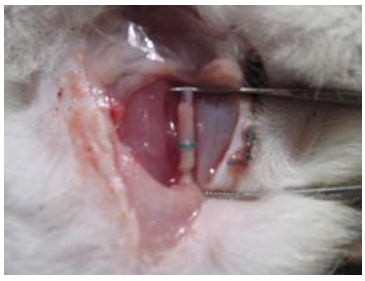

Fig. 10. In vivo model of prolonged local NO action on blood vessels: PHB-cylinder loaded with NO donor, FPTO, was implanted around carotid artery for sustain delivery of NO for 4 days.

8. Conclusions

Our team consisted of experts from different scientific areas: microbiology, polymeric chemistry, physiology, and medicine has realized a comprehensive research of biosynthesis, biodegradation, and biocompatibility of PHB and its copolymers and development of medical devices and therapeutic systems on the base of PHB and its copolymers. Nowadays, our team is focused on production of new PHB copolymers and development of new therapeutic systems based on PHB micro- and nanoparticles.

9. Acknowledgments

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