Introduction Currently, the intensive development of biodegradable and biocompatible materials for medical implication provoke comprehensive interdisciplinary studies on biopolymer structures and functions. The well-known and applicable biodegradable polymers are polylactides (PLA), polyglycolides (PGA), and their copolymers, poly-ε-caprolactone, poly(orthoesters), poly-β-maleic acid, poly(propylene fumarate), polyalkylcyanoacrylates, polyorthoanhydrides, polyphosphazenes, poly(propylene fumarate), some natural polysaccharides (starch, chitosan, alginates, agarose, dextrane, chondroitin sulfate, hyaluronic acid), and proteins (collagen, silk fibroin, fibrin, gelatin, albumin). Since some of these polymers should be synthesized through chemical stages (e.g. via lactic and glycolic acids) it is not quite correct to define them as the biopolymers. Besides biomedicine applications, the biodegradable biopolymers attract much attention as perspective materials in wide areas of industry, nanotechnologies, farming and packaging owing to the relevant combination of biomedical, transport, and physical-chemical properties. It is worth to emphasis that only medical area of these biopolymers includes implants and prosthesis, tissue engineering scaffolds, novel drug dosage forms in pharmaceutics, novel materials for dentistry and others. Each potentially applicable biopolymer arranges a wide multidisciplinary network, which usually includes tasks of searching for efficacy ways of biosynthesis reactions; economical problems associated with large-scale production; academic studies of mechanical, physicochemical, biochemical properties of the polymer and material of interest; technology of preparation and using this biopolymer; preclinical and clinical trials of these materials and products; a market analysis and perspectives of application of the developed products and many other problems. Poly((R)-3-hydroxybutyrate) (PHB) is an illustrative example for the one of centers for formation of the above mentioned scientific-technological network and a basis for the development of various biopolymer systems [1-2]. In recent decades an intense development of biomedical application of bacterial PHB in producing of biodegradable polymer implants and controlled drug release systems [3-6] needs for comprehensive understanding of the PHB biodegradation process. Examination of PHB degradation process is also necessary for development of novel friendly environment polymer packaging [7-9]. It is generally accepted that biodegradation of PHB both in living systems and in environment occurs via enzymatic and non-enzymatic processes that take place simultaneously under natural conditions. It is, therefore, important to understand both processes [6, 10]. Opposite to other biodegradable polymers (e.g. PGA and PLGA), PHB is considered to be moderately resistant to degradation in vitro as well as to biodegradation in biological media. The rates of degradation are influenced by the characteristics of the polymer, such as chemical composition, crystallinity, morphology and molecular weight [11, 12]. In spite of that PHB application in vitro and in vivo has been intensively investigated, the most of the available data are often incomplete and sometimes even contradictory. The presence of conflicting data can be partially explained by the fact that biotechnologically produced PHB with standardized

properties is relatively rare and is not readily available due to a wide variety of its biosynthesis sources and different manufacturing processes. Above inconsistencies can be explained also by excess applied trend in PHB degradation research. At most of the papers observed in this review, PHB degradation process has been investigated in the narrow framework of development of specific medical devices. Depending on applied biomedical purposes biodegradation of PHB was investigated under different geometry: films and plates with various thickness [13-16], cylinders [17-19], monofilament threads [20-22] and micro- and nanospheres [23, 24]. At these experiments PHB was used from various sources, with different molecular weight and crystallinity. Besides, different technologies of PHB devices manufacture affect such important characteristics as polymer porosity and surface structure [14, 15]. Reports regarding the complex theoretical research of mechanisms of hydrolysis, enzymatic degradation and biodegradation in vivo of PHB processes are relatively rare [13-15, 16, 25-27] that attaches great value and importance to these investigations. Nevertheless, the effect of thickness, size and geometry of PHB device, molecular weight and crystallinity of PHB on the mechanism of PHB hydrolysis and biodegradation was not yet well clarified. Hydrolytic and enzymatic degradation of PHB Nonenzymatic hydrolysis of PHB in vitro Examination of hydrolytic degradation of natural poly((R)-3-hydroxybutyrate) in vitro is a very important step for understanding of PHB biodegradation. There are several very profound and careful examinations of PHB hydrolysis that was carried out for 10-15 years [25-28]. Hydrolytic degradation of PHB was usually examined under standard experimental conditions simulating internal body fluid: in buffered solutions with pH=7.4 at 37°C but at seldom cases the higher temperature (55°C, 70°C and more) and other values of pH (from 2 to 11) were selected. The classical experiment for examination of PHB hydrolysis in comparison with hydrolysis of other widespread biopolymer, polylactic acid (PLA), was carried out by Koyama N. and Doi Y. [25]. They compared films (10 x 10 mm size, 50 μm thickness, 5 mg initial mass) from PHB (Mn = 300000, Mw = 650000) with polylactic acid (Mn = 9000, Mw = 21000) prepared by solvent casting and aged for 3 weeks to reach equilibrium crystallinity. They shown that hydrolytic degradation of natural PHB is the slow process. The mass of PHB film remained unchanged at 37°C in 10 mM phosphate buffer (pH=7.4) over a period of 150 days, while the mass of the PLA film rapidly decreased with time and reached 17% of the initial mass after 140 days. The rate of decrease in the Mn of the PHB was also much slower than the rate of decrease in the Mn of PLA. The Mn of the PHB decreased approximately to 65% of the initial value after 150 days, while the Mn of the PLA decreased to 20% (Mn = 2000) at the same time. As PLA used at this research was with low molecular weight it is worth to compare these data with the data of hydrolysis investigation with the same initial Mn as for PHB. In other work the mass loss of two polymer films (PLA and PHB) with the same thickness (40 μm) and molecular weight (Mw = 450000) was studied in vitro. It was shown that the mass of PLA film decreased to 87%, whereas the mass of PHB film

remained unchanged at 37°C in 25 mM phosphate buffer (pH=7.4) over a period of 84 days, but after 360 days the mass of PHB film was 64,9% of initial one [29-31]. The cleavage of polyester chains is known to be catalysed by the carboxyl end groups, and its rate is proportional to the concentrations of water and ester bonds that on the initial stage of hydrolysis are constant owing to the presence of a large excess of water molecules and ester bonds of polymer chains. Thus, the kinetics of nonenzymatic hydrolysis can be expressed by a simplified equation [32-33]: ln Mn = ln Mn0 - kht (1) where Mn and Mn0 are the number-average molecular weights of a polymer component at time t and zero, respectively and kh is effective hydrolysis constant. The average number of bond cleavage per original polymer molecule, N, is given by equation 2: N = (Mn0/Mn) - 1 = kd MmPn0 t, (2) where kd is the effective rate constant of hydrolytic depolymerization, and Pn0 is the initial number-average degree of polymerization at time zero, Mm is constant molecular mass of monomer. Thus, if the chain scission is completely random, the value of N is linearly dependent on time. The molecular weight decrease with time is the distinguishing feature of mechanism under nonenzymatic hydrolysis condition in contrast to enzymatic hydrolysis condition of PHB when Mn values remained almost unchanged. It was supposed also that water-soluble oligomers of PHB with molecular mass about 3 kDa may accelerate the hydrolysis rate of PHB homopolymer [25]. In contrast, Freier T. et al. [14] showed that PHB hydrolysis was not accelerated by the addition of pre-degraded PHB: the rate of mass and Mw loss of blends (70/30) from high-molecular PHB (Mw = 641000) and low-molecular PHB (Mw = 3000) was the same with degradation rate of pure high-molecular PHB. Meanwhile, the addition of amorphous atactic PHB (atPHB) (Mw = 10000) to blend with high-molecular PHB caused significant acceleration of PHB hydrolysis: the relative mass loss of PHB/atPHB blends was 7% in comparison with 0% mass loss of pure PHB; the decrease of Mw was 88% in comparison with 48% Mw decrease of pure PHB [14, 34]. We have showed that the rate of hydrolysis of PHB films depends on Mw of PHB. The PHB films of high molecular weight (Mw = 450000 and 1000000) degraded slowly as it was described above whereas films from PHB of low molecular weight (Mw = 150000 and 300000 kDa) lost weight relatively gradually and more rapidly [29-31]. To enhance the hydrolysis of PHB a higher temperature was selected for degradation experiments: 55°C, 70°C and more [25]. It was showed by the same research team that the weights of films (12 mm diameter, 65 μm thick) from PHB (Mn = 768 and 22 kDa, Mw = 1460 and 75 kDa) were unchanged at 55°C in 10 mM phosphate buffer (pH=7.4) over a period of 58 days. The Mn value decreased from 768 to 245 kDa for 48 days. The film thickness increased from 65 to 75 μm for 48 days, suggesting that water permeated the polymer matrix during the hydrolytic degradation. Examination of the surface and cross-section of PHB films before and after hydrolysis showed that surface after 48 days of hydrolysis was apparently unchanged, while the cross-section of the film exhibited a more porous structure (pore size 0.5 μm). It was shown also that the rate of hydrolytic degradation is not dependent upon the crystallinity of PHB film.

The observed data indicates that the nonenzymatic hydrolysis of PHB in the aqueous media proceeds via a random bulk hydrolysis of ester bonds in the polymer chain films and occurs throughout the whole film, since water permeates the polymer matrix [25-26]. Moreover, as over the whole degradation time the first-order kinetics was observed and the molecular weight distribution was unimodal, a random chain scission mechanism is very probable both on the crystalline surfaces and in the amorphous regions of the biopolymer [14, 35, 36]. For synthetic amorphous atPHB it was shown that its hydrolysis is the two-step process. First, the random chain scission proceeds that accompanying with a molecular weight decrease. Then, at a molecular weight of about 10000, mass loss begins [28]. The analysis of literature data shows a great spread in values of rate of PHB hydrolytic degradation in vitro. It can be explained by different thickness of PHB films or geometry of PHB devices used for experiment as well as by different sources, purity degree and molecular weight of PHB (Table 1). At 37°C and pH=7.4 the weight loss of PHB (unknown Mw) films (500 μm thick) was 3% after 40 days incubation [36-38], 0% after 52 weeks (364 days) and after 2 years (730 days) incubation (640 kDa PHB, 100 μm films) [14-15], 0% after 150 days incubation (650 kDa PHB, 50 μm film) [25], 7.5% after 50 days incubation (279 kDa PHB, unknown thickness of films) [37], 0% after 3 months (84 days) incubation (450 kDa PHB, 40 μm films), 12% after 3 months (84 days) incubation (150 kDa PHB, 40 μm films) [29-30], 0% after 180 days incubation of monofilament threads (30 μm in diameter) from PHB (470 kDa) [22-23]. The molecular weight of PHB dropped to 36% of the initial values after 2 years (730 days) of storage in buffer solution [15], to 87% of the initial values after 98 days [38], to 58% of the initial values after 84 days [29-30] (Table 1). Table 1 - Nonenzymatic hydrolysis of PHB in vitro Type of implant/ device Initial Mw, kDa Size/ Thickness, μm Condi-tions Relative mass loss, % Relative loss of Mw, % Time, Days Links Film 650 50 37°C, pH=7.4 0 35 150 25 Film 640 100 37°C, pH=7.4 0 64 730 15 Film 640 100 37°C, pH=7.4 0 45 364 14 Film 450 40 37°C, pH=7.4 0 42 84 29-30 Film 150 40 37°C, pH=7.4 12 63 84 29-30 Film 450 40 37°C, pH=7.4 35,1 - 360 31 Film 279 - 37°C, pH=7.4 7.5 - 50 36 Plate - 500 37°C, pH=7.4 3 - 40 30 Plate 380 1000 37°C, pH=7.4 0 - 28 43 Plate 380 2000 37°C, pH=7.4 0 8 98 43 Thread 470 30 37°C, pH=7.0 0 - 180 23 Thread - - 37°C, pH=7.2 0 - 182 22 Micro-spheres 50 250-850 37°C, pH=7.4 0 0 150 42 Thread 470 30 37°C, pH=5.2 0 - 180 22 Film 279 - 37°C, pH=10 100 - 28 36 Film 279 - 37°C, pH=13 100 - 19 36 Film 650 50 55°C, pH=7.4 0 68 150 25 Plate 380 2000 55°C, pH=7.4 0 61 98 43 Film 640 100 70°C, pH=7.4 - 55 28 14 Film 150 40 70°C, pH=7.4 39 96 84 29-30 Film 450 40 70°C, pH=7.4 12 92 84 29-30 Micro-spheres 50 250-850 85°C, pH=7.4 50 68 150 42 Micro-spheres 600 250-850 85°C, pH=7.4 25 - 150 42 In acidic or alkaline aqueous media PHB degrades more rapidly: 0% degradation after 140 days incubation in 0.01M NaOH (pH=11) (200 kDa, 100 μm film thickness) with visible surface changing [39], 0% degradation after 180 days incubation of PHB threads in phosphate buffer (pH=5.2 and 5.9) [23], complete PHB films biodegradation after 19 days (pH=13) and 28 days (pH=10) [37]. It was

demonstrated that after 20 weeks of exposure to NaOH solution, the surfaces of PHB samples became rougher due to, along with an increased density in their surface layers. From these results, one may surmise that the non-enzymatic degradation of PHAs progresses on their surfaces before noticeable weight loss occurring as illustrated in [39] and by the authors in Fig.1. It was shown also that treatment of PHB film with 1M NaOH caused a reduce in pore size on film surface from 1-5 μm to around 1 μm that indicates a partially surface degradation of PHB in alkaline media [40-41]. At higher temperature no weight loss of PHB films and threads was observed after 98 and 182 days incubation in phosphate buffer (pH=7.2) at 55°C and 70°C, respectively [22], 12% and 39% of PHB (450 and 150 kDa, respectively) films after 84 days incubation at 70°C [35,40], 50% and 25% after 150 days incubation of microspheres (250-850 μm diameter) from PHB (50 kDa and 600 kDa, respectively) [42]. (a) (b) (c) (d) Fig. 1 - AFM topographic images of PHB films (170 kDa) with a scan size of 18x18 μm: the rough surface of fresh-prepared sample (exposed to air) - (a); the smooth surface of fresh-prepared sample (exposed to glass) - (b); the sample exposed to phosphate buffer at 310K for 83 days - (c); the sample exposed to phosphate buffer at 343K for 83 days - (d) General magnificence is 300 [17] During degradation of PHB monofilament threads, films and plates the change of mechanical properties was observed under different conditions in vitro [22, 43]. It was shown that a number of mechanical indices of threads became worse: load at break lost 36%, strain at break lost 33%, Young's modulus didn't change, tensile strength lost 42% after 182 days incubation in phosphate buffer (pH=7.2) at 70°C. But at 37°C the changes were more complicated: at first load at break increased from 440 g to 510 g (16%) at 90th day and then decreased to the initial value on 182nd day, strain at break increased rapidly from 60 to 70% (in 17%) at 20th day and then gradually increased to 75% (in 25%) at 182nd day, Young's modulus didn't change [22]. For PHB films it was demonstrated a gradual 32% decrease in Young's modulus and 77% fall in tensile strength during 120 days incubation in phosphate buffer (pH=7.4) at 37°C [43]. For PHB plates more complicated changes were observed: at first tensile strength dropped in 13% for 1st day and then increased to the initial value at 28th day, Young's modulus dropped in 32% for 1st day and then remain unchanged up to 28th day, stiffness decreased sharply also in 40% for 1st day and then remain unchanged up to 28th day [44]. Enzymatic degradation of PHB in vitro The examination of enzymatic degradation of PHB in vitro is the following important step for understanding of PHB performance in animal tissues and in environment. The most papers observed degradation of PHB by depolymerases of its own bacterial producers. The degradation of PHB in vitro by depolymerase was thoroughly examined and mechanism of enzymatic PHB degradation was perfectly clarified by Doi Y. [25-26]. At these early works it was shown that 68-85% and 58% mass loss of PHB (Mw = 650-768 and 22 kDa, respectively) films (50-65 μm thick) occurred for 20 h under incubation at 37˚C in phosphate solution (pH=7.4) with depolymerase (1.5-3 μg/ml) isolated from A. faecalis. The rate (ke) of

enzymatic degradation of films from PHB (Mn = 768 and 22 kDa) was 0.17 and 0.15 mg/h, respectively. The thickness of polymer films dropped from 65 to 22 μm (32% of initial thickness) during incubation. The scanning electron microscopy examination showed that the surface of the PHB film after enzymatic degradation was apparently blemished by the action of PHB depolymerase, while no change was observed inside the film. Moreover, the molecular weight of PHB remained almost unchanged after enzymatic hydrolysis: the Mn of PHB decreased from 768 to 669 kDa or unchanged (22 kDa) [25-26]. The extensive literature data on enzymatic degradation of PHB by specific PHB depolymerases was collected in detail in review of Sudesh K., Abe H. and Doi Y. [45]. We would like to summarize some the most important data. But at first it is necessary to note that PHB depolymerase is very specific enzyme and the hydrolysis of polymer by depolymerase is the unique process. But in animal tissues and even in environment the enzymatic degradation of PHB is occurred mainly by nonspecific esterases [24, 46]. Thus, in the frameworks of this review, it is necessary to observe the fundamental mechanisms of PHB enzymatic degradation. The rate of enzymatic erosion of PHB by depolymerase is strongly dependent on the concentration of the enzyme. The enzymatic degradation of solid PHB polymer is heterogeneous reaction involving two steps, namely, adsorption and hydrolysis. The first step is adsorption of the enzyme onto the surface of the PHB material by the binding domain of the PHB depolymerase, and the second step is hydrolysis of polyester chains by the active site of the enzyme. The rate of enzymatic erosion for chemosynthetic PHB samples containing both monomeric units of (R)- and (S)-3-hydrohybutyrate is strongly dependent on both the stereocomposition and on the tacticity of the sample as well as on substrate specificity of PHB depolymerase. The water-soluble products of random hydrolysis of PHB by enzyme showed a mixture of monomers and oligomers of (R)-3-hydrohybutirate. The rate of enzymatic hydrolysis for melt-crystallized PHB films by PHB depolymerase decreased with an increase in the crystallinity of the PHB film, while the rate of enzymatic degradation for PHB chains in an amorphous state was approximately 20 times higher than the rate for PHB chains in a crystalline state. It was suggested that the PHB depolymerase predominantly hydrolyzes polymer chains in the amorphous phase and then, subsequently, erodes the crystalline phase. The surface of the PHB film after enzymatic degradation was apparently blemished by the action of PHB depolymerase, while no change was observed inside the film. Thus, depolymerase hydrolyses of the polyester chains in the surface layer of the film and polymer erosion proceeds in surface layers, while dissolution, the enzymatic degradation of PHB are affected by many factors as monomer composition, molecular weight and degree of crystallinity [45]. The PHB polymer matrix ultrastructure [47] plays also very important role in enzymatic polymer degradation [48]. At the next step it is necessary to observe enzymatic degradation of PHB under the conditions that modeled the animal tissues and body fluids containing nonspecific esterases. In vitro degradation of PHB films in the presence of various lipases as nonspecific esterases

was carried out in buffered solutions containing lipases [18, 49-50], in digestive juices (for example, pancreatin) [14], simulated body fluid [51] biological media (serum, blood etc.) [23] and crude tissue extracts containing a mixture of enzymes [24] to examine the mechanism of nonspecific enzymatic degradation process. It was noted that a Ser..His..Asp triad constitutes the active center of the catalytic domain of both PHB depolymerase [52] and lipases [53]. The serine is part of the pentapeptide Gly X1-Ser-X2-Gly, which has been located in all known PHB depolymerases as well as in lipases, esterases and serine proteases [52]. On the one hand, it was shown that PHB was not degraded for 100 days with a quantity of lipases isolated from different bacteria and fungi [49-50]. On the other hand, the progressive PHB degradation by lipases was shown [18, 40-41]. PHB enzymatic biodegradation was studied also in biological media: it was shown that with pancreatin addition no additional mass loss of PHB was observed in comparison with simple hydrolysis [14], the PHB degradation process in serum and blood was demonstrated to be similar to hydrolysis process in buffered solution [31], whereas progressive mass loss of PHB sutures was observed in serum and blood: 16% and 25%, respectively, after 180 days incubation [23], crude extracts from liver, muscle, kidney, heart and brain showed the activity to degrade the PHB: from 2% to 18% mass loss of PHB microspheres after 17 h incubation at pH 7.5 and 9.5 [24]. The weight loss of PHB (Mw = 285000) films after 45 days incubation simulated body fluid was about 5% [51]. The degradation rate in solution with pancreatin addition, obtained from the decrease in Mw of pure PHB, was accelerated about threefold: 34% decrease in Mw after incubation for 84 days in pancreatin (10 mg/ml in Sorensen buffer) vs. 11% decrease in Mw after incubation in phosphate buffer [14]. The same data was obtained for PHB biodegradation in buffered solutions with porcine lipase addition: 72% decrease in Mw of PHB (Mw = 450000) after incubation for 84 days with lipase (20 U/mg, 10 mg/ml in Tris-buffer) vs. 39% decrease in Mw after incubation in phosphate buffer [18]. This observation is in contrast to enzymatic degradation by PHB depolymerases which was reported to proceed on the surface of the polymer film with an almost unchanged molecular weight [24-25]. It has been proposed that for depolymerases the relative size of the enzyme compared with the void space in solvent cast films is the limiting factor for diffusion into the polymer matrix [54] whereas lipases can penetrate into the polymer matrix through pores in PHB film [40-41]. It was shown that lipase (0.1 g/l in buffer) treatment for 24 h caused significant morphological change in PHB film surface: transferring from native PHB film with many pores ranging from 1 to 5 μm in size into a pore free surface without producing a quantity of hydroxyl groups on the film surface. It was supposed that the pores had a fairly large surface exposed to lipase, thus it was degraded more easily [40-41]. It indicates also that lipase can partially penetrate into pores of PHB film but the enzymatic degradation proceeds mainly on the surface of the coarse polymer film which is achievable for lipase. Two additional effects reported for depolymerases could be of importance. It was concluded that segmental mobility in amorphous phase and

polymer hydrophobicity play an important role in enzymatic PHB degradation by nonspecific esterases [14]. Significant impairment of the tensile strength and other mechanical properties were observed during enzymatic biodegradation of PHB threads in serum and blood. It was shown that load at break lost 29%, Young's modulus lost 20%, and tensile strength didn't change after 180 days of threads incubation, the mechanical properties changed gradually [23]. Biodegradation of PHB by soil microorganisms Polymers exposed to the environment are degraded through their hydrolysis, mechanical, thermal, oxidative, and photochemical destruction, and biodegradation [7, 38, 55, 56]. 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 by microorganisms finally to CO2 and H2O. This property of PHB allows to manufacture biodegradable polymer objects for various applications [4]. 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 [4, 38, 55, 56]. Maergaert et al. isolated from soil more than 300 microbial strains capable of degrading PHB in vitro [55]. The bacteria detected on the degraded PHB films were dominated by the genera Pseudomonas, Bacillus, Azospirillum, Mycobacterium, and Streptomyces etc. 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 [57]. It was studied biodegradability of PHB films under aerobic, microaerobic and anaerobic condition in the presence and absence of nitrate by microbial populations of soil, sludge from anaerobic and nitrifying/denitrifying reactors, and sediment of a sludge deposit site, as well as to obtain active denitrifying enrichment culture degrading PHB (Fig. 2) [58]. Changes in molecular mass, crystallinity, and mechanical properties of PHB have been studied. A correlation between the PHB degradation degree and the molecular weight of degraded PHB was demonstrated. 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 [59]. 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 [58]. Fig. 2 - 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) [58] 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. [26] 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. As it was shown at single PHB crystals [47] 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 [60]. 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 [58, 61]. Biodegradation of PHB in vivo in animal tissues The first scientific works on biodegradation of PHB in vivo in animal tissues were carried out 15-20 years ago by Miller N.D. et al. and Saito T. et al [22, 24]. They are high-qualitative researches that disclosed many important characteristics of this process. As it was noted above the both enzymatic and non-enzymatic processes of biodegradation of PHB in vivo can occur simultaneously under normal conditions. But it doesn't mean that polymer biodegradation in vivo is a simple combination of non-enzymatic hydrolysis and enzymatic degradation. Moreover, in vivo the biodegradation (decrease of molecular weight and mass loss) of PHB) is a controversial subject in the literature. As it was noted above for in vitro PHB hydrolysis, the main reason for the controversy, is the use of samples made by various processing technologies and the incomparability of different implantation and animal models. The most of researches on PHB biodegradation was carried out with use of prototypes of various medical devices on the base of PHB: solid films and plates [13, 16, 18, 31, 62], porous patches [14, 15], porous scaffolds [63], electrospun microfiber mats [64], nonwoven patches consisted of fibers [65-69], screws [31], cylinders as nerve guidance channels and conduits [16, 20-21], monofilament sutures [22-23], cardiovascular stents [70], microspheres [24,71]. In vivo biodegradation was studied on various laboratory animals: rats [14, 18, 20-24, 64], mice [16, 75], rabbits [13, 62, 70, 72], minipigs [15], cats [20], calves [65], sheep [66-68], and even at clinical trials on patients [69]. It is obviously that these animals differ in level of metabolism very much: for example, only weight of these

animals differs from 10-20 g (mice) to 50 kg (calves). The implantation of devices from PHB was carried out through different ways: subcutaneously [13, 16, 18, 22, 23, 72], intraperitoneally on a bowel [14], subperiostally on the osseus skull [15-62], nerve wrap-around [19-21], intramuscularly [71-72], into the pericardium [66-69], into the atrium [65] and intravenously [24]. The terms of implantation were also different: 2.5 h, 24 h, 13 days, 2 months [24]; 7, 14, 30 days [21], 2, 7, 14, 21, 28, 55, 90, 182 days [22]; 1, 3, 6 months [13, 16, 19]; 3, 6, 12 months [65]; 6, 12 months [66]; 6, 24 months [69]; 3, 6, 9, 12, 18, 24 months [68]. The most entire study of PHB in vivo biodegradation was fulfilled by Gogolewski S. et al. and Qu X.-H. et al. [13,16]. It was shown that PHB lost about 1.6% (injection-molded film, 1.2 mm thick, Mw of PHB = 130 kDa) [16] and 6% (solvent-casting film, 40 μm thick, Mw = 534 kDa) [13] of initial weight after 6 months of implantation. But the observed small weight loss was partially due to the leaching out of low molecular weight fractions and impurities present initially in the implants. The Mw of PHB decreased from 130000 to 74000 (57% of initial Mw) [16] and from 534000 to 216000 (40% of initial Mw) [13] after 6 months of implantation. The polydispersity of PHB polymers narrowed during implantation. PHB showed a constant increase in crystallinity (from 60.7 to 64.8%) up to 6 months [16] or an increase (from 65.0 to 67.9%) after 1 month and a fall again (to 64.5%) after 6 month of implantation [13] which suggests the degradation process had not affected the crystalline regions. This data is in accordance with data of PHB hydrolysis [25] and enzymatic PHB degradation by lipases in vitro [14] where Mw decrease was observed. The initial biodegradation of amorphous regions of PHB in vivo is similar to PHB degradation by depolymerase [45]. Thus, the observed biodegradation of PHB showed coexistence of two different degradation mechanisms in hydrolysis in the polymer: enzymatically or non-enzymatically catalyzed degradation. Although non-enzymatical catalysis occurred randomly in homopolymer, indicated by Mw loss rate in PHB, at some point in a time, a critical molecular weight is reached whereupon enzyme-catalyzed hydrolysis accelerated degradation at the surface because easier enzyme/polymer interaction becomes possible. However considering the low weight loss of PHB, the critical molecular weight appropriate for enzymes predominantly does not reach, yet resulting low molecular weight and crystallinity in PHB could provide some sites for the hydrolysis of enzymes to accelerate the degradation of PHB [13, 16]. Additional data revealing the mechanism of PHB biodegradation in animal tissues was obtained by Kramp B. et al. in long-term implantation experiments. A very slow, clinically not recordable degradation of films and plates was observed during 20 month (much more than in experiments mentioned above). A drop in the PHB weight loss evidently took place between the 20th and 25th month. Only initial signs of degradation were to be found on the surface of the implant until 20 months after implantation but no more test body could be detected after 25 months [62]. The complete biodegradation in vivo in the wide range from 3 to 30 months of PHB was shown by other researches [65, 67-69, 73], whereas almost no weight loss and surface

changes of PHB during 6 months of biodegradation in vivo was shown [16, 22]. Residual fragments of PHB implants were found after 30 months of the patches implantation [66, 68]. A reduction of PHB patch size in 27% was shown in patients after 24 months after surgical procedure on pericardial closure with the patch [69]. Significantly more rapid biodegradation in vivo was shown by other researches [13, 20, 23, 43, 65]. It was shown that 30% mass loss of PHB sutures occurred gradually during 180 days of in vivo biodegradation with minor changes in the microstructure on the surface and in volume of sutures [23]. It was shown that PHB nonwoven patches (made to close atrial septal defect in calves) was slowly degraded by polynucleated macrophages, and 12 months postoperatively no PHB device was identifiable but only small particles of polymer were still seen. The absorption time of PHB patches was long enough to permit regeneration of a normal tissue [65]. The PHB sheets progressive biodegradation was demonstrated qualitatively at 2, 6 and 12 months after implantation as weakening of the implant surface, tearing/cracking of the implant, fragmentation and a decrease in the volume of polymer material [21, 43, 72]. The complete biodegradation of PHB (Mw = 150-1000 kDa) thin films (10-50 μm) for 3-6 months was shown and degradation process was described. 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 (Fig. 3) [18, 31]. Interesting data were obtained for biodegradation in vivo of PHB microspheres (0.5-0.8 μm in diameter). It was demonstrated indirectly that PHB loss about 8% of weight of microspheres accumulated in liver after 2 month of intravenous injection. It was demonstrated also a presence of several types PHB degrading enzymes in the animal tissues extracts [24]. Fig. 3 - Biodegradation of PHB films in vivo. Connective-tissue capsule with PHB thin films (outlined with broken line) 2 weeks (98% residual weight of the film) (left photograph) and 3 months (0% residual weight of the film) (left photograph) after subcutaneous implantation [18] Some researches studied a biodegradation of PHB threads with a tendency of analysis of its mechanical properties in vivo [22, 23]. It was shown that at first load at break index decreased rapidly from 440 g to 390 g (12%) at 15th day and then gradually increased to the initial value at 90th and remain almost unchanged up to 182nd day [22] or gradually decreased in 27% during 180 days [23], strain at break decreased rapidly from 60 to 50% (in 17% of initial value) at 10th day and then gradually increased to 70% (in 17% of initial value) at 182nd day [22] or didn’t change significantly during 180 days [23]. It was demonstrated that the primary reason of PHB biodegradation in vivo was a lysosomal and phagocytic activity of polynucleated macrophages and giant cells of foreign body reaction. The activity of tissue macrophages and nonspecific enzymes of body liquids made a main contribution to significantly more rapid rate of PHB biodegradation in

vivo in comparison with rate of PHB hydrolysis in vitro. The PHB material was encapsulated by degrading macrophages. Presence of PHB stimulated uniform macrophage infiltration, which is important for not only the degradation process but also the restoration of functional tissue. The long absorption time produced a foreign-body reaction, which was restricted to macrophages forming a peripolymer layer [23, 65, 68, 72]. Very important data that clarifies the tissue response that contributes to biodegradation of PHB was obtained by Lobler M. It was demonstrated an significant increase of expression of two specific lipases after 7 and 14 days of PHB contact with animal tissues. Moreover, liver specific genes were induced with similar results. It is striking that pancreatic enzymes are induced in the gastric wall after contact with biomaterials [46]. Saito T. et al. suggested the presence of at least two types of degradative enzymes in rat tissues: liver serine esterases with the maximum of activity in alkaline media (pH=9.5) and kidney esterases with the maximum of activity in neutral media [24]. The mechanism of PHB biodegradation by macrophages was demonstrated at cultured macrophages incubated with particles of low-molecular weight PHB [74]. It was shown that macrophages and, to a lesser level, fibroblasts have the ability to take up (phagocytize) PHB particles (1-10 μm). At high concentrations of PHB particles (>10 μg/mL) the phagocytosis is accompanied by toxic effects and alteration of the functional status of the macrophages but not the fibroblasts. This process is accompanied by cell damage and cell death. The elevated production of nitric oxide (NO) and tumor necrosis factor alfa (TNF-α) by activated macrophages was observed. It was suggested that the cell damage and cell death may be due to phagocytosis of large amounts of PHB particles; after phagocytosis, polymer particles may fill up the cells, and cause cell damage and cell death. It was demonstrated also that phagocytized PHB particles disappeared in time due to an active PHB biodegradation process (Fig. 4) [74]. Fig. 4 - Phagocytosis of microparticles of PHB in macrophages. TEM analysis of cultured macrophages in the presence (A) or absence (B) of 2 μg PHB microparticles/mL for 24 h. Bar in B represents 1 μm, for A and B [74]