

1 **Properties of PHA bi-, ter-, and quarter-polymers containing 4-hydroxybutyrate monomer**  
2 **units**

3  
4 Natalia Zhila<sup>a,b,\*</sup>, Ekaterina Shishatskaya<sup>a,b</sup>

5  
6 <sup>a</sup>Siberian Federal University, 79 Svobodnyi Avenue, Krasnoyarsk, 660041, Russian Federation

7 <sup>b</sup>Institute of Biophysics SB RAS, Federal Research Center “Krasnoyarsk Science Center SB  
8 RAS”, Akademgorodok, Krasnoyarsk, 660036, Russian Federation  
9

10  
11  
12  
13 \*Corresponding author. Tel.: +7 391 2494428; fax: +7 391 2433400

14 *E-mail address:* nzhila@mail.ru (Natalia Zhila)  
15  
16  
17  
18

19 **Abstract**

20 The present study investigates physicochemical, mechanical, and biological properties of  
21 polyhydroxyalkanoate (PHA) copolymers containing 4-hydroxybutyrate (4HB) synthesized in  
22 *Cupriavidus eutrophus* B10646 culture. In poly(3-hydroxybutyrate/4-hydroxybutyrate)  
23 [P(3HB/4HB)] bipolymers, 4HB varied between 10.4 and 75.0 mol.%; in poly(3-  
24 hydroxybutyrate/3-hydroxyvalerate/4-hydroxybutyrate) terpolymers, 4HB constituted 28.7-55.6  
25 mol.%; and in poly(3-hydroxybutyrate/3-hydroxyvalerate/4-hydroxybutyrate/3-  
26 hydroxyhexanoate) quaterpolymers, 4HB varied between 9.3 and 13.3 mol.%. The degree of  
27 crystallinity of P(3HB/4HB) copolymers decreased consistently with an increase in 4HB content,  
28 reaching 38%. The incorporation of 3-hydroxyvalerate and 3-hydroxyhexanoate into copolymers  
29 enhanced that effect. The effect of 4HB monomer units on temperature properties of copolymers  
30 was exhibited as lowering of the melting temperature and crystallization temperature, which  
31 improved the processing-related properties of the copolymers. All copolymers containing 4HB  
32 showed enhanced elongation at break compared to poly(3-hydroxybutyrate). Polymer films  
33 prepared from PHAs with different chemical composition had similar microstructure and  
34 porosity and had no toxic effect on mouse fibroblast NIH 3T3 cells, proving their high  
35 biocompatibility.  
36

37 *Keywords:* polyhydroxyalkanoates, 4-hydroxybutyrate monomer units, physicochemical  
38 and mechanical properties  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51

## 1           **1. Introduction**

2           Development of new materials is among the priorities for critical technologies at the  
3 global level. Special emphasis is placed on developing and investigating materials for biomedical  
4 applications, which are intended to come into contact with the internal environment of living  
5 organisms and which are necessary to enhance the efficacy of reconstructive processes in  
6 damaged tissues and organs [1]. The diversity of polymers that have widely varying properties,  
7 the possibility of constructing hybrids with different substances, and the availability of numerous  
8 techniques for processing them provide the basis for producing an extensive range of novel  
9 materials with new valuable properties. Polymers of hydroxy-derived carbonic acids of microbial  
10 origin, polyhydroxyalkanoates (PHAs), are a useful product of biotechnology. PHAs are  
11 biodegradable, biocompatible, and thermoplastic polymers with various chemical compositions,  
12 which do not undergo abiotic hydrolysis in aqueous medium and whose properties (molecular  
13 weight, crystallinity, mechanical strength, and biodegradability) vary considerably depending on  
14 their monomer composition and proportions of monomer units [2-4].

15           Of the various PHAs, poly(3-hydroxybutyrate/4-hydroxybutyrate) copolymers  
16 [P(3HB/4HB)] deserve special attention. They are elastomeric materials with enhanced  
17 elongation and tensile strength. The first products approved by the FDA for clinical uses were  
18 P(3HB/4HB)-based devices designed by Tepha (U.S.) ([http://www.tepha.com/pipeline-](http://www.tepha.com/pipeline-drugdelivery.htm)  
19 [drugdelivery.htm](http://www.tepha.com/pipeline-drugdelivery.htm)). P(3HB/4HB) copolymers can be synthesized by both wild-type and  
20 genetically modified strains under specialized cultivation conditions, in the culture medium  
21 supplemented with such precursors of 4-hydroxybutyrate (4HB) monomers as  $\gamma$ -hydroxybutyric  
22 acid,  $\gamma$ -butyrolactone, 1,4-butanediol, etc. These substances, however, inhibit the growth of  
23 microorganisms, decreasing cell biomass production, total copolymer yields, and the 4HB  
24 fraction of the copolymer. Therefore, there are only relatively limited data available regarding  
25 the properties and potential for wide-scale application of these copolymers. Published studies  
26 mainly report results of synthesis and investigation of P(3HB/4HB) copolymers comprising two

1 components, with different 4HB fractions [5-9]. A number of papers addressed the effect of 4HB  
2 monomer concentration on the temperature and molecular-weight characteristics of P(3HB/4HB)  
3 copolymers and their mechanical properties [3, 10-13]. There are few published data on PHA  
4 terpolymers containing 4HB. Several studies described poly(3-hydroxybutyrate/3-  
5 hydroxyvalerate/4-hydroxybutyrate) [(P(3HB/3HV/4HB))] copolymers with different monomer  
6 fractions and their temperature and molecular-weight properties [14-17]. Until recently, no data  
7 on the properties of PHA quaterpolymers containing 4HB monomer units have been found in the  
8 available literature.

9 The authors of this study have assembled a collection of bacterial strains capable of  
10 synthesizing high yields of PHAs on various substrates and studied PHA synthesis under  
11 different conditions of carbon nutrition. Based on this, they managed to synthesize not only poly-  
12 3-hydroxybutyrate but also copolymers of various compositions: 3HB/diethylene glycol  
13 [P(3HB/DEG)] copolymer [18], bipolymers of 3HB with 3-hydroxyvalerate (3HV) and 3-  
14 hydroxy-4-methylvalerate [19], and a series of PHA ter- and quarterpolymers composed of  
15 various short- and medium-chain-length monomers in different proportions, including ones  
16 containing various concentrations of 4-hydroxybutyrate [20-23].

17 The purpose of this study was to investigate and compare physical, mechanical, and  
18 biological properties of PHA bi-, ter-, and quarter-polymers containing various concentrations of  
19 4HB.

## 20 **2. Experimental**

### 21 *2.1. Materials*

22 Samples of PHAs of different composition were synthesized by using *Cupriavidus*  
23 *eutrophus* B10646 wild strain, at the Institute of Biophysics SB RAS. Cells were batch-cultured  
24 under strictly aseptic conditions, following the previously developed technology [20-21]. PHAs  
25 of different compositions were synthesized using precursor substrates such as valeric acid,  
26 hexanoic acid and  $\gamma$ -butyrolactone. Polymer was extracted from cells with chloroform, and the

1 extracts were precipitated using hexane. The extracted polymers were re-dissolved and  
2 precipitated again 3-4 times to prepare homogeneous specimens.

### 3 *2.2. Analysis of PHA structure*

4  $^1\text{H}$  NMR spectra of PHA were recorded at room temperature in  $\text{CDCl}_3$  on a BRUKER  
5 AVANCE III 600 spectrometer (Germany) operating at 600.13 MHz.

### 6 *2.3. Analysis of physicochemical properties of PHAs*

7 Molecular weight and molecular-weight distribution of PHAs were examined with a gel  
8 permeation chromatograph (“Agilent Technologies” 1260 Infinity, U.S.) with a refractive index  
9 detector, using an Agilent PLgel Mixed-C column.

10 Thermal analysis of PHA specimens was performed using a DSC-1 differential scanning  
11 calorimeter (METTLER TOLEDO, Switzerland). The specimens were heated at a rate of 5  
12  $^{\circ}\text{C}/\text{min}$  to 200  $^{\circ}\text{C}$ , then cooled to -20  $^{\circ}\text{C}$ , held for 20 minutes and re-heated to 320  $^{\circ}\text{C}$ . Glass  
13 transition temperature ( $T_g$ ), crystallization temperature ( $T_c$ ), melting point ( $T_{melt}$ ) and thermal  
14 degradation temperature ( $T_{degr}$ ) were determined from peaks in thermograms using the “StarE”  
15 software.

16 X-Ray structure analysis and determination of crystallinity of copolymers were  
17 performed employing a D8 ADVANCE X-Ray powder diffractometer equipped with a  
18 VANTEC fast linear detector (Bruker, AXS, Germany). Calculations were done by using the  
19 Eva program of the diffractometer software.

### 20 *2.4. Analysis of PHA surface microstructure*

21 Films of PHAs were prepared by casting chloroform solution (2% w/v) on degreased  
22 glass and subsequent drying at room temperature for 2-3 days in a dust-free box. The film discs  
23 were 100 mm in diameter and 0.04 mm thick.

24 The microstructure of the surface of PHA films was analyzed using scanning electron  
25 microscopy (S 5500, Hitachi, Japan).

26 The roughness of film surface was determined using atomic-force microscopy (AFM) in

1 semicontact mode (Smart SPM™, AIST-NT, Zelenograd, Russia).

2 Surface properties of the polymer films were examined using a DSA-25E drop shape  
3 analyzer (Krüss, Germany) and software DSA-4 for Windows.

#### 4 *2.5. Analysis of physical/mechanical properties of PHAs*

5 Physical and mechanical properties of the films prepared from PHAs with different  
6 compositions were investigated using an Instron 5565 electromechanical tensile testing machine  
7 (U.K.). Dumbbell-shaped samples 50 mm long, 6.1 mm wide, and 25–30 µm thick were prepared  
8 for studying physical and mechanical properties of the films. The speed of the crosshead was 3  
9 mm/min at ambient temperature. Young's modulus (E, MPa), tensile strength (σ, MPa) and  
10 elongation at break (ε, %) were automatically calculated by the Instron software (Bluehill 2,  
11 Elancourt, France).

#### 12 *2.6. Cell attachment assay*

13 Films were cut into disks 10 mm in diameter, using a mold cutter, and sterilized with  
14 H<sub>2</sub>O<sub>2</sub> plasma in the Sterrad NX system (Johnson & Johnson, USA) for 45 min. The ability of  
15 ultrafine PHA films to facilitate cell attachment was studied using NIH 3T3 mouse fibroblast  
16 cells. Morphology of cells attached to film surface was determined using DAPI and FITC  
17 fluorescent dyes (DNA and cytoplasm markers).

18 Characterization was done using procedures similar to previously described ones [20-21].

### 19 **3. Results and Discussion**

20 Strain *Cupriavidus eutrophus* B10646 cultivated under specialized conditions of carbon  
21 nutrition synthesized polymers with different proportions of 4HB monomer units: P(3HB/4HB)  
22 copolymers with 4HB 10.4-75.0 mol.% (3HB constituting the other fraction); P(3HB/3HV/4HB)  
23 terpolymers with 4HB 28.7-55.6 mol.% (with 3HV 10.1 -20.5 and 3HB 27.4-51.5 mol.%), and  
24 poly(3-hydroxybutyrate/3-hydroxyvalerate/4-hydroxybutyrate/3-hydroxyhexanoate)  
25 [(P(3HB/3HV/4HB/3HHx))] quaterpolymers with 4HB 9.3 - 13.3 mol.% (with 3HV 6.4 -20.9,  
26 3HHx 1.4-2.5, and 3HB 63.9-82.9 mol.%) (Table 1).

1 The chemical composition of monomers in the PHAs was identified using NMR  
2 spectrometry. Figure 1 shows examples of NMR spectra characterizing the chemical  
3 composition of PHA bi-, ter-, and quaterpolymers.

### 4 *3.1. Physicochemical properties of PHA copolymers with different compositions*

5 The proportions of monomers influenced physicochemical properties of PHAs, including  
6 their degrees of crystallinity and molecular-weight and temperature characteristics (Table 1).

#### 7 *3.1.1. Degree of crystallinity*

8 Degree of crystallinity ( $C_x$ ), which is determined by the ratio of amorphous to crystalline  
9 regions, is a fundamental property of polymeric materials, influencing their fine structure,  
10 thermomechanical properties, techniques employed to process polymers into products, and  
11 properties of these products. PHAs are semi-crystalline materials, and PHA crystallization is  
12 determined by the structure of the carbon chain. PHA crystallization occurs at different  
13 temperatures ( $T_c$ ), and some portions of the bulk are not crystallized. Thus, these polymers  
14 comprise two phases (crystalline and amorphous ones), and their ratio is largely determined by  
15 the composition and proportions of monomers. However, the available information on the  
16 degrees of crystallinity of PHAs with different concentrations of 4HB is too scarce to  
17 definitively determine the effect of this monomer on  $C_x$ .

18 Incorporation of 4HB monomer units into the carbon chain of 3-hydroxybutyrate (3HB)  
19 considerably changed the ratio of amorphous to crystalline regions in all copolymers, decreasing  
20  $C_x$  to different extents (Table 1). The P(3HB/4HB) bipolymers containing between 10.4 and 75.0  
21 mol.% 4HB showed the degree of crystallinity below 55%, and this value consistently decreased  
22 as the proportion of 4HB increased. The copolymers with 4HB below 50% (10.4, 15.0 and 29.3  
23 mol.%) had the degree of crystallinity about 50% (53, 50 and 46%); in the copolymer consisting  
24 on nearly equal molar fractions of 4HB and 3HB (Sample 4, Table 1), the amorphous region was  
25 larger than the crystalline one ( $C_x$  was 40%). Finally, in the copolymer with 4HB constituting 75  
26 mol.%, the degree of crystallinity dropped to 30%. Mitomo et al. investigated P(3HB/4HB)

1 copolymers with 4HB between 10 and 94 mol.% and reported a decrease in  $C_x$  to 18-50%  
2 relative to P(3HB) (72%), but the authors did not find any clear relationship between this  
3 decrease and the percentage of 4HB [24]. Other studies also presented contradictory data on the  
4 effects of the molar fraction of 4HB in P(3HB/4HB) on  $C_x$ . For instance, in a study by  
5 Ramachandran and Amirul,  $C_x$  reached 41% in the sample with a very low 4HB fraction (3  
6 mol.%) and 2-10% in copolymers with higher proportions of 4HB (15-40 mol.%) [13]. Iqbal and  
7 Amirul investigated a wider range of P(3HB/4HB) copolymers, which contained 4HB between  
8 10 and 65 mol.%; the  $C_x$  of the copolymers with 4HB increasing from 10 to 30 mol.% dropped  
9 dramatically (from 31.6 to 12.9%) while the samples with higher 4HB percentage were  
10 amorphized, and crystalline regions were undetectable [12].

11 A more noticeable decrease in the degree of crystallinity was observed in  
12 P(3HB/3HV/4HB) terpolymers (Samples 6-8, Table 1). Even in the copolymers that did not  
13 contain very high proportions of 4HB (28.7 and 38.4 mol.%) and 3HV (10.1-20.5 mol.%), which  
14 also decreased  $C_x$ , the degree of crystallinity was below 50% (35-33%). Thus, 4HB and 3HV  
15 together had a stronger effect on the ratio of amorphous to crystalline regions in the copolymer  
16 than either of these monomers alone. The  $C_x$  of the terpolymer containing 4HB 55.6 mol.% (with  
17 3HB and 3HV 27.4 and 17.0 mol.%, respectively) was 26%.

18 In the P(3HB/3HV/4HB/3HHx) quaterpolymers, the proportions of 4HB were not very  
19 high (9.3, 10.1, and 13.3 mol.%; the major component was 3HB (63.9 – 82.9 mol.%), with the  
20 other three monomers (4HB, 3HV, and 3HHx) totaling between 17.1 and 36.1 mol.%. The  $C_x$  of  
21 these copolymers was below 50%. A search of the literature did not reveal any data on the  $C_x$  of  
22 the PHA terpolymers and quaterpolymers of the same or similar composition. There are data,  
23 however, showing that incorporation of other monomer units (3HV, 4HB or 3HHx) into the  
24 carbon chain of 3HB leads to formation of different types of the crystalline lattice, depending on  
25 the contents of the incorporated monomers [6, 25-27]. Our results suggest that the degree of  
26 crystallinity of PHAs can be decreased by synthesizing P(3HB/4HB) bipolymers, and that the

1 degree of crystallinity decreases more noticeably with an increase in the 4HB content. The  $C_x$   
2 can also be decreased by incorporating a greater number of monomer units other than 3HB, i.e.  
3 by synthesizing terpolymers and quaterpolymers containing not only 4HB but also 3HV and  
4 3HHx, which contribute to achieving a balance between the amorphous and crystalline regions in  
5 the polymer.

### 6 *3.1.2. Molecular weight properties*

7 PHA molecular weight is a highly variable parameter, which is determined by a number  
8 of factors such as the type of the PHA-producing microorganism, carbon source, duration of  
9 cultivation, and technique of polymer extraction. For instance, molecular weight of P(3HB) may  
10 vary between 200 and 3000 kDa [24, 28-29]. Analysis of P(3HB/4HB) copolymers did not reveal  
11 any clear relationship between the chemical composition and 4HB content, on the one hand, and  
12 weight average molecular weight ( $M_w$ ), on the other. The  $M_w$  values of different copolymers  
13 varied between 570 and 830 kDa, with no correlation with the increasing 4HB fraction, and these  
14 values were close to those of P(3HB). However, number average molecular weight ( $M_n$ ) was 1.5-  
15 2.0 times lower than the  $M_n$  of P(3HB). That also influenced the value of polydispersity, which  
16 increased to 3.0-4.7 as 4HB content was increased, suggesting higher non-uniformity of the  
17 polymer – the presence of a greater number of fragments with different degrees of  
18 polymerization. A number of studies [12-13, 24, 29-30] addressed molecular weight properties  
19 of P(3HB/4HB) copolymers with different percentages of 4HB (5 to 94 mol.%) but did not reveal  
20 any direct relationship between 4HB content and weight average molecular weight ( $M_w$ ) of the  
21 polymer. In different studies, the  $M_w$  varied between 100-200 and 400-600 kDa, tending to  
22 decrease somewhat in the polymers with 4HB higher than 40-50 mol.%. The number average  
23 molecular weight ( $M_n$ ) reported by different authors for the copolymers containing comparable  
24 proportions of 4HB (23-24 mol.%) varied widely, between 104 and 590 kDa [7, 11]. That also  
25 affected polydispersity of P(3HB/4HB), which varied between 0.6 – 1.0-2.0 [30] and 2.5-3.5 [12-  
26 13].



1 No direct relationship was found between the chemical composition and 4HB content, on  
2 the one hand, and  $M_w$  and  $M_n$ , on the other, in terpolymers either (Table 1). The  $M_w$  of different  
3 samples ranged between 490 and 580 kDa and the  $M_n$  between 160 and 180 kDa. Polydispersity,  
4 which shows the proportions of fragments with different degrees of polymerization in the  
5 polymer, ranged between 2.9 and 3.4 and was generally higher than P(3HB) polydispersity  
6 (Table 1). Terpolymers consisting of the same monomer units were investigated in a number of  
7 studies [14-17, 31]. In those studies, 4HB content of the copolymers varied widely (between 10  
8 and 94 mol.%). The  $M_w$  and  $\bar{D}$  values varied between 177 and 1100 kDa and between 1.13 and  
9 6.46, respectively, and they were not related to monomer proportions and 4HB content.

10 Similar results were obtained in the study of quaterpolymers (Table 1).  $M_w$  varied  
11 between 580 and 790 kDa; increased polydispersity ranged between 4.5 and 5.3 in different  
12 samples. The highest values were measured in the sample with the lowest 3HB content and the  
13 highest total content of the three other monomers, including 4HB. Thus, 4HB content was  
14 generally found to affect  $M_n$  and polydispersity.

### 15 3.1.3. Temperature properties

16 The melting temperature ( $T_{melt}$ ) of PHAs, including the P(3HB) homopolymer, is about  
17 100 °C lower than their temperature of onset of thermal degradation ( $T_{degr}$ ). This is an essential  
18 technological property, as it enables fabrication of polymer products from melts. Incorporation of  
19 4HB monomer units into the 3HB chain produced different effects on temperature properties of  
20 PHA copolymers, depending on 4HB content and presence of other monomers (3HV, 3HHx) in  
21 the polymer.

22 All P(3HB/4HB) bipolymers showed melting temperatures that were somewhat lower  
23 than  $T_{melt}$  of P(3HB) (178 °C). The  $T_{melt}$  values of the P(3HB/4HB) copolymers that contained  
24 4HB between 10.4 and 75.0 mol.% ranged between 150 and 162 °C, and the copolymer  
25 containing 4HB 10.4 mol.% had the lowest  $T_{melt}$ . In contrast to  $T_{melt}$ ,  $T_{degr}$  values were similar in  
26 all bipolymers, irrespective of the 4HB content, ranging between 295 and 299 °C, which was

1 comparable with the  $T_{degr}$  of P(3HB) (295 °C). Not all PHA types showed glass transition. The  
2 glass transition temperature ( $T_g$ ) of P(3HB/4HB) copolymers containing 4HB 10.4 and 15.0  
3 mol.% was (3.4 °C) and (-2.6 °C), respectively; the thermograms of the copolymers with higher  
4 4HB contents (29.3 – 75.0 mol.%) taken under measurement conditions did not show any glass  
5 transition regions (Samples 3-5, Table 1). The highest crystallization temperature was exhibited  
6 by pure P(3HB), and of the copolymers, Samples 3 and 4 had the highest  $T_c$  (Table 1). The  $T_c$  of  
7 the copolymers with relatively low 4HB contents (10.4-15.0 mol.%) was about 57-66 °C; as the  
8 4HB content increased, the  $T_c$  rose to 88-97°C. The lower crystallization temperature of the  
9 copolymers compared to P(3HB) suggests that the copolymers remain highly ductile for longer  
10 time periods and, thus, are more readily processable. There are literature data both confirming and  
11 contradicting our results. The temperature properties of P(3HB/4HB) have been examined by very  
12 many researchers, but the published data vary greatly, even for copolymers containing similar  
13 proportions of monomer units. For instance, in a study by Mitomo et al., the melting temperature  
14 ( $T_{melt}$ ) of P(3HB/4HB) copolymers containing 4HB between 19 and 94 mol.% consistently  
15 decreased with the increase in the 4HB fraction [24]. The  $T_{melt}$  of the copolymer with the lowest  
16 4HB fraction was 158 °C, dropping dramatically, to 44 and 54 °C, when the 4HB content was  
17 increased to 74 and 94 mol.%, respectively, and the  $T_g$  decreased by one order of magnitude,  
18 from -4 to -40°C. A similar  $T_{melt}$  decrease, to 40-54 °C, was observed when the 4HB content of  
19 the copolymer was increased to 75-100 mol.% [7, 14]. Doi et al., however, found that the  $T_{melt}$  of  
20 the copolymer with the high 4HB fraction (84 mol.%) was at least 130°C [5]. A number of  
21 authors showed that  $T_{melt}$  and  $T_g$  varied depending on 4HB content: as 4HB content was increased  
22 from 10-40 to 50-90 mol.%, both parameters dropped from 150-160 and -2 -10 °C to 40-50 and -  
23 35 -48 °C, respectively [6, 29]. The authors of another study [13], however, did not reveal any  
24 effect of the increase in the 4HB content from 3 to 40 mol.% on the  $T_{melt}$  of the copolymers,  
25 which was about 145-155 °C for all samples. Iqbal and Amirul reported a somewhat lower

1 melting temperature (121- 132°C) for the P(3HB/4HB) copolymers with comparable 4HB  
2 contents (20 to 30 mol.%) [12].

3 The values of  $T_{melt}$  obtained for terpolymers and quaterpolymers were 9-17 °C lower than  
4 the  $T_{melt}$  of P(3HB), decreasing as the 4HB content increased. At the same time, the  $T_{degr}$  were  
5 comparable with the corresponding parameter of P(3HB). Determination of the  $T_g$  and  $T_c$  in the  
6 study of terpolymers and quaterpolymers gave the following results. Two terpolymers with  
7 different proportions of monomer units had the same  $T_g$ , -5 °C. However, the copolymer with the  
8 highest 4HB content, 55.6 mol.%, showed no peak characterizing glass transition (Sample 8,  
9 Table 1). For the quaterpolymers with 3HB as the major component (63.9, 74.7, and 82.9 mol.%)  
10 and the total content of the other monomer units (3HV, 4HB, and 3HHx) varied but kept below  
11 50% (36.1, 25.3, and 17.1 mol.%, respectively), the  $T_g$  was -0.7, -3.1, and -4.4 °C (Samples 9-11,  
12 Table 1). The  $T_c$  of quaterpolymers (51-70 °C) was generally higher than the  $T_c$  of P(3HB). The  
13 literature search showed the following. Madden et al. investigated temperature properties of  
14 3HB/3HV/4HB with 3HB as the major component (74-93 mol.%) and low contents of 4HB (5-13  
15 mol.%) and 3HV (2-10 mol.%) and revealed a drop in the  $T_{melt}$  from 160 to 100°C and energy of  
16 crystallization from 70 to 49 J/g [31]. It is important that the decrease in the  $T_{melt}$  and the  $T_{degr}$  of  
17 the terpolymers and quaterpolymers studied did not significantly decrease the difference between  
18 these parameters. Thus, the PHA copolymers investigated in this study, whatever their  
19 composition and monomer proportions, retained one of the essential properties of PHAs –  
20 thermoplasticity.

### 21 3.2. *Physical/mechanical properties of PHAs with different compositions*

22 Surface properties of polymer products are important for evaluating their potential  
23 performance characteristics. To investigate surface properties and mechanical properties of 4HB-  
24 containing PHAs with different compositions, we prepared  $25 \pm 0.3$ - $\mu\text{m}$  thick smooth transparent  
25 films.

26

### 1           3.2.1. Characterization of the surface properties

2           Results of studying the microstructure of polymer film surface are shown in Figure 2 and  
3 Table 2. SEM images of the samples prepared from polymers with different chemical  
4 composition and differing in their physicochemical properties looked dissimilar; the surfaces of  
5 copolymer films, in contrast to the surface of P(3HB)-based ones, were developed (to different  
6 degrees), porous, and uneven. The surface of the P(3HB) films was smoother and denser, with a  
7 few small pores, of diameter of no more than 0.5  $\mu\text{m}$ . The surface structure of the films prepared  
8 from bipolymers was noticeably different from the surface structure of P(3HB) film (Fig. 3f) in  
9 that it was rougher and had pores of diameter between 0.5 and 5  $\mu\text{m}$  and prominent circular  
10 structures (Fig. 3a, b). The surface of the films prepared from terpolymers and quaterpolymers  
11 was also rougher, with very many pores of different diameters (between 0.5 and 6  $\mu\text{m}$ ) (Fig. 3c,  
12 d, e).

13           A significant parameter, which indirectly characterizes biocompatibility and influences  
14 cell adhesion and viability, is hydrophilic/hydrophobic balance of the surface [32]. It is evaluated  
15 by measuring contact angles for water. These measurements provide the basis for determining  
16 such important parameters of the surface as cohesive forces, surface tension, and interfacial free  
17 energy. Morphological characteristics of films with different compositions influenced the values  
18 of contact angles for water and parameters determined from them: surface energy and polar  
19 component of surface free energy (Table 2).

20           Different contact angles for water were obtained for films prepared from P(3HB/4HB)  
21 bipolymers, 4HB-containing terpolymers, quaterpolymers, and P(3HB): 78.6 – 92.5°, 81.7-96.6°,  
22 88.9 – 99.7°, and 97.4°, respectively. The values of surface free energy were also different: 36.7  
23 – 44.5  $\text{erg}/\text{cm}^2$ , 20.7 – 39.3  $\text{erg}/\text{cm}^2$ , 34.5 – 40.9  $\text{erg}/\text{cm}^2$ , and 30.4  $\text{erg}/\text{cm}^2$ , respectively. The  
24 values of the polar component of surface free energy increased in copolymers, in PHA  
25 bipolymers and terpolymers in particular, compared with P(3HB), indirectly indicating a more  
26 hydrophilic surface.

1 Another important property necessary to characterize the surface is roughness, which  
2 determines adhesion. Nanometer-scale roughness can determine adhesion, spreading, and motile  
3 activity of cells and affect synthesis of specific proteins [33]. However, some data suggest that  
4 cells are attached better to rough surfaces than to polished ones, whereas other data indicate that  
5 changes in roughness are not accompanied by any cellular effects [34]. The data in Table 3 show  
6 considerable differences between the values of roughness of the films with different  
7 compositions. Roughness is determined from the arithmetic average of the roughness profile  
8 (Ra) and root mean squared roughness (Rq).

9 The values of surface roughness of the films prepared from PHA copolymers differed  
10 considerably depending on the monomer composition and proportions (Table 3). The  
11 P(3HB/4HB) (25.0/75.0 mol.%) copolymer showed the highest value of the Ra – 568.2 nm,  
12 which was almost 8 times higher than the corresponding parameter of P(3HB) films – 71.8 nm.  
13 The lowest value of surface roughness – 92.9 nm – was determined for the P(3HB/4HB)  
14 (70.7/29.3) bipolymers. The P(3HB/4HB) (25.0/75.0) bipolymer had the highest Rq: 681.1 nm.  
15 Our results are in good agreement with the data reported by other authors [35-36], suggesting  
16 that incorporation of 3HV monomer units into the P(3HB) chain increases the film surface  
17 roughness. The surfaces of the films of polylactic acid (PLA) and P(3HB/3HHx) were smoother  
18 than the surface of the films based on the P(3HB/4HB/3HHx) terpolymers [37]. However, some  
19 data suggest that the increase in the 4HB content of the copolymer and oxygen plasma treatment  
20 decreased surface roughness, making the film surface smoother [11, 38]. Thus, results obtained  
21 in the present study show that copolymers differing in chemical composition and proportions of  
22 monomers can be used to prepare films with markedly dissimilar surface microstructure and  
23 properties.

### 24 *3.2.2 Mechanical properties of films*

1 Physical/mechanical properties of PHAs were studied using films prepared from  
2 solutions of the copolymers. Table 3 presents some results of measuring mechanical properties  
3 of films prepared from PHAs of various compositions.

4 All copolymer films showed considerably higher elastic properties, expressed as  
5 elongation at break, than P(3HB) films, but lower mechanical strength, expressed as Young's  
6 modulus and tensile strength. Elongation at break of P(3HB/4HB) films consistently increased  
7 from 5.7 to 323.4% as 4HB content increased from 10.4 to 75.0 mol.%, while elongation at  
8 break of P(3HB) films was no more than 2-3%. At the same time, P(3HB/4HB) films showed  
9 some decrease in tensile strength and a dramatic decrease in Young's modulus, which dropped to  
10 between 242.9 and 975.7 MPa, relative to Young's modulus of P(3HB) films – 1764.0 MPa.  
11 Analysis of the literature data, including reviews by Laycock, also suggested that elasticity of  
12 polymer films increased with an increase in 4HB content of the copolymers, but the data on  
13 mechanical properties of P(3HB/4HB) bipolymers reported by different authors varied widely [3,  
14 39]. For instance, Ramachandran and Amirul investigated copolymers with 4HB content of no  
15 more than 20-40 mol.% and reported their Young's modulus values of between 10 and 119 MPa,  
16 tensile strength of between 1 and 12 MPa, and elongation at break of between 1 and 300-400%  
17 [13]. Comparable values of the three parameters of copolymers with the same 4HB content were  
18 reported by Huong et al. [30]. By contrast, Iqbal and Amirul reported lower values of mechanical  
19 properties for copolymers containing higher percentages of 4HB (47 and 65 mol.%) [12]. These  
20 contradictions are most likely associated with different processes of preparing films; moreover,  
21 most of the authors do not provide geometric properties of their samples.

22 Films prepared from terpolymers containing 3HB, 4HB, and 3HV also showed enhanced  
23 elasticity and an even greater decrease in mechanical strength (Table 3). The data reported in the  
24 literature for P(3HB/3HV/4HB) terpolymers are very contradictory. In the copolymer with 4HB  
25 content of 84 mol.% [14, 37], elongation at break reached 300%, which was higher by an order  
26 of magnitude than in P(3HB/3HV). Young's modulus increased to 392 MPa in the copolymer

1 with 3HV 23 mol.% and 4HB 66 mol.%, which was 13 times higher than Young's modulus of  
2 P(3HB/4HB). The copolymer containing 3HV 34 mol.% and 4HB 55 mol.% showed an increase  
3 in Young's modulus to 618 MPa.

4 For P(3HB/3HV/4HB/3HHx) quaterpolymers, which had a higher degree of crystallinity,  
5 elongation at break was somewhat lower than for bipolymers and terpolymers, but although it  
6 was no more than 100%, it was significantly higher than elongation at break of P(3HB).  
7 Mechanical strength parameters were lower for all quaterpolymer samples. Young's modulus  
8 dropped to 127.8-419.4 MPa and tensile strength to 7.3-11.7 MPa, and these values were two  
9 orders of magnitude lower than the values of the corresponding parameters of P(3HB). We did  
10 not reveal any direct relationship between physical/mechanical properties of the quaterpolymers  
11 and proportions of monomers, including 4HB, in them, but we did observe a dramatic increase in  
12 elongation at break and a decrease in Young's modulus compared to P(3HB), suggesting higher  
13 ductility of the quaterpolymers.

### 14 *3.3. Biological properties of PHAs with different compositions*

15 Biological properties of bi-, ter-, and quaterpolymer films are illustrated by photographs  
16 showing fibroblasts stained with fluorescent dyes – markers of nuclear DNA (DAPI) and cell  
17 cytoplasm (phalloidin conjugated to FITC) (Fig. 3). Polystyrene of culture plates was used as  
18 control. DAPI and FITC staining didn't reveal any cytotoxic effect of P(3HB) and PHA bi-, ter-,  
19 and quaterpolymers. Morphology of physiologically active cells showed that the best results  
20 were obtained on the PHA films.

21 Thus, none of the PHA types investigated in this study showed cytotoxicity, i.e. the study  
22 proved their high biocompatibility and better performance as cell scaffolds compared to P(3HB)  
23 and polystyrene.

### 24 **Conclusions**

25 In this study, we compared properties of PHA bi-, ter-, and quaterpolymers that contained  
26 different concentrations of 4HB monomer units. 4HB monomer units produced the strongest

1 effect on the degree of crystallinity of PHAs, reducing this parameter to 30-40% compared to the  
2 high-crystallinity P(3HB) (67%). Synthesis of PHA ter- and quaterpolymers that, in addition to  
3 4HB, contained 3HV and 3HHx monomer units increased the effect of 4HB on  $C_x$ . Copolymers  
4 containing 4HB were more ductile than homogenous P(3HB): their values of elongation at break  
5 were higher than that of P(3HB). However, their mechanical strength was lower. All copolymers  
6 containing 4HB were highly biocompatible and suitable for fabricating cell scaffolds for cellular  
7 and tissue engineering.

### 8 **Acknowledgement**

9 The research was supported by the Russian Science Foundation (grant No. 17-15-01352)

### 10 **References**

- 11 [1] Biomaterials, artificial organs and tissue engineering, L.L. Hench, J.R. Jones (Eds.)  
12 CRC Press, BocaRaton, 2005.
- 13 [2] K. Sudesh, H. Abe, Y. Doi, Synthesis, structure and properties of  
14 polyhydroxyalkanoates: biological polyesters, Prog. Polym. Sci. 25 (2000) 1503-1555.
- 15 [3] B. Laycock, P. Halley, S. Pratt, A. Werker, P. Lant, The chemomechanical properties  
16 of microbial polyhydroxyalkanoates, Prog. Polym. Sci. 38 (2013) 536-583.
- 17 [4] T.G. Volova, E.I. Shishatskaya, A.J. Sinskey, Degradable Polymers: Production,  
18 Properties and Applications, Nova Science Publishers, New York, 2013.
- 19 [5] Y. Doi, A. Segawa, M. Kunioka, Biosynthesis and characterization of poly(3-  
20 hydroxybutyrate-co-4-hydroxybutyrate) in *Alcaligenes eutrophus*, Int. J. Biol. Macromol. 12  
21 (1990) 106-11.
- 22 [6] Y. Saito, Y. Doi, Microbial synthesis and properties of poly(3-hydroxybutyrate-co-4-  
23 hydroxybutyrate) in *Comamonas acidovorans*, Int. J. Biol. Macromol. 16 (1994) 99-104.
- 24 [7] S. Vigneswari, S. Vijaya, M.I.A. Majid, K. Sudesh, C.S. Sipaut, M.N.M. Azizan,  
25 A.A. Amirul, Enhanced production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) copolymer



1 with manipulated variables and their properties, J. Ind. Microbial. Biotechnol. 36 (2009) 547-  
2 556.

3 [8] D.H. Park, B.S. Kim, Production of poly(3-hydroxybutyrate) and poly(3-  
4 hydroxybutyrate-co-4-hydroxybutyrate) by *Ralstonia eutropha* from soybean oil, New  
5 Biotechnol. 28 (2011) 719-724.

6 [9] J.M.B.T. Cavaleiro, R.S. Raposo, M.C.M.D. de Almeida, M.T. Cesário, C. Sevrin,  
7 C. Grandfils, M.M.R. da Fonseca, Effect of cultivation parameters on the production of poly(3-  
8 hydroxybutyrate-co-4-hydroxybutyrate) and poly(3-hydroxybutyrate-4-hydroxybutyrate-3-  
9 hydroxyvalerate) by *Cupriavidus necator* using waste glycerol, Bioresour. Technol. 111 (2012)  
10 391-397.

11 [10] S. Chanprateep, Y. Kotakura, S. Visetkoop, H. Shimizu, S. Kulpreecha, S. Shioya,  
12 Characterization of new isolated *Ralstonia eutropha* strain A-04 and kinetic study of  
13 biodegradable copolyester poly(3-hydroxybutyrate-co-4-hydroxybutyrate) production, J. Ind.  
14 Microbiol. Biotechnol. 35 (2008) 1205-1215.

15 [11] S. Chanprateep, K. Buasri, A. Muangwong, P. Utiswannakul, Biosynthesis and  
16 biocompatibility of biodegradable poly(3-hydroxybutyrate-co-4-hydroxybutyrate), Polym.  
17 Degrad. Stab. 95 (2010) 2003-2012.

18 [12] N.Md. Iqbal, A.A. Amirul, Synthesis of P(3HB-co-4HB) copolymer with target-  
19 specific 4HB molar fractions using combinations of carbon substrates, J. Chem. Technol.  
20 Biotechnol. 89 (2014) 407-418.

21 [13] H. Ramachandran, A.A. Amirul, Bioconversion of glycerine pitch into a novel  
22 yellow-pigmented P(3HB-co-4HB) copolymer: synergistic effect of ammonium acetate and  
23 polymer characteristics, Appl. Biochem. Biotechnol. 172 (2014) 891-909.

24 [14] S. Chanprateep, S. Kulpreecha, Production and characterization of biodegradable  
25 terpolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-4-hydroxybutyrate) by *Alcaligenes*  
26 sp. A-04. J. Biosci. Bioeng. 101 (2006) 51-56.

- 1 [15] M. Koller, P. Hesse, R. Bona, C. Kutschera, A. Atlić, G. Braunegg, Biosynthesis of  
2 high quality polyhydroxyalkanoate co-and terpolyesters for potential medical application by the  
3 archaeon *Haloferax mediterranei*, *Macromol. Symposia* 253 (2007) 33-39.
- 4 [16] T.M.F. Azira, A.A. Nursolehah, Y. Norhayati, M.I.A. Majid, A.A. Amirul,  
5 Biosynthesis of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-4-hydroxybutyrate) terpolymer  
6 by *Cupriavidus* sp. USMAA2-4 through two-step cultivation process, *World J. Microbiol.*  
7 *Biotechnol.* 27 (2011) 2287-2295.
- 8 [17] H. Ramachandran, N.Md. Iqbal, C.S. Sipaut, A.A.-A. Abdullah, Biosynthesis and  
9 Characterization of poly (3-hydroxybutyrate-co-3-hydroxyvalerate-co-4-hydroxybutyrate)  
10 terpolymer with various monomer compositions by *Cupriavidus* Sp. USMAA2-4, *Appl.*  
11 *Biochem. Biotechnol.* 164 (2011) 867-877.
- 12 [18] T. Volova, N. Zhila, E. Kiselev, E. Shishatskaya, A study of synthesis and properties  
13 of poly-3-hydroxybutyrate/diethylene glycol copolymers, *Biotechnol. Prog.* 32 (2016) 1017-  
14 1028.
- 15 [19] O. Vinogradova, T. Volova, Biosynthesis and properties of PHA containing  
16 monomers 3-hydroxy-4-methylvalerate, *J. Siberian Federal University. Biology* 9 (2016) 1145-  
17 152.
- 18 [20] T.G. Volova, E.G. Kiselev, E.I. Shishatskaya, N.O. Zhila, A.N. Boyandin, D.A.  
19 Syrvacheva, O.N. Vinogradova, G.S. Kalacheva, A.D. Vasiliev, I.V. Peterson, Cell growth and  
20 accumulation of polyhydroxyalkanoates from CO<sub>2</sub> and H<sub>2</sub> of a hydrogen-oxidizing bacterium,  
21 *Cupriavidus eutrophus* B-10646, *Bioresour. Technol.* 146 (2013) 215-222.
- 22 [21] T. Volova, E. Kiselev, O. Vinogradova, E. Nikolaeva, A. Chistyakov, A. Sukovatiy,  
23 E. Shishatskaya, A glucose-utilizing strain, *Cupriavidus eutrophus* B-10646: growth kinetics,  
24 characterization and synthesis of multicomponent PHAs, *PLoS One* 9 (2014) 1–15.

- 1 [22] T.G. Volova, O.N. Vinogradova, N.O. Zhila, I.V. Peterson, E.G. Kiselev, A.D.  
2 Vasiliev, A.G. Sukovatiy, E.I. Shishatskaya, Properties of a novel quaterpolymer  
3 P(3HB/4HB/3HV/3HHx). *Polymer* 101 (2016) 67-74.
- 4 [23] T.G. Volova, O.N. Vinogradova, N.O. Zhila, E.G. Kiselev, I.V. Peterson, A.D.  
5 Vasil'ev, A.G. Sukovaty, E.I. Shishatskaya, Physicochemical properties of multicomponent  
6 polyhydroxyalkanoates: novel aspects, *Polymer Science, Series A* 59 (2017) 76-85.
- 7 [24] H. Mitomo, W.-C. Hsieh, K. Nishiwaki, K. Kasuya, Y. Doi, Poly(3-  
8 hydroxybutyrate-co-4-hydroxybutyrate) produced by *Comamonas acidovorans*, *Polymer* 42  
9 (2001) 3455-3461.
- 10 [25] M. Scandola, G. Ceccorulli, M. Pizzoli, M. Gazzano, Study of the crystal phase and  
11 crystallization rate of bacterial poly( $\beta$ -hydroxybutyrate-co- $\beta$ -hydroxyvalerate), *Macromol.* 25  
12 (1992) 1405-1410.
- 13 [26] T. Iwata, Y. Doi, Crystals structure and biodegradation of aliphatic polyesters  
14 crystals, *Macromol.* 30 (1997) 5290-5296.
- 15 [27] T. Iwata, Y. Doi, S. Nakayama, H. Sasatsuki, S. Teramachi, Structure and enzymatic  
16 degradation of poly(3-hydroxybutyrate) copolymer single crystals with an extracellular PHB  
17 depolymerase from *Alcaligenes faecalis* T1, *Int. J. Biol. Macromol.* 25 (1999) 169-176.
- 18 [28] D. Byrom, Polyhydroxyalkanoates, in: D.P. Mobley (Ed.), *Plastic from Microbes:*  
19 *Microbial Synthesis of Polymers and Polymer Precursors*, Hanser, Munich, 1994, pp. 5-33.
- 20 [29] S. Chanprateep, Current trends in biodegradable polyhydroxyalkanoates, *J. Biosci.*  
21 *Bioeng.* 110 (2010) 621-632.
- 22 [30] K.H. Huong, S. Kannusamy, S.Y.H. Lim, A.A. Amirul, Biosynthetic enhancement  
23 of single-stage poly(3-hydroxybutyrate-co-4-hydroxybutyrate) production by manipulating the  
24 substrate mixtures, *J. Ind. Microbiol. Biotechnol.* 42 (2015) 1291-1297.

- 1 [31] L.A. Madden, A.J. Anderson, J. Asrar, P. Garrett, Production and characterization of  
2 poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-4-hydroxybutyrate) synthesized by *Ralstonia*  
3 *eutropha* in fed-batch cultures, *Polymer* 41 (2000) 3499-3505.
- 4 [32] W. Ou, H. Qiu, Z. Chen, K. Xu, Biodegradable block poly(ester-urethane)s based on  
5 poly(3-hydroxybutyrate-co-4-hydroxybutyrate) copolymers, *Biomaterials* 32 (2011) 3178-3188.
- 6 [33] S.P. Xavier, P.S.P. Carvalho, M.M. Beloti, A.L. Rosa, Response of rat bone marrow  
7 cells to commercially pure titanium submitted to different surface treatments, *J. Dent.* 31 (2003)  
8 173-80.
- 9 [34] A.P. Boskhomdzhev, *Izucheniye biodestruktsii i biosovmestivosti polimernykh*  
10 *system na osnove polioxialkanoatov (A study of biodegradation and biocompatibility of*  
11 *polyhydroxyalkanoates-based polymer systems)*, Summary of PhD thesis, Moscow, A.N. Bakh  
12 Institute of Biochemistry RAS, 2010.
- 13 [35] A. Bera, S. Dubey, K. Bhayani, D. Mondal, S. Mishra, P.K. Ghosh, Microbial  
14 synthesis of polyhydroxyalkanoate using seaweed-derived crude levulinic acid as co-nutrient,  
15 *Int. J. Biol. Macromol.* 72 (2015) 487-494.
- 16 [36] L.H. Poley, A.P.L. Siqueira, M.G. da Silva, R. Sanchez, R. Prioli, A.M. Mansanares,  
17 H. Vargas, Photothermal methods and atomic force microscopy images applied to the study of  
18 poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) dense membranes,  
19 *J. Appl. Polym. Sci.* 97 (2005) 1491-1497.
- 20 [37] X. Wei, Y.J. Hu, W.P. Xie, R.L. Lin, G.Q. Chen, Influence of poly(3-  
21 hydroxybutyrate-co-4-hydroxybutyrate-co-3-hydroxyhexanoate) on growth and osteogenic  
22 differentiation of human bone marrow-derived mesenchymal stem cells, *J. Biomed. Mater. Res.*  
23 *A.* 90 (2009) 894-905.
- 24 [38] J. Zhang, K. Kasuya, A. Takemura, A. Isogai, T. Iwata, Properties and enzymatic  
25 degradation of poly(acrylic acid) grafted polyhydroxyalkanoate films by plasma-initiated  
26 polymerization, *Polym. Degrad. Stab.* 98 (2013) 1458-1464.

1 [39] B. Laycock, M.V. Arcos-Hernandez, A. Langford, J. Buchanan, P.J. Halley, A.  
2 Werker, P.A. Lant, S. Pratt, Thermal properties and crystallization behavior of fractionated  
3 blocky and random polyhydroxyalkanoate copolymers from mixed microbial cultures, J. Appl.  
4 Polym. Sci. 11 (2014) 791-808

5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45

1 Table 1 Chemical composition and physicochemical properties of PHA copolymers with  
 2 different proportions of monomers

| Specimen no.                           | PHA composition, mol.% |      |      |     | $M_w$ , kDa | $M_n$ , kDa | D   | $C_x$ , % | $T_g$ , °C | $T_c$ , °C | $T_{melt}$ , °C | $T_{degr.}$ , °C |
|--|------------------------|------|------|-----|-------------|-------------|-----|-----------|------------|------------|-----------------|------------------|
| homopolymer of poly(3-hydroxybutyrate) |                        |      |      |     |             |             |     |           |            |            |                 |                  |
|  | P(3HB) 100             |      |      |     | 920         | 368         | 2.5 | 67        | n.d.       | 107        | 178             | 295              |
| PHA bipolymers                         |                        |      |      |     |             |             |     |           |            |            |                 |                  |
| 3HB/4HB                                |                        |      |      |     |             |             |     |           |            |            |                 |                  |
| 1                                      | 89.6                   | 10.4 |      |     | 570         | 150         | 3.8 | 53        | 3.4        | 66         | 150             | 297              |
| 2                                      | 85.0                   | 15.0 |      |     | 750         | 250         | 3.0 | 50        | -2.6       | 57         | 158             | 296              |
| 3                                      | 70.7                   | 29.3 |      |     | 830         | 220         | 3.8 | 46        | n.d.       | 96         | 162             | 298              |
| 4                                      | 43.9                   | 56.1 |      |     | 770         | 190         | 4.1 | 40        | n.d.       | 97         | 162             | 299              |
| 5                                      | 25.0                   | 75.0 |      |     | 700         | 150         | 4.7 | 30        | n.d.       | 88         | 158             | 295              |
| PHA terpolymers                        |                        |      |      |     |             |             |     |           |            |            |                 |                  |
| 3HB/3HV/4HB                            |                        |      |      |     |             |             |     |           |            |            |                 |                  |
| 6                                      | 50.8                   | 20.5 | 28.7 |     | 580         | 180         | 3.2 | 35        | -5         | 64         | 166             | 278              |
| 7                                      | 51.5                   | 10.1 | 38.4 |     | 490         | 170         | 2.9 | 33        | -5         | 61         | 164             | 280              |
| 8                                      | 27.4                   | 17.0 | 55.6 |     | 540         | 160         | 3.4 | 26        | n.d.       | 25         | 166             | 296              |
| PHA quaterpolymers                     |                        |      |      |     |             |             |     |           |            |            |                 |                  |
| 3HB/3HV/4HB/3HHx                       |                        |      |      |     |             |             |     |           |            |            |                 |                  |
| 9                                      | 63.9                   | 20.9 | 13.3 | 1.9 | 790         | 150         | 5.3 | 45        | -0.7       | 51         | 169             | 292              |
| 10                                     | 74.7                   | 12.7 | 10.1 | 2.5 | 580         | 130         | 4.5 | 44        | -3.1       | 70         | 165             | 290              |
| 11                                     | 82.9                   | 6.4  | 9.3  | 1.4 | 760         | 150         | 5.1 | 39        | -4.4       | 63         | 161             | 289              |

3  
 4 n.d.- non detected  
 5

6  
 7  
 8  
 9

1 Table 2 Surface properties of the films prepared from solutions of P(3HB/4HB),  
 2 P(3HB/3HV/4HB), and P(3HB/3HV/4HB/3HHx) copolymers with different monomer contents  
 3 (numbers according to Table 1)

| Specimen no. | Contact angle for water, ° | Contact angle for diiodomethane, ° | Surface free energy, erg/cm <sup>2</sup> | Polar component of surface free energy, erg/cm <sup>2</sup> |
|--------------|----------------------------|------------------------------------|--|---|
| P(3HB)       | 97.4±2.6                   | 58.5±1.4                           | 30.4±1.0                                 | 1.2±0.2   |
| 1            | 89.8±1.6                   | 48.9±1.1                           | 36.8±1.1                                 | 1.9±0.1   |
| 2            | 88.0±2.4                   | 49.6±0.7                           | 36.9±0.2                                 | 2.4±0.1   |
| 3            | 78.6±1.2                   | 42.0±1.1                           | 44.5±1.1                                 | 4.2±0.1   |
| 4            | 92.5±1.3                   | 47.8±1.4                           | 36.7±1.1                                 | 1.2±0.1   |
| 5            | 90.6±1.3                   | 45.5±1.1                           | 38.4±1.1                                 | 1.5±0.2   |
| 6            | 83.7±5.2                   | 54.5±1.1                           | 36.0±0.5                                 | 4.2±0.2   |
| 7            | 96.6±1.1                   | 80.5±2.3                           | 20.7±0.4                                 | 3.4±0.1   |
| 8            | 81.7±0.8                   | 48.6±2.1                           | 39.3±0.5                                 | 4.2±0.1   |
| 9            | 88.9±3.7                   | 45.4±1.9                           | 38.7±1.2                                 | 1.9±0.2   |
| 10           | 89.0±1.8                   | 40.2±1.2                           | 40.9±1.1                                 | 1.4±0.0   |
| 11           | 99.7±2.4                   | 50.2±1.0                           | 34.5±1.2                                 | 0.3±0.0   |

4

5

6

7

8

9

10

11

12

1 Table 3 Physical/mechanical properties of films prepared from PHAs with different  
 2 compositions (numbers according to Table 1)

| Specimen no. | Tensile strength, MPa | Young's modulus, MPa | Elongation at break, % | Ra – arithmetic average of the roughness profile, nm | Rq – root mean squared roughness, nm |
|--------------|-----------------------|----------------------|------------------------|--|--------------------------------------|
| P(3HB)       | 20.9                  | 1764.0               | 3.2                    | 71.8   | 80.3                                 |
| 1            | 14.8                  | 975.7                | 5.7                    | 92.9   | 113.1                                |
| 3            | 7.8                   | 242.9                | 30.7                   | 177.6  | 248.1                                |
| 5            | 15.4                  | 424.8                | 323.4                  | 568.2  | 681.1                                |
| 7            | 5.3                   | 130.5                | 47.6                   | 126.5  | 170.7                                |
| 8            | 8.8                   | 34.3                 | 365.4                  | 184.5  | 221.8                                |
| 9            | 7.3                   | 127.8                | 93.7                   | 132.5  | 172.9                                |
| 11           | 11.7                  | 419.4                | 49.0                   | 283.8  | 348.5                                |

3  
 4  
 5  
 6  
 7  
 8  
 9  
 10  
 11  
 12  
 13  
 14  
 15  
 16



1           **Figure Legends**

2           Figure 1. <sup>1</sup>H NMR spectra of copolymer samples: a – P(3HB); b – P(3HB/56.1 mol. %-  
3   4HB); c – P(3HB/20.5 mol. %-3HV/28.7 mol. %-4HB); d – P(3HB/20.9 mol. %-3HV/13.3 mol. %-  
4   4HB/1.9 mol. %-3HHx).

5           Figure 2. SEM images of the films prepared from PHAs with different compositions: A –  
6   P(3HB/29.3 mol %-4HB), B – P(3HB/75.0 mol %-4HB), C - P(3HB/10.1 mol. %-3HV/38.4  
7   mol. %-4HB), D - P(3HB/17.0 mol. %-3HV/55.6 mol. %-4HB), E - P(3HB/20.9 mol. %-3HV/13.3  
8   mol. %-4HB/1.9 mol. %-3HHx), F – P(3HB).

9           Figure 3. FITC and DAPI fluorescent staining of NIH 3T3 mouse fibroblast cells  
10   cultivated on films of PHAs of different composition at Day 3 of cultivation.