



## Properties of a novel quaterpolymer P(3HB/4HB/3HV/3HHx)



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### ARTICLE INFO

#### Article history:

Received 18 May 2016

Received in revised form

18 July 2016

Accepted 12 August 2016

Available online 15 August 2016

#### Keywords:

Polyhydroxyalkanoates

Quaterpolymers

Physicochemical and mechanical properties

### ABSTRACT

*Cupriavidus eutrophus* B10646 was used to synthesize a series of polyhydroxyalkanoate (PHA) quaterpolymers composed of the short-chain-length 3-hydroxybutyrate (3HB), 4-hydroxybutyrate (4HB), and 3-hydroxyvalerate (3HV) and the medium-chain-length 3-hydroxyhexanoate (3HHx). The molar fraction of 3HB in the quaterpolymers varied between 63.5 and 93.1 mol.%, 3HV – between 1.1 and 24.6 mol.%, 4HB – between 2.4 and 15.6 mol.%, and 3HHx – between 0.4 and 4.8 mol.%. The properties of PHA quaterpolymers were significantly different from those of the P(3HB) homopolymer: they had much lower degrees of crystallinity (up to 30–45%), and lower melting points and thermal decomposition temperatures, with the interval between these temperatures remaining practically unchanged. Films prepared from PHA quaterpolymers were rougher and more porous than P(3HB) films; they showed higher values of elongation at break (up to 6–113%), i.e. were more elastic. Films prepared from PHA quaterpolymers were biocompatible and had no toxic effect on mouse fibroblast NIH 3T3 cells.

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## 1. Introduction

Polymers of hydroxy derived alkanic acids (polyhydroxyalkanoates, PHAs) are valuable products of biotechnology. PHAs are synthesized by prokaryotes as energy and carbon storage. PHA producers accumulate them intracellularly under the conditions limiting microbial growth and synthesis of primary metabolites by nutrient (nitrogen, phosphorus, oxygen, etc.) deficiency [1]. PHAs have a wide range of useful properties, including biocompatibility and biodegradability. They can be used to fabricate various products – from degradable packaging to high-tech devices for biomedical applications [2–4].

PHAs include polymers with various chemical structures, which differ in their basic physicochemical properties. PHAs are divided into three groups: short-chain-length monomers, which consist of 3–5 carbon atoms; medium-chain-length monomers, which

consist of 6–14 carbon atoms; and long-chain-length monomers, which consist of 14 and more carbon atoms [5].

The list of PHAs keeps growing. PHA copolymers are more attractive materials for fabricating various products than the highly crystalline homopolymer of 3-hydroxybutyric acid [P(3HB)]. PHA copolymers have aroused considerable interest, and a great number of genetically modified and a few natural PHA producers capable of synthesizing them have been described by now. Synthesis of new PHA types with enhanced processing ability is usually aimed at producing polymers with lower degrees of crystallinity, which show elastomer properties. These include short-chain-length copolymers (SCL-PHAs) comprising 3- and 4-hydroxybutyrate (C<sub>4</sub>) and 3-hydroxyvalerate (C<sub>5</sub>) monomers and copolymers consisting of both short-chain-length and medium-chain-length monomers such as 3-hydroxyhexanoate (3HHx) (C<sub>6</sub>) or 3-hydroxyoctanoate (3HO) (C<sub>8</sub>) etc. [1,6]. MCL-PHAs containing 3-hydroxyoctanoate monomers exhibit the characteristics of elastomers with poor tensile strength [7].

Based on their properties, PHA copolymers comprising short- and medium-chain-length monomers (SCL/MCL-PHAs) occupy an intermediate position between the SCL- and MCL-PHAs. Physical

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and mechanical properties of PHAs, such as tensile strength, Young modulus and elongation at break, are determined by the proportions of monomers in the polymer. As potential applications of PHAs are very wide (medicine, pharmacology, agriculture, municipal engineering, etc.), they will require polymers with different properties. It is very important to determine the relationship between the chemical composition and properties of PHAs to be able to find a proper application for each PHA.

However, synthesis of PHA copolymers is a complex and difficult task of biotechnology, as it usually requires that the culture medium be supplemented with additional carbon sources – precursors of the target monomers, most of which inhibit the growth of microorganisms. Hence, the cell biomass production and PHA yields are reduced [8,9]. Difficulties in achieving regulated and reproducible synthesis of PHA copolymers hinder accumulation of data on the effects of monomer composition on the physicochemical properties of PHA copolymers. A considerable amount of literature has been published on synthesis and properties of PHAs consisting of two monomers; there has been much less research on PHA terpolymers.

Data on synthesis and properties of PHA quaterpolymers are limited. Tan et al. [10] described the ability of *Pseudomonas putida* PGA1 grown on saponified palm oil and fatty acid derivatives to synthesize multicomponent PHAs that contained medium-chain-length monomers with even numbered carbon chains (C<sub>6</sub>, C<sub>8</sub>, C<sub>10</sub>, C<sub>12</sub>, C<sub>14</sub>). Other authors [11–13] showed that recombinant *Pseudomonas* strains harboring cloned genes of the synthesis of short-chain-length PHAs from *Ralstonia* and other PHA producers were capable of synthesizing 3-hydroxybutyrate copolymers with various medium-chain-length monomers, whose properties were similar to those of low-density polyethylene. A number of studies describe quaterpolymers consisting of such monomers as P(3HB/3HP/3HHx/3HO) [14], P(3HB/3HV/3HHD/3HOD) [15], P(3HB/3HV/3HHx/3HO) [16]. However, these works do not provide comprehensive information on physical and mechanical properties of the PHAs; moreover, some of the monomers are present as minor components. In a relatively recent study by Mizuno et al. [17], recombinant *Ralstonia eutropha* PHB<sup>-4</sup> carrying synthase gene from *Pseudomonas* sp. (PhaC1Ps) was used to synthesize PHA quaterpolymer of a novel composition that was mainly constituted by 3HB monomers (92–99 mol.%) and minor fractions of 3HV (0.7–3.0 mol.%), 3H4MV (0.3–0.6 mol.%), and 3H3PhP (4.2–12.2 mol.%) from the complex carbon substrate that contained such precursors as 3-hydroxy-3-phenylpropionic acid (3P3PhP), 3-phenylpropionic acid (3PhP), cinnamic acid (CA), 5-phenylvaleric acid (5PhV), and 6-phenylhexanoic acid (6PhHx). Those PHAs had lower melting temperature, enthalpy of fusion, and number average molecular weight than P(3HB), but the authors did not include the data on the degree of crystallinity and mechanical properties of these copolymers.

In a previous study conducted by our team, two wild-type strains (*Wautersia eutropha* H16 and B5786) were grown under autotrophic conditions on CO<sub>2</sub> and in heterotrophic culture on fructose. The addition of valerate or hexanoate or octanoate as a precursor substrate resulted in the synthesis of PHA copolymers consisting of short-chain-length monomers of 3-hydroxybutyrate and 3-hydroxyvalerate as major monomers (57–98 mol.% and 21–68 mol.%, respectively) and minor fractions of medium-chain-length monomers (3HHx, 3HO, and 3HHp) – between 0.10 and 2–3 mol.% [18]. The properties of those PHAs varied depending on the molar fractions of the monomers: C<sub>x</sub> between 49 and 71%; T<sub>m</sub> and T<sub>d</sub> between 146 and 168 and between 210 and 268 °C, respectively. Relatively recently, in the culture of the strain *C. eutrophus* 10646, which is tolerant to precursor substrates (valerate, hexanoate,  $\gamma$ -butyrolactone), we achieved synthesis of a

novel SCL/MCL-PHA: P(3HB/3HV/4HB/3HHx) with high molar fractions of 3HB (48.2–78.9 mol.%) and 4HB (20.0–51.3 mol.%) but with extremely low molar fractions of 3HV and 3HHx (0.2–0.4 mol.%) [19].

The purpose of the present study was to investigate properties of the PHA quaterpolymer P(3HB/4HB/3HV/3HHx) containing short- and medium-chain-length monomers.

## 2. Experimental

### 2.1. Materials

Samples of PHA were synthesized at the Institute of Biophysics of the Russian Academy of Sciences. PHA quaterpolymer containing short-chain-length and medium-chain-length monomers were synthesized using *Cupriavidus eutrophus* B10646. PHA synthesis was performed based on previously obtained data on the physiological effect of toxic precursor substrates ( $\gamma$ -butyrolactone, valeric acid, hexanoic acid) and the effect of their concentrations on specific growth rate of cells, cell biomass yield and total yield of PHA [19–21].

### 2.2. Analysis of the chemical composition of PHAs

Polymer was extracted from cells with chloroform, and the extracts were precipitated using hexane. The extracted polymers were re-dissolved and precipitated again 3–4 times to prepare homogeneous specimens. The composition of PHAs was analyzed with a GC-MS (6890/5975C, Agilent Technologies, U.S.). To further confirm the PHA composition, <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of PHA in CDCl<sub>3</sub> were obtained at room temperature on a BRUKER AVANCE III 600 spectrometer operated at 600.13 MHz of <sup>1</sup>H NMR spectra and 150.90 MHz of <sup>13</sup>C NMR spectra.

### 2.3. Analysis of physicochemical properties of PHA

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

Thermal analysis of PHA specimens was performed using a DSC-1 differential scanning calorimeter (METTLER TOLEDO, Switzerland). Samples were preheated to 60 °C and cooled to 25 °C. The specimens were heated to temperatures from 25 °C to 300 °C, at 5 °C/min (measurement precision 1.5 °C). The thermograms were analyzed using the STARE v11.0 software.

X-Ray structure analysis and determination of crystallinity of copolymers were performed employing a D8 ADVANCE X-Ray powder diffractometer equipped with a VANTEC fast linear detector (Bruker, AXS, Germany). Calculations were done by using the Eva program of the diffractometer software. The measurement precision was 2%.

### 2.4. Analysis of PHA surface microstructure and physical/mechanical properties

To investigate PHA properties, the polymer was processed into films. Films were prepared by casting chloroform solution (2% w/v) on degreased glass and subsequent drying at room temperature for 2–3 days in a dust-free box. The film discs were 100 mm in diameter and 0.04 mm thick.

The microstructure of the surface of PHA films was analyzed using scanning electron microscopy (S 5500, Hitachi, Japan). Prior to microscopy, the samples were sputter coated with platinum (at 10 mA, for 40 s), with an Emitech K575X sputter coater.

The roughness of film surface was determined using atomic-force microscopy (AFM) in semicontact mode (Smart SPM™, AIST-NT, Zelenograd, Russia).

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

Physical/mechanical properties of films were investigated using an Instron 5565 electromechanical tensile testing machine (U.K.). Young's modulus ( $E$ , MPa), tensile strength ( $\sigma$ , MPa) and elongation at break ( $\epsilon$ , %) were automatically calculated by the Instron software (Bluehill 2, Elancourt, France), with measurement precision of 10%.

All the characterization was performed in similar procedures as previous described [19,20].

### 3. Results and discussion

Quaterpolymers of different compositions were synthesized in *C. eutrophus* B10646 culture, under specialized conditions. The molar fraction of 3HB in the quaterpolymers varied between 63.5 and 93.1 mol.%, 3HV – between 1.1 and 24.6 mol.%, 4HB – between 2.4 and 15.6 mol.%, and 3HHx – between 0.4 and 4.8 mol.% (Table 1). Thus, the lowest total content of the three monomer units other than the major 3-hydroxybutyrate (3HB) was 6.9 mol.% and the highest 36.5 mol.%. The monomer composition of the PHAs was determined by chromatography-mass spectrometry, and  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectroscopy. Fig. 1A shows the ion chromatogram with mass spectra of the monomers; Fig. 1 B,C shows  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of one PHA – P(3HB/3HV/4HB/3HHx) (63.5/19.4/12.3/4.8 mol.%). Another proof that was a quaterpolymer was the presence of the corresponding signals from methyl ( $-\text{CH}_3$ ), methylene ( $-\text{CH}_2$ ), and methine ( $-\text{CH}$ ) carbon nuclei in its  $^{13}\text{C}$  NMR spectrum (Fig. 1C). Identification of chemical shifts was performed by comparing with the previously published spectral data for 3HB-3HV-3H4MV-3HHx [22] and 3HB-3HV-4HB polymers [23].

#### 3.1. Physicochemical properties of PHA quaterpolymers

Thermal properties of PHAs and their ability to crystallize in their native state are their most significant parameters, as they determine the thermomechanical properties of the polymers and, hence, their ability to be processed from the melts. PHAs, like many other polymers, have a heat distortion temperature somewhat lower than the thermal degradation temperature. Thus, polymers cannot exist in the gaseous state, and the main type of phase equilibrium in them is a condensed state – crystalline, glassy, viscoelastic, and liquid. The ability of a PHA to crystallize is determined by the inner properties of its chains. In a number of polymers, crystallization develops only partly for various reasons. Thus, most of the polymers, including PHAs, are semi-crystalline materials.

The relative fractions of monomers influenced physicochemical properties of PHAs, including their degrees of crystallinity, molecular-weight properties, and thermal characteristics (Table 1).

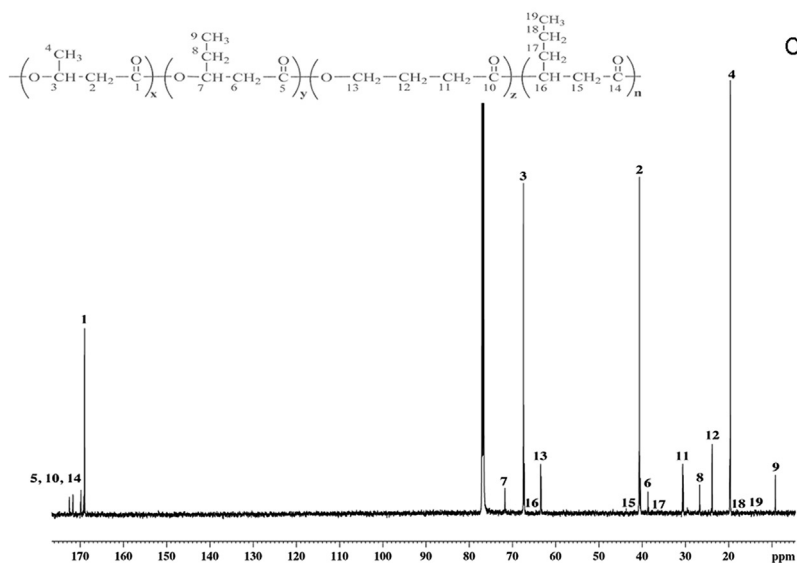
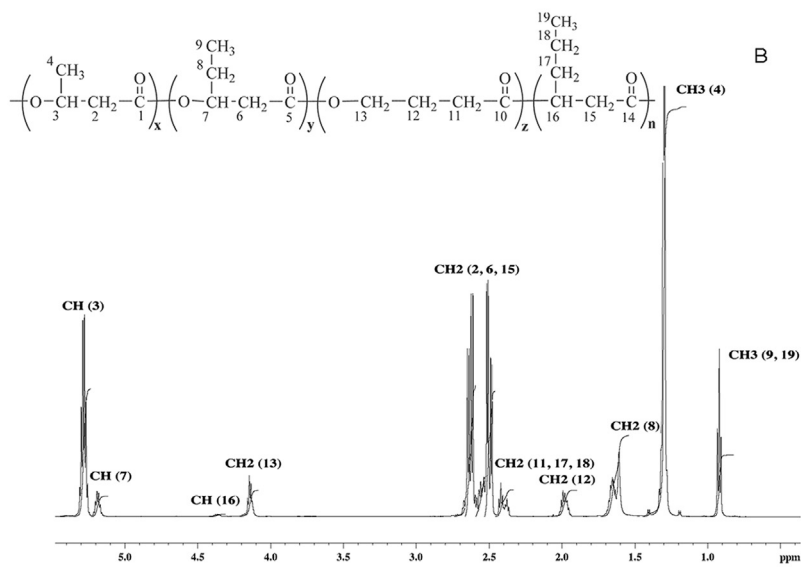
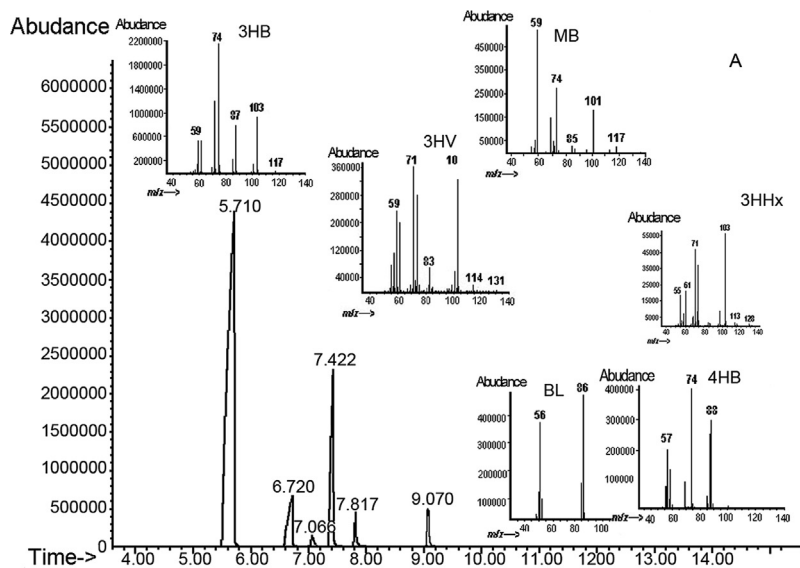
One of the most important macroscopic parameters characterizing polymer properties is molecular weight, which determines the processability of the material. PHAs produced by microbial synthesis show higher molecular weights than chemically synthesized PHAs [24]. PHA molecular weight is a variable parameter, depending on physiological and biochemical properties of the PHA producing strain, the conditions of carbon nutrition, and the method of polymer recovery. For instance, the number average molecular weight ( $M_n$ ) of P(3HB) synthesized by *Comamonas acidovorans* is no more than 58 kDa [25] while the  $M_n$  of the polymer synthesized by recombinant strain *Ralstonia eutropha* PHB-4 is 1580 kDa [26]. The  $M_n$  values of the P(3HB/4HB) also vary widely, but its average  $M_n$  is lower than that of the P(3HB) homopolymer. The  $M_n$  values of P(3HB/4HB) containing 23–24 mol.% 4HB reported by different authors range between 104 and 590 kDa [27,28]. Similar values are reported for P(3HB/3HV) and P(HB/3HHx). Results of examining molecular-weight properties of PHAs are given in Table 1.  $M_n$  values varied between 72 and 223 kDa, being 1.5–5.0 times lower than the  $M_n$  values of P(3HB). The sample with the highest total content of 3HV/4HB/3HHx monomers (36.5 mol.%) (No. 7) showed the lowest  $M_n$  and  $M_w$  values (72 and 437 kDa, respectively). The  $M_n$  and  $M_w$  of the polymers with lower molar fractions of 4HB and 3HHx were higher. The effect of 3HV monomers on the temperature parameters was weaker: the polymer with the highest molar fraction of 3HV (24.6 mol.%) and low 4HB and 3HHx (sample No. 6) had the  $M_w$  of 814 kDa, which was comparable with the  $M_w$  of P(3HB). The polymers with higher molar fractions of 3HV, 4HB, and 3HHx also showed higher polydispersity, ranging between 3.03 and 6.07 versus 2.52 in P(3HB). The higher  $\bar{D}$  values suggested heterogeneity of the fragments of polymer carbon chains. The decrease in the  $M_w$  of quaterpolymers is consistent with the data on PHA bi- [21,27,28] and terpolymers, with the most substantial decrease in  $M_w$  detected in the PHAs containing MCL monomers 3HHx and 3HO [10,29].

Important properties of PHAs are their melting temperature and thermal degradation temperature. For quaterpolymers, thermograms were taken within a wide temperature range, including glass transition temperature ( $T_g$ ), crystallization temperature ( $T_c$ ), melting point ( $T_{melt}$ ) and thermal degradation temperature ( $T_{degr}$ ) (Table 1). The melting temperature and thermal degradation temperature of the samples were 166–173 and 259–286°C, respectively, and these values were insignificantly lower than those of P(3HB). No relationship was found between  $T_{melt}$  and  $T_{degr}$  and PHA composition. A decrease in the melting temperature (to 53–54 °C) was also observed for PHA quaterpolymers with another composition, which contained medium-chain-length monomers with even numbers of carbon atoms ( $C_6$ ,  $C_8$ ,  $C_{10}$ ,  $C_{12}$ ,  $C_{14}$ ) [10]. It is

**Table 1**  
Physicochemical properties of films of PHA quaterpolymers with different molar fractions of monomer units.

Specimen no.	PHA composition, mol.%				$T_g$ , °C	$T_c$ , °C	$T_{melt}$ , °C	$T_{degr}$ , °C	$C_x$ , %	$M_n$ , kDa	$M_w$ , kDa	$\bar{D}$
	3HB	3HV	4HB	3HHx								
P(3HB)	100.0	0.0	0.0	0.0	–	–	178	295	76	365	920	2.52
1	93.1	1.1	5.4	0.4	–4.3	53.7	166	259	42	102	476	4.67
2	89.7	4.9	2.9	2.5	–0.7	52.8	168	284	42	178	787	4.42
3	79.5	4.5	15.6	0.4	–	–	169	285	45	183	554	3.03
4	77.5	19.6	2.4	0.5	–1.3	68.7	171	284	37	223	817	3.66
5	74.7	17.8	3.7	3.8	–2.3	55.7	173	270	37	126	542	4.30
6	70.6	24.6	4.3	0.5	–3.7	70.0	169	272	34	139	814	5.85
7	63.5	19.4	12.3	4.8	–8.6	84.0	168	286	30	72	437	6.07

– peak is absent.



important that although the melting temperature and the thermal degradation temperature decreased in all PHA specimens, the interval between these two parameters remained almost the same. Thus, the PHAs with different monomer compositions all exhibited thermoplasticity – one of the most important properties of PHAs. The composition of the quaterpolymers did not exert any significant effect on their temperature properties. For instance, the  $T_{melt}$  values of the specimens with the lowest (63.5 mol.%) and highest (93.1 mol.%) molar fractions of 3HB, i.e. with the highest (36.5 mol.%) and lowest (6.9 mol.%) molar fractions of the other three monomers (3HB, 3HV, 3HHx), were very similar – 166 and 168 °C (samples No. 1 and 7). The  $T_{melt}$  of the polymer containing the highest molar fraction of 3HB (sample No. 6) was 169 °C. The glass transition temperature of the quaterpolymers ranged between –8.6 and –0.7 °C. The thermogram of P(3HB) did not show a  $T_g$  peak (Suppl. Fig. 1A). According to the literature data, the  $T_g$  of P(3HB) may vary between –3 and 5 °C [30,31] and the  $T_g$  of PHA bipolymers may vary between –1 and –48 °C, depending on the type and molar fraction of the second monomer (4HB, 3HV, 3HO, 3HHx) [1,32]. Kato et al. [33] showed that the  $T_g$  and  $M_w$ , of the MCL-PHAs consisting of monomers with the carbon chains comprising between 6 and 12 carbon atoms could vary by a factor of almost 10. There has been a lot of controversy in the literature about the temperature properties of PHAs. The  $T_{melt}$  for P(3HB) reported in the literature ranges between 162 °C [34] and 197 °C [35]. The data reported on the  $T_{melt}$  of P(3HB/3HV) copolymers are contradictory. For instance, the  $T_{melt}$  of a P(3HB/3HV) copolymer with 6 mol.% 3HV was determined as 186 °C [35], but in a study by Zhao and Chen, the copolymer of a similar composition had the  $T_{melt}$  of 156 °C [34], while Zhang et al. reported the value of 170 °C [36]. The  $T_{melt}$  of the copolymers with 20 mol.% 3HV was 114 °C as reported in a study by Avella et al. [37] but 145 °C as measured by Tsuge [38]. The data on the  $T_{melt}$  of other PHA types are also inconsistent. According to the literature data, the  $T_{melt}$  of P(3HB/4HB) containing between 2 and 7 mol.% 4HB may range between 114 and 172 °C. As the molar fraction of 4HB was increased to 75–100%, the  $T_{melt}$  dropped to 40–54 °C [28,30,39]. Another study, however, showed that the  $T_{melt}$  of P(3HB/4HB) containing 84 mol.% 4HB was at least 130 °C [40]. Similar differences between the data and the trend to a decrease in  $T_{melt}$  and  $T_{degr}$  were reported for PHA terpolymers [19,20,36].

The monomer composition of the quaterpolymers exerted the most significant effect on their degrees of crystallinity (Table 1 and Suppl. Fig. 1B), which were generally considerably lower (under 50%) than the degree of crystallinity of P(3HB) (76%). Sample No. 7, which had the lowest molar fraction of 3HB (63.5 mol.%) and the highest total content of 4HB, 3HV, and 3HHx (36.5 mol.%), had the lowest  $C_x$  (30%). The polymers with the low total content of these three monomers (between 6.9 and 20.5 mol.%) had the highest  $C_x$  (42–45%, Samples No. 1–3). Thus, as the total proportion of the monomer units other than 3HB increased, the  $C_x$  value dropped, and the most substantial decrease, to 30 and 34%, was observed in the polymers in which 3HV, 4HB, and 3HHx totaled about 29.4 and 36.5 mol.%. Hence, in all the PHA quaterpolymers, the crystalline phase decreased and the amorphous, disordered regions increased, indicating higher processing ability of the material. Literature data on the degree of crystallinity of PHA copolymers are limited and rather contradictory. PHA copolymers differ from P(3HB) and between each other in crystallization kinetics, which is largely determined by their monomer composition. For instance, P(3HB/

3HV) copolymers are isodimorphous due to co-crystallization of 3HB and 3HV monomer units; the presence of 3HB influences the rate of crystallization and the size of the spherulites. If the 3HV molar fraction is lower than 35–40 mol.%, 3HV monomers can crystallize in the 3HB lattice; if the 3HV molar fraction is higher than 40 mol.%, 3HB monomers can crystallize in the 3HV lattice [41]. P(3HB/4HB) copolymers with low molar fractions of 4HB (below 29 mol.%) have the P(3HB)-type crystalline lattice; only at high 4HB monomer concentrations (78–100 mol.%), the P(4HB)-type lattice was observed [42]. The 3HHx monomers do not significantly influence the crystallographic parameters of P(3HB/3HHx), as 3HHx monomers are not incorporated in the P(3HB) lattice, but as the molar fraction of 3HHx increases, the rates of crystallization and crystal growth decrease dramatically [43,44]. The authors of those studies suggested that 3HHx monomers were not crystallized into the lattice but were located on it. The available data on the  $C_x$  relationship to the PHA composition, especially to the composition of PHA copolymers, are fragmentary. Even the  $C_x$  of P(3HB) reported by different authors varies between 59 and 86% [25,45,46]. Noda et al. [47] showed that the presence of a 20–22% 3HV molar fraction exerted very little influence on the degree of crystallinity of the copolymer, but Dai et al. [48] reported that the degree of crystallinity of the copolymer containing 29 and 32 mol.% 3HV dropped dramatically (to 5 and 9%, respectively). Even fewer data are available on the degree of crystallinity of 3HB/3HHx copolymers, and they are also contradictory. In their study, Noda et al. showed that the copolymer containing 12–18 mol.% 3HHx had the degree of crystallinity of 38–40% [47], while Fukui et al. reported the same degree of crystallinity for the copolymer that contained a much smaller fraction of 3HHx (1.5 mol.%) [49]. The lowest  $C_x$  values were reported for PHA bipolymers ( $C_x$  9–20%) and terpolymers ( $C_x$  30–50%) containing 4HB monomer units [20,25,50].

### 3.2. Physical/mechanical properties of PHA quaterpolymers

In order to investigate physical/mechanical properties of the PHAs, dense smooth films were prepared from the chloroform solutions of PHAs. The films differed considerably in their mechanical strength (Table 2). The physical/mechanical properties, namely, the ratio between strength and elasticity parameters, of quaterpolymers differed considerably from those of P(3HB). At the same time, one of the parameters of mechanical strength (tensile strength) was comparable with the tensile strength of P(3HB) in 5 out of 7 samples, and only in 2 samples, it was lower by a factor of almost two. The difference was more noticeable in Young's modulus, which was the lowest (102.47 MPa) in the 3HB/3HV/4HB/3HHx = 63.5/19.4/12.3/4.8 polymer – one with the lowest 3HB molar fraction (63.5 mol.%) and the highest total content of the other three monomers (36.5 mol.%). The specimens with a somewhat higher 3HB fraction, which varied between 70.6 and 79.5 mol.%, and the lower total content of the other three monomers (between 20.5 and 29.4 mol.%), showed similar values of Young's modulus – 337.01–352.59 MPa. That was 6 times lower than Young's modulus of P(3HB). Elongation at break (an indicator of elasticity) was considerably higher in all copolymer specimens. The highest values reached 103.21–113.32% and the lowest 6.34–16.68%, i.e. they were 40–80 and 3–8 times higher, respectively, than elongation at break of P(3HB). Having reviewed the available literature, we did not find any data on strength parameters of PHA copolymers of the same composition. There are data,

**Fig. 1.** Ion chromatogram (A) with mass spectra (with retention times: methyl-3-hydroxybutyrate (3HB) – 5.710; butyrolactone (BL) – 6.720; methyl-4-methoxybutyrate (MB) – 7.066; methyl-3-hydroxyvalerate (3HV) – 7.422; methyl-4-hydroxybutyrate (4HB) – 7.817; methyl-3-hydroxyhexanoate – 9.070 min),  $^1\text{H}$  NMR spectrum (B) and  $^{13}\text{C}$  NMR spectrum (C) of quaterpolymer P(3HB/3HV/4HB/3HHx) (63.5/19.4/12.3/4.8 mol.%).

**Table 2**  
Physical/mechanical properties of films of PHA quaterpolymers with different molar fractions of monomer units (numbers according to Table 1).

Specimen no.	Tensile strength, MPa	Young's modulus, MPa	Elongation at break, %	Ra – arithmetic mean surface roughness, nm	Rq – root mean square roughness, nm
P(3HB)	16.70 ± 1.52	2071.20 ± 352.50	2.50 ± 0.35	71.749	80.283
1	13.35 ± 0.97	665.71 ± 72.45	18.68 ± 2.45	–	–
2	21.72 ± 2.84	1083.87 ± 98.26	6.34 ± 0.70	–	–
3	7.51 ± 0.85	346.73 ± 37.83	37.75 ± 4.80	158.257	198.504
4	17.16 ± 2.10	906.03 ± 79.50	13.67 ± 1.55	–	–
5	14.29 ± 1.71	337.01 ± 42.66	113.32 ± 9.35	189.619	244.497
6	11.04 ± 1.23	352.59 ± 39.23	56.25 ± 6.20	305.577	375.110
7	7.12 ± 1.04	102.47 ± 10.50	103.21 ± 11.60	120.908	157.535

– not measured.

however, suggesting that incorporation of 3HHx and/or 4HB monomer units into the carbon chain of 3-hydroxybutyrate increased elasticity but decreased mechanical strength of polymer products fabricated from PHA [6,19,20,28]. Thus, the quaterpolymers produced in this study are comparable with P(3HB) in some physicochemical properties, are inferior to P(3HB) in mechanical strength, and are superior to P(3HB) in elasticity.

### 3.3. Morphology and characterization of the surface of the films prepared from PHA quaterpolymers

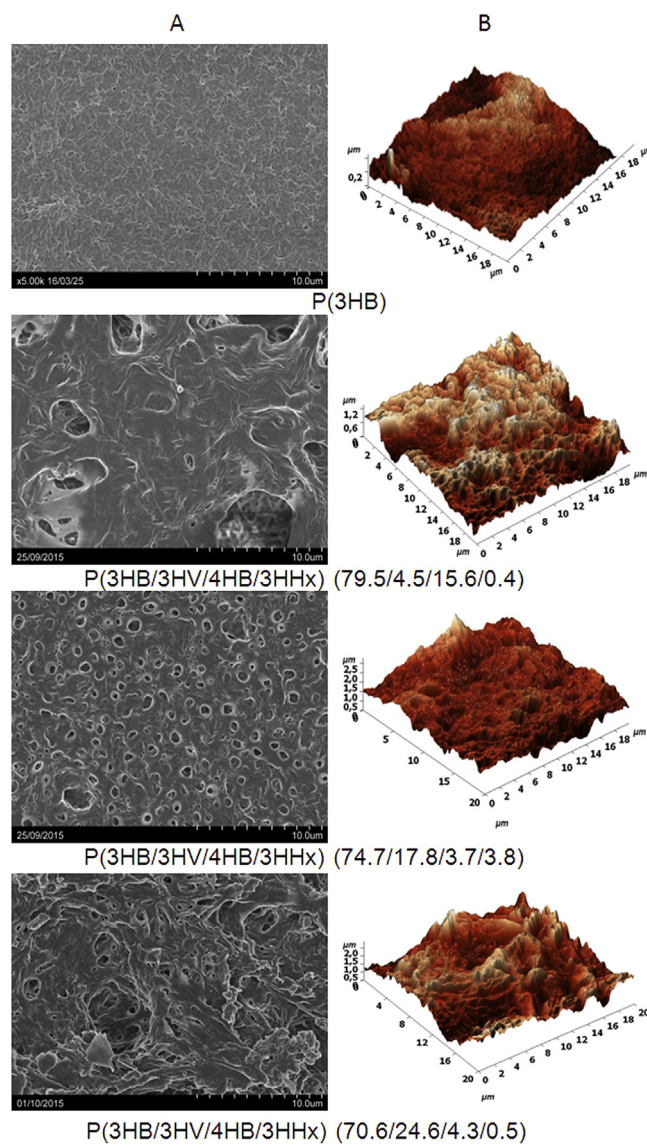
SEM and AFM images of the surfaces of PHA specimens prepared from PHAs with different chemical compositions that had dissimilar physicochemical properties are shown in Fig. 2. The surface of the films prepared from quaterpolymers [P(3HB/3HV/4HB/3HHx)] was rougher than the surface of P(3HB) films, with numerous pores of different diameters (1–6 μm) (Fig. 2A). As the molar fraction of 4HB was increased, the pores became more numerous and of larger size (between 3 and 6 μm); their shape and size became more diverse.

Analysis of the atomic-force microscopy images (Fig. 2B) suggested that the root mean square roughness (Rq) of all copolymer films was 2.0–4.7 times higher than the Rq of P(3HB) films (Table 2). The highest value of Rq (375.110 nm) was determined for the P(3HB/3HV/4HB/3HHx) = 70.6/24.6/4.3/0.5 (mol.%) specimen. The surface roughness of the films used as cell scaffolds may determine cell attachment, spreading, and motility; it may also influence the synthesis of specific proteins. However, while some data suggest that cells are attached better to rough surfaces than to polished ones, other data state that changes in roughness are not accompanied by any cellular effects [51]. In a previous study, we observed considerable differences between the roughness of the films of PHAs consisting of 3HB/3HV/4HB or 3HB/3HV/3HHx monomers and the roughness of P(3HB) films, but we did not reveal any direct relationship of the roughness to the monomer composition [20].

An important parameter indirectly characterizing biocompatibility and influencing cell attachment and viability is the hydrophilic/hydrophobic balance of the surface [52]. It is evaluated by measuring contact angles for water or diiodomethane. Results of these measurements are used to determine surface energy and polar and dispersive components of surface free energy. The water contact angle for quaterpolymers varied between 87.62 and 102.52° and was generally close to the water contact angle for P(3HB) films (97.42°); the other parameters of the homopolymer and quaterpolymers films were similar, too (Table 3).

### 3.4. Cytotoxicity assay of films of PHA tetrapolymers with different molar fractions of monomer units

Biological properties of PHA films (their adhesive properties and



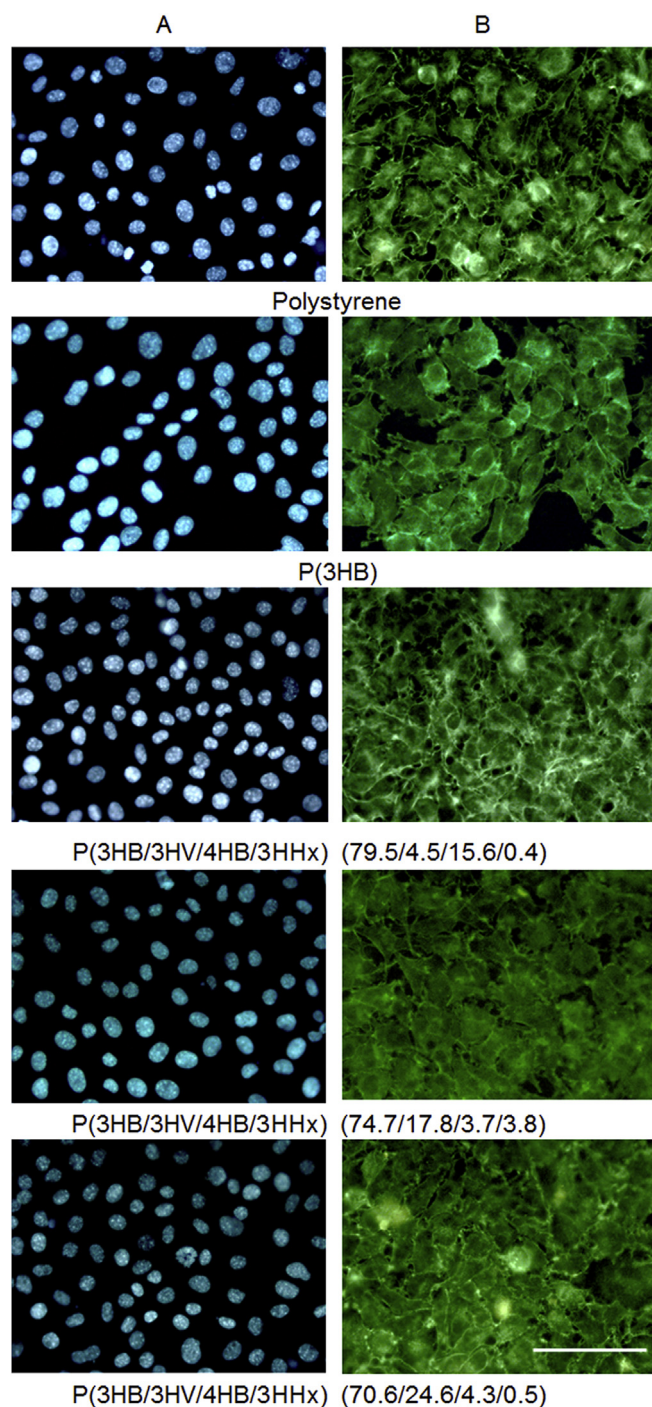
**Fig. 2.** SEM (A) and AFM (B) images of the films prepared from PHA quaterpolymers with different molar fractions of monomers (bar = 10 μm).

ability to facilitate cell proliferation) were studied in the culture of NIH 3T3 mouse fibroblast cells. MTT assay showed that none of the PHA specimens produced any cytotoxic effect in direct contact testing. The fibroblasts attached to the surface of the polymer films retained their normal morphology, were metabolically active, and proliferated without any toxic changes during the entire observation period. After 24 h of cultivation, cell counts were comparable

**Table 3**

Surface properties of films of PHA quaterpolymers with different molar fractions of monomer units (numbers according to Table 1).

Specimen no.	Water contact angle, $\theta$ , °	Diiodomethane contact angle, $\theta$ , °	Surface free energy, erg/cm <sup>2</sup>	Polar component of surface free energy, erg/cm <sup>2</sup>
P(3HB)	97.42 ± 2.63	58.52 ± 1.44	30.43 ± 1.01	1.23 ± 0.18
1	100.10 ± 2.64	56.86 ± 1.73	30.96 ± 0.61	0.60 ± 0.12
2	102.52 ± 2.54	51.27 ± 1.25	33.67 ± 1.36	0.30 ± 0.87
3	87.62 ± 1.91	49.94 ± 1.25	36.80 ± 1.21	2.56 ± 0.23
4	100.62 ± 2.42	57.15 ± 1.14	30.75 ± 0.72	0.55 ± 0.54
5	101.06 ± 2.43	44.18 ± 1.10	37.80 ± 0.68	0.12 ± 0.42
6	96.50 ± 1.33	53.76 ± 1.37	33.04 ± 0.55	0.88 ± 0.36
7	97.98 ± 1.91	67.56 ± 2.02	25.82 ± 1.25	1.58 ± 0.42

**Fig. 3.** Morphology of NIH 3T3 fibroblast cells cultivated on films of PHA quaterpolymers with different proportions of monomers (Day 3): DAPI (A) and FITC (B) staining. Bar = 50  $\mu$ m.

on all films of PHA copolymers, polystyrene (control), and on P(3HB) films (Suppl. Fig. 2). Some differences in the number of viable cells were recorded at Day 3, but they were not statistically significant.

Results of investigating the morphology of fibroblasts cultivated on the PHA films by using fluorescent dyes – a nuclear DNA marker (DAPI) and a cytoplasm marker (FITC) – were consistent with results of MTT assay. After three days of cultivation, more than 80% of the surface of each film made of PHA tetrapolymers was covered by a confluent monolayer of cells (Fig. 3).

#### 4. Conclusion

In this work, we investigated PHA quaterpolymers composed of different fractions of the short-chain-length 3-hydroxybutyrate, 4-hydroxybutyrate, and 3-hydroxyvalerate and the medium-chain-length 3-hydroxyhexanoate. Physicochemical, physical/mechanical, and biological properties of the quaterpolymers in which the total content of the three monomers other than the major 3-hydroxybutyrate varied between 6.9 and 36.5 mol.% were investigated. The properties of PHA quaterpolymers were significantly different from those of the P(3HB) homopolymer: they had much lower degrees of crystallinity (reaching 30–45%) and lower melting points and thermal decomposition temperatures, with the interval between these temperatures remaining practically unchanged.

#### Acknowledgement

The research was supported by the state budget allocated to the fundamental research at the Russian Academy of Sciences (project No. 01201351505).

#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.polymer.2016.08.048>.

#### References

- [1] K. Sudesh, H. Abe, Y. Doi, Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters, *Prog. Polym. Sci.* 25 (2000) 1503–1555.
- [2] S. Chanprateep, Current trends in biodegradable polyhydroxyalkanoates, *J. Biosci. Bioeng.* 110 (2010) 621–632.
- [3] G.-Q. Chen, Industrial production of PHA, in: G.-Q. Chen, A. Steinbüchel (Eds.), *Plastics from Bacteria. Natural Functions and Applications*, Springer-Verlag, Berlin Heidelberg, 2010, pp. 121–132.
- [4] T.G. Volova, E.I. Shishatskaya, A.J. Sinskey, *Degradable Polymers: Production, Properties and Applications*, Nova Science Publishers, New York, 2013.
- [5] A. Steinbüchel, H.E. Valentin, Diversity of bacterial polyhydroxyalkanoic acids, *FEMS Microbiol. Lett.* 128 (1995) 219–228.
- [6] B. Laycock, P. Halley, S. Pratt, A. Werker, P. Lant, The chemomechanical properties of microbial polyhydroxyalkanoates, *Prog. Polym. Sci.* 38 (2013) 536–583.
- [7] A.K. Singh, N. Mallick, Enhanced production of SCL-LCL-PHA co-polymer by sludge-isolated *Pseudomonas aeruginosa* MTCC 7925, *Lett. Appl. Microbiol.* 46 (2008) 350–357.

- [8] K. Bhubalan, W.-H. Lee, C.-Y. Loo, T. Yamamoto, T. Tsuge, Y. Doi, K. Sudesh, Controlled biosynthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate) from mixtures of palm kernel oil and 3HV-precursors, *Polym. Degrad. Stab.* 93 (2008) 17–23.
- [9] S. Chanprateep, Y. Kotakura, S. Visetkoop, H. Shimizu, S. Kulpreecha, S. Shioya, Characterization of new isolated *Ralstonia eutropha* strain A-04 and kinetic study of biodegradable copolyester poly(3-hydroxybutyrate-co-4-hydroxybutyrate) production, *J. Ind. Microbiol. Biotechnol.* 35 (2008) 1205–1215.
- [10] I.K.P. Tan, K. Sudesh Kumar, M. Theanmalar, S.N. Gan, B. Gordon III, Saponified palm kernel oil and its major free fatty acids as carbon substrates for the production of polyhydroxyalkanoates in *Pseudomonas putida* PGA1, *Appl. Microbiol. Biotechnol.* 47 (1997) 207–211.
- [11] H. Abe, Y. Doi, H. Aoki, T. Akehata, Solid-state structures and enzymatic degradability for melt-crystallized films of copolymers of (R)-3-hydroxybutyric acid with different hydroxyalkanoic acids, *Macromolecules* 31 (1998) 1791–1797.
- [12] T. Fukui, Y. Doi, Efficient production of polyhydroxyalkanoates from plants oils by *Alcaligenes eutrophus* and recombinant strain, *Appl. Microbiol. Biotechnol.* 49 (1998) 333–336.
- [13] H. Matsusaki, H. Abe, K. Taguchi, T. Fukui, Y. Doi, Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyalkanoates) by recombinant bacteria expressing the PHA synthase gene *phC1* from *Pseudomonas* sp. 61-3, *Appl. Microbiol. Biotechnol.* 53 (2000) 401–409.
- [14] P.R. Green, J. Kemper, L. Schechtman, L. Guo, M. Satkowski, S. Fiedler, A. Steinbüchel, B.H.A. Rehm, Formation of short chain length/medium chain length polyhydroxyalkanoate copolymers by fatty acid  $\beta$ -oxidation inhibited *Ralstonia eutropha*, *Biomacromolecules* 3 (2002) 208–213.
- [15] A.K. Singh, N. Mallick, Exploitation of inexpensive substrates for production of a novel SCL-LCL-PHA co-polymer by *Pseudomonas aeruginosa* MTCC 7925, *J. Ind. Microbiol. Biotechnol.* 36 (2009) 347–354.
- [16] A. Rathinasabapathy, B.A. Ramsay, J.A. Ramsay, F. Pérez-Guevara, A feeding strategy for incorporation of canola derived medium-chain-length monomers into the PHA produced by wild-type *Cupriavidus necator*, *World J. Microbiol. Biotechnol.* 30 (2014) 1409–1416.
- [17] S. Mizuno, S. Katsumata, A. Hiroe, T. Tsuge, Biosynthesis and thermal characterization of polyhydroxyalkanoates bearing phenyl and phenylalkyl side groups, *Polym. Degrad. Stab.* 109 (2014) 379–384.
- [18] T.G. Volova, G.S. Kalacheva, A. Steinbüchel, Biosynthesis multi-component polyhydroxyalkanoates by the bacterium *Wautersia eutropha*, *Macromol. Symp.* 269 (2008) 1–7.
- [19] T. Volova, E. Kiselev, E. Shishatskaya, N. Zhila, A. Boyandin, D. Syrvacheva, O. Vinogradova, G. Kalacheva, A. Vasiliev, I. Peterson, Cell growth and PHA accumulation from CO<sub>2</sub> and H<sub>2</sub> of a hydrogen-oxidizing bacterium, *Cupriavidus eutrophus* B-10646, *Bioresour. Technol.* 146 (2013) 215–222.
- [20] T.G. Volova, E.G. Kiselev, O.N. Vinogradova, E.D. Nikolaeva, A.A. Chistyakov, A.G. Sukovaty, E.I. Shishatskaya, A glucose-utilizing strain, *Cupriavidus eutrophus* B-10646: growth kinetics, characterization and synthesis of multicomponent PHAs, *PLoS One* 9 (2014) 1–15.
- [21] T.G. Volova, D.A. Syrvacheva, N.O. Zhila, A.G. Sukovaty, Synthesis of P(3HB-co-3HHx) copolymers containing high molar fraction of 3-hydroxyhexanoate monomer by *Cupriavidus eutrophus* B10646, *J. Chem. Technol. Biotechnol.* 91 (2016) 416–425.
- [22] K.-H. Chia, T.-F. Ooi, A. Saika, T. Tsuge, K. Sudesh, Biosynthesis and characterization of novel polyhydroxyalkanoate polymers with high elastic property by *Cupriavidus necator* PHB<sup>4</sup> transformant, *Polym. Degrad. Stab.* 95 (2010) 2226–2232.
- [23] T.M.F. Azira, A.A. Nursolehah, Y. Norhayati, M.I.A. Majid, A.A. Amirul, Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-4-hydroxybutyrate) terpolymer by *Cupriavidus* sp. USMAA2-4 through two-step cultivation process, *World J. Microbiol. Biotechnol.* 27 (2011) 2287–2295.
- [24] G.-Q. Chen, Plastics completely synthesized by bacteria: polyhydroxyalkanoates, in: G.-Q. Chen, A. Steinbüchel (Eds.), *Plastics from Bacteria. Natural Functions and Applications*, Springer-Verlag, Berlin Heidelberg, 2010, pp. 17–37.
- [25] H. Mitomo, W.-C. Hsieh, K. Nishiwaki, K. Kasuya, Y. Doi, Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) produced by *Comamonas acidovorans*, *Polymer* 42 (2001) 3455–3461.
- [26] N. Tanadchangsang, A. Kitagawa, T. Yamamoto, H. Abe, T. Tsuge, Identification, biosynthesis, and characterization of polyhydroxyalkanoate copolymer consisting of 3-hydroxybutyrate and 3-hydroxy-4-methylvalerate, *Biomacromolecules* 10 (2009) 2866–2874.
- [27] S. Chanprateep, K. Buasri, A. Muangwong, P. Utiswannakul, Biosynthesis and biocompatibility of biodegradable poly(3-hydroxybutyrate-co-4-hydroxybutyrate), *Polym. Degrad. Stab.* 95 (2010) 2003–2012.
- [28] S. Vigneswari, S. Vijaya, M.I.A. Majid, K. Sudesh, C.S. Sipaut, M.N.M. Azizan, A.A. Amirul, Enhanced production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) copolymer with manipulated variables and their properties, *J. Ind. Microb. Biotechnol.* 36 (2009) 547–556.
- [29] Y.-M. Wong, C.J. Brigham, C.K. Rha, A.J. Sinskey, K. Sudesh, Biosynthesis and characterization of polyhydroxyalkanoate containing high 3-hydroxyhexanoate monomer fraction from crude palm kernel oil by recombinant *Cupriavidus necator*, *Biores. Technol.* 121 (2012) 320–327.
- [30] H.-H. Wang, X.-R. Zhou, Q. Liu, G.-Q. Chen, Biosynthesis of polyhydroxyalkanoates homopolymers by *Pseudomonas putida*, *Appl. Microbiol. Biotechnol.* 89 (2011) 1497–1507.
- [31] W.P. Xie, G.-Q. Chen, Production and characterization of terpolyesterpoly(3-hydroxybutyrate-co-4-hydroxybutyrate-co-3-hydroxyhexanoate) by recombinant *Aeromonas hydrophila* 4AK4 harboring genes *phaPCJ*, *Biochem. Eng. J.* 38 (2008) 384–389.
- [32] L.L. Madison, G.W. Huisman, Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic, *Microbiol. Mol. Biol. Rev.* 63 (1999) 21–53.
- [33] M. Kato, H.J. Bao, C.-K. Kang, T. Fukui, Y. Doi, Production of a novel copolyester of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids by *Pseudomonas* sp. 61-3 from sugars, *Appl. Microbiol. Biotechnol.* 45 (1996) 363–370.
- [34] W. Zhao, G.Q. Chen, Production and characterization of terpolyester poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate) by recombinant *Aeromonas hydrophila* 4AK4 harboring genes *phaAB*, *Process. Biochem.* 42 (2007) 1342–1347.
- [35] S. Akhtar, C.W. Pouton, L.J. Notarianni, Crystallization behaviour and drug release from bacterial polyhydroxyalkanoates, *Polymer* 33 (1992) 117–126.
- [36] H.F. Zhang, L. Ma, Z.H. Wang, G.Q. Chen, Biosynthesis and characterization of 3-hydroxyalkanoate terpolyesters with adjustable properties by *Aeromonas hydrophila*, *Biotechnol. Bioeng.* 104 (2009) 582–589.
- [37] M. Avella, E. Martuscelli, M. Raimo, Properties of blends and composites based on poly(3-hydroxybutyrate) (P3HB) and poly(3-hydroxybutyrate-hydroxyvalerate) (PHBV) copolymers, *J. Mater. Sci.* 35 (2000) 523–545.
- [38] T. Tsuge, Metabolic improvements and use of inexpensive carbon sources in microbial production of polyhydroxyalkanoates, *J. Biosci. Bioeng.* 94 (2002) 579–584.
- [39] S. Chanprateep, S. Kulpreecha, Production and characterization of biodegradable terpolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-4-hydroxybutyrate) by *Alcaligenes* sp. A-04, *J. Biosci. Bioeng.* 101 (2006) 51–56.
- [40] Y. Doi, A. Segawa, M. Kunioka, Biosynthesis and characterization of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in *Alcaligenes eutrophus*, *Int. J. Biol. Macromol.* 12 (1990) 106–111.
- [41] M. Scandola, G. Ceccorulli, M. Pizzoli, M. Gazzano, Study of the crystal phase and crystallization rate of bacterial poly( $\beta$ -hydroxybutyrate-co- $\beta$ -hydroxyvalerate), *Macromolecules* 25 (1992) 1405–1410.
- [42] Y. Saito, Y. Doi, Microbial synthesis and properties of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in *Comamonas acidovorans*, *Int. J. Biol. Macromol.* 16 (1994) 99–104.
- [43] T. Iwata, Y. Doi, Crystals structure and biodegradation of aliphatic polyesters crystals, *Macromolecules* 30 (1997) 5290–5296.
- [44] T. Iwata, Y. Doi, S. Nakayama, H. Sasatsuki, S. Teramachi, Structure and enzymatic degradation of poly(3-hydroxybutyrate) copolymer single crystals with an extracellular PHB depolymerase from *Alcaligenes faecalis* T1, *Int. J. Biol. Macromol.* 25 (1999) 169–176.
- [45] S. Nakamura, Y. Doi, M. Scandola, Microbial synthesis and characterization of poly(3-hydroxybutyrate-co-4-hydroxybutyrate), *Macromolecules* 25 (1992) 4237–4241.
- [46] R. Luo, J. Chen, L. Zhang, G. Chen, Polyhydroxyalkanoate copolymers produced by *Ralstonia eutropha* PHB4 harboring a low-substrate-specificity PHA synthase *PhaC2<sub>ps</sub>* from *Pseudomonas stutzeri* 1317, *Biochem. Eng. J.* 32 (2006) 218–225.
- [47] I. Noda, P.R. Green, M.M. Satkowski, L.A. Schechtman, Preparation and properties of a novel class of polyhydroxyalkanoate copolymers, *Biomacromolecules* 6 (2005) 580–586.
- [48] Yu Dai, Z. Yuan, K. Jack, J. Keller, Production of targeted poly(3-hydroxyalkanoates) copolymers by glycogen accumulating organisms using acetate as sole carbon source, *J. Biotechnol.* 129 (2007) 489–497.
- [49] T. Fukui, H. Abe, Y. Doi, Engineering of *Ralstonia eutropha* for production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from fructose and solid-state properties of the copolymer, *Biomacromolecules* 3 (2002) 618–624.
- [50] T.G. Volova, N.O. Zhila, E.I. Shishatskaya, P.V. Mironov, A.D. Vasil'ev, A.G. Sukovaty, A.J. Sinskey, The Physicochemical properties of polyhydroxyalkanoates with different chemical structures, *Polym. Sci. Ser. A* 55 (2013) 427–437.
- [51] S.P. Xavier, P.S.P. Carvalho, M.M. Beloti, A.L. Rosa, Response of rat bone marrow cells to commercially pure titanium submitted to different surface treatments, *J. Dent.* 31 (2003) 173–180.
- [52] W. Ou, H. Qiu, Z. Chen, K. Xu, Biodegradable block poly(ester-urethane)s based on poly(3-hydroxybutyrate-co-4-hydroxybutyrate) copolymers, *Biomaterials* 32 (2011) 3178–3188.