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

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## Abstract

*Cupriavidus eutrophus* B10646 was used to synthesize a series of polyhydroxyalkanoates (PHA) tetrapolymers 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 copolymers 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 tetrapolymers 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. Films prepared from PHA tetrapolymers were rougher and more porous than P(3HB) films; they showed higher values of elongation at break (up to 100-200%), i.e. were more elastic. Films prepared from PHA tetrapolymers were biocompatible and had no toxic effect on mouse fibroblast NIH 3T3 cells.

*Keywords: polyhydroxyalkanoates, tetrapolymers, physicochemical and mechanical properties, fibroblast cells, MTT assay, viability*

## 1. Introduction

Polymers of hydroxy derived alkanoic 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. 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. 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 [5-6]. 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 tetrapolymers are limited. Tan et al. [7] 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

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with even numbered carbon chains ($C_6$, $C_8$, $C_{10}$, $C_{12}$, $C_{14}$). Other authors [8-10] 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. In a relatively recent study by Mizuno et al. [11], recombinant *Ralstonia eutropha* PHB-4 carrying synthase gene from *Pseudomonas* sp. (PhaC1Ps) was used to synthesize PHA tetrapolymer that was mainly constituted by 3HB monomers (92-99 mol.%) and minor fractions of 3HV (0.7-3.0), 3H4MV (0.3-0.6), and 3H3PhP (4.2-12.2 mol.%) from the complex carbon substrate that contained such precursors as 3-hydroxy-3-phenilpropionic acid (3P3PhP), 3-phenilpropionic acid (3PhP), cinnamic acid (CA), 5-phenilvaleric acid (5PhV), and 6-phenilhexanoic acid (6PhHx). Those PHAs had lower melting temperature, enthalpy of fusion, and number average molecular weight than P(3HB).

In a previous study conducted by our team, two wild-type strains (*Wautersia eutropha* H16 and B5786) were grown under autotrophic conditions on $CO_2$ 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.% [12]. The properties of those PHAs varied depending on the molar fractions of the monomers: $C_x$ between 49 and 71 %; $T_m$ and $T_d$ between 146 and 168 and between 210 and 268°C, respectively.

The purpose of the present study was to investigate properties of PHA tetrapolymers containing short- and medium-chain-length monomers.

**2. Experimental**

2.1. Materials

1    Samples of PHA were synthesized at the Institute of Biophysics of the Russian Academy

2    of Sciences. PHA tetrapolymers containing short-chain-length and medium-chain-length

3    monomers were synthesized using *Cupriavidus eutrophus* B10646. PHA synthesis was

4    performed on the basis of previously data obtained on the physiological effect of toxic precursor

5    substrates (γ-butyrolactone; valeric acid, hexanoic acid) and the effect of their concentrations on

6    specific growth rate of bacteria, cell biomass yield and total yield of PHA [13-15].

7

8    2.2. Analysis of PHA structure

9    Polymer was extracted with chloroform and then precipitated with hexane. Composition

10   of extracted polymer samples was analyzed with a GC-MS (6890/5975C, Agilent Technologies,

11   U.S.). $^1$H NMR spectra of copolymer were recorded at room temperature in $CDCl_3$ on a

12   BRUKER AVANCE III 600 spectrometer operating at 600.13 MHz.

13

14   2.3. Analysis of physicochemical properties of PHA

15   Molecular weight and molecular-weight distribution of PHAs were examined using a gel

16   permeation chromatograph (Agilent Technologies 1260 Infinity, U.S.) with a refractive index

17   detector, using an Agilent PLgel Mixed-C column.

18   Thermal analysis of PHA specimens was performed using a DSC-1 differential scanning

19   calorimeter (METTLER TOLEDO, Switzerland). Samples were preheated to 60°C and cooled to

20   25°C. The specimens were heated to temperatures from 25°C to 300°C, at 5°C×min-1

21   (measurement precision 1.5°C). The thermograms were analyzed using the STARe v11.0

22   software.

23   X-Ray structure analysis and determination of crystallinity of copolymers were performed

24   employing a D8 ADVANCE X-Ray powder diffractometer equipped with a VANTEC fast linear

25   detector (Bruker, AXS, Germany). Calculations were done by using the Eva program of the

26   diffractometer software.

1        2.4. Analysis of PHA microstructure and physical/mechanical properties

2        To investigate PHA properties, the polymer was processed into films. Films were

3 prepared by casting chloroform solution (2% w/v) on degreased glass and subsequent drying at

4 room temperature for 2-3 days in a dust-free box. The film discs were 100 mm in diameter and

5 0.04 mm thick.

6        The microstructure of the surface of PHA films was analyzed using scanning electron

7 microscopy (S 5500, Hitachi, Japan). Prior to microscopy, the samples were sputter coated with

8 platinum (at 10 mA, for 40 s), with an Emitech K575X sputter coater.

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

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

11        Surface properties of the polymer films and 3D constructs were examined using a DSA-

12 25E drop shape analyzer (Krüss, Germany) and software DSA-4 for Windows.

13        Physical/mechanical properties of films were investigated using an Instron 5565

14 electromechanical tensile testing machine (U.K.). Young's modulus (E, MPa), tensile strength

15 ($\sigma$, MPa) and elongation at break ($\varepsilon$, %) were automatically calculated by the Instron software

16 (Bluehill 2, Elancourt, France).

17
18        2.5. Assays of PHA biocompatibility

19        Films were cut into disks of 10 mm diameter, using a mold cutter. The samples were

20 packed using an NS 1000 shrink-wrapping machine (Hawo Gmbh, Germany) and sterilized with

21 $H_2O_2$ plasma in the Sterrad NX system (Johnson & Johnson, U.S.) for 45 min. The ability of

22 ultrafine PHA films to facilitate cell attachment was studied using NIH 3T3 mouse fibroblast

23 cells. Cell cytoplasm and nuclear DNA molecules were stained with phalloidin conjugated with

24 fluorescein (FITC) and DAPI, respectively (Sigma–Aldrich). The cells were analyzed using a

25 Leica DM6000 B fluorescence microscope. Cell viability was evaluated using MTT assay at Day

26 3 after cell seeding onto films. Optical density of the samples was measured at wavelength 540

1 nm, using a Bio-Rad 680 microplate reader (Bio-Rad LABORATORIES Inc., U.S.

2 All the characterization was performed in similar procedures as previous described [13-

3 14].

4 **3. Results and Discussion**

5 Copolymers of different compositions were synthesized in *C. eutrophus* B-10646 culture,

6 under specialized conditions. The molar fraction of 3HB in the copolymers varied between 63.5

7 and 93.1 mol.%, 3HV – between 1.1 and 24.6 mol.%, 4HB – between 2.4 and 15.6 mol.%, and

8 3HHx – between 0.4 and 4.8 mol.% (Table 1). Thus, the lowest total content of the three

9 monomer units other than the major 3-hydroxybutyrate (3HB) was 6.9 mol.% and the highest

10 36.5 mol.%. The monomer composition of the PHAs was determined by chromatography-mass

11 spectrometry and $^1$H NMR spectroscopy. Figure 1 shows the ion chromatogram with mass

12 spectra of the monomers and a $^1$H NMR spectrum of one PHA – P(3HB/3HV/4HB/3HHx)

13 (63.5/19.4/12.3/4.8 mol.%).

14

15 3.1. Physicochemical properties of PHA tetrapolymers

16 Thermal properties of PHAs and their ability to crystallize in their native state are their

17 most significant parameters, as they determine the thermomechanical properties of the polymers

18 and, hence, their ability to be processed from the melts. PHAs, like many other polymers, have a

19 heat distortion temperature somewhat lower than the thermal degradation temperature. Thus,

20 polymers cannot exist in the gaseous state, and the main type of phase equilibrium in them is a

21 condensed state – crystalline, glassy, viscoelastic, and liquid. The ability of PHA to crystallize is

22 determined by the inner properties of its chains. In a number of polymers, crystallization

23 develops only partly for various reasons. Thus, most of the polymers, including PHAs, are semi-

24 crystalline materials.

1    The relative fractions of monomers influenced physicochemical properties of PHAs,

2    including their degrees of crystallinity, molecular-weight properties, and thermal characteristics

3    (Table 1).

4    One of the most important macroscopic parameters characterizing polymer properties is

5    molecular weight, which determines the processability of the material. PHAs produced by

6    microbial synthesis show higher molecular weights than chemically synthesized PHAs [16].

7    PHA molecular weight is a variable parameter, depending on physiological and biochemical

8    properties of the PHA producing strain, the conditions of carbon nutrition, and the method of

9    polymer recovery. For instance, the number average molecular weight ($M_n$) of P(3HB)

10   synthesized by *Comamonas acidovorans* is no more than 58 kDa [17] while the $M_n$ of the

11   polymer synthesized by recombinant strain *Ralstonia eutropha* PHB⁻4 is 1580 [18]. The $M_n$

12   values of the P(3HB/4HB) copolymer also vary widely, but its average $M_n$ is lower than that of

13   the P(3HB) homopolymer. The $M_n$ values of P(3HB/4HB) containing 23-24 mol.% 4HB

14   reported by different authors range between 104 and 590 kDa [19-20]. Similar values are

15   reported for P(3HB/3HV) and P(HB/3HHx). Results of examining molecular-weight properties

16   of PHA tetrapolymers are given in Table 1. $M_n$ values varied between 72 and 223 kDa, being

17   1.5-5.0 times lower than the $M_n$ values of P(3HB). The P(3HB/3HV/4HB/3HHx) specimen

18   containing 63.5/19.4/12.3/4.8 mol.% of the monomers showed the lowest $M_n$ and $M_w$ values – 72

19   and 437 kDa, respectively. The $M_n$ and $M_w$ of the polymers with lower molar fractions of 4HB

20   and 3HHx were higher. The polymers with higher molar fractions of 3HV, 4HB, and 3HHx also

21   showed higher polydispersity, ranging between 3.03 and 6.07 versus 2.52 in P(3HB). The higher

22   Đ values suggested heterogeneity of the fragments of polymer carbon chains. The decrease in the

23   $M_w$ of PHA tetrapolymers is consistent with the data on PHA copolymers [15, 19-20] and

24   terpolymers [2, 14].

25   Important properties of PHAs are their melting temperature ($T_m$) and thermal degradation

26   temperature ($T_{degr}$). The $T_m$ for P(3HB) reported in the literature ranges between 162°C [21] and

1  197°C [22]. The data reported on the $T_m$ of P(3HB/3HV) copolymers are contradictory. For

2  instance, the $T_m$ of a P(3HB/3HV) copolymer with 6 mol.% 3HV was determined as 186°C [22],

3  but in a study by Zhao and Chen, the copolymer of a similar composition had the $T_m$ of 156°C

4  [21], while Zhang et al. reported the value of 170°C [23]. The $T_m$ of the copolymers with 20

5  mol.% 3HV was 114°C as reported in a study by Avella et al. [24] but 145°C as measured by

6  Tsuge [25]. The data on the $T_m$ of other PHA types are also inconsistent. According to the

7  literature data, the $T_m$ of P(3HB/4HB) copolymers containing between 2 and 7 mol.% 4HB may

8  range between 114 and 172°C. As the molar fraction of 4HB was increased to 75-100%, the $T_m$

9  dropped to 40-54°C [20, 26-27]. Another study, however, showed that the $T_m$ of P(3HB/4HB)

10  copolymer containing 84 mol.% 4HB was at least 130°C [28]. Similar differences between the

11  data and the trend to a decrease in $T_m$ and $T_{degr}$ were reported for PHA terpolymers [13-14, 23].

12      For PHA tetrapolymers, thermograms were taken within a wide temperature range,

13  including melting point ($T_m$) and thermal degradation temperature ($T_{degr}$) (Table 1). The melting

14  temperature of P(3HB/3HV/4HB/3HHx) (93.1/1.1/5.4/0.4 mol.%) was 166°C, i.e. 12°C lower

15  than the melting temperature of P(3HB); moreover, this PHA specimen had the lowest

16  thermal degradation temperature – 259°C, which was 36°C lower than that of P(3HB). It is

17  important that although the melting temperature and the thermal degradation temperature

18  decreased in all PHA specimens, the interval between these two parameters remained almost

19  the same. A decrease in the melting temperature (to 53-54°C) was also observed for PHA

20  tetrapolymers with another composition, which contained medium-chain-length monomers

21  with even numbers of carbon atoms ($C_6$, $C_8$, $C_{10}$, $C_{12}$, $C_{14}$) [7]. Thus, the PHA tetrapolymers

22  with different monomer compositions all exhibited thermoplasticity – one of the most

23  important properties of PHAs.

24      The monomer composition of the PHA tetrapolymers exerted the most significant effect

25  on their degrees of crystallinity (Table 1), which were generally considerably lower (under 50%)

26  than the degree of crystallinity of P(3HB) (76%). As the total proportion of the monomer units

1    other than 3HB increased, the $C_x$ value dropped, and the most substantial decrease, to 30 and

2    34%, was observed in the polymers in which 3HV, 4HB, and 3HHx totaled about 29.4 and 36.5

3    mol.%. Thus, in all the PHA tetrapolymers, the crystalline phase decreased and the amorphous,

4    disordered regions increased, indicating higher processing ability of the material. Literature data

5    on the degree of crystallinity of PHA copolymers are limited and rather contradictory. The $C_x$ of

6    P(3HB) reported by different authors varied between 59 and 86% [17, 29-30]. Noda et al. [31]

7    showed that the presence of a 20-22% 3HV molar fraction exerted very little influence on the

8    degree of crystallinity of the copolymer, but Dai et al. [32] reported that the degree of

9    crystallinity of the copolymer containing 29 and 32 mol.% 3HV dropped dramatically (to 5 and

10   9%, respectively). Even fewer data are available on the degree of crystallinity of 3HB/3HHx

11   copolymers, and they are also contradictory. In their study, Noda et al. showed that the

12   copolymer containing 12-18 mol.% 3HHx had the degree of crystallinity of 38-40% [31], while

13   Fukui et al. reported the same degree of crystallinity for the copolymer that contained a much

14   smaller fraction of 3HHx (1.5 mol.%) [33]. The lowest $C_x$ values were reported for PHA

15   copolymers (9-20%) and terpolymers (30-50%) containing 4HB monomer units [14, 17, 34].

16

17        3.2. Physical/mechanical properties of PHA tetrapolymers

18        In order to investigate physical/mechanical properties of the PHAs, dense smooth films

19   were prepared from the chloroform solutions of PHAs. The films differed considerably in their

20   mechanical strength (Table 2). Strength parameters, i.e. tensile strength and Young's modulus, of

21   PHA copolymers were considerably lower than those of the P(3HB) homopolymer. This

22   difference was more noticeable in Young's modulus, which was the lowest (102.47 MPa) in the

23   3HB/3HV/4HB/3HHx = 63.5/19.4/12.3/4.8 polymer − one with the lowest 3HB molar fraction

24   (63.5 mol.%) and the highest total content of the other three monomers (36.5 mol.%). The

25   specimens with a somewhat higher 3HB fraction, which varied between 70.6 and 93.1 mol.%,

26   and the lower total content of the other three monomers (between 6.9 and 29.4 mol.%), showed

9

1    similar values of Young's modulus – 337.01 – 352.59 MPa. That was 6 times lower than

2    Young's modulus of P(3HB). The values of tensile strength of the specimens examined were

3    also lower than the tensile strength of P(3HB), although to a lesser extent, reaching 7.12 – 14.29

4    MPa. By contrast, elongation at break (an indicator of elasticity) was considerably higher in all

5    copolymer specimens. The highest values reached 103.21-113.32% and the lowest 37.75-

6    56.25%, i.e. they were 40-80 and 15-20 times higher, respectively, than elongation at break of

7    P(3HB). Having reviewed the available literature, we did not find any data on strength

8    parameters of PHA copolymers of the same composition. There are data, however, suggesting

9    that incorporation of 3HHx and/or 4HB monomer units into the carbon chain of 3-

10   hydroxybutyrate increased elasticity but decreased mechanical strength of polymer products

11   fabricated from PHA co- and terpolymers [13-14, 20, 35]. Thus, PHA tetrapolymers containing

12   3HHx, 4HB, and 3HV showed enhanced elasticity and decreased mechanical strength.

13

14       3.3. Morphology and characterization of the surface of the films prepared from PHA

15   tetrapolymers

16       SEM and AFM images of the surfaces of PHA specimens prepared from PHAs with

17   different chemical compositions that had dissimilar physicochemical properties are shown in

18   Figure 2. The surface of the films prepared from PHA tetrapolymers [P(3HB/3HV/4HB/3HHx)]

19   was rougher than the surface of P(3HB) films, with numerous pores of different diameters (1 to 6

20   μm) (Fig. 2a). As the molar fraction of 4HB was increased, the pores became more numerous

21   and of larger size (between 3 and 6 μm); their shape and size became more diverse.

22       Analysis of the atomic-force microscopy images (Fig. 2b) suggested that the root mean

23   square roughness (Rq) of all copolymer films was 2.0 to 4.7 times higher than the Rq of P(3HB)

24   films (Table 2). The highest value of Rq (375.110 nm) was determined for the

25   P(3HB/3HV/4HB/3HHx) = 70.6/24.6/4.3/0.5 (mol.%) specimen. The surface roughness of the

26   films used as cell scaffolds may determine cell attachment, spreading, and motility; it may also

1   influence the synthesis of specific proteins. However, while some data suggest that cells are

2   attached better to rough surfaces than to polished ones, other data state that changes in roughness

3   are not accompanied by any cellular effects [36]. In a previous study, we observed considerable

4   differences between the roughness of the films of PHA terpolymers consisting of 3HB/3HV/4HB

5   or 3HB/3HV/3HHx monomers and the roughness of P(3HB) films, but we did not reveal any

6   direct relationship of the roughness to the monomer composition [14].

7        An important parameter indirectly characterizing biocompatibility and influencing cell

8   attachment and viability is the hydrophilic/hydrophobic balance of the surface [37]. It is

9   evaluated by measuring contact angles for water or diiodomethane. Results of these

10  measurements are used to determine surface energy and polar and dispersive components of

11  surface free energy. The water contact angle for PHA tetrapolymers varied between 87.62 and

12  101.06° and was generally close to the water contact angle for P(3HB) films (97.4°); the other

13  parameters of the homopolymer and copolymer films were similar, too (Table 3).

14

15       3.4. Cytotoxicity assay of films of PHA tetrapolymers with different molar fractions of

16  monomer units

17       Biological properties of PHA films (their adhesive properties and ability to facilitate cell

18  proliferation) were studied in the culture of NIH 3T3 mouse fibroblast cells. MTT assay showed

19  that none of the PHA specimens produced any cytotoxic effect in direct contact testing. The

20  fibroblasts attached to the surface of the polymer films retained their normal morphology, were

21  metabolically active, and proliferated without any toxic changes during the entire observation

22  period. After 24 h of cultivation, cell counts were comparable on all films of PHA copolymers,

23  polystyrene (control), and on P(3HB) films (Supplementary Fig.). Some differences in the

24  number of viable cells were recorded at Day 3, but they were not statistically significant.

25       Results of investigating the morphology of fibroblasts cultivated on the PHA films by

26  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).

**Conclusion**

In this work, we investigated PHA tetrapolymers 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 PHA copolymers in which the total content of the three monomer other than the major 3-hydroxybutyrate varied between 6.9 and 36.5 mol.% were investigated. The properties of PHA tetrapolymers 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. All films prepared from PHA tetrapolymers with different compositions were biocompatible and had no toxic effect on mouse fibroblast NIH 3T3 cells.

**Acknowledgement**

**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] 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.

[6] 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.

[7] 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, App. Microbiol. Biotechnol. 47 (1997) 207-211.

[8] 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, Macromol. 31 (1998) 1791-1797.

[9] T. Fukui, Y. Doi, Efficient production of polyhydroxyalkanoates from plants oils by *Alcaligenes eutrophus* and recombinant strain, Appl. Microbiol. Biotechnol. 49 (1998) 333-336.

[10] 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.

[11] 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.

1    [12] T.G. Volova, G.S. Kalacheva, A. Steinbüchel, Biosinthesis multi-component

2    polyhydroxyalkanoates by the bacterium *Wautersia eutropha*, Macromol. Symposia 269 (2008)

3    1-7.

4    [13] T. Volova, E. Kiselev, E. Shishatskaya, N. Zhila, A. Boyandin, D. Syrvacheva, O.

5    Vinogradova, G. Kalacheva, A. Vasiliev, I. Peterson, Cell growth and PHA accumulation from

6    $CO_2$ and $H_2$ of a hydrogen-oxidizing bacterium, *Cupriavidus eutrophus* B-10646, Bioresour.

7    Technol. 146 (2013) 215-222**.**

8    [14] T.G. Volova, E.G. Kiselev, O.N. Vinogradova, E.D. Nikolaeva, A.A. Chistyakov,

9    A.G. Sukovatyi, E.I. Shishatskaya, A glucose-utilizing strain, *Cupriavidus eutrophus* B-10646:

10   growth kinetics, characterization and synthesis of multicomponent PHAs, Plos One 9 (2014) 1-15.

11   [15] T.G. Volova, D.A. Syrvacheva, N.O. Zhila, A.G. Sukovatiy, Synthesis of P(3HB-co-

12   3HHx) copolymers containing high molar fraction of 3-hydroxyhexanoate monomer by

13   *Cupriavidus eutrophus* B10646, J. Chem. Technol. Biotechnol. 91 (2016) 416-425.

14   [16] G.-Q. Chen, Plastics completely synthesized by bacteria: polyhydroxyalkanoates, in:

15   G.-Q. Chen, A. Steinbüchel (Eds), Plastics from Bacteria. Natural Functions and Applications.

16   Springer-Verlag, Berlin Heidelberg, 2010, pp. 17-37.

17   [17] H. Mitomo, W.-C. Hsieh, K. Nishiwaki, K. Kasuya, Y. Doi, Poly(3-

18   hydroxybutyrate-*co*-4-hydroxybutyrate) produced by *Comamonas acidovorans*, Polymer 42

19   (2001) 3455-3461.

20   [18] N. Tanadchangsaeng, A. Kitagawa, T. Yamamoto, H. Abe, T. Tsuge, Identification,

21   biosynthesis, and characterization of polyhydroxyalkanoate copolymer consisting of 3-

22   hydroxybutyrate and 3-hydroxy-4-methylvalerate, Biomacromolecules 10 (2009) 2866-2874.

23   [19] S. Chanprateep, K. Buasri, A. Muangwong, P. Utiswannakul, Biosynthesis and

24   biocompatibility of biodegradable poly(3-hydroxybutyrate-co-4-hydroxybutyrate), Polym.

25   Degrad. Stab. 95 (2010) 2003-2012.

[20] 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. Microbial. Biotechnol. 36 (2009) 547-556.

[21] 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.

[22] S. Akhtar, C.W. Pouton, L.J. Notarianni, Crystallization behaviour and drug release from bacterial polyhydroxyalkanoates, Polymer 33 (1992) 117-126.

[23] 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.

[24] 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.

[25]. T. Tsuge, Metabolic improvements and use of inexpensive carbon sources in microbial production of polyhydroxyalkanoates, J. Biosci. Bioeng. 94 (2002) 579-584.

[26] 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.

[27] 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.

[28] Doi Y., Segawa A., Kunioka M. Biosynthesis and characterization of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in Alcaligenes eutrophus //Int J Biol Macromol. 1990. V. 12. P. 106-11.

1    [29] S. Nakamura, Y. Doi, M. Scandola, Microbial synthesis and characterization of

2    poly(3-hydroxybutyrate-co-4-hydroxybutyrate), Macromolecules 25 (1992) 4237-4241.

3    [30] R. Luo, J. Chen, L. Zhang, G. Chen, Polyhydroxyalkanoate copolyesters produced

4    by *Ralstonia eutropha* PHB¯4 harboring a low-substrate-specificity PHA synthase PhaC2$_{Ps}$ from

5    *Pseudomonas stutzeri* 1317, Biochem. Eng. J. 32 (2006) 218-225.

6    [31] I. Noda, P.R. Green, M.M. Satkowski, L.A. Schechtman, Preparation and properties

7    of a novel class of polyhydroxyalkanoate copolymers, Biomacromolecules 6 (2005) 580-586.

8    [32] Yu Dai, Z. Yuan, K. Jack, J. Keller Production of targeted poly(3-

9    hydroxyalkanoates) copolymers by glycogen accumulating organisms using acetate as sole

10   carbon source, J. Biotechnol. 129 (2007) 489-497.

11   [33] T. Fukui, H. Abe, Y. Doi, Engineering of *Ralstonia eutropha* for production of

12   poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate from fructose and solid-state properties of the

13   copolymer, Biomacromolecules 3 (2002) 618-624.

14   [34] T.G. Volova, N.O. Zhila, E.I. Shishatskaya, P.V. Mironov, A.D. Vasil'ev, A.G.

15   Sukovatyi, A.J. Sinskey, The Physicochemical properties of polyhydroxyalkanoates with

16   different chemical structures, Polymer Science, Ser. A 55 (2013) 427-437.

17   [35] B. Laycock, P. Halley, S. Pratt, A. Werker, P. Lant, The chemomechanical

18   properties of microbial polyhydroxyalkanoates, Prog. Polym. Sci. 38 (2013) 536-583.

19   [36] S.P. Xavier, P.S.P. Carvalho, M.M. Beloti, A.L. Rosa, Response of rat bone marrow

20   cells to commercially pure titanium submitted to different surface treatments, J. Dent. 31 (2003)

21   173-180.

22   [37] W. Ou, H. Qiu, Z. Chen, K. Xu , Biodegradable block poly(ester-urethane)s based

23   on poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) copolymers, Biomaterials 32 (2011) 3178-

24   3188.

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1        Table 1 Physicochemical properties of films of PHA tetrapolymers with different molar

2    fractions of monomer units

| Specimen No. | PHA composition, mol.% | | | | $T_{melt.}$, °C | $T_{degr.}$, °C | $C_x$, % | $M_n$, kDa | $M_w$, kDa | Đ |
|---|---|---|---|---|---|---|---|---|---|---|
| | 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 | 166 | 259 | 42 | 102 | 476 | 4.67 |
| 2 | 89.7 | 4.9 | 2.9 | 2.5 | 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 | 171 | 284 | 37 | 223 | 817 | 3.66 |
| 5 | 74.7 | 17.8 | 3.7 | 3.8 | 173 | 270 | 37 | 126 | 542 | 4.30 |
| 6 | 70.6 | 24.6 | 4.3 | 0.5 | 169 | 272 | 34 | 139 | 814 | 5.85 |
| 7 | 63.5 | 19.4 | 12.3 | 4.8 | 168 | 286 | 30 | 72 | 437 | 6.07 |

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1        Table 2 Physical/mechanical properties of films of PHA tetrapolymers with different

2    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 | 2071.20 | 2.50 | 71.749 | 80.283 |
| 3 | 7.51 | 346.73 | 37.75 | 158.257 | 198.504 |
| 5 | 14.29 | 337.01 | 113.32 | 189.619 | 244.497 |
| 6 | 11.04 | 352.59 | 56.25 | 305.577 | 375.110 |
| 7 | 7.12 | 102.47 | 103.21 | 120.908 | 157.535 |

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1   Table 3 Surface properties of films of PHA tetrapolymers with different molar fractions

2   of monomer units (numbers according to Table 1).

| Specimen No. | Water contact angle, θ, ° | Diiodomethane contact angle, θ, ° | Surface free energy, erg/cm$^2$ | Polar component of surface free energy, erg/cm$^2$ |
|---|---|---|---|---|
| P(3HB) | 97.42±2.63 | 58.52±1.44 | 30.43±1.01 | 1.23±0.18 |
| 3 | 87.62±1.91 | 49.94±1.25 | 36.80±1.21 | 2.56±0.23 |
| 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 |

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1    Figure Legends

2    Fig. 1. Ion chromatogram (a) with mass spectra and $^1$H NMR spectrum (b) of

3    P(3HB/3HV/4HB/3HHx) (63.5/19.4/12.3/4.8 mol.%)

4    Fig. 2. SEM (a) and AFM (b) images of the films prepared from PHA tetrapolymers with

5    different molar fractions of monomers (bar =10 μm).

6    Fig. 3. Morphology of NIH 3T3 fibroblast cells cultivated on films of PHA tetrapolymers

7    with different proportions of monomers (Day 3): DAPI (A) and FITC (B) staining. Bar = 50 μm.

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