Biosynthesis and properties of P(3HB/3HV/3H4MV) produced by using *Cupriavidus eutrophus* B-10646

Tatiana Volova[1,2,*], Olga Menshikova[2], Natalia Zhila[1,2], Alexander Vasiliev[1,3], Evgeniy Kiselev[1,2], Ivan Peterson[4], Ekaterina Shishatskaya[1,2], Sabu Thomas[1]

[1]Siberian Federal University, 79 Svobodnyi Avenue, Krasnoyarsk, 660041, Russian Federation
[2]Institute of Biophysics SB RAS, Federal Research Center "Krasnoyarsk Science Center SB RAS", Akademgorodok 50/50, Krasnoyarsk, 660036, Russian Federation
[3]L.V. Kirenskii Institute of Physics SB RAS, Federal Research Center "Krasnoyarsk Science Center SB RAS", Akademgorodok 50/38, Krasnoyarsk, 660036, Russian Federation
[4]Institute of Chemistry and Chemical Technology SB RAS, Federal Research Center "Krasnoyarsk Science Center SB RAS", Akademgorodok 50/24, Krasnoyarsk, 660036, Russian Federation

*Corresponding author. Tel.: +7 391 2494428; fax: +7 391 2433400
E-mail address: volova45@mail.ru (Tatiana G. Volova)

Abstract

Synthesis of poly(3-hydroxybutyrate/3-hydroxyvalerate/3-hydroxy-4-methylvalerate) [P(3HB/3HV/3H4MV)] has been studied in batch culture of *Cupriavidus eutrophus* B-10646 under nitrogen deficiency and varied conditions of carbon nutrition. A study has been conducted to determine the effect of the carbon substrate (sugars, butyric acid) and concentration of methylvalerate as a precursor substrate necessary for synthesis of 3H4MV monomer units on cell concentration, total polymer yield, and 3H4MV content of the copolymer. The results suggest that methylvalerate concentration in the culture medium must not be higher than 1 g L^{-1}, and that methylvalerate must be carefully dosed, with at least 10-12 h intervals between supplementations. Bacterial cells were cultivated in the modes that enabled production of a series of copolymers with molar fractions of 3H4MV of between 2.7 and 11.3 mol.%. Degrees of crystallinity, molecular weight characteristics, and thermal properties of the copolymers have been investigated as dependent on proportions of monomers. Results suggest that the molar fraction of 3H4MV has the strongest effect on the crystalline to amorphous region ratio. P(3HB/3HV/3H4MV) specimens synthesized in this study had a degree of crystallinity of below 50%, which remained unchanged for 2.5 years.

Keywords: *polyhydroxyalkanoates, P(3HB/3HV/3H4MV) copolymers, synthesis, physicochemical properties*

Introduction

P(3HB/3HV/3H4MV) copolymers are relatively new representatives of the class of microbial reserve macromolecules synthesized by prokaryotic microorganisms under specific conditions of unbalanced growth. These are so-called polyhydroxyalkanoates (PHAs) – polyesters capable of being degraded in biological media to end products: to CO_2 and H_2O under aerobic conditions and to CH_4 and H_2O under anaerobic conditions. PHAs are characterized by high biological compatibility, thermoplasticity, and ability to be processed into polymer products from different phase states. Therefore, they are attractive materials for medical, pharmaceutical, agricultural, and municipal engineering applications [1-4].

PHAs can be used as a basis for producing a wide range of high-molecular-weight materials with diverse properties, which would differ considerably in their thermal and molecular weight characteristics and degree of crystallinity. These materials could be then processed into products with various physical

and mechanical properties [5-9]. PHA copolymers are more promising materials, but it is difficult to achieve the controlled and reproducible synthesis of multicomponent polymers, which hinders the collection of data on the effect of the monomer composition of PHAs on their properties.

Studies reporting the synthesis of PHA copolymers containing 3-hydroxy-4-methylvalerate monomer units were published relatively recently. The authors of those studies assumed that these polymers had better processing properties than other PHA types and were not prone to ageing [10, 11]. These copolymers were mainly produced by genetically modified strains [10-13], wild-type microorganisms being used in fewer studies [14-17]. The review of the literature showed the scantiness of the data and the difficulty of achieving both high cell concentrations and large yields of the copolymer, on the one hand, and high molar fractions of 3-hydroxy-4-methylvalerate, on the other (Table 1). Published studies mainly report data on the achieved cell biomass yields, total production of the P(3HB/3HV/3H4MV) copolymer, and the monomer composition of the copolymer. Properties of this type of copolymers have been studied insufficiently. A number of papers report studies of molecular weight properties, some of them present thermal parameters, and just a few give values of the degree of crystallinity of the polymer. No available studies have presented integrated results, including production characteristics of the cultures and copolymer composition and properties.

The purpose of this study was to investigate the process of production of P(3HB/3HV/3H4MV) copolymers in the culture of *Cupriavidus eutrophus* B-10646 and synthesize polymers with different contents of 3-hydroxy-4-methylvalerate (3H4MV) and investigate their properties.

Materials and methods

The strain and composition of the nutrient medium

Bacterial strain *Cupriavidus eutrophus* B-10646 was investigated in this study. This strain has a broad organotrophic potential and is capable of synthesizing PHA copolymers at high yields. The strain is registered in the Russian National Collection of Industrial Microorganisms.

Bacterial cells were cultivated using Schlegel's mineral medium: $Na_2HPO_4 \times H_2O$ – 9.1; KH_2PO_4 – 1.5; $MgSO_4 \times H_2O$ – 0.2; urea – 1.0 (g L^{-1}). A solution of iron citrate (5 g L^{-1}), which was used as a source of iron, was added to reach a concentration of 5 ml/L. Hoagland's trace element solution was used: 3 ml of standard solution per 1 L of the medium. The composition of the standard solution was as follows: H_3BO_3 – 0.288; $CoCl_2 \times 6H_2O$ – 0.030; $CuSO_4 \times 5H_2O$ – 0.08; $MnCl_2 \times 4H_2O$ – 0.008; $ZnSO_4 \times 7H_2O$ – 0.176; $NaMoO_4 \times 2H_2O$ – 0.050; $NiCl_2$ – 0.008 (g L^{-1}). The main carbon source was fructose (Panreac, E.U.) or glucose (China), or butyric acid (Panreac, E.U.). The precursor for synthesis of 3H4MV copolymers was 4-methylvaleric acid (Sigma-Aldrich, China). Butyric acid and 4-methylvaleric acid were neutralized with a 33% KOH solution (pH 7.0±0.2) and then sterilized by filtration using an Opticap XL300 Millipore Express SHC membrane (U.S.).

Bacterial culture

Cells were grown under strictly aseptic conditions in two-phase batch culture. Cultivation was performed at a temperature of 30 °C, with pH maintained at 7.0. In Phase 1, cells were cultured under nitrogen deficiency, by using 1 g L^{-1} of urea as a source of nitrogen, in order to induce polymer accumulation. In Phase 2, the nitrogen-free culture medium was used. The culture medium for synthesis of the homopolymer of 3-hydroxybutyric acid contained a sole carbon substrate; the culture media for synthesis of copolymers were supplemented with a second carbon substrate (4-methylvalerate) as a precursor for the synthesis of the 3H4MV monomers.

Cell biomass increase in the culture was evaluated by periodically measuring culture optical density and determining cell concentration (by weighing the dry matter). Cell concentration in the culture medium was also monitored by converting the optical absorbance at 440 nm of culture broth to dry cell weight by using a standard curve prepared previously. The bacterial cells were harvested by centrifugation (6000g, 15 min), washed twice with distilled water and dried at 105°C for 24 h.

Nitrogen concentration in the culture medium was analyzed using a photometric method, with Nessler's reagent. Fructose concentration was determined using the resorcinol method [18]. Glucose concentration was determined using the 'Glucose – FKD' kit ('Farmatsevtika i klinicheskaya diagnostika', Moscow, Russia), which contained chromogenic enzyme substrate and a calibrator (a 10 mmol L^{-1} glucose solution). Optical density of the study sample and calibration sample were compared photometrically with the optical density of the blank, with optical path length 10 mm at wavelength 490 nm. Glucose concentration in the samples was calculated using the following formula:

$$C = (E_0/E_k) \times 10,$$

where C is glucose concentration, mmol L^{-1}; E_0 is optical density of the sample tested, units of optical density; E_k is optical density of the calibration sample, units of optical density; 10 is glucose concentration in the calibrator, mmol L^{-1}.

Butyric acid and 4-methylvaleric acid concentrations in the culture medium were controlled using a gas chromatography analysis (GC-MS 6890/5975C, Agilent Technologies, U.S.) of the culture medium samples, which was done after preliminary extraction with chloroform from acidified samples (pH 2-3).

Analysis of the chemical composition of P(3HB/3HV/3H4MV) copolymers

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 polymers was analyzed with a GC-MS (6890/5975C, Agilent Technologies, U.S.). To further reconfirm the composition, ^{1}H NMR spectra of PHA in $CDCl_3$ were obtained at room temperature on a BRUKER AVANCE III 600 spectrometer operating at 600.13 MHz of ^{1}H NMR spectra.

Analysis of microstructure and properties of copolymers films

To investigate the copolymer 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 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.

Surface properties were studied with a Drop Shape Analyzer – DSA-25E (KRÜSS GmbH, Germany) for measuring contact angles of water and diiodomethane drops by the Owens, Wendt, Rabel and Kaelble method: surface free energy (SFE) and its dispersion and polar components were measured, and the data were processed using the DSA-4 software.

Analysis of physicochemical properties of P(3HB/3HV/3H4MV) copolymers

Molecular weight and molecular-weight distribution of polymers were examined using a gel permeation chromatography. An Agilent Technologies 1260 Infinity chromatograph, equipped with a refractive index detector and an Agilent PLgel Mixed-C column, was employed. Chloroform was the eluent. Calibration was done using polystyrene standards (Fluka, Switzerland, Germany). Molecular weights (weight average, M_w, and number average, M_n) and polydispersity ($Đ = M_w/M_n$) were determined.

Thermal analysis of P(3HB/3HV/3H4MV) specimens was performed using a DSC-1 differential scanning calorimeter (METTLER TOLEDO, Switzerland). Powdered samples (4.0 ±0.2 mg each) were placed into an aluminum crucible and compressed prior to measurement. Every sample was measured at least 3 times. The specimens were heated at a rate of 5 °C/min to 200 °C, then cooled to -20 °C, held for 20 minutes, and re-heated to 320 °C. Glass transition temperature (T_g), crystallization temperature (T_c), melting point (T_{melt}) and thermal degradation temperature (T_{degr}) were determined from peaks in thermograms using the "StarE" 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, using CuKa radiation (Bruker, AXS, Germany). In order to determine the degree of crystallinity of P(3HB/3HV/3H4MV), 3 film samples 2 cm in diameter and 0.15 mm thick were prepared from a 2% polymer solution in chloroform. The samples had a circular shape because during measurement the sample spins in a direction perpendicular to the surface. The scan step was 0.016°, measurement time in each step 114 s, and scanning range from 5° to 60° (from 48° to 60° there only was a uniformly decreasing background); the registered parameter was intensity of X-rays scattered by the sample; 55°/0.016°=3438 times. The degree of crystallinity was calculated as a ratio of the total area of crystalline peaks to the total area of the radiograph (the crystalline + amorphous components). Measurement accuracy: point measurement accuracy ± 0.4 PPS, with the lowest intensity 1.5 PPS and the highest intensity 32 PPS; the error in determination of the degree of crystallinity, which was calculated based on multiple measurements, was 2% or less.

Results and Discussion

A study of P(3HB/3HV/3H4MV) synthesis in the C. eutrophus B-10646 *culture*

First, in order to determine non-inhibiting concentrations of the precursor substrate necessary for the production of 3H4MV monomer units, we studied the effect of 4-methylvalerate concentration on the growth of *C. eutrophus* B-10646 cells and synthesis of P(3HB/3HV/3H4MV). For bacterial cells to be able to synthesize PHA copolymers, the nutrient broth must contain not only the main carbon substrate

but also precursors of the target monomer units. Precursor substrates, however, usually inhibit the growth of PHA producing strains and PHA synthesis. In our experiment, 4-methylvalerate fed into the bacterial culture in the phase of polymer synthesis at a concentration of 0.5 g L^{-1} did not significantly inhibit the cell growth and polymer production (Fig. 1). Over 96 h of the culture, cell concentration reached 6.9 g L^{-1}, which was comparable to the cell concentration in the control culture, where cells were grown on the sole carbon substrate, fructose (7.3 g L^{-1}). The total polymer yield reached 5.5 g L^{-1}, which corresponded to its intracellular content of 80% and was close to the control value (83-85%). As potassium 4-methylvalerate concentration was increased to 1.0 g L^{-1}, we observed inhibition of the cell growth and copolymer synthesis, which dropped to 4.7 g L^{-1} and 65%, respectively. The content of 3H4MV monomer units in the copolymer was about 6 mol.%. A concentration of potassium 4-methylvalerate of 1.5 g L^{-1} had a strong inhibitory effect on the bacterial growth and especially on polymer synthesis, whose intracellular concentration dropped to 20%; the 3H4MV content was no more than 1 mol.% (Fig. 1). A chromatogram with mass spectra and a ^{1}H NMR spectrum of one P(3HB/3HV/3H4MV) specimen is shown at Fig. 2a and Fig.2b respectively. In the ^{1}H spectrum of 3H4MV three peaks (3, 7 and 12) of the methine protons in the main chain were elucidated. In the side chain of 3H4MV other methine proton (peak 13) was assigned to the carbon C13. The methylene protons of the carbons C2, C6 and C6 observed as peaks (2), (6) and (11), respectively. Finally, terminal methyl groups (peaks 4, 9 and 14) were definitely identified.

Our results are consistent with the literature data suggesting a toxic effect of 4-methylvalerate on microorganisms and describing difficulties of producing polymers with a high content of 3H4MV monomer units simultaneously with achieving high cell concentrations and considerable polymer yields. For instance, cultivation of the recombinant strain *Ralstonia eutropha* PHB$^-$4 on fructose and 4-methylvalerate resulted in cell concentration of 1.5 g L^{-1} and copolymer yield of 46% [10]. In a study of the recombinant strain *C. necator* PHB$^-$4, the yields of 3HB/3H4MV and cell biomass were about 40% and 1.6 g L^{-1}, respectively [13]. Selection of leucine resistant mutants did not result in high yields: cell concentration and polymer yield were 1.5-1.7 g L^{-1} and 51-55%, respectively, and 3HV and 3H4MV molar fractions were low (about 0.4-1.7 and 0.5-0.9 mol.%, respectively). Supplementation of the fructose-containing culture medium for growing the recombinant strain *R. eutropha* PHB$^-$4 with leucine at a concentration of 10 g L^{-1} caused cell concentration to increase to 7.1-7.3 g L^{-1} but copolymer content to drop to 29-36%; the 3H4MV molar fraction was no higher than 3.0 mol.% [11]. The wild-type strain *Azotobacter chroococcum* 7B cultivated on sucrose supplemented with 4-methylvalerate synthesized rather high yields of the copolymer (71-79%), but cell concentration was no more than 3.7 g L^{-1}, and the molar fraction of 3H4MV was extremely low (0.14-0.60 mol.%) [19]. Thus, synthesis of P(3HB/3HV/3H4MV) containing a large molar fraction of 3H4MV in the culture producing high cell concentrations and copolymer yields seems problematic.

Another question that needed to be answered was how rapidly bacterial cells grown on fructose as the main carbon substrate consumed 4-methylvalerate. Fructose, whose initial concentration in the medium was 20 g L^{-1}, was consumed gradually, as cell concentration increased. After 24 h of cultivation (in the phase when the copolymer was synthesized at the highest rate), the culture medium was supplemented with 4-methylvalerate at concentrations of 0.5 and 1.0 g L^{-1}. For 3-4 h after it was added to the medium, its concentration remained unchanged; after 6 h, it decreased to 0.23 and 0.45 g L^{-1}; after 12 and 24 h, only trace amounts of 4-methylvalerate were detected in the medium (data not shown). Our results suggested that a single dose of 4-methylvalerate must not be more than 0.5-1.0 g L^{-1} and the time interval between supplementations must be at least 10-12 h

As microorganisms can synthesize copolymers when grown on different substrates – fructose [13, 15], valerate, propionate [14], and soybean oil [20], we investigated the total yields of P(3HB/3HV/3H4MV) copolymers and molar fractions of 3H4MV in them in experiments with fructose or glucose, or butyric acid as the sole carbon substrate (Fig. 3). We added once 0.5 g L^{-1} of 4-methylvalerate to every culture medium containing one of the substrates after 24 h of cultivation.

In the cultures with sugars (glucose or fructose), comparable cell concentrations and copolymer yields were attained. After the addition of 4-methylvalerate, cell concentration and copolymer production were increasing for some time, reaching 6.8-7.0 g L^{-1} and 78-80%, respectively, by the end of the experiment. The content of 3H4MV in the copolymer showed a different trend (Fig. 3a, b). In 24 h after the addition of 4-methylvalerate, monomer concentration reached its peak (6.0 mol.%), but then it declined, and after 96 h it constituted 2-3 mol.%. In the culture with the butyric acid as the carbon substrate, the molar fraction of 3H4MV was higher: 10.2 mol.% after 48 h and no less than 6 mol.% at the

end of the experiment (Fig. 3c). The cell concentration and total copolymer yield were comparable to those obtained in cultures with sugars.

Thus, the butyric acid should be used as the sole carbon substrate in the culture of *C. eutrophus* B-10646 to achieve production of P(3HB/3HV/3H4MV) copolymers with the highest 3H4MV molar fraction. The disadvantage of using this substrate is the necessity to periodically add doses of the butyric acid to the culture medium, as at concentrations above 1.5-2.0 g L^{-1} it exerts an inhibitory effect on bacterial cells.

Literature data suggest that methylvalerate content can be varied by using different conditions of carbon nutrition. For instance, Lau et al. [14] reported that the use of valerate or sodium propionate as the main substrate and isocaproic acid as the precursor substrate in the culture of the wild-type strain *Burkholderia* sp. considerably increased molar fractions of 3HV (from 10 to 84 mol.%) and 3H4MV (from 1 to 44 mol.%). However, the cell concentration and copolymer yield were very low: 1.3-1.8 g L^{-1} and 1-5%, respectively.

Based on our results (Fig. 3), we synthesized a series of P(3HB/3HV/3H4MV) copolymers with large molar fractions of 3H4MV, by growing bacterial cells in two-phase batch culture on the butyric acid, with 4-methylvalerate added in controlled doses. A single dose of 4-methylvalerate was 0.5 g L^{-1}, and the time interval between additions was varied between 10-12 and 20-24 h. Depending on how many times the precursor substrate was added, the resulting copolymers contained between 2.7 and 11.3 mol.% 3H4MV (Table 2). In addition to 3H4MV, the copolymers also contained monomer units of 3-hydroxyvalerate (3HV), whose molar fraction varied between 3.5 and 18.1 mol.% (Table 2).

Physicochemical properties of P(3HB/3HV/3H4MV) copolymers with different proportions of monomers

After producing a series of P(3HB/3HV/3H4MV) copolymers, we investigated the effects of the proportions and composition of monomers on the physicochemical properties of the copolymers. To do this, we prepared films of P(3HB/3HV/3H4MV) copolymers, which differed noticeably in their surface topography (Fig. 4).

SEM images clearly showed that all films of P(3HB/3HV/3H4MV) copolymers had more pores of sizes between 2-3 and 8-10 μm than films prepared from P(3HB) and P(3HB/3HV), and the number and size of the pores increased with an increase in the 3H4MV molar fraction. However, no definite relationship was found between the 3H4MV content of P(3HB/3HV/3H4MV) and film surface properties characterized by measuring contact angles of water and diiodomethane (Table 3). The contact angle of diiodomethane, though, did increase above 60° with an increase in the 3H4MV molar fraction, compared to 49.7° and 39.2° for P(3HB) and P(3HB/3HV). At the same time, the liquid surface tension of the polar component increased by one order of magnitude and the liquid surface tension of the dispersion component decreased about 1.5-fold.

Physicochemical properties of P(3HB/3HV/3H4MV) films varied depending on the monomer proportion and content of 3H4MV (Table 2). The molecular weight (M_w) of copolymer films was somewhat lower than that of P(3HB) films, and their number average molecular weight (M_n) was significantly lower. Therefore, polydispersity of P(3HB/3HV/3H4MV) was 2.0-2.5 times higher than that of P(3HB). For the copolymers containing 3HV (between 3.5 and 18.1 mol.%), the increase in the 3H4MV molar fraction from 2.7 to 11.3 mol.% did not cause any significant changes in their molecular weight characteristics except the decrease in M_n values and increase in Đ values (4.0-6.2) mentioned above. The molecular weight parameters are among the most important characteristics of high-molecular-weight compounds, which determine processablity of polymers. That is probably why PHA copolymer research is mainly focused on these characteristics. A number of studies addressed the molecular weight characteristics of P(3HB/3HV/3H4MV) with different molar fractions of 3H4MV. Lau et al. [15] reported that as 3H4MV was increased from 1 to 19 mol.%, M_n dropped from 213 to 91 kDa and M_w decreased from 657 to 274 kDa, although polydispersity did not change. However, the study by Tanadchangsaeng et al. [10] showed that in the specimens containing 0.4-0.9 mol.% 3HV and 0.7-12.9 mol.% 3H4MV, number average molecular weight and polydispersity varied between 100 and 400 kDa and between 1.4 and 4.1, respectively. The authors of another study [11] investigated copolymers with a considerably lower molar fraction of 3H4MV (0.8-3.1 mol.%) and found that their M_n ranged between 98 and 251 kDa, with polydispersity values being rather low (1.6-1.9). Higher values of M_n (298-421 kDa) and Đ (2.2-2.5) were reported by Chia et al. [12] for P(3HB/3H4MV/3HHx) and P(3HB/3HV/3H4MV/3HHx) specimens with low molar fractions of 3H4MV (1-3 mol.%); the authors, though, concluded that incorporation of methylvalerate monomers decreased the molecular weight of the polymer relative to P(3HB) and P(3HB/3HV).

The study of thermal properties of P(3HB/3HV/3H4MV) showed a consistent decrease in the melting temperature with the increase in the 3H4MV molar fraction (Table 2). In specimens containing between 3.3 and 11.3 mol.% 3H4MV (4-7 in Table 2), melting regions in thermograms (Fig. 5a) are noticeably shifted to the left compared to the specimen with a lower molar fraction of this monomer (2.7 mol.%, Specimen 3 in Table 2) and to T_{melt} of P(3HB/3HV) and P(3HB). Thermal behavior of copolymers containing methylvalerate monomers is characterized by the presence of two peaks in the melting region, with the difference between them of between 9 and 19 degrees (Fig. 5a). In contrast to T_{melt}, no significant differences were found between temperatures of thermal degradation. All specimens, irrespective of their 3H4MV and 3HV contents, showed T_{degr} of between 293 and 297 °C, which was comparable with the values for the homopolymer and copolymers with 3-hydroxyvalerate. Not all gradually heated specimens showed a peak in the glass transition region (Fig. 5a). No glass transition peaks were present in the thermograms of the P(3HB) specimen and the copolymer with the lowest 3H4MV content (2.7 mol.%) (Specimens 1 and 3 in Table 2). In the other P(3HB/3HV/3H4MV) specimens, T_g varied between -0.15 and 1.40, with no direct relationship with the polymer composition and monomer proportions. We did not find any definite relationship between copolymer composition and monomer proportions and the temperature of crystallization (Table 2, Fig. 5a). The T_c of some of the specimens was above 100 °C. These were P(3HB) and P(3HB/3HV/3H4MV) specimens with 3H4MV molar fractions of 2.7 and 4.0 mol.% (Specimens 1, 3, and 5 in Table 2). The T_c of other terpolymers was considerably lower, between 44.16 and 67.68 °C. It is difficult to compare results obtained in the present study and literature data since detailed data are scant (Table 1). P(3HB/3H4MV) that contained 19 mol.% 3H4MV showed T_g, T_{melt}, and T_{degr} of 3.3, 160.8, and 306 °C, respectively [15]. A study by Saika et al. [11] showed that as the 3H4MV molar fraction of the copolymers increased from 0.8 to 3.1 mol.%, their T_{melt} decreased slightly (from 146 to 137 °C and from 159 to 151 °C) while T_g remained the same (3 °C). Authors of another study [10] reported that as the 3H4MV molar fraction was increased to 38 mol.%, both T_{melt} and T_g tended to decrease.

Analysis of radiograms of PHA specimens with different composition revealed several dissimilarities between them (Fig. 5b). X-Ray showed a decrease in intensities of diffraction maxima at 2θ = 16°, 30°, 38° and 45°, with the most significant decrease at 2θ = 16° and 30°, for all P(3HB/3HV/3H4MV) specimens compared to P(3HB/3HV) and P(3HB): intensities of diffraction maxima of P(3HB) and P(3HB/3HV) decreased from 4200 and 3500 to 1600 and 2300, respectively, and intensities of diffraction maxima of the five specimens containing 3H4MV decreased from 2800 and 2300 to 400-1400 (Specimens 3-7, Fig. 5b). P(3HB/3HV/3H4MV) specimens also showed changes in the 18-22° region, with additional maxima observed at 2θ = 22° and a certain decrease in intense peaks at 2θ = 18°. X-Ray diffraction analysis of PHAs with different compositions (Table 2, Fig. 5b) showed that the degree of crystallinity of P(3HB/3HV/3H4MV) copolymers was considerably lower than that of the high-crystallinity homopolymer (76%) and P(3HB/3HV). The C_x of P(3HB/3HV/3H4MV) copolymers was 42-44%, i.e. the amorphous region was greater than the crystalline region. At the same time, we did not reveal any relationship between C_x and the 3H4MV content in the range between 2 and 11 mol.%. Thus, 3H4MV monomer units produce a stronger effect on the crystalline to amorphous region ratio than 3-hydroxyhexanoate and 3-hydroxyvalerate monomer units [7-9, 21]. In P(3HB/3HV), 3HB and 3HV monomers co-crystallize in the hydroxybutyrate or hydroxyvalerate lattice, depending on the monomer ratio, and the increase in 3HV content to above 40-50 mol.% does not cause a decrease in C_x below 45-50%. Only fragmentary data are available for the effect of 3H4MV monomers on the degree of crystallinity of copolymers. In a study by Tanadchangsaeng et al. [22], cultivation of the recombinant strain *Ralstonia eutropha* PHB$^-$4 on fructose with varied concentrations of 4-methylvalerate produced polymers with molar fractions of 3H4MV and 3HV of 46 mol.% and 1.0 mol.%, respectively, with low cell concentration (1.4 g L^{-1}) and copolymer yield of 53%. The degree of X-ray crystallinity of fractionated P(3HB-co-3H4MV) films decreased from 60 to 13% as the 3H4MV fraction increased from 0 to 39 mol.%. The results reported by Bonartsev et al. [19] suggesting a decrease in C_x of P(3HB/3HV/3H4MV) with a very low molar fraction of 3H4MV (0.14-0.6 mol.%) to 49% seem dubious.

There are literature data on the effect of another monomer – 3-hydroxy-2-methylvalerate (3HMV) – on PHA properties: a decrease in the degree of crystallinity of P(3HB/3HV/3HMV) terpolymers synthesized by glycogen accumulating organisms (GAO) grown on acetate. An enriched GAO culture was obtained in a lab-scale reactor operated under alternating anaerobic and aerobic conditions with acetate fed at the beginning of the anaerobic period. The C_x value varied between 11 and 19% in the specimens containing 4-12 mol.% 3HMV and 16-25 mol.% 3HV [16]. In another study, Dai et al. [23]

cultivated *Defluviicoccus vanus*-related glycogen accumulating organisms in the medium with acetate supplemented with propionate and synthesized P(3HB/3HV/3HMV/3HMB) quaterpolymers containing 32-36 and 15-41 mol.% 3HV and 3HMV, respectively, with a C_x of 14-15%. Thus, incorporation of methylvalerate monomers into the carbon chain of 3-hydroxybutyrate/3-hydroxyvalerate, even though methylvalerate content is relatively low, results in a significant decrease in the degree of crystallinity of the copolymer.

Crystallinity is a most important property of polymers, which essentially determines the processability of polymers into specialized products. The most widespread and best-studied short-chain-length polymers – poly-3-hydroxybutyrate and 3-hydroxybutyrate/3-hydroxyvalerate copolymers – are bioresorbed *in vivo* quite slowly, i.e. they stay in the organism for long periods of time and may cause tissue inflammation in the implantation site [24]. In contrast to PHA copolymers, in these PHA types, especially P(3HB), the crystalline region is greater than the amorphous region, and, thus, products fabricated using these PHAs have a high degree of crystallinity and high hydrophobicity and are prone to ageing [25]. We measured the degree of crystallinity of P(3HB) films that had been kept at room temperature for three years and found that it had increased from the initial value of 76% to 95% (unpublished data). The degree of crystallinity of films prepared from P(3HB/3HV/3H4MV) containing 6.2 mol.% 3H4MV (42%) had not changed over 2.5 years, in contrast to P(3HB) films, whose C_x had increased from 72% to 89% over the same time period. These findings suggest that copolymers containing 3H4MV are more stable and have better processability.

Conclusions

Synthesis of P(3HB/3HV/3H4MV) was investigated in batch culture of *C. eutrophus* B-10646 under different conditions of carbon nutrition and as dependent on the concentration of the precursor substrate – 4-methylvalerate. Then, bacterial cells were cultivated in the modes that enabled the production of a series of copolymers with molar fractions of 3H4MV of between 2.7 and 11.3 mol.%. Degrees of crystallinity, molecular weight characteristics, and thermal properties of the copolymers were investigated as dependent on proportions of monomers. Results suggested that the molar fraction of 3H4MV had a strong effect on the crystalline to amorphous region ratio.

Conflict of interest

No conflict of interest to declare.

Acknowledgment

This study was financially supported by Project "Agropreparations of the new generation: a strategy of construction and realization" (Agreement No 14.Y26.31.0023) in accordance with Resolution No 220 of the Government of the Russian Federation of April 9, 2010, "On measures designed to attract leading scientists to the Russian institutions of higher learning".

References

1 Sudesh K, Abe H, Doi Y. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. Prog Polym Sci 2000; 25:1503-1555.

2 Chanprateep S. Current trends in biodegradable polyhydroxyalkanoates. J Biosci Bioeng 2010; 110:621-632.

3 Chen G-Q. Industrial production of PHA. In: Chen G-Q, Steinbüchel A, editors. Plastics from bacteria. Natural functions and applications, Berlin: Springer; 2010, p. 121–132.

4 Volova TG, Shishatskaya EI, Sinskey AJ. Degradable polymers: production, properties and applications. New York: Nova Science Pub, Inc, USA; 2013.

5 Madden L, Anderson AJ, Asrar J, Garrett P. Production and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) synthesized by *Ralstonia eutropha* in fed-batch cultures. Polymer 2000; 41:3499-3505.

6 Chanprateep S, Kulpreecha S. Production and characterization of biodegradable terpolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-4-hydroxybutyrate) by *Alcaligenes* sp. A-04. J Biosci Bioeng 2006; 101:51-56.

7 Laycock B, Halley P, Pratt S, Werker A, Lant P. The chemomechanical properties of microbial polyhydroxyalkanoates. Prog Polym Sci 2013; 38:536-583.

8 Volova TG, Zhila NO, Shishatskaya EI, Mironov PV, Vasil'ev AD, Sukovatyi AG, Sinskey AJ. The physicochemical properties of polyhydroxyalkanoates with different chemical structures. Polymer Science, Ser A. 2013; 55:427–437.

9 Volova TG, Vinogradova ON, Zhila NO, Kiselev EG, Peterson IV, Vasil'ev AD, Sukovatyi AG, Shishatskaya EI. Physicochemical properties of multicomponent polyhydroxyalkanoates: Novel aspects. Polymer Science, Ser A. 2017; 59:98–106.

10 Tanadchangsaeng N, Kitagawa A, Yamamoto T, Abe H, Tsuge T. Identification, biosynthesis, and characterization of polyhydroxyalkanoate copolymer consisting of 3-hydroxybutyrate and 3-hydroxy-4-methylvalerate. Biomacromolecules 2009; 10:2866–2874.

11 Saika A, Watanabe Y, Sudesh K, Abe H, Tsuge T. Enhanced incorporation of 3-hydroxy-4-methylvalerate unit into biosynthetic polyhydroxyalkanoate using leucine as a precursor. AMB Express 2011; 1:6.

12 Chia K-H, Ooi T-F, Saika A, Tsuge T, Sudesh K. Biosynthesis and characterization of novel polyhydroxyalkanoate polymers with high elastic property by *Cupriavidus necator* PHB$^-$4 transformant. Polymer Degrad Stab 2010; 95:2226-2232.

13 Lau N-S, Sudesh K. Revelation of the ability of Burkholderia sp. USM (JCM 15050) PHA synthase to polymerize 4-hydroxybutyrate monomer. AMB Express 2012; 2:41.

14 Lau N-S, Chee J-Y, Tsuge T, Sudesh K. Biosynthesis and mobilization of a novel polyhydroxyalkanoate containing 3-hydroxy-4-methylvalerate monomer produced by *Burkholderia sp*. USM (JCM15050). Bioresour Technol 2010; 101:7916–7923.

15 Lau N-S, Tsuge T, Sudesh K. Formation of new polyhydroxyalkanoate containing 3-hydroxy-4-methylvalerate monomer in *Burkholderia* sp. Appl Microbiol Biotechnol 2011; 89:1599–1609.

16 Dai Y, Yuan Z, Jack K, Keller J. Production of targeted poly(3-hydroxyalkanoates) copolymers by glycogen accumulating organisms using acetate as sole carbon source. J Biotechnol 2007; 29:489–497.

17 Ling SC, Tsuge T, Sudesh K. Biosynthesis of novel polyhydroxyalkanoate containing 3-hydroxy-4-methylvalerate by *Chromobacterium* sp. USM2. J Appl Microbiol 2011; 111:559–571.

18 Ermakov AI, Arasimovich VV, Smirnova-Ikonnikova MI, Yarosh NP, Lukovnikova GA. Metody biokhimicheskogo issledovaniya rastenii (Methods of Biochemical Plant Research). Leningrad: Kolos; 1972 (in Russian).

19 Bonartsev Yakovlev SG, Zernov AL, Ivanova EV, Akoulina EA, Kuznetsova ES, Zhuikov VA, Alekseeva SG, Podgorskii VV, Bessonov IV, Kopitsyna MN, Morozov AS, Milanovskiy EY, Tyugay ZN, Bykova GS, Kirpichnikov MP, Shaitan KV. Biosynthesis of poly(3-hydroxybutyrateco-3-hydroxy-4-methylvalerate) by Strain *Azotobacter chroococcum* 7B. Acta Naturae 2016; 8:77-87.

20 Watanabe Y, Ichinomiya Y, Shimada D, Saika A, Abe H, Taguchi S, Tsuge T. Development and validation of an HPLC-based screening method to acquire polyhydroxyalkanoate synthase mutants with altered substrate specificity. J Biosci Bioeng 2012; 113:286–292.

21 Volova TG, Syrvacheva DA, Zhila NO, Sukovatiy AG. Synthesis of P(3HB-co-3HHx) copolymers containing high molar fraction of 3-hydroxyhexanoate monomer by *Cupriavidus eutrophus* B10646. J Chem Technol Biotechnol 2016; 91:416-425.

22 Tanadchangsaeng N, Tsuge T, Abe H. Comonomer compositional distribution, physical properties, and enzymatic degradability of bacterial poly(3-hydroxybutyrate-*co*-3-hydroxy-4-methylvalerate) copolyesters. Biomacromolecules 2010; 11:1615–1622.

23 Dai Y, Lambert L, Yuan Z, Keller J. Characterisation of polyhydroxyalkanoate copolymers with controllable four-monomer composition. J Biotechnol 2008; 134:137–145.

25 Engelberg I, Kohn J. Physico-mechanical properties of degradable polymers used in medical applications: a comparative study. Biomaterials 1991; 12:292-304.

24 Stock UA, Nagashima M, Khalil PN, Nollert GD, Herden T, Sperling JS, Moran A, Lien J, Martin DP, Schoen FJ, Vacanti JP, Mayer JE. Tissue-engineered valved conduits in the pulmonary circulation. J Thorac Cardiovasc Surg 2000; 119:732 –740.

25. Saika A, Watanabe Y, Sudesh K, Tsuge T. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxy-4-methylvalerate) by recombinant *Escherichia coli* expressing leucine metabolism-related enzymes derived from *Clostridium difficile*. *J Biosci Bioeng* 2014;117:670-675.

26 Saika A, Ushimaru K, Mizuno S, Tsuge T. Genome-based analysis and gene dosage studies provide new insight into 3-hydroxy-4-methylvalerate biosynthesis in *Ralstonia eutropha*. *J Bacteriol* 2015;197:1350-1359.

Table 1 Summary of polyhydroxyalkanoates containing 3H4MV synthesis and their properties

Species	Biomass (g L^{-1})	PHA content (% of dry biomass)	Content of monomers, mol.%			M_w (kDa)	Đ	C_x (%)	T_{melt} (°C)	T_{degr} (°C)	Reference
			3HB	3HV	3H4MV						
wild-type strain *A. chroococcum* 7B	2.6-3.7	71-79	99.40-99.96	–	0.04-0.60	620-1390	2.0	58.0-49.6	169.9/177.3	-	[19]
wild-type and recombinant strain *Burkholderia* sp.	3.7-6.1	13-24	81-99	–	1-19	274-657	3.0-3.1	–	162.3-160.8	298-306	[15]
recombinant strain *C. necator* PHB^{-4}	1.6	40	97	–	3	–	–	–	–	–	[13]
wild-type strain *Chromobacterium* sp. USM2	0.8-1.9	1-49	78-98	–	2-22	–	–	–	–	–	[17]
recombinant strain *E. coli* BL21	0.5-2.9	17-36	86.0-99.4	–	0.6-14.0	–	–	–	–	–	[25]
recombinant strain *R. eutropha* PHB^{-4}	1.4-2.6	53-70	53-89	1	11-46	289-1443	1.7-3.9	–	91-165	–	[22]
recombinant strains *R. eutropha* PHB^{-4}, 1F2, KNK-DCD1	1.33-7.24	32-65	92.0-99.5	0.3-7.4	0.1-4.1	–	–	–	–	–	[26]
recombinant strains *R. eutropha* PHB^{-4} и 1F2	1.6-7.6	29-55	95.8-99.3	0.3-1.7	0.3-3.1	156.8-475.0	1.6-1.9	–	137/151-146/159	–	[11]
glycogen accumulating organisms	–	14-41	63-80	16-25	3HMV: 4-12	140-390	1.6-2.2	11-19	96-170	–	[16]
wild-type strain *R.eutropha* H16, recombinant strain *R. eutropha* PHB^{-4}	1.2-3.1	38-71	60.4-99.0	0.1-1.6	0.6-38.9	130-1840	1.3-4.1	–	120/135-157/169	–	[10]

- no data

Table 2 The composition and properties of P(3HB/3HV/3H4MV) specimens containing different proportions of monomers

n/n	Composition of PHA, mol.%			$M_{w,}$ kDa	$M_{n,}$ kDa	Đ	$C_{x,}$ %	$T_{melt,}$ °C	$T_{degr,}$ °C	$T_{c,}$ °C	$T_{g,}$ °C
	3HB	3HV	3H4MV								
1	100	0.0	0.0	920	368	2.5	76	176	293	112.70	n.d.
2	80.5	19.5	0.0	520	122	4.3	59	168	295	67.50	1.26
3	93.8	3.5	2.7	859	139	6.2	44	171	297	110.64	n.d.
4	79.2	17.5	3.3	609	143	4.3	42	146/162	293	65.35	0.24
5	88.0	8.0	4.0	769	135	5.7	43	162/171	295	103.77	1.40
6	75.7	18.1	6.2	664	166	4.0	42	150/163	294	44.16	-0.15
7	80.7	8.0	11.3	691	163	4.2	41	144/163	297	67.68	0.61

n.d. – not detected

Table 3 The structure and properties of the surface of P(3HB/3HV/3H4MV) films

Composition of PHA, mol.%			Water contact angle water (θ)	Contact angle of diiodomethane (θ)	Liquid surface tension, (mN/m)	Liquid surface tension – polar component, (mN/m)	Liquid surface tension – dispersion component, (mN/m)
3HB	3HV	3H4MV					
100	0.0	0.0	92.7±1.9	49.7±1.2	36.3±0.6	1.3±0.4	35.0±0.6
80.5	19.5	0.0	83.3±2.0	39.2±1.4	42.8±0.4	2.8±0.1	40.0±0.3
93.8	3.5	2.7	84.9±1.7	48.5±1.3	38.3±0.2	3.2±0.1	35.1±0.1
79.2	17.5	3.3	89.3±1.5	54.5±1.0	34.2±0.6	2.5±0.3	31.7±0.3
88.0	8.0	4.0	87.2±1.8	49.7±0.9	37.0±0.5	2.6±0.1	34.4±0.4
75.7	18.1	6.2	95.1±2.1	65.6±1.2	27.4±0.6	2.1±0.1	25.4±0.5
80.7	8.0	11.3	72.2±2.0	61.5±1.5	38.4±0.2	10.7±0.1	27.7±0.1

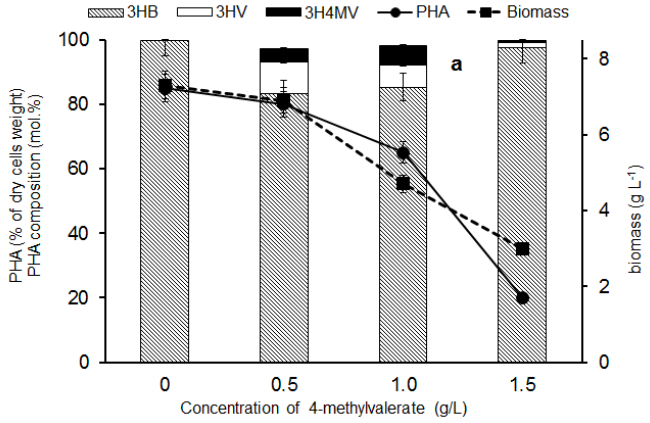


Fig. 1 Effect of 4-methylvalerate concentration on cell concentration, copolymer yield (%), and monomer composition (mol.%) of *C. eutrophus* B-10646 culture in the phase of synthesis of P(3HB/3HV/3H4MV) copolymers.

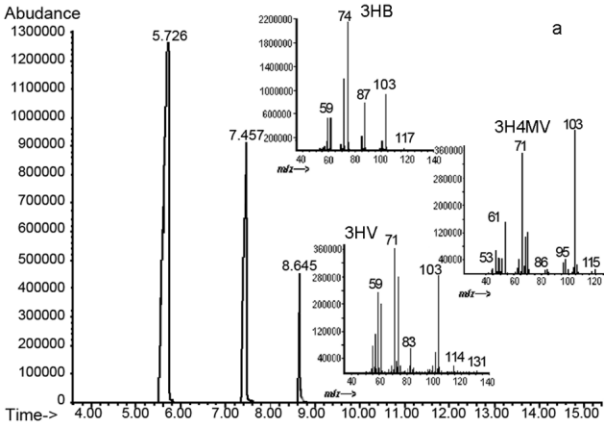


Fig. 2 Chromatogram with mass spectra (a) and ^{1}H NMR spectrum (b) of one P(3HB/3HV/3H4MV) specimen.

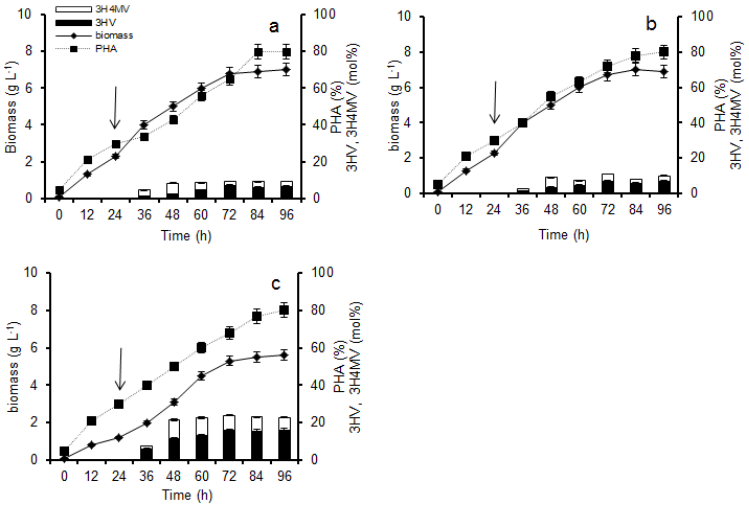


Fig. 3 Parameters of *C. eutrophus* B-10646 culture and synthesis of P(3HB/3HV/3H4MV) copolymers in experiments with fructose (a), glucose (b) or butyric acid (c) as a sole carbon substrate and a single addition of 4-methylvalerate (the time of supplementation is shown by arrows).

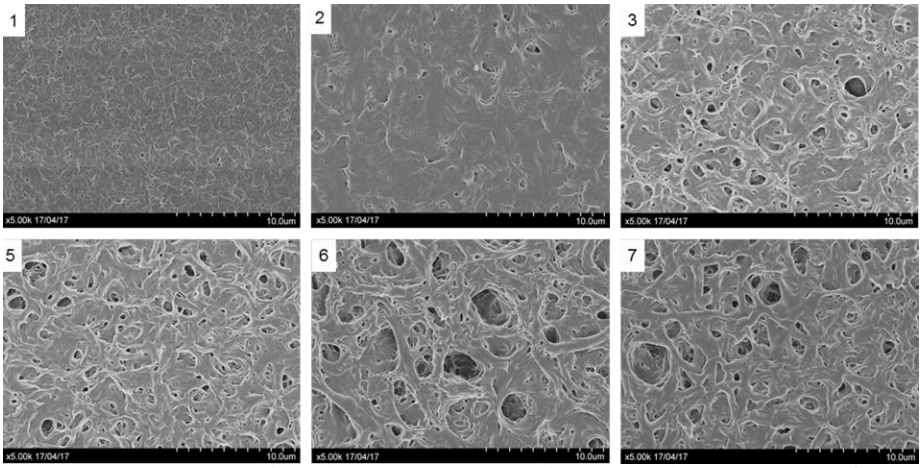
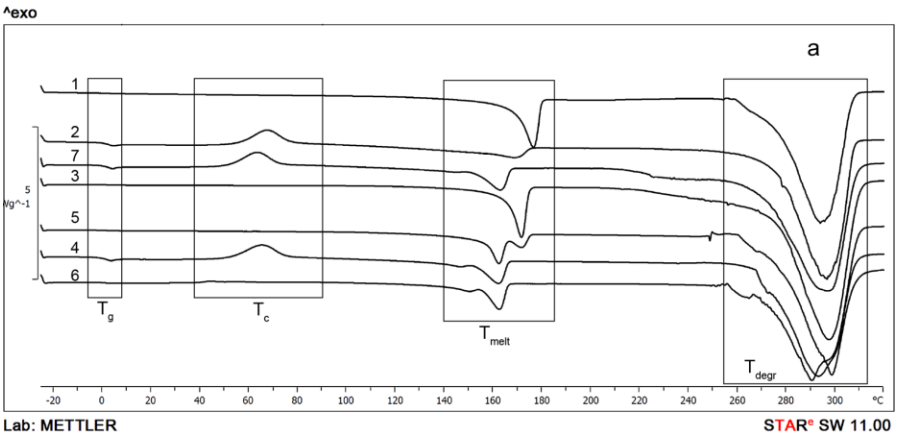


Fig. 4 SEM images of surface topography of polymer films prepared from PHAs with different composition. Bar=10 μm (numbered according to Table 2)



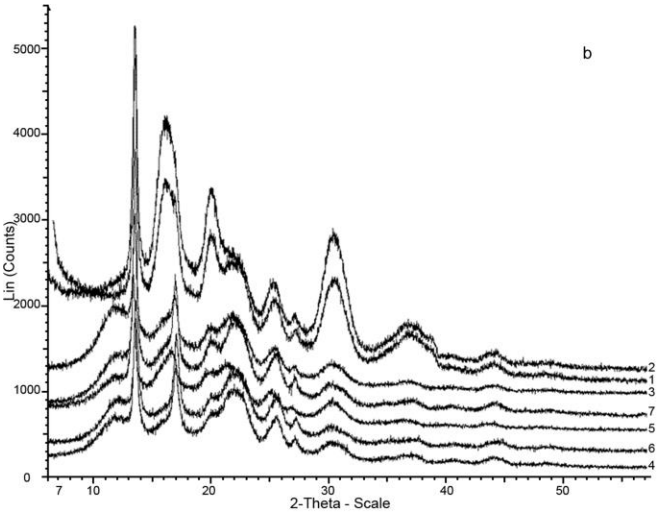


Fig. 5 Thermal properties (a) and X-Ray (b) of PHA specimens with different chemical composition: 1 – P(3HB), 2 – P(3HB/3HV), 3-7 – P(3HB/3HV/3H4MV) (numbered according to Table 2)