

# Biosynthesis and properties of P(3HB-co-3HV-co-3H4MV) produced by using the wild-type strain *Cupriavidus eutrophus* B-10646

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## Abstract

**BACKGROUND:** Polyhydroxyalkanoates (PHA) containing 3-hydroxy-4-methylvalerate (3H4MV) have better processing properties than other PHA types and were not prone to ageing. The main challenge in the production of these polymers is to simultaneously achieve high cell biomass, high polymer content, and high molar fraction of 3H4MV. The aim of this study was to investigate the production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxy-4-methylvalerate) [P(3HB-co-3HV-co-3H4MV)] by *Cupriavidus eutrophus* B-10646, synthesize polymers with different contents of 3H4MV and investigate their properties.

**RESULTS:** It was shown that 4-methylvalerate (4MV, the precursor substrate) concentration in the culture medium must not be higher than 1 g L<sup>-1</sup>, and that 4MV must be carefully dosed, with at least 10–12 h intervals between supplementations. A series of polymers with molar fractions of 3H4MV between 2.7 and 11.3 mol% was synthesized. Degrees of crystallinity, molecular weight characteristics, and thermal properties of the polymers have been investigated as dependent on proportions of monomers.

**CONCLUSION:** The content of 3H4MV depends on the concentration of 4MV and conditions of carbon nutrition. The molar fraction of 3H4MV had a strong effect on the crystalline to amorphous region ratio. PHA containing 3H4MV specimens synthesized in this study had a degree of crystallinity of below 50%, which remained unchanged for 2.5 years.

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**Keywords:** polyhydroxyalkanoates; 3-hydroxy-4-methylvalerate; synthesis; physicochemical properties

## INTRODUCTION

Poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxy-4-methylvalerate) [P(3HB-co-3HV-co-3H4MV)] copolymers are relatively new representatives of the class of microbial reserve macromolecules synthesized by microorganisms under specified conditions of unbalanced growth. These are so-called polyhydroxyalkanoates (PHAs) – polyesters capable of being degraded to CO<sub>2</sub> and H<sub>2</sub>O under aerobic conditions and to CH<sub>4</sub> and H<sub>2</sub>O under anaerobic conditions. PHA 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.<sup>1–6</sup>

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.<sup>7–11</sup> 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 monomer composition of PHAs on their properties.

Studies reporting the synthesis of PHA copolymers containing 3H4MV monomer units were published relatively recently. The authors of those studies assumed that these polymers had better

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processing properties than other PHA types and were not prone to ageing.<sup>12,13</sup> This interesting fact was addressed in a study by Tanadchangsang *et al.*<sup>12</sup>: the authors investigated the ageing effect of P(3HB-co-3H4MV) at room temperature for 180 days. They found that fatal deterioration was not observed for copolymer containing 3H4MV molar fraction higher than 14 mol%. These copolymers were mainly produced by genetically modified strains,<sup>12–15</sup> wild-type microorganisms being used in fewer studies.<sup>16–19</sup> A 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 3H4MV, on the other (Table 1). Published studies mainly report data on the cell biomass yields achieved, total production of the P(3HB-co-3HV-co-3H4MV) copolymer, and the monomer composition of the copolymer. Properties of this type of copolymer 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 aim of this study was to investigate the production of P(3HB-co-3HV-co-3H4MV) by *Cupriavidus eutrophus* B-10646, synthesize polymers with different contents of 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. The strain is registered in the Russian National Collection of Industrial Microorganisms. Bacterial cells were cultivated using Schlegel's mineral medium as described previously.<sup>20</sup> The main carbon source was fructose (Panreac, EU) or glucose (China), or butyric acid (Panreac, EU). 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% aqueous KOH solution (pH  $7.0 \pm 0.2$ ) and then they were filter-sterilized.

### Bacterial culture

Schlegel's mineral medium was used as a basic solution for growing cells:  $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$  – 9.1;  $\text{KH}_2\text{PO}_4$  – 1.5;  $\text{MgSO}_4 \cdot \text{H}_2\text{O}$  – 0.2;  $\text{NH}_4\text{Cl}$  – 1.0 ( $\text{g L}^{-1}$ ). A solution of iron citrate ( $5 \text{ g L}^{-1}$ ), which was used as a source of iron, was added to reach a concentration of  $5 \text{ mL L}^{-1}$ . Hoagland's trace element solution was used at 3 mL of standard solution per 1 L of the medium. The standard solution contains  $\text{H}_3\text{BO}_3$  – 0.288;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  – 0.030;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  – 0.08;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  – 0.008;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  – 0.176;  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$  – 0.050;  $\text{NiCl}_2$  – 0.008 ( $\text{g L}^{-1}$ ). Fructose or glucose ( $10$ – $12 \text{ g L}^{-1}$ ) or butyric acid ( $2 \text{ g L}^{-1}$ ) were used as carbon sources. As the cell biomass increased and the substrate was used up, the culture medium was supplemented with additional portions of the substrate in order to maintain its concentration in the medium.

Cells were grown under aseptic conditions in two-phase batch culture. In Phase 1, cells were cultured under nitrogen deficiency: the amount of nitrogen supplied in this stage was 50% of the cell's physiological requirements. By the end of Phase 1, the bacteria consumed all nitrogen and in Phase 2, cells were cultured in nitrogen-free medium; the other parameters were the same as in Phase 1. Cultivation was performed at  $30^\circ\text{C}$  and 200 rpm.

Cell biomass increase was evaluated by periodically measuring culture optical density and determining cell concentration (by weighing the dry matter). Analyses for the determination of optical density and cell dry biomass were carried out following procedures described previously.<sup>21</sup>

Nitrogen concentration in the culture medium was analyzed with Nessler's reagent. Concentration of fructose in medium was determined using the resorcinol method.<sup>22</sup> Glucose concentration was determined using the 'Glucose – FKD' kit ('Farmatsevtika i klinicheskaya diagnostika', Moscow, Russia) as described in Volova *et al.*<sup>23</sup> Butyric acid and 4-methylvaleric acid concentrations in the medium were controlled using a gas chromatography analysis (GC-MS 6890/5975C, Agilent Technologies, USA).

### Analysis of the chemical composition of P(3HB-co-3HV-co-3H4MV) copolymers

To extract the polymer, freeze-dried biomass was submerged in chloroform (1:15–20, w/v) and was allowed to stay at room temperature for at least 72 h. After filtration, the chloroform extract was concentrated by rotary evaporation process and the dissolved PHA was precipitated by adding a double volume of hexane. Both lyophilized cells and extracted PHAs were treated with chloroform and methanol/sulfuric acid solution (85:15 (v/v)) and incubated at  $80^\circ\text{C}$  for 160 min. The composition of polymers was analyzed with a GC-MS (6890/5975C, Agilent Technologies, USA). To reconfirm the composition,  $^1\text{H}$  NMR spectra of PHA in  $\text{CDCl}_3$  were obtained on a Bruker Avance III 600 spectrometer operating at 600.13 MHz.

### Analysis of microstructure and properties of copolymers films

To investigate the copolymer properties, the polymer was processed into films, which were prepared by casting chloroform solution (2% w/v) on degreased glass. The film discs were 100 mm in diameter and 0.04 mm thick.

The microstructure of the film surface 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. To study surface properties contact angles of water and diiodomethane drops were measured with a Drop Shape Analyzer – DSA-25E (KRÜSS GmbH, Germany) and surface free energy (SFE) and its dispersion and polar components were processed using the DSA-4 software.

### Analysis of physicochemical properties of P(3HB-co-3HV-co-3H4MV) copolymers

Molecular weights (weight average,  $M_w$ , and number average,  $M_n$ ) and polydispersity ( $D = M_w/M_n$ ) were determined using gel permeation chromatography (Agilent Technologies 1260 Infinity chromatograph, equipped with a refractive index detector and an Agilent PLgel Mixed-C column). Chloroform was used as the mobile phase. Polystyrene standards (Fluka, Switzerland, Germany) were used to make a calibration curve.

Glass transition temperature ( $T_g$ ), crystallization temperature ( $T_c$ ), melting point ( $T_m$ ) and thermal degradation temperature ( $T_{\text{degr}}$ ) of polymers were determined using a DSC-1 differential scanning calorimeter (Mettler Toledo, Switzerland). About  $4.0 \pm 0.2 \text{ mg}$  of each sample was heated at a rate of  $5^\circ\text{C min}^{-1}$  to  $200^\circ\text{C}$ , then cooled to  $-20^\circ\text{C}$ , held for 20 min, and re-heated to  $320^\circ\text{C}$ .

The X-ray diffraction spectra were obtained using a D8 Advance X-Ray powder diffractometer equipped with a VANTEC fast linear detector, using CuK $\alpha$  radiation (Bruker, AXS, Germany).

**Table 1.** Literature data on synthesis and properties of PHAs containing 3H4MV monomers

Species	Biomass (g L <sup>-1</sup> )	PHA content (% of CDM)	Content of monomers, mol.%			<i>M<sub>w</sub></i> (kDa)	<i>D</i>	<i>C<sub>x</sub></i> (%)	<i>T<sub>m</sub></i> (°C)	<i>T<sub>degr</sub></i> (°C)	Reference
			3HB	3HV	3H4MV						
wild-type strain <i>A. chroococcum</i> 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	–	26
wild-type and recombinant strain <i>Burkholderia</i> sp.	3.7–6.1	13–24	81–99	–	1–19	274–657	3.0–3.1	–	162.3–160.8	298–306	17
recombinant strain <i>C. necator</i> PHB <sup>-4</sup>	1.6	40	97	–	3	–	–	–	–	–	15
wild-type strain <i>Chromobacterium</i> sp. USM2	0.8–1.9	1–49	78–98	–	2–22	–	–	–	–	–	19
recombinant strain <i>E. coli</i> BL21	0.5–2.9	17–36	86.0–99.4	–	0.6–14.0	–	–	–	–	–	31
recombinant strain <i>R. eutropha</i> PHB <sup>-4</sup>	1.4–2.6	53–70	53–89	1	11–46	289–1443	1.7–3.9	–	91–165	–	27
recombinant strains <i>R. eutropha</i> PHB <sup>-4</sup> , 1F2, KNK-DCD1	1.33–7.24	32–65	92.0–99.5	0.3–7.4	0.1–4.1	–	–	–	–	–	32
recombinant strains <i>R. eutropha</i> PHB <sup>-4</sup> and 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	–	13
glycogen accumulating organisms	–	14–41	63–80	16–25	3HMV: 4–12	140–390	1.6–2.2	11–19	96–170	–	18
wild-type strain <i>R. eutropha</i> H16, recombinant strain <i>R. eutropha</i> PHB <sup>-4</sup>	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	–	12

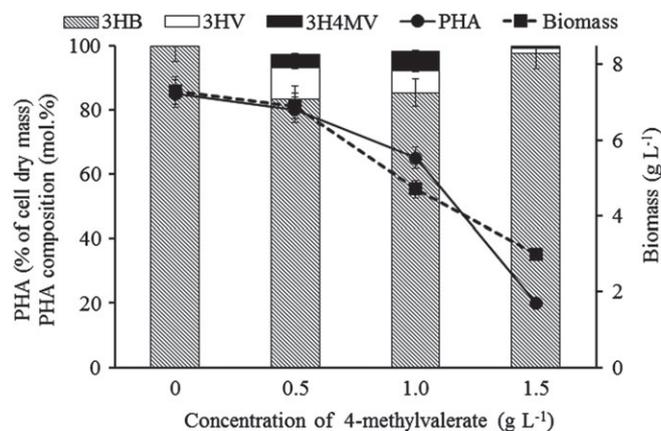
—, no data.

All the characterization was performed using similar procedures to those previously described.<sup>21,23,24</sup>

## RESULTS AND DISCUSSION

### Study of P(3HB-co-3HV-co-3H4MV) synthesis in the *C. eutrophus* B-10646 culture

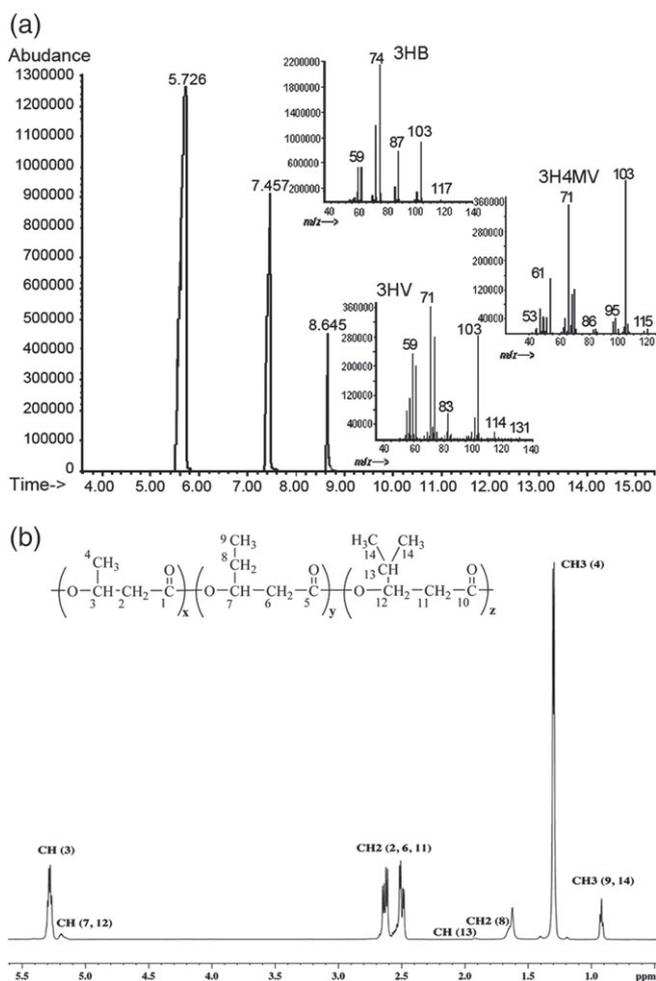
First, in order to determine non-inhibiting concentrations of the precursor substrate necessary for the production of 3H4MV monomer units, the effect of 4-methylvalerate (4MV) concentration on the growth of *C. eutrophus* B-10646 cells and synthesis of P(3HB-co-3HV-co-3H4MV) was studied. For bacterial 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.<sup>25</sup> In our experiment, 4MV fed into the bacterial culture in the phase of polymer synthesis at a concentration of 0.5 g L<sup>-1</sup> did not significantly inhibit cell growth and polymer production (Fig. 1). Over 96 h of the culture, cell concentration reached 6.9 g L<sup>-1</sup>, which was comparable with the cell concentration in the control culture, where cells were grown on the sole carbon substrate, fructose (7.3 g L<sup>-1</sup>). The total polymer yield reached 5.5 g L<sup>-1</sup>, which corresponded to its intracellular content of 80% of cell dry mass (CDM) and was close to the control value (83–85% of CDM). As potassium 4MV concentration was increased to 1.0 g L<sup>-1</sup>, inhibition of the cell growth and copolymer synthesis was observed, which dropped to 4.7 g L<sup>-1</sup> and 65% of CDM, respectively. The content of 3H4MV monomer units in the copolymer was about 6 mol%. A concentration of potassium 4MV of 1.5 g L<sup>-1</sup> had a strong inhibitory effect on the bacterial growth and especially on polymer synthesis, whose intracellular concentration dropped to



**Figure 1.** Effect of 4MV concentration on cell concentration, copolymer fraction in CDM (%), and monomer composition (mol%) of *C. eutrophus* B-10646 culture in the phase of synthesis of P(3HB-co-3HV-co-3H4MV) copolymers.

20% of CDM; the 3H4MV content was no more than 1 mol% (Fig. 1). A chromatogram with mass spectra and a <sup>1</sup>H NMR spectrum of one P(3HB-co-3HV-co-3H4MV) specimen is shown at Fig. 2(a, b) respectively. In the <sup>1</sup>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 the other methine proton (peak 13) was assigned to the carbon C13. The methylene protons of the carbons C2, C6 and C6 were observed as peaks (2), (6) and (11), respectively. Finally, terminal methyl groups (peaks 4, 9 and 14) were definitely identified.

The results obtained are consistent with the literature data suggesting a toxic effect of 4MV on microorganisms and describing



**Figure 2.** Chromatogram showing mass spectra (a) and <sup>1</sup>H NMR spectrum (b) of one P(3HB-co-3 HV-co-3H4MV) specimen.

difficulties of producing polymers with a high content of 3H4MV monomer units simultaneously with achieving high cell concentrations and a considerable mass fraction of PHA of CDM. For instance, cultivation of the recombinant strain *Ralstonia eutropha* PHB<sup>-4</sup> on fructose and 4MV resulted in cell concentration of 1.5 g L<sup>-1</sup> and PHA content of 46% of CDM.<sup>12</sup> In a study of the recombinant strain *C. necator* PHB<sup>-4</sup>, the mass fraction of P(3HB-co-3H4MV) in CDM and cell biomass yield were about 40% and 1.6 g L<sup>-1</sup>, respectively.<sup>15</sup> Selection of leucine resistant mutants did not result in high yields: cell concentration and polymer content were 1.5–1.7 g L<sup>-1</sup> and 51–55% of CDM, respectively, and 3 HV 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<sup>-4</sup> with leucine at a concentration of 10 g L<sup>-1</sup> caused cell concentration to increase to 7.1–7.3 g L<sup>-1</sup> but copolymer content to drop to 29–36% of CDM; the 3H4MV molar fraction was no higher than 3.0 mol%.<sup>13</sup> The wild-type strain *Azotobacter chroococcum* 7B cultivated on sucrose supplemented with 4MV synthesized rather high contents of the copolymer (71–79% of CDM), but cell concentration was no more than 3.7 g L<sup>-1</sup>, and the molar fraction of 3H4MV was extremely low (0.14–0.60 mol%).<sup>26</sup> Thus, synthesis of P(3HB-co-3 HV-co-3H4MV) containing a large molar fraction of 3H4MV in the culture producing high cell concentrations and copolymer content seems problematic.

Another question that needed to be answered was how rapidly bacterial cells grown on fructose as the main carbon substrate consumed 4MV. Fructose, whose initial concentration in the medium was 20 g L<sup>-1</sup>, 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 4MV at concentrations of 0.5 and 1.0 g L<sup>-1</sup>. 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<sup>-1</sup>; after 12 and 24 h, only trace amounts of 4MV were detected in the medium (data not shown). The results obtained suggested that a single dose of 4MV must not be more than 0.5–1.0 g L<sup>-1</sup> and the time interval between supplementations must be at least 10–12 h.

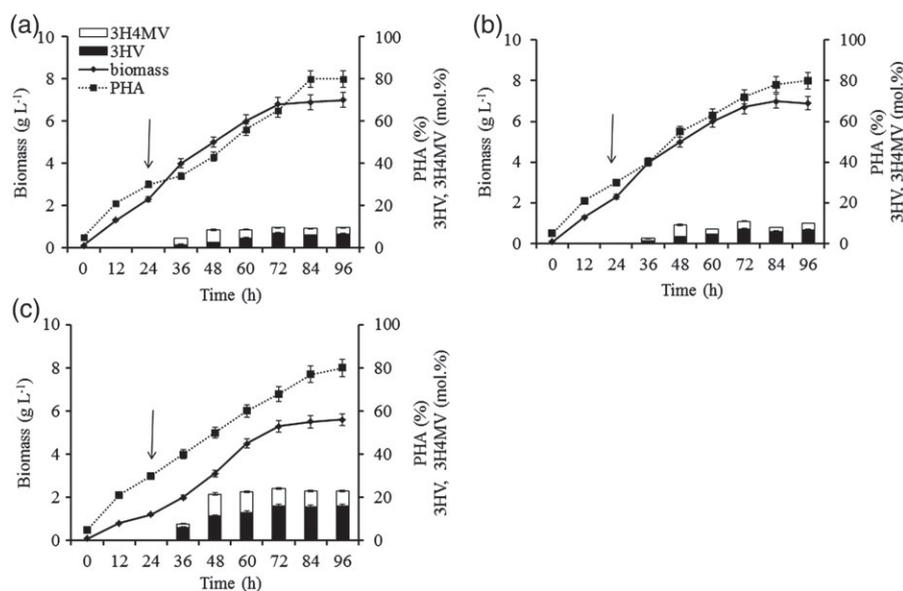
As microorganisms can synthesize copolymers when grown on different substrates – fructose,<sup>15,17</sup> valerate, propionate,<sup>16</sup> and soybean oil,<sup>24</sup> the total yields of P(3HB-co-3 HV-co-3H4MV) copolymers and molar fractions of 3H4MV in them were investigated in experiments with glucose or fructose, or butyric acid as the sole C-substrate (Fig. 3). This was done by adding once 0.5 g L<sup>-1</sup> of 4MV 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 4MV, cell concentration and copolymer production were increasing for some time, reaching 6.8–7.0 g L<sup>-1</sup> and 78–80% of CDM, respectively, by the end of the experiment. The content of 3H4MV in the copolymer showed a different trend (Fig. 3(a) and (b)). At 24 h after the addition of 4MV, monomer concentration reached its peak (6.0 mol%), but then declined, and after 96 h was 2–3 mol%. In the culture with the butyric acid, 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. 3(c)). The cell concentration and total mass fraction of PHA in CDM were comparable with those obtained in cultures with sugars.

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

Literature data suggest that 3H4MV content can be varied by using different conditions of carbon nutrition. For instance, Lau et al.<sup>16</sup> 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 3 HV (from 10 to 84 mol%) and 3H4MV (from 1 to 44 mol%). However, the cell concentration and copolymer content were very low, 1.3–1.8 g L<sup>-1</sup> and 1–5% of CDM, respectively.

Based on these results (Fig. 3), a series of P(3HB-co-3 HV-co-3H4MV) copolymers was synthesized with high molar fraction of 3H4MV in PHA, by growing bacterial cells in two-phase batch culture on butyric acid, with 4MV added in controlled doses. A single dose of 4MV was 0.5 g L<sup>-1</sup>, 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 HV, whose molar fraction varied between 3.5 and 18.1 mol% (Table 2).



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

**Table 2.** The composition and properties of P(3HB-co-3HV-co-3H4MV) specimens containing different proportions of monomers

n/n	Composition of PHA, mol.%			$M_w$ , kDa	$M_n$ , kDa	$\bar{D}$	$C_x$ , %	$T_m$ , °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

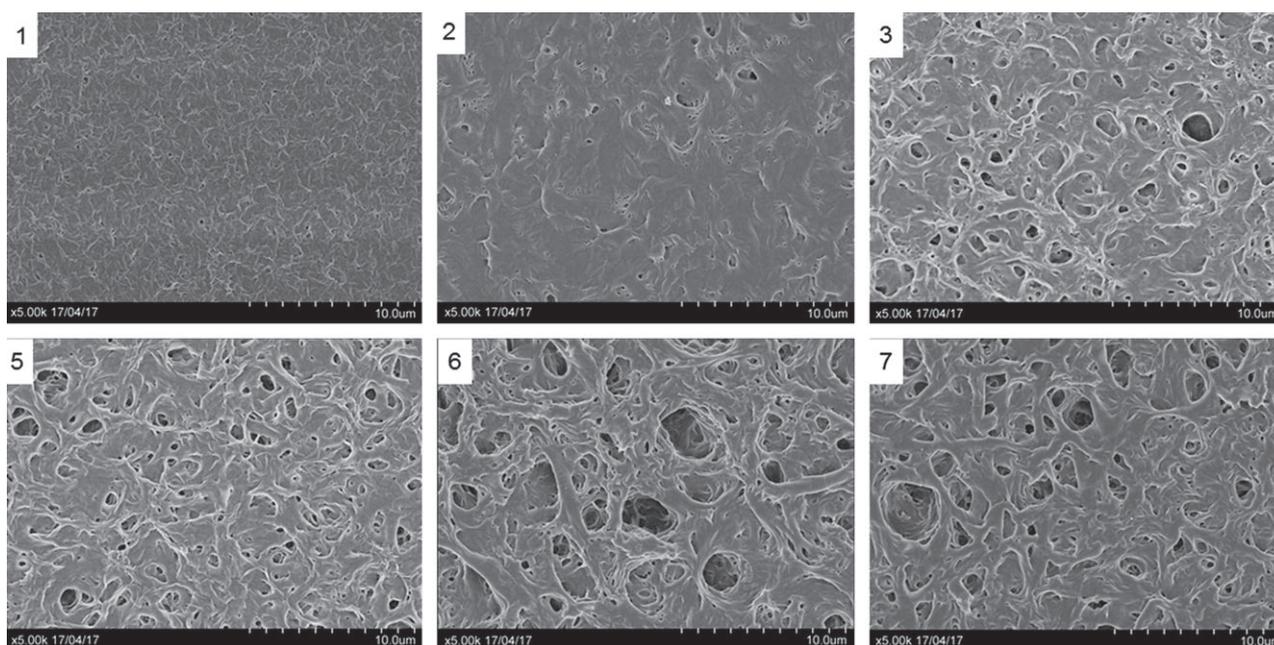
n.d. = not detected.

### Physicochemical properties of P(3HB-co-3HV-co-3H4MV) copolymers with different proportions of monomers

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

SEM images clearly showed that all films of P(3HB-co-3HV-co-3H4MV) copolymers had more pores of sizes between 2–3 and 8–10  $\mu\text{m}$  than films prepared from P(3HB) and P(3HB-co-3HV), and the number and size of the pores increased with increase in the 3H4MV content. However, no definite relationship was found between the 3H4MV content of P(3HB-co-3HV-co-3H4MV) and film surface properties characterized by measuring contact angles of diiodomethane and water (Table 3). The contact angle of diiodomethane, though, did increase above 60° with increase in the 3H4MV content, compared with 49.7° and 39.2° for P(3HB) and P(3HB-co-3HV). 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-co-3HV-co-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-co-3HV-co-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  $\bar{D}$  values (4.0–6.2) mentioned above. The molecular weight parameters are among the most important characteristics of high-molecular-weight compounds, and determine processibility 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-co-3HV-co-3H4MV) with different content of 3H4MV. Lau *et al.*<sup>17</sup> 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 Tanadchangsang *et al.*<sup>12</sup> showed that in specimens containing 0.4–0.9 mol% 3HV and 0.7–12.9 mol% 3H4MV, number average molecular weight and



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

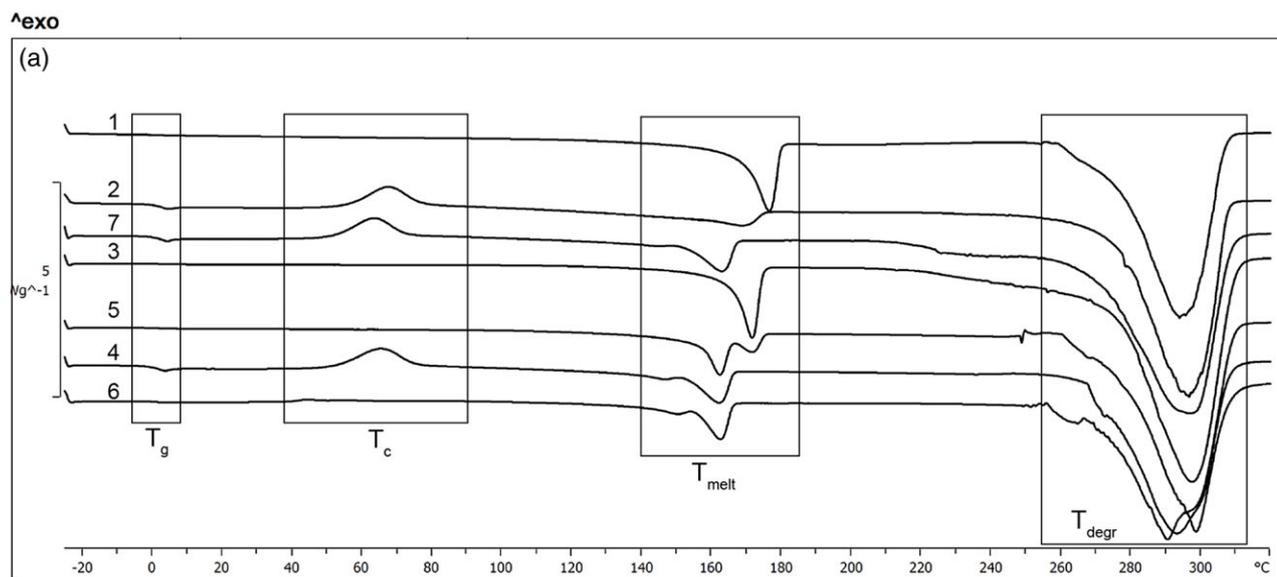
**Table 3.** The structure and properties of the surface of P(3HB-co-3 HV-co-3H4MV) films

Composition of PHA, mol.%			Water contact angle water ( $\theta$ )	Contact angle of diiodomethane ( $\theta$ )	Liquid surface tension, (mN/m)	Liquid surface tension – polar component, (mN/m)	Liquid surface tension – dispersion component, (mN/m)
3HB	3 HV	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

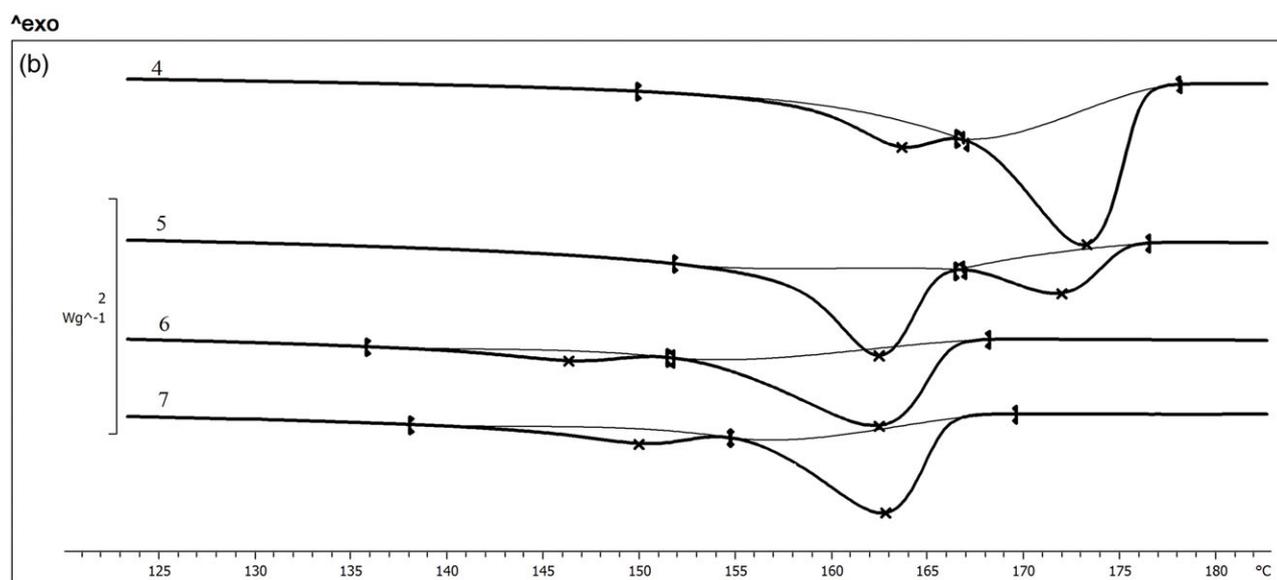
polydispersity varied between 100 and 400 kDa and between 1.4 and 4.1, respectively. The authors of another study<sup>13</sup> investigated copolymers with a considerably lower molar fraction of 3H4MV (0.8–3.1 mol%) and found that their  $M_n$  ranged between 98 and 251 kDa, with polydispersity values being rather low (1.6–1.9). Higher values of  $M_n$  (298–421 kDa) and  $\mathcal{D}$  (2.2–2.5) were reported by Chia *et al.*<sup>14</sup> for P(3HB-co-3H4MV-co-3HHx) and P(3HB-co-3 HV-co-3H4MV-co-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-co-3 HV).

The study of thermal properties of P(3HB-co-3 HV-co-3H4MV) showed a consistent decrease in the  $T_m$  with increase in the 3H4MV content (Table 2). In specimens containing between 3.3 and 11.3 mol% 3H4MV (4–7 in Table 2), melting regions in thermograms (Fig. 5(a)) are noticeably shifted to the left compared with the specimen with a lower molar fraction of this monomer (2.7 mol%, Specimen 3 in Table 2) and to  $T_m$  of P(3HB-co-3 HV) and P(3HB). Thermal behavior of copolymers containing 3H4MV

monomers is characterized by the presence of two peaks in the melting region, with the difference between them 9 to 19° (Fig. 5(a, b)). In contrast to  $T_m$ , no significant differences were found between temperatures of thermal degradation. All specimens, irrespective of their 3H4MV and 3 HV contents, showed  $T_{degr}$  of between 293 and 297°C, which was comparable with the values for the homopolymer and copolymers with 3 HV. Not all gradually heated specimens showed a peak in the glass transition region (Fig. 5(a)). 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-co-3 HV-co-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. 5(a)). The  $T_c$  of some of the specimens was above 100°C. These were P(3HB) and P(3HB-co-3 HV-co-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



Lab: METTLER

STAR<sup>®</sup> SW 11.00

Lab: METTLER

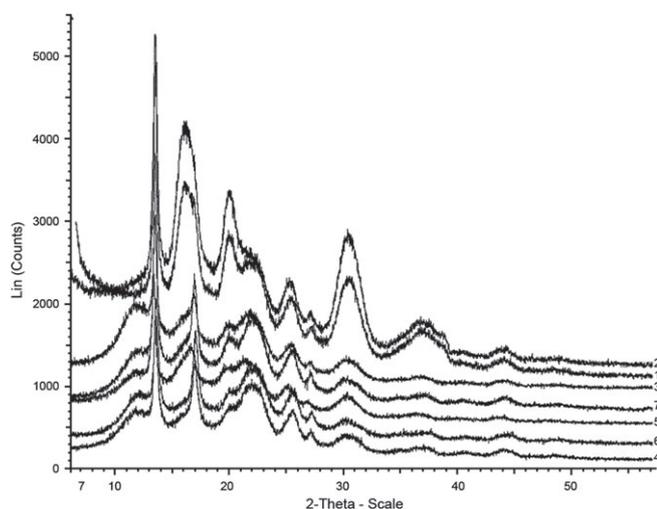
STAR<sup>®</sup> SW 11.00

**Figure 5.** Thermal properties (a) of PHA specimens with different chemical compositions, and the melting regions of Samples 4–7 (b): 1 – P(3HB), 2 P(3HB-co-3 HV), 3–7 – P(3HB-co-3 HV-co-3H4MV) (numbered according to Table 2) × – melting temperature peaks in Fig. 5b.

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-co-3H4MV) that contained 19 mol% 3H4MV showed  $T_g$ ,  $T_m$ , and  $T_{degr}$  of 3.3, 160.8, and 306°C, respectively.<sup>17</sup> A study by Saika *et al.*<sup>13</sup> showed that as the 3H4MV molar fraction of the copolymers increased from 0.8 to 3.1 mol%, their  $T_m$  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<sup>12</sup> reported that as the 3H4MV molar fraction was increased to 38 mol%, both  $T_m$  and  $T_g$  tended to decrease.

Analysis of radiograms of PHA specimens with different composition revealed several dissimilarities between them (Fig. 6). X-Rays showed a decrease in intensities of diffraction maxima at  $2\theta = 16^\circ$ ,  $30^\circ$ ,  $38^\circ$  and  $45^\circ$ , with the most significant decrease at  $2\theta = 16^\circ$  and  $30^\circ$ , for all P(3HB-co-3 HV-co-3H4MV) specimens compared with P(3HB-co-3 HV) and P(3HB): intensities

of diffraction maxima of P(3HB) and P(3HB-co-3 HV) 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. 6). P(3HB-co-3 HV-co-3H4MV) specimens also showed changes in the 18–22° region, with additional maxima observed at  $2\theta = 22^\circ$  and a certain decrease in intense peaks at  $2\theta = 18^\circ$ . X-Ray diffraction analysis of PHAs with different compositions (Table 2, Fig. 6) showed that the degree of crystallinity ( $C_x$ ) of P(3HB-co-3 HV-co-3H4MV) copolymers was considerably lower than that of the high-crystallinity homopolymer (76%) and P(3HB-co-3 HV). The  $C_x$  of P(3HB-co-3 HV-co-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



**Figure 6.** X-Ray of PHA specimens with different chemical composition: 1 – P(3HB), 2 – P(3HB-co-3 HV), 3–7 – P(3HB-co-3 HV-co-3H4MV) (numbered according to Table 2).

a stronger effect on the crystalline to amorphous region ratio than 3HHx and 3HV monomer units.<sup>9–11,23</sup> In P(3HB-co-3 HV), 3HB and 3HV monomers co-crystallize in the 3HB or 3HV 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 Tanadchangsang *et al.*<sup>27</sup> cultivation of the recombinant strain *Ralstonia eutropha* PHB<sup>-4</sup> on fructose with varied concentrations of 4MV produced polymers with molar fractions of 3H4MV and 3HV of 46 mol% and 1.0 mol%, respectively, with low cell concentration ( $1.4 \text{ g L}^{-1}$ ) and copolymer yield of 53%.  $C_x$  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.*<sup>26</sup> suggesting a decrease in  $C_x$  of P(3HB-co-3 HV-co-3H4MV) with a very low content 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  $C_x$  of P(3HB-co-3 HV-co-3HMV) terpolymers synthesized by glycogen accumulating organisms (GAO) grown on acetate. The  $C_x$  value varied between 11 and 19% in the specimens containing 4–12 mol% 3HMV and 16–25 mol% 3HV.<sup>18</sup> In another study, Dai *et al.*<sup>28</sup> cultivated *Deffluviococcus vanus*-related glycogen accumulating organisms in the medium with acetate supplemented with propionate and synthesized P(3HB-co-3 HV-co-3HMV-co-3HMB) quaterpolymers containing 32–36 and 15–41 mol% 3HV and 3HMV, respectively, with a  $C_x$  of 14–15%. Thus, incorporation of 3H4MV monomers into the carbon chain of P(3HB-co-3 HV), even though 3H4MV content is relatively low, results in a significant decrease in  $C_x$  of the copolymer.

Crystallinity is a most important property of polymers, and essentially determines the processability of polymers into specialized products. The most widespread and best-studied short-chain-length polymers – P(3HB) and P(3HB-co-3 HV) copolymers – are bioresorbed *in vivo* slowly, i.e. they stay in the organism for a long time and may cause tissue inflammation in the implantation site.<sup>29</sup> 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.<sup>30</sup> Having studied the effect of 3H4MV monomer units on the stability of the properties of the films, we found that the  $C_x$  value of P(3HB) films that had been kept at room temperature for 3 years had increased from the initial value of 76% to 95% (unpublished data). The degree of crystallinity of films prepared from P(3HB-co-3 HV-co-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-co-3 HV-co-3H4MV) was investigated in batch cultures of *C. eutrophus* B-10646 under different conditions of carbon nutrition and as dependent on the concentration of the precursor substrate – 4MV. Then, bacterial cells were cultivated in the modes that enabled the production of copolymers containing 3H4MV from 2.7 to 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.

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