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Biosynthesis of Poly-3-Hydroxybutyrate–3-Hydroxyvalerate Copolymer by *Azotobacter chroococcum* Strain 7B

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Abstract—The ability of *Azotobacter chroococcum* strain 7B, producer of polyhydroxybutyrate (**PHB**), to synthesize its copolymer poly-3-hydroxybutyrate–3-hydroxyvalerate (**PHB–HV**) was studied. It was demonstrated, for the first time, that *A. chroococcum* strain 7B was able to synthesize PHB–HV with various molar rates of **HV** in the polymer chain when cultivated on medium with sucrose and carboxylic acids as precursors of HV elements in the PHB chain, namely, valeric (13.1–21.6 mol %), propanoic (3.1 mol %), and hexanoic (2.1 mol %) acids. Qualitative and functional differences between PHB and PHB–HV were demonstrated by example of the release kinetic of methyl red from films made of synthesized polymers. Maximal HV incorporation into the polymer chain (28.8 mol %) was recorded when the nutrient medium was supplemented with 0.1% peptone on the background of 20 mM valerate. These results suggest that that the studied strain can be regarded as a potential producer of not only PHB but also PHB–HV.

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First and foremost, the interest in bacterial polyhydroxyalkanoate (**PHA**) polymers—polyhydroxybutyrate (**PHB**) and its copolymers—is determined by the fact that they are close to synthetic thermoplastics in their main characteristics and concurrently display several unique properties, such as a high biocompatibility with body organs and tissues and the ability to biodegrade forming nontoxic products. Over 300 PHA producers have been characterized; approximately 100 various hydroxyalkanoic acids (in addition to 3-hydroxybutyric acid) have been detected as components of PHAs [1]; the effect of carbon nutrition conditions on PHA synthesis were actively studied in the context of the possibility to synthesize not only single-component, but also multicomponent PHAs composed of short- and medium-chain monomers with a chain length of C_4 to C_{16}. No PHAs containing the monomers longer than C_{16} have been found. It has been shown that the cosubstrate, alkanoic acids, is the main player in determining the PHA composition, while peptone and phosphorus compounds significantly influence the PHA yield [2–5].

As a rule, organic acids or alcohols with an odd number of carbon atoms are used as either main or additional sources of carbon to produce copolymers by microbiological synthesis: in metabolism, they are transformed into a five-carbon compound, valeryl-CoA, which is utilized for synthesizing 3-hydroxyvalerate (**HV**) [6]. In particular, the methylotrophic bacterium *Methylobacterium extorquens* synthesizes the copolymer PHB–HV when growing on methanol using propanol, propionate, pentanol, and valerate as cosubstrates [7]. However, some microorganisms synthesize only PHB even when cultivated on the sources with an odd number of carbon atoms. For example, the majority of purple sulfur bacteria fail to incorporate HV into the polymer when using propionate, valerate, and heptanoate as carbon sources [8].

The PHB–HV copolymer is currently best studied and is manufactured by Monsanto (United States; trade name Biopol; producer, a commercial strain of *Ralstonia eutropha*). According to literature data, bacteria of the genus *Azotobacter* are also capable of synthesizing PHB–HV copolymer [9, 10]. For *A. vinelandii* strain UWD, the main HV source for the copolymer is β-oxidation of the *n*-alkanoates with an odd number of carbon atoms; in this case, sucrose is used as the main source of carbon. Valeric acid is incorporated into the copolymer via the β-oxidation pathway: valeryl-CoA > 3-ketovaleryl-CoA > D-3-hydroxyvaleryl-CoA > HV; thus, 3-ketovaleryl-CoA, an intermediate product of valeric acid β-oxidation does not cleave further to give acetyl-CoA and propionyl-CoA but rather is used for the synthesis of PHA with the involvement of acetoacetyl-CoA reductase [10, 11]. *A. vinelandii* strain UWD synthesizes the PHB–HV copolymer when grown on valeric, heptanoic, and nonanoic acids but incorporates only trace HV amounts (<1%) when grown on propanoic acid. Another *Azotobacter* species, *A. salinestris,* is able to synthesize PHB with a molar rate of HV to 10% when grown only on sugars without the addition of valeric acid [12]. Indian researchers [13] have recently isolated *A. chroococcum* strain MAL-201, which is capa-

ble of synthesizing the copolymer concurrently containing HV and polyethylene glycol (PHB–HV–PEG) when grown on the medium with glucose supplemented with sodium salt of valeric acid and PEG.

Incorporation of HV into the PHB homopolymer chain considerably improves the physicochemical properties of the copolymer. The melting temperature of PHB–HV is lower than that of PHB, which provides for increasing the technological "processability window" (the difference between the melting temperature and the temperature of thermal degradation commencement); it is more plastic, extensible, and resilient due to a decrease in the value of Young's modulus with an increase in the HV molar fraction in PHB–HV polymer chain; therefore, the polymer can find a wider application [14–18]. Recently, PHB and its copolymers attract ever-increasing attention as a potential material for polymeric systems with a controllable drug release [19–21].

The goal of this work was to study the possibility of PHB–HV biosynthesis by *A. chroococcum* strain 7B cultivated on the medium with sucrose supplemented with carboxylic acids, to determine the molar percent of HV incorporation into the PHB–HV polymer chain, and to assess the qualitative functional difference between the synthesized copolymer and homopolymer comparing the release kinetic of a model drug incorporated into the polymeric matrix from biopolymer film systems.

MATERIALS AND METHODS

Research object. *A. chroococcum* strain 7B, producer of PHB biodegradable thermoplastic able to overproduce PHB (to 80% of cell dry weight), was used in the work.

Growth conditions. The strain was isolated from the wheat rhizosphere (sod-podzolic soil) and maintained on Ashby's medium, containing 0.2 g/l $K_2HPO_4 \cdot 3H_2O$, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.2 g/l NaCl, 0.006 g/l $Na_2MoO_4 \cdot 2H_2O$, 5.0 g/l $CaCO_3$, 20 g/l sucrose, and 20 g/l agar. All experiments were performed under laboratory conditions. For PHB–HV synthesis in cells, the culture was grown in shaker flasks (containing 100 ml of the medium) at 30°C in Burk's medium, containing 0.4 g/l $MgSO_4 \cdot 7H_2O$, 0.01 g/l $FeSO_4 \cdot 7H_2O$ – 0.01, $Na_2MoO_4 \cdot 2H_2O$, 0.006 g/l $Na_2MoO_4 \cdot 2H_2O$, 0.5 g/l sodium citrate, 0.1 g/l $CaCl_2$, 1.05 g/l $K_2HPO_4 \cdot 3H_2O$, 0.2 g/l KH_2PO_4, and 30 g/l sucrose. Peptone was added at a concentration of 0.1%. As HV precursors in PHB chain, carboxylic acids valeric, propanoic, hexanoic, octanoic, nonanoic, and dodecanoic) were added as sodium salts at a concentration of 10 or 20 mM after 10-h incubation of the culture [22]; the experiment was performed for 48 h.

Microscopy. A Biomed 1 (Biomed, Russia) light microscope was used.

Optical density was measured by nephelometry in an FEK-56 (Russia) photoelectrocolorimeter using a cuvette with an optical path length of 1 mm and an optical filter no. 6.

Study of polymer composition by gas liquid chromatography (GLC). The specimen (4 mg) was dissolved in chloroform (2 ml) in twist cap vials supplemented with 3% solution of sulfuric acid in methanol (2 ml), mixed, kept in a thermostat at 70°C for 3.5 h, cooled to a room temperature, and washed with water to pH 7. The bottom layer was transferred to another vial to assay the methyl esters contained in chloroform solution in a Shimadzu 2010 (Japan) chromatograph equipped with a QP 2010 mass selective detector using an MDN-1 capillary column (bonded methyl silicone) with a length of 30 m and a diameter of 0.35 mm. Chromatography mode: thermostat, 100°C, injector, 200°C; flow splitting, 1 : 10; interface, 210°C; detector, 200°C; and carrier gas, helium at a rate of 0.8 ml/min. Temperature gradient: 100°C for 2 min; heating at 5°/min to 120°C; at 20°/min to 260°C; and isotherm for 2 min. Detector mode: mass identification from 33 to 250 m/z.

Study of the polymer composition by nuclear magnetic resonance (NMR). ^{1}H-NMR spectra of 1–2% PHB and PHB–HV solutions in deuterated chloroform were recorded in an MSL-300 (Bruker, Germany) spectrometer at a working frequency of 300 MHz. Chemical shifts were measured relative to the signal of $CDCl_3$ residual protons, 7.20 ppm. Number of accumulations NS = 40. The percent content of elementary HV elements in the PHB–HV copolymer was calculated according to the ratio of the integral signal intensity from HV methyl group (0.89 ppm) to the sum of integral signal intensities from the methyl groups of HV and hydroxybutyrate (1.27 ppm).

Production of matrix-type PHA films containing drug. Four polymer specimens were used: (1) PHB with a molecular weight of 1220 kDa; (2) PHB, 340 kDa; (3) P(HB–HV 2.5%), 820 kDa; and (4) P(HB–HV 17%), 1300 kDa. The films were produced by pouring polymer solution (1 wt/vol%) into petri dishes with subsequent evaporation of the solvent at room temperature. Chloroform solution of a model drug, methyl red, was mixed with polymer solution before pouring. The content of methyl red in PHB and PHB–HV films was 4 wt % and film thickness was 20 μm. Film fragments of the same shape with a weight of 20 mg were placed into the glass vials containing 30 ml of 0.1 M phosphate buffer pH 7.4. The vials were placed into a shaker (250 rpm) at 37°C. The dynamics of methyl red release was recorded by periodically sampling the solution and determining methyl red concentration by spectrophotometry at 430 nm in a DU-650 (Beckman, United States) spectrophotometer.

Production of high purified PHB and PHB–HV from bacterial biomass. The polymer isolation and purification from *A. chroococcum* comprised the following stages: (1) PHB and PHB–HV extraction with chloroform in a shaker for 12 h at 37°C; (2) separation

Table 1. Synthesis of the PHB–HV copolymer by *A. chroococcum* 7B on the sucrose-containing medium supplemented with carboxylic acids

Substrate	Yield of biomass, g/l medium	Total PHA content, % of dry cell weight	HV content, mol %	PHA molecular weight, kDa
Sucrose (S), 3%	7.9	81.3	0	1650
S + 20 mM propanoic acid	2.3	63.9	3.1	914
S + 10 mM valeric acid	4.5	75.8	13.1	1490
S + 20 mM valeric acid	3.2	72.3	21.6	1300
S + 10 mM hexanoic acid	3.5	67.7	2.1	Not determined
S + 10 mM heptanoic acid	3.9	60.0	Traces	″
S + 10 mM octanoic acid	3.7	60.2	0	″
S + 10 mM nonanoic acid	6.8	67.4	0	″
S + 10 mM dodecanoic acid	2.1	61.1	0	″
S + 10 mM propanoic acid + + 20 mM acetic acid	2.5	65.0	2.5	820

of PHB and PHB–HV solutions from cell debris by filtration; (3) PHB and PHB–HV precipitation from chloroform solution with isopropanol; (4) subsequent repeated dissolution in chloroform and precipitation with isopropanol; and (5) drying at 60°C.

HBV and PHB–HV molecular weights were determined using an Ubblohde capillary glass viscometer (capillary diameter, 0.56 mm) [23]. For this purpose, 10 ml of solution was poured into the viscometer and placed strictly vertically into a thermostat. Viscosity was determined at 30 ± 0.5°C. The flow time was measured 15–20 min after the placement into the thermostat. The mean flow times of PHB and PHB–HV solutions were determined in four to six replicates. When determining characteristic viscosity, the measurements were made for four to five concentrations of solutions. Different PHB and PHB–HV concentrations were obtained by diluting the initial solutions with chloroform. The concentration of studied specimens was within the range of 50–20 mg/100 ml chloroform and the flow time was from 40 to 115 s. Specific viscosity was calculated as $\eta_{sp} = (t - t_0)/t_0$, where t_0 is the flow time for solvent (s) and t is the flow time for polymer (s).

Molecular weight was calculated according to the Mark–Houwink–Kun equation [24].

To determine the characteristic viscosity [η], the experimentally determined numerical values were plotted using the coordinate system with the concentration of polymer solution (C) on the abscissa and the values of reduced viscosity (η_{sp}/C) on the ordinate. The [η] value was obtained by extrapolating the obtained line to the ordinate axis; the accuracy of determination was ~1%. The determination accuracy for molecular weight calculated according to the Mark–Houwink–Kun equation was 2–5%.

In total, five experiments were performed, each in triplicate. The tables list the averaged data.

RESULTS AND DISCUSSION

The effect of carboxylic acids on bacterial growth and PHB and PHB–HV contents in cells. The HV incorporation into PHB–HV was assessed using two methods—GLC and ^{1}H-NMR. Table 1 lists the data on PHB–HV biosynthesis by the *A. chroococcum* 7B culture grown in the medium containing sucrose as a main source of carbon and supplemented with several carboxylic acids as additional sources of carbon for synthesis of the copolymer. These data were obtained by GLC. The HV incorporation into the copolymer was observed when using valeric, propanoic, and hexanoic acids. The best results were obtained in the case of the medium with sucrose supplemented with 20 or 10 mM valeric acid. In these variants, the resulting PHB–HV copolymer displayed a high rate of HV—21.6 and 13.1 mol %, respectively. However, note that the addition of valeric acid inhibited the culture growth. In particular, the biomass yield in the control variants on sucrose was 7.9 g/l versus the variant with 20 and 10 mM valeric acid, when the yield considerably decreased (to 3.2 and 4.5 g/l, respectively). In the variants with the addition of propanoic and hexanoic acids to nutrient medium, PHB–HV was also synthesized; however, the HV molar percent in the copolymer was significantly lower, reaching only 3.1 mol % in the presence of 20 mM propanoic acid and 2.1 mol % in the case of 10 mM hexanoic acid. Molecular weight of the synthesized PHB–HV was lower than that of PHB homopolymer; presumably, this is connected

with the addition of organic acids to the sugar-containing growth medium. It is evident from Table 1 that the addition of 10 or 20 mM valeric acid, 20 mM propanoic acid, or the combined addition of 10 mM propanoic and 20 mM acetic acids gave the PHB–HV with molecular weights of 1490, 1300, 914, and 820, respectively. Earlier [25, 26] we observed a decrease in the molecular weight of the PHB produced by the *A. chroococcum* culture grown in the presence of organic acids in the sugar-containing medium or in the medium with molasses containing organic acid impurities.

HV incorporation into the PHB synthesized by *Azotobacter* and change in the copolymer molecular weight have been observed by several researchers [2, 4, 10, 13]. The data by Page and Manchak [10] are most close to our results; they reported that the addition of 10, 20, or 30 mM valerate to the medium for cultivating *A. vinelandii* UWD resulted in the synthesis of the polymer with a high HV content (18, 22, and 25 mol %, respectively) and inhibition of the culture growth increasing with the valerate concentration in the medium. When *A. vinelandii* UWD was grown on propanoic acid, HV was also incorporated into the polymer chain giving PHB–HV. Again, similar to our data, the molar content of HV was low, less than 1%. The most well-known PHA producers—*Ralstonia eutropha* and *Alcaligenes latus*—also synthesized the copolymer with a lower percent content of HV in the medium with propionate as compared with the medium containing valerate. The pathway of PHB–HV synthesis in the presence of propionate involves 3-ketothiolase and proceeds via condensation of acetyl-CoA and propionyl-CoA to give 3-ketovaleryl-CoA; the last compound is then reduced to 3-hydroxyvaleryl-CoA, which, in turn, is a substrate for PHA polymerase [27–29]. All the PHB–HV specimens that we obtained were additionally assayed for the HV incorporation into PHB by ^{1}H-NMR. These experiments confirmed that PHB contained valeric acid. The 1H-NMR data on HV incorporation into PHB practically did not differ from the GLC data on the molar percent of HV incorporation into the copolymer. Figures 1 and 2 show the ^{1}H-NMR spectra of copolymer specimens with maximal HV incorporation (17 mol %; the variant with 20 mM valerate in the medium) and minimal HV incorporation (2.5 mol %; 20 mM propionate) as well as a specimen of PHB homopolymer (the variant with sucrose only). The spectrum in Fig. 1 (a and b) displays the signal of the 3-HV methyl group at a chemical shift of 0.89 ppm versus the spectrum of PHB homopolymer (Fig. 1c), lacking this signal. Figure 2 (a–c) demonstrates the presence of HV in the polymer chains of the same variants according to GLC assay. The percent content of HV in PHB–HV in the presence of 20 mM valerate was 21.6 mol % and in the case of 20 mM propionate, 3.1 mol %. In the control (pure PHB), the peak corresponding to valerate was absent.

Thus, the addition of valeric, propanoic, or hexanoic acid to cultivation medium makes it possible to produce the PHB–HV copolymer with various HV percent contents in the polymer chain. According to literature data [16, 26], the properties of the produced copolymers with different HV contents (mol %) in PHB are in a wide range of thermomechanical characteristics, namely, melting temperature of 145°C (20 mol % HV) to 170°C (3 mol % HV), Young's modulus of 1.2 GPa (20 mol % HV) to 2.9 GPa (3 mol % HV), crystallinity of 50% (20 mol % HV) to 69% (3 mol % HV), and tensile strength of 32 MPa (20 mol % HV) to 38 MPa (3 mol % HV) versus the PHB homopolymer with its melting temperature of 179°C, Young's modulus of 3.5 GPa, crystallinity of 79%, and tensile strength of 40 MPa.

The effect of peptone on bacterial growth and PHB and PHB–HV content in cells. To decrease the inhibiting effect of added carboxylic acids on the bacterial growth during synthesis of the PHB–HV copolymer (Table 1), we supplemented the growth medium with peptone, because, according to literature data, this could enhance the growth of *Azotobacter* and considerably elevate the content of polymer in their cells [2].

Table 2 shows the data on *A. chroococcum* growth and the contents of PHB and PHB–HV in its cells in the medium with sucrose and carboxylic acids supplemented with 0.1% peptone. The addition of peptone failed to increase both the cell yield and total yield of the polymer; however, a pronounced cell pleomorphism was observed (Fig. 3). Presumably, this was connected with specific physiological and biochemical features of the used strain. Nonetheless, the addition of peptone influenced the HV content in PHB: the HV molar percent in the copolymer increased more than twofold in the variant with 10 mM valerate, reaching 27.0 mol % versus 11.0 mol % HV in PHB in the medium without peptone. The trend of increase in HV molar percent retained when peptone was added on the background of 20 mM valerate (from 22.6 mol % without peptone to 28.8 mol % with peptone); however, 20 mM valeric acid yet considerably inhibited the culture growth both without and with peptone.

Kinetics of model drug release from polymer matrix. To assess the functional difference between the synthesized copolymer and homopolymer, we studied the release kinetics of a model drug from the polymer matrix of biopolymer systems. For this purpose, we produced film matrix systems of prolonged action containing a model drug (methyl red) involving the PHB homopolymer with molecular weights of 1220 and 340 kDa, copolymer (PHB–HV 17 mol %) with a molecular weight of 1300 kDa, and copolymer (PHB–HV 2.5 mol %) with a molecular weight of 820 kDa. Methyl red, 2-[[4-(diamino)phenyl]azo]-benzoic acid, was chosen as a model drug, because it is readily soluble in chloroform, matches a moderately lipophilic drug in its physicochemical characteristics, and

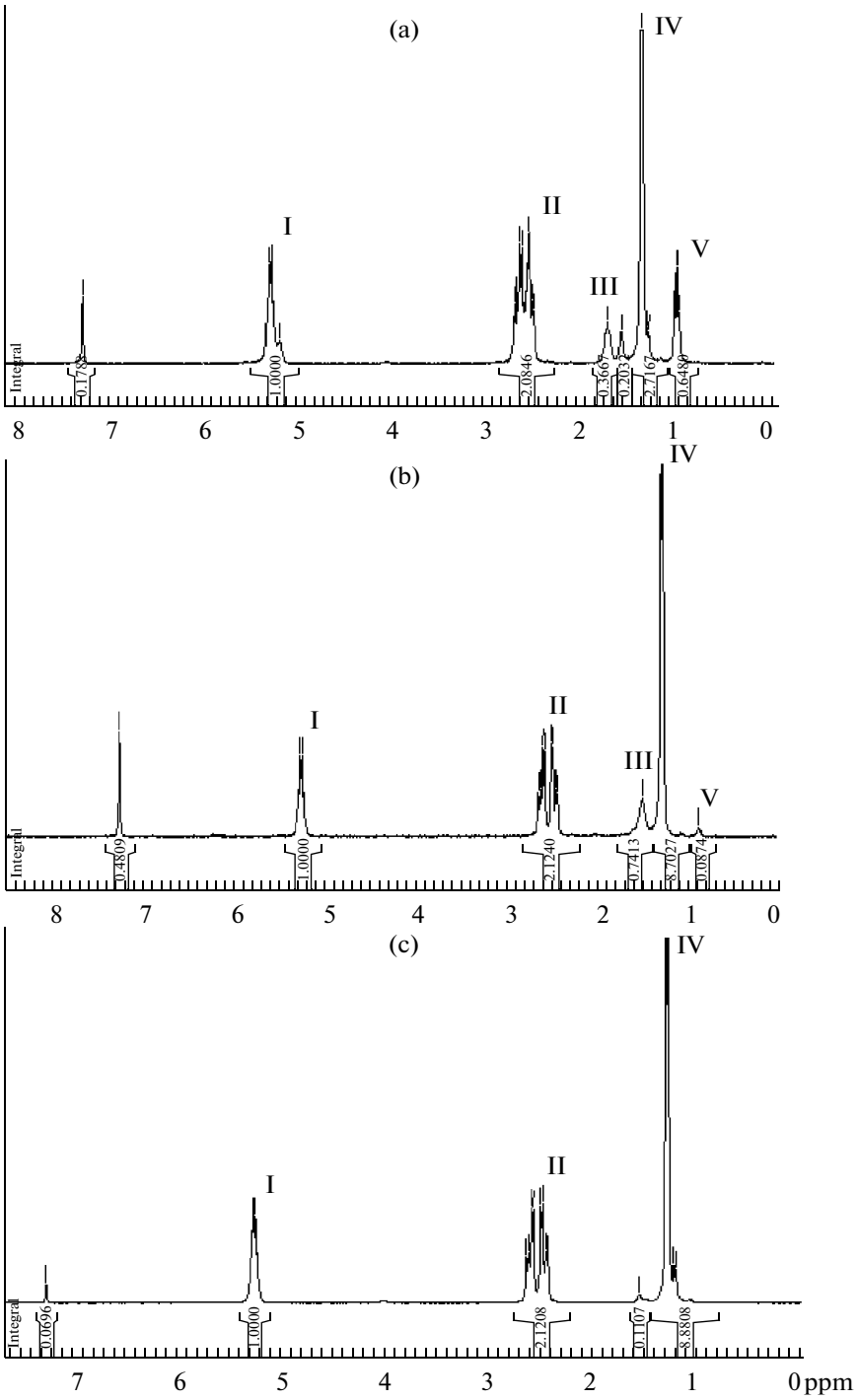

Fig. 1. ^{1}H-NMR spectra of the PHB–HV copolymer with the HV content of (a) 17% and (b) 2.5% and (c) PHB homopolymer: I is CH, II is CH_2(b), III is CH_2(s)HV, IV is CH_3(s)HB, V is CH_3(s)HV, s is side chain, and b is polymer backbone.

is easily detectable by spectrophotometry at 430 nm when released from polymer matrix.

The release kinetics of this model drug (methyl red, 4 wt %) from films into the environment, depending on molecular weight and chemical composition of the polymer matrix, was monitored in in vitro buffer system. The mass transfer of this substance from PHB and PHB–HV films into solution followed a two-stage pattern. An initially high release rate then dropped (a plateau region in the curve, Fig. 4). Since the hydrolyses of both PHB and PHB–HV are slow [30], it is likely that the erosion of polymer matrix caused by hydrolysis does not influence the release of model drug within the considered time intervals. It cannot be

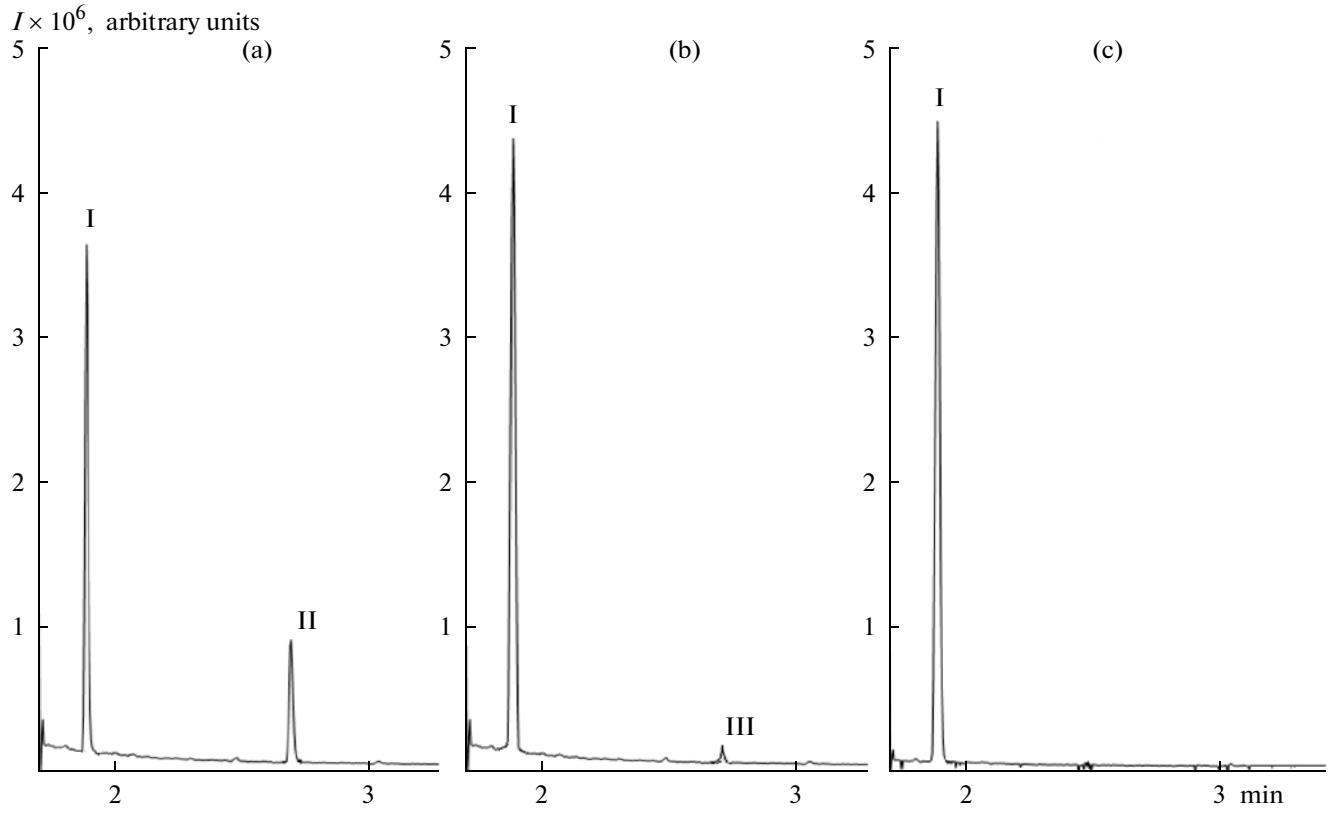

Fig. 2. Chromatograms characterizing chemical composition of the studied biopolymer: (a) I is PHB and II is PHB–HV with 21.6 mol % of HV, (b) III is PHB–HV with 3.1 mol % of HV, and (c) is the absence of the peak corresponding to HV.

excluded that the drug is released due to diffusion through water channels or the formed network of pores [32].

As is evident from Fig. 4, 19% of methyl red passed into solution from the film of PHB 340 kDa over 150 h and 14%, from the film of PHB 1220 kDa, i.e., the difference is 5%. However, 57% of methyl red passed into solution from the film of PHB–HV 2.5 mol % 820 kDa and almost 100% passed from the film of PHB–HV 17 mol % 1300 kDa. This suggests that the polymer composition to a greater degree influences the release rate of model drug as compared with the molecular weight of PHB homopolymer. Interestingly, the release of methyl red into solution even at a low HV content in the copolymer (2.5 mol %) is considerably increased as compared with the homopolymer. Presumably, this is connected with that incorporation of comonomer into homopolymer decreases both the degree of crys-

Table 2. Synthesis of the PHB–HV copolymer by *A. chroococcum* 7B on the sucrose-containing medium supplemented with carboxylic acids and 0.1% peptone

Substrate	Without peptone			With peptone		
	Yield of biomass, g/l medium	Total PHA content, % of dry cell weight	HV content, mol %	Yield of biomass, g/l medium	Total PHA content, % of dry cell weight	HV content, mol %
Sucrose (S), 3%	7.5	81.0	0	7.0	80.0	0
S + 20 mM propanoic acid	2.5	60.4	2.9	3.9	59.8	2.5
S + 10 mM valeric acid	5.2	70.0	11.0	5.0	75.0	27.0
S + 20 mM valeric acid	3.7	72.0	22.6	3.2	72.5	28.8
S + 10 mM hexanoic acid	3.9	65.3	0	3.5	68.8	0
S + 10 mM octanoic acid	3.2	61.0	0	3.5	64.2	0
S + 10 mM dodecanoic acid	2.9	59.0	0	2.2	60.0	0
S + 10 mM propanoic acid + + 20 mM acetic acid	3.3	67.0	2.1	4.3	67.7	2.3

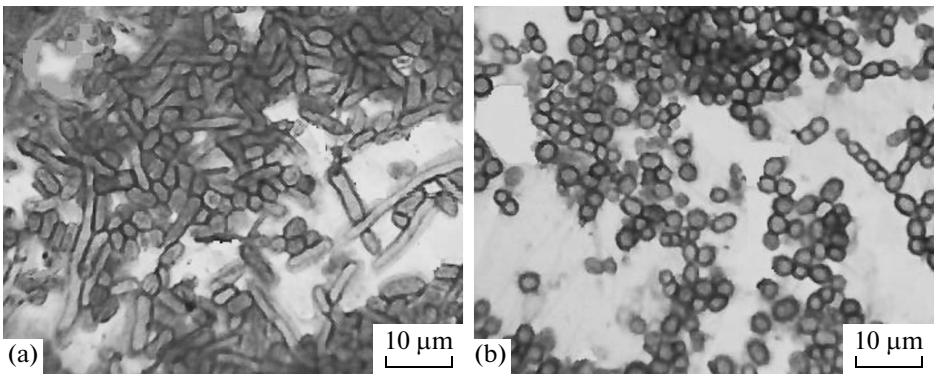

Fig. 3. *A. chroococcum* 7B culture after 48-h cultivation on Burk's medium with sucrose and (a) peptone and (b) without peptone.

tallinity [32] and the size of spherulites [33], thereby allowing water to enter faster the polymer matrix through amorphous regions, i.e., increasing the rate of polymer swelling.

Thus, we have for the first time demonstrated that *A. chroococcum* strain 7B is able to synthesize the PHB–HV copolymer with a HV content in the polymer chain in the range of 2.1–21.6 mol % at a high yield of both the polymer (67.7–75.8%) and cell biomass (2.2–4.5 g/l) when using valeric, propanoic, and hexanoic acids as an additional source of carbon. Not only qualitative (molar percent of HV incorporation into PHB), but also functional distinctions between the synthesized homopolymer and PHB–HV copolymer have been shown by the example of the release kinetics of a model drug. It has been found that the release kinetics of methyl red into the environment depends on both the composition of copolymer and molecular weight of homopolymer; moreover, the presence of HV monomers in the polymer considerably accelerates the release of model drug. The addition of peptone (0.1%) to the medium with 10 mM valerate can elevate the HV incorporation into PHB (27.0 mol %) on the background of a good culture growth (5.0 g/l). These results suggest that the studied strain is a promising producer of both PHB and PHB–HV.

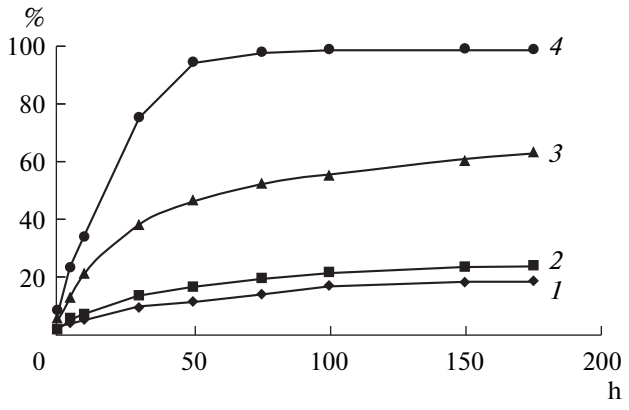

Fig. 4. The release kinetics of methyl red from polymer matrix depending on the polymer molecular weight (kDa) and composition (% of release): (*1*) PHB 1220 kDa, (*2*) PHB 340 kDa, (*3*) P(HB–HV 2.5%) 820 kDa, and (*4*)) P(HB–HV 17%) 1300 kDa.

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