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Preparative Biochemistry and Biotechnology

ISSN: 1082-6068 (Print) 1532-2297 (Online) Journal homepage: http://www.tandfonline.com/loi/lpbb20

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To cite this article: A. P. Bonartsev, I. I. Zharkova, S. G. Yakovlev, V. L. Myshkina, T. K. Mahina, V. V. Voinova, A. L. Zernov, V. A. Zhuikov, E. A. Akoulina, E. V. Ivanova, E. S. Kuznetsova, K. V. Shaitan & G. A. Bonartseva (2017) Biosynthesis of poly(3-hydroxybutyrate) copolymers by Azotobacter chroococcum 7B: A precursor feeding strategy, Preparative Biochemistry and Biotechnology, 47:2, 173-184, DOI: 10.1080/10826068.2016.1188317

To link to this article: http://dx.doi.org/10.1080/10826068.2016.1188317

Accepted author version posted online: 23 May 2016.
Published online: 23 May 2016.

PREPARATIVE BIOCHEMISTRY AND BIOTECHNOLOGY
2017, VOL. 47, NO. 2, 173–184
http://dx.doi.org/10.1080/10826068.2016.1188317

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Biosynthesis of poly(3-hydroxybutyrate) copolymers by *Azotobacter chroococcum* 7B: A precursor feeding strategy

A. P. Bonartsev[a,b,c], I. I. Zharkova[b], S. G. Yakovlev[a,c], V. L. Myshkina[a], T. K. Mahina[a], V. V. Voinova[b,c], A. L. Zernov[a,c], V. A. Zhuikov[a,c], E. A. Akoulina[a], E. V. Ivanova[b], E. S. Kuznetsova[b], K. V. Shaitan[b], and G. A. Bonartseva[a]

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ABSTRACT

A precursor feeding strategy for effective biopolymer producer strain *Azotobacter chroococcum* 7B was used to synthesize various poly(3-hydroxybutyrate) (PHB) copolymers. We performed experiments on biosynthesis of PHB copolymers by *A. chroococcum* 7B using various precursors: sucrose as the primary carbon source, various carboxylic acids and ethylene glycol (EG) derivatives [diethylene glycol (DEG), triethylene glycol (TEG), poly(ethylene glycol) (PEG) 300, PEG 400, PEG 1000] as additional carbon sources. We analyzed strain growth parameters including biomass and polymer yields as well as molecular weight and monomer composition of produced copolymers. We demonstrated that *A. chroococcum* 7B was able to synthesize copolymers using carboxylic acids with the length less than linear 6C, including poly (3-hydroxybutyrate-*co*-3-hydroxy-4-methylvalerate) (PHB-4MHV) using *Y*-shaped 6C 3-methylvaleric acid as precursor as well as EG-containing copolymers: PHB–DEG, PHB–TEG, PHB–PEG, and PHB–HV–PEG copolymers using short-chain PEGs (with $n \leq 9$) as precursors. It was shown that use of the additional carbon sources caused inhibition of cell growth, decrease in polymer yields, fall in polymer molecular weight, decrease in 3-hydroxyvalerate content in produced PHB–HV–PEG copolymer, and change in bacterial cells morphology that were depended on the nature of the precursors (carboxylic acids or EG derivatives) and the timing of its addition to the growth medium.

KEYWORDS

Azotobacter chroococcum 7B; biosynthesis; poly(3-hydroxyalkanoates); poly(3-hydroxybutyrate); poly(ethylene glycol); precursor feeding

Introduction

The widespread use of biodegradable polymers in medicine makes the study of their biosynthesis, especially relevant in modern biotechnology.[1–3] Some medical applications of these polymers include implants and devices for regenerative medicine,[4,5] tissue engineering,[6,7] novel drug dosage forms in biopharmaceutics,[8–10] novel materials for dentistry and maxillofacial surgery.[4,6,7,11] Furthermore, biodegradable biopolymers are used in the food packaging industry and agriculture. Many biodegradable polymers are the products of chemical synthesis (e.g., lactic acid and their copolymers that are widely used in medicine), but the products of biodegradation of non-natural synthetic polymers and these polymers themselves may be toxic (including chemical and immune toxicity) to animal tissues.[12,13] Unlike most natural polymers (e.g., alginates, chitosan, dextran, collagen, fibroin, albumin, etc.), poly-3-hydroxyalkanoates (PHAs): poly(3-hydroxybutyrate) (PHB) and their copolymers are produced by biotechnological techniques: the biosynthesis allows controlling chemical structure and, therefore, the physical–chemical properties of these polymers.[2,3] PHB and its copolymers display several unique properties, such as great mechanical and diffusion properties, these polymers are biocompatible and biodegradable (without toxic byproducts generation) when tested in cell cultures and laboratory animals as well as in medical applications.[2,3,6,14–16] PHAs have a unique nanostructure. Being a semicrystalline polymer, PHAs are able to form different nanostructures: lamellae and spherulites. PHA biodegradation and biocompatibility are strongly dependent on this semicrystalline polymer morphology.[17,18] Nowadays, about 100 various hydroxyalkanoic acids besides PHB were detected as PHA components; and over 300 producers of PHAs were characterized.[19–25] The process of PHA isolation and deep purification for biomedical applications is relatively simple, because of high levels of PHB accumulation in bacterial cells of strain producer and the polymer solubility in organic solvents. Therefore, PHAs are used in various fields of medicine: hernioplasty, orthopedic surgery, maxillofacial surgery, dentistry, cardiovascular surgery, biopharmaceutics, etc.[2,3,6–8,26,27]

Unfortunately, some physical–chemical properties of PHB homopolymer: high hydrophobicity, brittle mechanics, high crystallinity degree, and low rate of biodegradation limit its biomedical application. These factors limit development of PHB-based blood vessel prostheses.[14,15,28,29] A number of various carbon sources were used to produce PHAs and different strategies were used to improve production of these biopolymers.[21,22,24,25,30–32] The effect of carbon nutrition conditions on PHA synthesis was studied in the context of regulation of PHAs' molecular weight and the possibility to

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synthesize PHAs consisting of not only one component but also from several monomer types to improve polymer physical–chemical and biomedical properties.[23–25,30–36] However, to understand better the process of PHA biosynthesis, one good strategy is to explore the maximal biopolymers' biosynthetic opportunities of a particular highly effective bacterial producer.

In this paper, we analyzed the biosynthetic capabilities of strain *Azotobacter chroococcum* 7B as a producer of PHAs and different approaches to regulate biosynthesis of biopolymers by this high-production strain using carboxylic acids and ethylene glycol (EG) derivatives as additional carbon sources.

Experimental

Materials

Ethylene glycol derivatives: diethylene glycol (DEG), triethylene glycol (TEG), poly(ethylene glycol) 200 g/mol (PEG 200), poly(ethylene glycol) 300 g/mol (PEG 300), poly (ethylene glycol) 400 g/mol (PEG 400), poly(ethylene glycol) 1,000 g/mol (PEG 1000); sodium salt of propionic (propanoic) acid (PA); sodium salt of valeric (pentanoic) acid (VA), sodium salt of 4-methylvaleric acid (4MVA), sodium salt of hexanoic acid (HxA), sodium salt of heptanoic acid (HpA), sodium salt of octanoic acid (OA), sodium salt of nonanoic acid (NA), sodium salt of dodecanoic acid (DA); components of growth media: $K_2HPO_4 \cdot 3H_2O$, $MgSO_4 \cdot 7H_2O$, NaCl, $Na_2MoO_4 \cdot 2H_2O$, $CaCO_3$, $FeSO_4 \cdot 7H_2O$, sodium citrate, $CaCl_2$, KH_2PO_4, sucrose, agar, phosphate buffer saline. All materials were purchased from Sigma-Aldrich (USA) and used as received.

Growth conditions

The 7B strain of *A. chroococcum*: nitrogen-fixing nonsymbiotic bacterium able to accumulate PHB up to 80% of cell dry weight was used in the work as PHAs producer.[32,37–39] 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. For PHB biosynthesis, the culture was grown in shaker flasks with 100 mL of the medium at 30°C at 250 rpm shaking 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 17 g/L (50 mM) sucrose as main carbon source; the microbiological incubator shaker Innova 43 (New Brunswick Scientific, USA) was used.

For PHB copolymer biosynthesis, the additional carbon sources were added in culture medium. As a 3HV precursor in PHB–HV copolymer chain, VA was added as sodium salt at a concentration of 5, 10, 20, 35, and 150 mM at different time points, after 0, 4, 12, and 18 h of incubation of the culture. The concentrations from 5 to 35 mM were selected as optimal according to early obtained data.[32,39] As 3-hydroxy-4-methylvalerate (3H4 MV) precursor in PHB-3H4 MV copolymer chain, 4MVA was added as sodium salt at a concentration of 20 mM after 12 h of incubation of the culture. The other alkanoic acids (HxA, HpA, OA, NA, DA) were added as sodium salts also at a concentration of 20 mM after 12 h of incubation of the culture. The concentration of 20 mM was selected as optimal according to early obtained data.[32,40] PEG derivatives (DEG, TEG, PEG 200, PEG 300, PEG 400) were added at a concentration of 150 mM. The further increase in PEG concentration can inhibit growth and reduce 3HV incorporation, molecular weight, and PHA production.[41] Thus, we used the maximal optimal concentration of PEG. The each experiment was performed for 72 h with control of optical density by nephelometry; strain growth, and polymer accumulation in cells by light microscopy [microscope Biomed 1 (Biomed, Russia) was used]. The following parameters of copolymer biosynthesis were determined: the biomass yield and the polymer yield.[32,39]

All culture media, working solutions, reagent solutions (including carboxylic acids and PEGs) as well as laboratory glassware used in the experimental work were sterilized by autoclaving with use of laboratory autoclave VK-75-01 (Tumenskii ZMOI, RF). All experiments were performed under laboratory conditions.

Isolation and purification polymers from bacterial biomass

The polymer isolation and purification from *A. chroococcum* biomass comprised the following stages: (1) polymer extraction with chloroform in a shaker for 12 h at 37°C; (2) polymer solution separation from cell debris by filtration; (3) polymer precipitation with isopropanol from chloroform solution; (4) to remove any additives and contaminants, subsequent repeated cycles of dissolution in chloroform and precipitation with isopropanol for four to five times; and (5) drying at 60°C.[32,39]

Study of polymer monomer composition

Proton (^{1}H) NMR spectra of PHB and its copolymers solutions in deuterated chloroform were recorded in an MSL-300 (Bruker, Germany) spectrometer at a working frequency of 400 MHz. Chemical shifts in parts per million (ppm) were measured from 0.00 ppm relative to the signal of chloroform-d ($CDCl_3$) residual protons, 7.27 ppm. The experimental parameters were as follows: 1% (w/v) polymer in chloroform-d, 313 K, 2.5 s acquisition time, and 4,000 Hz spectral width. The percent content of elementary 3HV elements in the PHB–HV and PHB–HV–PEG copolymer was calculated according to the ratio of the integral signal intensity from the 3HV methyl group (0.89 ppm) to the sum of integral signal intensities from the methyl groups of 3HV, PEG, and 3-hydroxybutyrate (1.27 ppm).[32] The percent content of elementary 3H4 MV elements in the P(3HB-3H4 MV) copolymer was calculated according to the ratio of the integral signal intensity from the 3H4 MV 4-methyl group (g) at a chemical shift of 0.90 ppm and –CH (f) group at a chemical shift of 1.91 ppm to the sum of integral signal

intensities from the methyl and –CH groups of 3H4 MV and 3-hydroxybutyrate (1.27 ppm).[40] The percent content of elementary EG elements in the PHB–PEG and PHB–HV–PEG copolymers was calculated according to the ratio of the sum of integral signal intensities from EG-CH_2– groups (3.61, 3.70, 3.66, 3.73, 4.24 ppm) to the sum of integral signal intensities from the methyl group of 3HV, PEG, and 3-hydroxybutyrate (1.27 ppm).[34]

Study of polymer molecular weight

Gel permeation chromatography (GPC) technique was used to determine the molecular weights of produced polymers. The detection system consisted of a Waters 2414 differential refractive index detector and a UV detector and a waters 1525 pump, connected to four Waters styragel columns (Waters, USA) (Styragel HT 6E, 4.6×300 mm) placed in series was used. Chloroform was the eluent, at a flow rate of 1.0 mL/min.[34] The M_w determined by GPC was correlated with data estimated by viscosimetry: the viscosity of the PHB solution in chloroform was measured at 30°C on an RT RheoTec viscometer (RheoTec, Germany); the molecular mass was calculated using the Mark–Kuhn–Houwink equation according to Myshkina et al.[39]

Statistical analysis

The software package SPSS/PC+Statistics™ 12.1 (SPSS) was used to perform statistical evaluation of obtained data. To ensure consistency of statistical analysis, the samples were analyzed in triplicate ($n = 3$). One-way ANOVA was used for all statistical analyses. In Figures, data are averaged with the standard error to the mean (±SD) and considered significant for $p < 0.05$; in Tables, data are reported as the median ($n = 3$).

Results and discussion

Production of PHB copolymers using precursor feeding approach

Azotobacter chroococcum produced homopolymer PHB with a high molecular weight (up to 1.63×10^5 Da) with sucrose as the sole carbon source for polymer biosynthesis. We used a precursor feeding approach to produce various PHB copolymers: data on PHB and its copolymers biosynthesis by the *A. chroococcum* 7B culture grown in a medium containing sucrose as the primary carbon source and supplemented with valeric and propionic acids and a range of EG derivatives (DEG, TEG, PEG 200, PEG 300, PEG 400, and PEG 1000) as precursors and additional sources of carbon for synthesis of the copolymers are listed in Tables 1 and 2. Previously, it was shown that *A. chroococcum* could not use carboxylic acids and EG derivatives as sole carbon sources, even though these substrates were tolerated in the presence of glucose. Earlier, we have shown that 3-hydroxyvalerate (3HV) monomers is incorporated into the PHB–HV copolymer when using valeric and propanoic acids as additional carbon sources, whereas copolymer biosynthesis failed when using hexanoic, heptanoic, octanoic, nonanoic, and dodecanoic acids.[32] Earlier, it was shown that when alcohols or organic acids with an odd number of carbon atoms were used as either primary or additional carbon sources, the biosynthesis of PHB copolymers was performed.[19,20] In the current study, we confirmed these data (Table 1).

The 3HV incorporation into copolymer was earlier confirmed by ^{1}H-NMR. The analysis of ^{1}H-NMR spectra indicated that the copolymer is a multiblock copolymer.[32] It was shown for *Azotobacter* bacteria that valerate is incorporated into the copolymer through the β-oxidation pathway: VA → valeryl-CoA → 3-ketovaleryl-CoA → D-3-hydroxyvaleryl-CoA → 3HV. The pathway of PHB–HV synthesis in the presence of propionate involves 3-ketothiolase and proceeds through condensation of acetyl-CoA and propionyl-CoA to give 3-ketovaleryl-CoA. The last compound is reduced then to 3-hydroxy-valeryl-CoA, which, in turn, is a substrate for PHA polymerase (Fig. 1).[42] In these cases, sucrose was used as the main carbon source.

We used also a derivative of valeric acid, 4MVA, as the additional carbon source with sucrose as the main carbon source. We found that this method resulted in the biosynthesis of the novel copolymer PHB-4MHV. Incorporation of 3H4 MV into copolymer chains was confirmed by ^{1}H-NMR. The ^{1}H-NMR spectrum displays the signal of the 3H4 MV 4-methyl group (g) at a chemical shift of 0.90 ppm and –CH (f) group at a chemical shift of 1.91 ppm versus the spectrum of the PHB or PHB–HV, which lacked these signals (Fig. 2).[32]

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

Substrate	Time of alkanoic acid addition (h)	Yield of biomass (g/L medium)	Total PHA content (% of dry cell weight)	Molecular weight of PHA ($\times 10^6$ Da)	3HA content (mol%)
Sucrose, 50 mM	–	4.7	81.2	1.63	0
S + 20 mM VA	0	3.2	67.7	0.82	9.08
S + 20 mM VA	12	3.5	70.5	1.27	21.28
S + 20 mM PA	12	2.2	63.3	0.89	2.92
S + 20 mM HxA	12	2.7	64.3	1.02	0
S + 20 mM HpA	12	3.1	57.0	1.17	0
S + 20 mM OA	12	2.9	57.2	1.21	0
S + 20 mM NA	12	4.1	64.1	1.45	0
S + 20 mM DA	12	1.7	57.9	1.07	0
S + 20 mM 4MVA	0	2.6	71.2	0.62	0.04
S + 20 mM 4MVA[a]	12	3.4	76.7	1.30	0.59

PHB, poly(3-hydroxybutyrate); VA, valeric (pentanoic) acid; PA, propionic (propanoic) acid; HxA, hexanoic acid; HpA, heptanoic acid; OA, octanoic acid; NA, nonanoic acid; DA, dodecanoic acid; 4MVA, 4-methylvaleric acid.
[a]For this case the 1H NMR spectrum is shown.

Table 2. Synthesis of the PHB copolymers by *A. chroococcum* 7B on the sucrose-containing medium supplemented with EG *derivatives* and PPG.

Substrate	Time of valeric acid/PEG (PPG) addition (h)	Yield of biomass (g/L medium)	Total PHA content (% of dry cell weight)	Molecular weight of PHA (× 10^6 Da)	3HV content (mol%)	EG/PG content (mol%)	Molar ratio PEG/PHB
Sucrose, 50 mM	-/-	4.7	81.2	1.630	0	0	0
S + 150 mM DEG[a]	-/18	1.3	31.2	0.108	0	0.24	1.55
S + 150 mM TEG[a]	-/18	2.0	42.7	0.054	0	0.28	0.59
S + 150 mM PEG 200	-/18	2.2	45.2	0.035	0	0.72	0.70
S + 150 mM PEG 300[a]	-/0	1.9	31.8	0.227	0	0.19	0.81
S + 150 mM PEG 300	-/4	2.6	37.2	0.258	0	0.16	0.77
S + 150 mM PEG 300	-/12	3.6	52.4	0.218	0	0.15	0.63
S + 150 mM PEG 300	-/18	4.1	63.1	0.246	0	0.12	0.51
S + 150 mM PEG 300	-/24	4.0	62.7	0.542	0	0.03	0.13
S + 150 mM PEG 400	-/18	2.6	44.5	0.030	0	0.01	0.04
S + 150 mM PEG 1000	-/18	4.5	75.7	1.480	0	0	–
S + 20 mM VA + 150 mM PEG 300	12/0	2.6	49.5	0.219	4.69	0.15	0.63
S + 20 mM VA + 150 mM PEG 300	12/4	3.1	53.8	0.152	5.34	0.19	0.56
S + 20 mM VA + 150 mM PEG 300	12/12	3.3	63.3	0.163	6.81	0.13	0.42
S + 20 mM VA + 150 mM PEG 300[a]	12/18	3.2	66.7	0.147	7.53	0.23	0.67
S + 20 mM VA + 150 mM PEG 400	12/12	1.7	38.7	0.030	6.46	0.02	0.01
S + 150 mM PPG	-/12	3.3	79.8	0.621	0	0	0

PHB, poly(3-hydroxybutyrate); EG, ethylene glycol; PHA, poly-3-hydroxyalkanoates; PEG, poly(ethylene glycol); DEG, diethylene glycol; TEG, triethylene glycol; VA, valeric (pentanoic) acid; PPG, poly(propylene glycol); PG, propylene glycol.
[a]For these cases the 1H NMR spectrum are shown.

We propose that the obtained copolymer is a multiblock copolymer like PHB–HV and assume that the 3H4 MV incorporation into the polymer chain occurs in analogy to the process for VA through the β-oxidation pathway: 4MVA → 4-methyl-valeryl-CoA → 3-keto-4-methylvaleryl-CoA → D-3-hydroxy-4-methylvaleryl-CoA → 3H4 MV.

Besides novel PHA biosynthesis using the carboxylic acids feeding strategy, EG monomer incorporation into the PHA polymer was also generated when DEG, TEG, PEG-200, PEG-300 were added to the growth medium. The analysis of PHB–PEG and PHB–HV–PEG copolymers by ^{1}H-NMR spectroscopy confirmed incorporation of EG elements into the PHA polymer. Five weak ^{1}H NMR signals at 3.66 ppm (the highest signal) and at 3.61, 3.70, 3.73, and 4.24 were observed that correspond to protons of EG repeat units (Figs. 3a–c and 4a, b). The signals at 4.24 and 3.73 ppm were assigned to protons a and b, respectively, of esterified PEG chain segments; peaks at 3.61 and 3.70 ppm were due to protons e and d of terminal free hydroxyl EG units. The highest peak was the sum of signals from protons of median EG units of PEG (Figs. 3b, c and 4b). ^{1}H NMR signals in the 3.6–3.8 ppm spectral region were not observed in PHB and

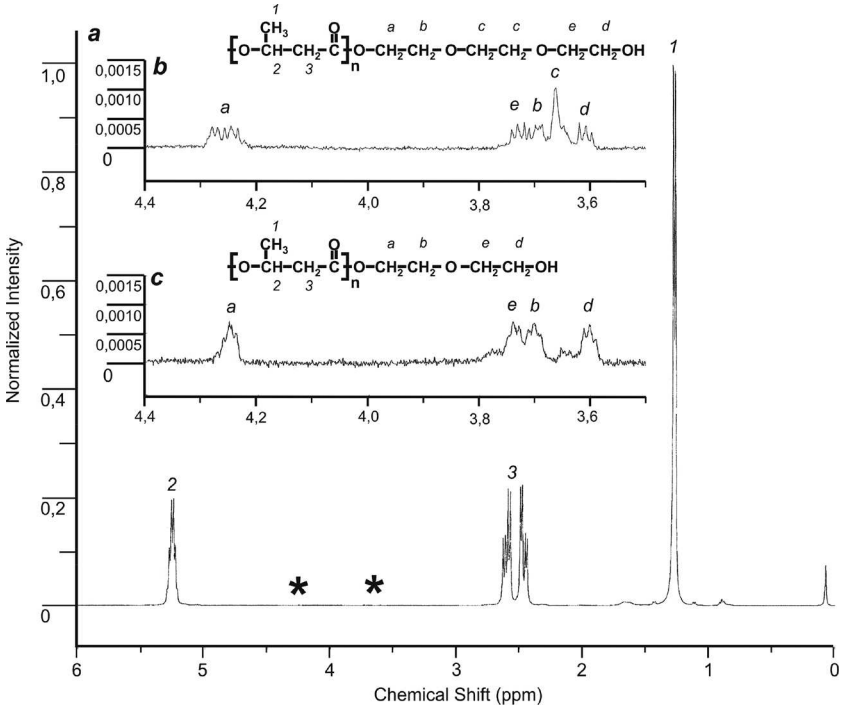

Figure 1. ^{1}H-NMR spectra of the PHB–TEG (**b**), PHB–DEG (**c**): (**a**) PHB chain: 1 is CH_3(*s*), 2 is CH(*b*), 3 is CH_2(*b*), *s* is a side chain, and *b* is a polymer backbone; *see zoomed graph section on (**b**, **c**); (**b**) TEG chain: "a" is linking –O–CH_2 (4.24 ppm), "b" is following CH_2 (3.73), "c" is the integral signal from median [–O–CH_2–CH_2–] group (3.66 ppm), "e" and "d" are tail –CH_2– (3.70 ppm) and –CH_2-OH (3.61 ppm) groups, respectively; (**c**) DEG chain: "a" is linking –O–CH_2 (4.24 ppm), "b" is following CH_2 (3.73), "e" and "d" are tail –CH_2– (3.70 ppm) and –CH_2-OH (3.61 ppm) groups, respectively. *Note*: PHB, poly(3-hydroxybutyrate); TEG, triethylene glycol; DEG, diethylene glycol.

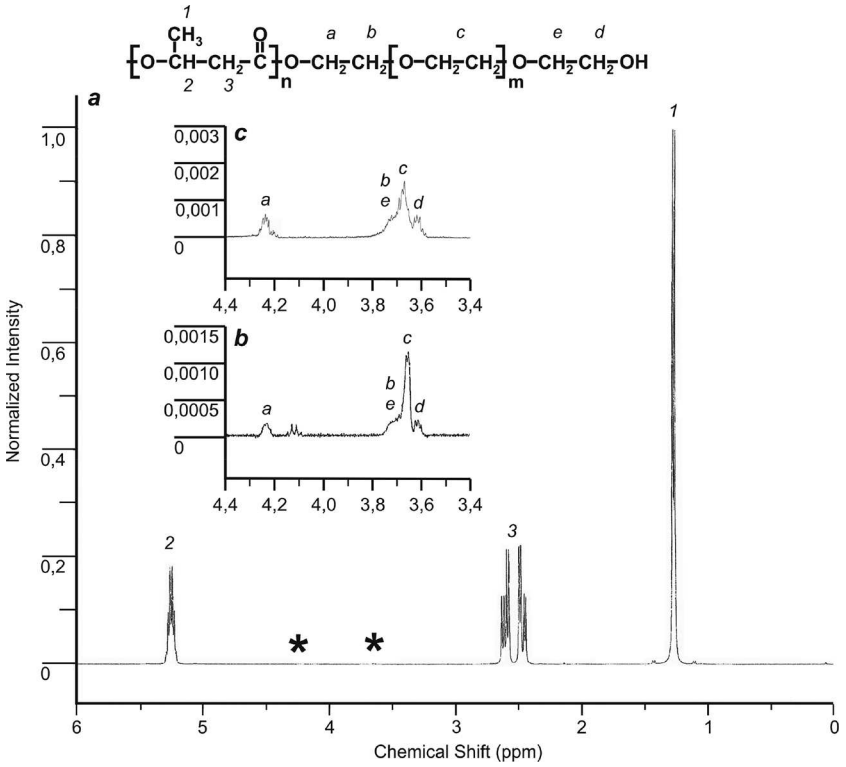

Figure 2. ^{1}H-NMR spectra of the PHB–PEG copolymer: (**a**) PHB chain: 1 is CH_3(*s*), 2 is CH(*b*), 3 is CH_2(*b*), *s* is a side chain, and *b* is a polymer backbone; *see zoomed graph section on (**b, c**); (**b, c**) PEG chain: "a" is linking –O–CH_2 (4.24 ppm), "b" is following CH_2 (3.73), "c" is the integral signal from median [–O–CH_2–CH_2–] group (3.66 ppm), "e" and "d" are tail –CH_2– (3.70 ppm) and –CH_2-OH (3.61 ppm) groups, respectively. *Note*: PHB, poly(3-hydroxybutyrate); PEG, poly(ethylene glycol).

PHB–HV synthetized in the absence of PEG.[32] These data indicate the formation of covalent bond between the carboxylate chain terminus of PHA and the PEG chain segments. The covalent binding of PEG to the PHA chain was further confirmed by ^{1}H-NMR spectra of the obtained PHB–TEG and PHB–DEG copolymers (Fig. 5a–c). Clearly, the signal of the

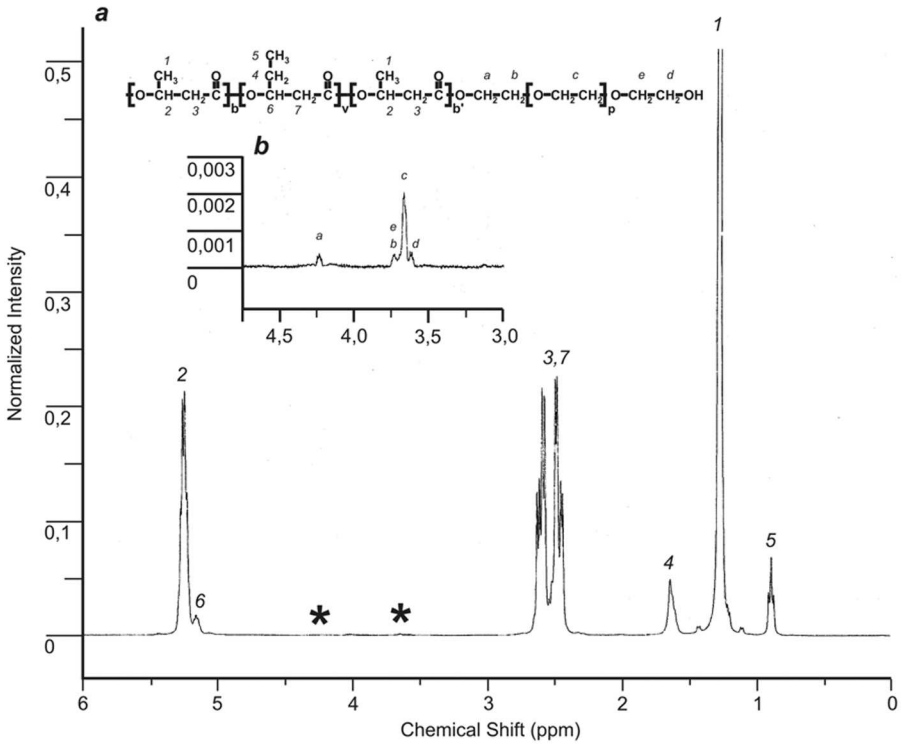

Figure 3. ^{1}H-NMR spectra of the PHB–HV–PEG copolymer: (**a**) PHB chain: 1 is CH_3(*s*), 2 is CH(*b*), 3 is CH_2(*b*), poly(3-hydroxyvalerate) chain: 4 is CH_2(*s*), 5 is CH_3(*s*), 6 is CH(*b*), 7 is CH_2(*b*), *s* is a side chain, and *b* is a polymer backbone; *See zoomed graph section on (**b**); (**b**) PEG chain: "a" is linking –O–CH_2 (4.24 ppm), "b" is following CH_2 (3.73), "c" is integral signal from backbone [–O–CH_2–CH_2–] group (3.66 ppm), "e" and "d" are tail –CH_2– (3.70 ppm) and –CH_2-OH (3.61 ppm) groups, respectively. *Note*: PHB, poly(3-hydroxybutyrate); PEG, poly(ethylene glycol).

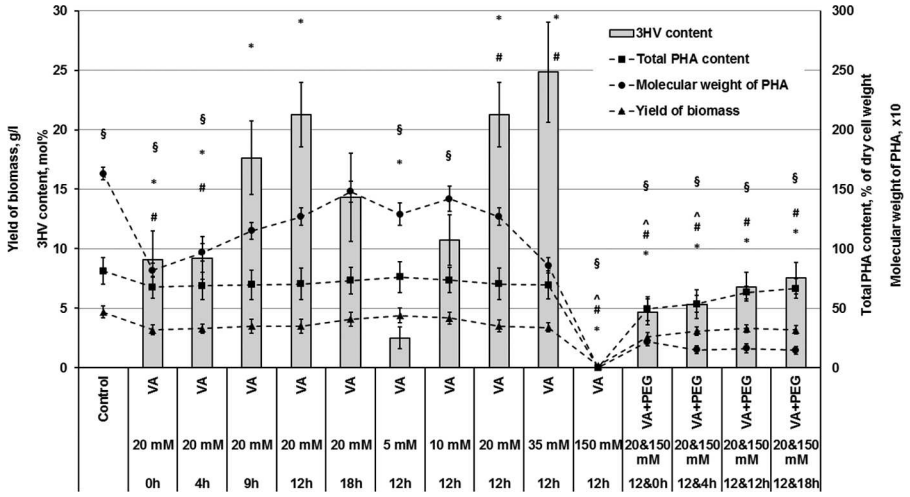

Figure 4. Effect of VA addition in the growth medium on the yield of biomass, total polymer content, molecular weight of polymer, and 3HV content in the chain of PHA. ^, #, * $p < 0.05$ of total polymer content, yield of biomass, and molecular weight, respectively, versus Control; § $p < 0.05$ of molecular weight versus group VA 20 mM 12 h. *Note*: VA, valeric (pentanoic) acid; PHA, poly-3-hydroxyalkanoates.

median EG unit of TEG at 3.66 ppm of PHB–TEG was much weaker (Fig. 5b) than accumulating signals at 3.66 of four median EG units of PHB–PEG (Fig. 3b). The peak at 3.66 ppm of PHB–DEG was totally absent, whereas all other signals of EG elements (including the signal at 4.24 ppm of EG units bound to PHA chain) were detected (Fig. 5c). Thus, the obtained copolymers are diblock or triblock copolymers of PHA and PEG: PEG is attached only to one end of the PHA chain. The maximum incorporation of EG monomers (0.19 mol%) in PHA was obtained with PEG-300 adding on 0 h at 150 mM concentration. This value indicates that there are 0.81 molecules of PEG-300 per 1 molecule of PHB ($M_w = 2.27 \times 10^5$ Da). We have introduced this dimensionless parameter, the PEG/PHB molar ratio, as the number of PEG molecules divided by the number of PHA molecules. This parameter facilitates a better understanding of our data. The application of PEG for production of novel PHA copolymers is a promising way in biotechnology. In pharmacology and

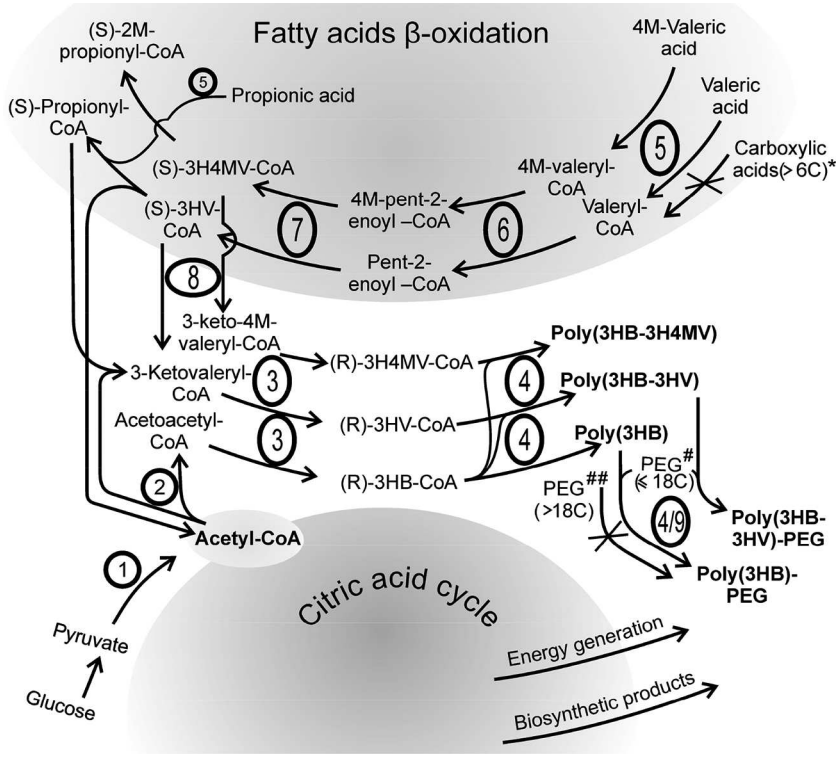

Figure 5. Design of biosynthetic pathways for poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)-PEG in *Azotobacter chroococcum* 7B. (1) pyruvate dehydrogenase complex; (2) β-ketothiolase; (3) NADPH-dependent acetoacetyl-CoA reductase; (4) short-chain-length PHA polymerase; (5) fatty acyl-CoA synthetase; (6) fatty acyl-CoA dehydrogenase; (7) enoyl-CoA hydratase; (8) NADH-dependent acetoacetyl-CoA reductase; (9) nonenzymatic nucleophilic attack of terminal PEG-hydroxyl group leading to PEG-end-capping of nascent PHB–HV chain. *Carboxylic acids (>6C): hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid, dodecanoic acid. #PEG (≤18C): DEG, TEG, PEG 200, PEG 300, PEG 400; ##PEG (>18C): PEG 1000. *Note*: PEG, poly(ethylene glycol); PHB, poly(3-hydroxybutyrate); DEG, diethylene glycol; TEG, triethylene glycol.

bioengineering, PEG is often used for chemical modification (PEGylation) of polymer nanoparticles, liposomes, and biopharmaceuticals. PEGylation of biodegradable polymers (e.g., PLGA) (poly(lactic-co-glycolic acid) is also used for the improvement of polymer biocompatibility.[43–47]

In light of the above, the addition of DEG, TEG, and PEG into culture leaded to the formation of a PHA–PEG (DEG, TEG) diblock copolymer, where the carboxylate (–COOH) terminus of PHA chains are covalently linked by an ester bond to a PEG chain, which may be caused by change in PHA biosynthesis involving the enzyme system. PEG binding to the PHA polymer chain may occur during the PHA polymers synthesis, suggesting possible interaction of PEG with PHA polymerase enzyme and the polymer itself (Fig. 1). As it was shown earlier that PEG and PHA have miscible nature; production of PHB/PEG blends is possible.[45,48,49] Moreover, decrease in PHA molecular weight could be attributed to PEG limiting the length of polymer chain.[33] PEG was effectively used to control molecular weight of PHAs produced by *Ralstonia eutrophus*,[33,50] *Alcaligenes latus*,[51,52] and *Pseudomonas oleovorans*.[53] PEG chain attachment with a covalent bond (resonance at 4.24 ppm) at the terminal position of a PHB and PHB–HV chain could lead to break in the elongating PHA chain. Enzymatic PHB–PEG copolymer synthesis was also partially confirmed when comparing the effect of EG derivatives with different chain lengths on copolymer biosynthesis. Introduction of DEG, TEG, and PEG 200 residues to the PHB chain was significantly lower than PEG 300, whereas the presence of PEG 400 and PEG 1000 residues in the PHB chain was negligible (Table 2). The failure of PEG-400 and PEG-1000 residues' incorporation into the PHB chain can be also explained by excessively long chains of PEG-400 and PEG-1000 oligomers that can hinder PHB polymerase from using PEG for copolymer biosynthesis. The PHB polymerase expressed in *A. chroococcum* by *phbC* gene is the polymerase of short-side-chain PHAs, e.g., 3-hydroxybutyrate and 3HV. This PHA polymerase cannot incorporate medium-chain-length 3-hydroxyalkanoates that longer than 3HV (e.g., 3-hydroxyhexanoate or 3-hydroxyoctanoate) into PHB chain.[54] The limitation of long-chain PEG (with $n > 7$) attachment to the PHA chain is probably connected with length restriction properties of *A. chroococcum* PHB polymerase.

Thus, the observed data demonstrated that *A. chroococcum* cannot produce PHB copolymers by incorporation of 3-hydroxycarboxylic acid residues with a chain linear length of more than 5C into the growing polymer chain. This limitation apparently indicates fairly high specificity of biochemical pathway of PHA block copolymer biosynthesis, and correspondingly high specificity of PHB synthase with respect to substrates for the biopolymer biosynthesis. Even the incorporation of 3H4 MV (the forked Y-shaped 6C reside) in contrast with failed incorporation of 3-hydroxyhexanoic acid (the linear 6C reside) in the chain can confirm the high specificity of PHB synthase to the length of substrate molecules for polymer biosynthesis. On the contrary, a number of EG derivatives (DEG, TEG, PEG-200, and PEG-300) can bond to the end of a polymer chain during biosynthesis to form diblock copolymers that suggests the possibility of covalent attachment of polymers with significantly different physical–chemical properties to PHAs. Surprisingly, there is also the limitation of long-chain PEG (with $n > 7$) attachment to the PHA chain that can indicate the specific mechanism of copolymer biosynthesis by PHB synthase. However, some researchers provided evidence in favor of the nonspecific process of PHA–PEG copolymer formation.[50] These data may indirectly confirm the findings of other researchers, who demonstrated the biochemical processes of PHB linking with other biopolymers such as proteins.[55] It should be noted that PEG performs also a number of important cell functions by covalent binding to proteins.[56–58] It is possible that earlier suggestion about other specific physiological roles of PHB (other than the role of energy depot)[55] is connected with the possibility of PHB to form covalent bonds with other biopolymers.

Effect of carboxylic acid and EG derivatives on copolymer production

Precursor feeding approach with the use of carboxylic acid and EG *derivatives* allows not only producing PHB copolymers but also regulate the chemical structure of obtained copolymers: monomer content and molecular weight within certain limits. The maximal 3HV content (24.8 mol%) in the copolymer was obtained when using 35 mM valeric acid (Fig. 6). We confirmed our previously obtained data on dependence of 3HV content in PHB–HV copolymer from cultivation conditions of strain producer[32]: 3HV content even linearly depended on VA concentration in cultural medium and nonlinearly depended on time of VA addition to cultural medium (Table 1, Fig. 6). Addition of VA, HxA, and 4MVA to medium caused relatively moderate decrease in M_w of produced copolymer: the PHB–HV and PHB-4MHV copolymers had lower M_w compared to PHB homopolymer. This effect on PHA M_w can be explained by some inhibitory action of carboxylic acids to polymer biosynthesis (Table 1, Fig. 6).[42]

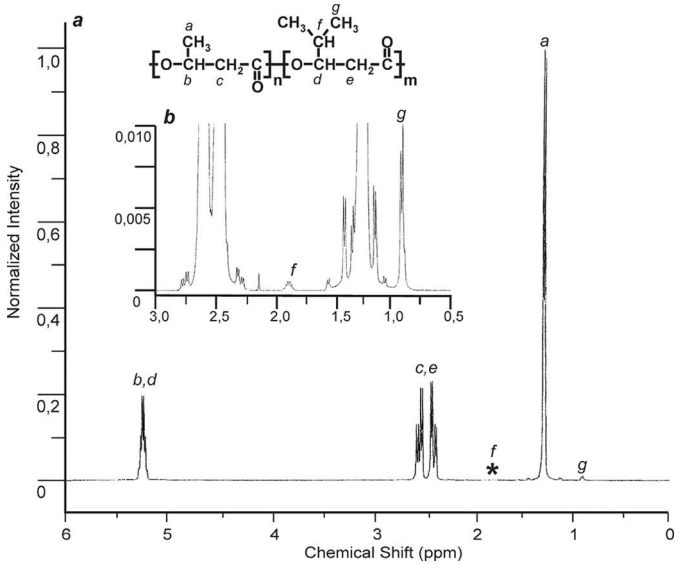

Figure 6. ^{1}H-NMR spectra of the P(3HB-3H4 MV) copolymer: (**a**) PHB chain: 1 is CH_3(*s*), 2 is CH(*b*), 3 is CH_2(*b*), P3H4 MV chain: 4 is CH_2(*s*), 5 is CH_3(*s*), 6 is CH(*b*), 7 is CH_2(*b*), *s* is a side chain, and *b* is a polymer backbone; *see zoomed graph section on (**b**). *Note*: PHB, poly(3-hydroxybutyrate).

Thus, the valeric and propionic acids feeding technique allows producing the PHB–HV copolymer with various 3HV contents in the polymer chain and, in this manner, regulating physical–chemical properties of PHB–HV polymer. The PHB–HV physical–chemical properties (crystallinity, melting temperature, impact strength, flexibility) and biodegradation kinetics vary considerably depending on 3HV content in the copolymer. Therefore, the PHB–HV polymer is well suited for broader applications.[54,59,60]

We observed that simultaneous addition of PEG-300 and sucrose at the initial time point led to a higher PEG content in produced copolymers in contrast to adding PEG-300 after 18 h, at which point the PHB polymer chain was partially synthesized (Table 2). But this dependence was not observed when the medium was supplemented together with both PEG-300 and 3HV (Table 2). Adding PEG to the medium also resulted in a significant drop in the molecular weight of produced polymers, but the timing of PEG 300 addition on the polymer molecular weight was not observed. However, combined PEG 300 and VA addition to the medium caused the greater molecular weight decrease in produced copolymer in comparison with the sole addition of PEG at each time point (Table 2, Fig. 7). Combined VA and PEG 300 addition to the medium at different time points caused also the great drop in 3HV content in PHB–HV–PEG copolymer in comparison with 3HV content in PHB–HV copolymer that was produced at the same conditions (20 mM VA at 12 h) without PEG addition (Fig. 6).

The formation of low-molecular weight PHB–PEG and PHB–HV–PEG copolymers by *A. chroococcum* may be attributed to the interaction of PEG with the PHA molecules itself as was the case for *R. eutrophus*.[50] However, addition of PEG-400 to the medium caused a pronounced decrease in M_w of produced polymers despite the failure in PHB–PEG copolymer formation, which indicates some other mechanism of PHA biosynthesis failure. A threefold decrease in 3HV content of produced PHB–HV–PEG copolymer in comparison with PHB–HV can be also explained by the inhibitory effect of PEG on PHB polymerase (Table 2, Fig. 7).

The effect of carboxylic acids and EG derivatives on cell growth and biopolymer production

Our data indicate that carboxylic acids and EG *derivatives* when using as additives to the culture medium caused inhibition of cell growth, decrease in polymer content in cells, and decrease in polymer yield. The inhibition of strain growth and polymer accumulation, when adding various carboxylic acids (propanoic, heptanoic, hexanoic, etc.) to the culture medium was also demonstrated earlier.[32] Particularly, the biomass yield in the control variants on sucrose was 4.7 g/L versus the variant with 20 and 35 mM valeric acid, in which case, we observed significantly decreased yields (3.5 and 3.4 g/L, respectively) (Fig. 6). Moreover, the level of this decrease depended not only on concentration but also on the nature of the additive. Thus, the biomass yield in medium supplemented with 150 mM DEG and PEG 300 was 1.3 and 3.6 g/L, respectively (Table 2), whereas the addition of VA at the same concentration (150 mM) caused a total inhibition of strain growth (Table 1). The biomass yield and polymer content in medium with various EG *derivatives* (DEG, TEG, PEG 200, PEG 300, PEG 400, PEG 1000) differed significantly. The same variations in growth parameters were reported when using various carboxylic acids as additional carbon sources.[32] Notably, a moderate effect of length of EG *derivative* chain on strain growth and polymer content was observed: The highest values were seen in the case of PEG 300, whereas DEG cased the maximal inhibition of strain growth (Table 2). The inhibition of cell growth and polymer accumulation caused by addition of DEG, TEG, and PEG 200 to the culture medium and a drop in M_w of produced polymers

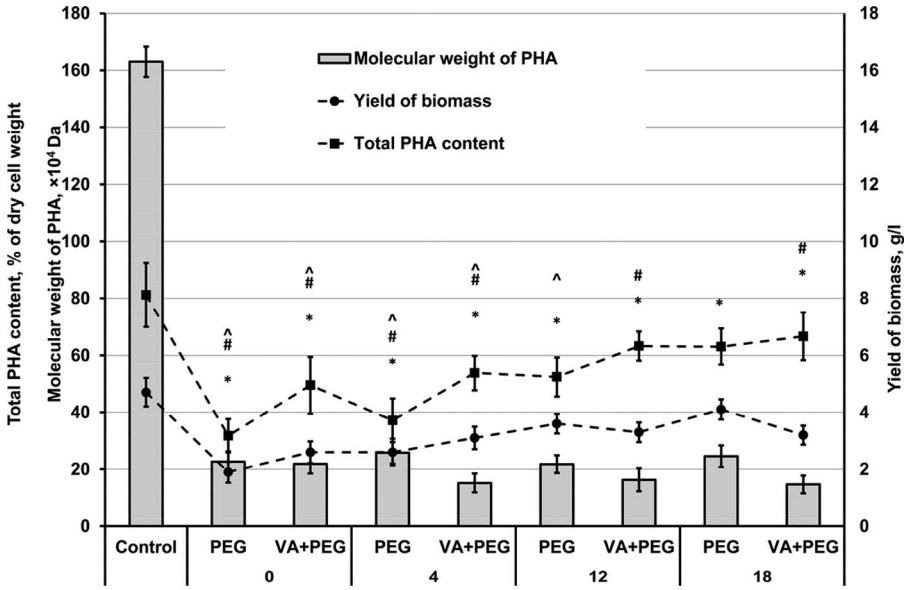

Figure 7. Effect of time addition of PEG in the growth medium on the yield of biomass, total polymer content, and molecular weight of polymer with or without VA adding to medium at 12 h. ^, #, *—$p < 0.05$ of total polymer content, yield of biomass, and molecular weight, respectively, versus Control. *Note*: PEG, poly(ethylene glycol); VA, valeric (pentanoic) acid.

may be linked to its excessive inhibitory effect on polymer biosynthesis by PHB synthase.

The time of addition of EG *derivatives* to the culture medium was of critical importance (Fig. 7): an initial addition of PEG-300 caused a twofold decrease in biomass yield and polymer content compared to adding PEG-300 after 18 h of strain cultivation. This effect can be explained by the general inhibitory impact of PEG on strain growth and polymer biosynthesis; the earlier PEG addition leaded to the more pronounced inhibition of cell metabolism including polymer biosynthesis. This is also the case for combined use of PEG 300 and VA: the earlier PEG 300 is added to the medium, the lower the biomass yield and polymer content (Table 2, Fig. 7). Using a combination of VA and PEG in our experiments yielded interesting information. The combined adding of PEG 300 at different time points and VA at 12 h caused less inhibition of strain growth in comparison with the sole PEG 300 addition at the same time points (Table 2, Fig. 7). This difference may be coupled with various nature of medium additive: VA is a carbon source and, besides, the use in copolymer biosynthesis can be used for energy generation and synthesis pathways (Fig. 1), whereas PEG is hardly can be used in general metabolic pathways in the bacterial cells. Therefore, under the PEG inhibition press, the VA addition could partially recover the biomass yield and polymer content, because 20 mM VA at 12 h, which is utilized for copolymer biosynthesis only to a small extent, is a relatively significant addition of carbon source in growth medium with partially expended 50 mM sucrose. However, without PEG addition, this effect cannot be revealed.

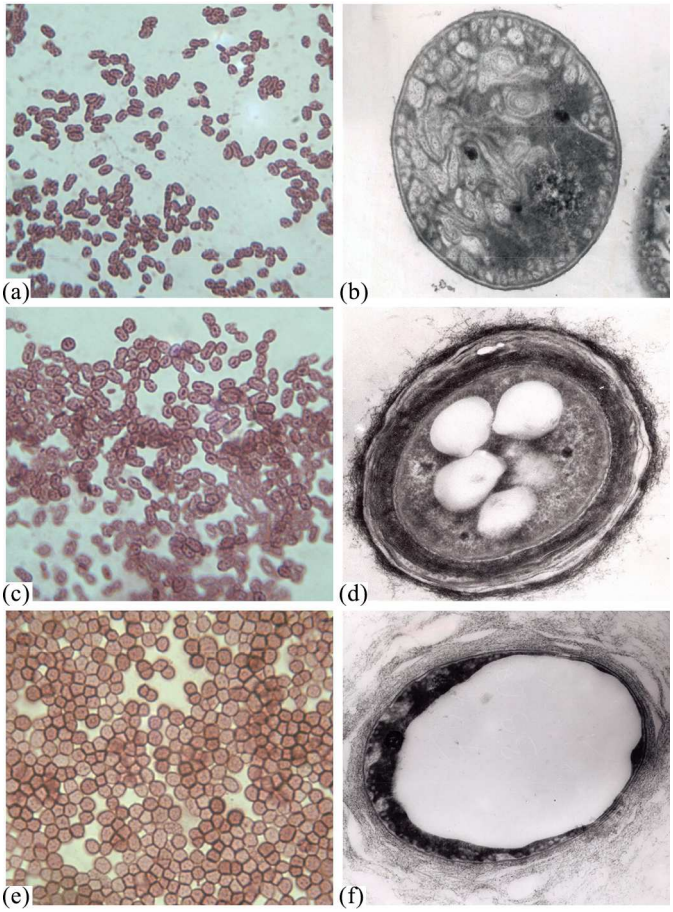

Figure 8. Bacterial cell morphology of strain producer *A. chroococcum* during growth in control conditions (a) sucrose, 21 h light microscopy (LM, ×900); (b) sucrose, 48 h (LM, ×900); (c) sucrose, 72 h (LM, ×900); (d) sucrose, 21 h transmission electron microscopy (TEM ×50,000); (e) sucrose, 48 h (TEM, ×50,000); (f) sucrose, 72 h (TEM, ×50,000).

The effect of carboxylic acids and EG derivatives A. chroococcum *cell morphology*

It was shown earlier that bacteria from genus *Azotobacter* demonstrated various morphological cell forms. The diversity of forms of *Azotobacter* is largely a consequence of its pleomorphism that partly due to the appearance of involutive forms (for example, giant inflated cells). The appearance of morphologically different cells in cultures of *Azotobacter* dependent on composition of the medium. By changing the composition of the medium and the conditions of culture, it is possible to induce changes in the phases of development of *Azotobacter*, and in the morphology of these bacteria.[61,62] At an early age, the size of the cells varies within the limits 2.0–7.0 μm × 1.0–2.5 μm (Fig. 8a and 8d). In individual cases, the length reaches 10–12 μm. Multiplication proceeds by simple division with the formation of a transverse septum and sometimes a constriction is observed. Young *Azotobacter* cells are motile and have peritrichous flagella. In young cells of *Azotobacter*, the plasma is finely granulated. When the cells age, they lose their motility, shorten, and assume an almost coccoid form and grains appear in the plasma that sharply refract the light (Fig. 8b and 8e). In special conditions for polymer production and accumulation, the polymer-containing grains filled most of the cytoplasm volume of *A.* cell (up to 85%), the cell organelles and biostructures pushed to the periphery adjacent to the membrane (Fig. 8b and 8e).

The addition of both carboxylic acids and EG *derivatives* caused the marked change in cell morphology of strain producer *A. chroococcum* (Fig. 9). Due to high propensity for pleomorphism for *Azotobacter* cells, the effect of carboxylic acids and EG *derivatives* on cell morphology appeared quite clearly. The addition of low concentration (5 mM) of valeric acid even did not change the cell morphology (Fig. 9a) but high valeric acid concentration (35 mM) caused the pronounced change in the cell morphology: coccoid form transformed into bacillary form (Fig. 9b). The addition of 20 mM 4MVA caused even formation of filamentous cells although the coccoid and bacillary forms were also present (Fig. 9b). The addition of PEG led to the formation of long filamentous cells (Fig. 9f) or a mixture of cells with different morphologies: filamentous and coccoid (Fig. 9f and 9g). These additives probably can act as other well-known stress-inducing agents (acids, alkalines, peptone) that caused change in bacterial cell morphology.[61,62]

Thus, the use of carboxylic acids and EG *derivatives* as additional carbon sources resulted in inhibition of strain growth that depends on nature, concentration, and time of its addition.

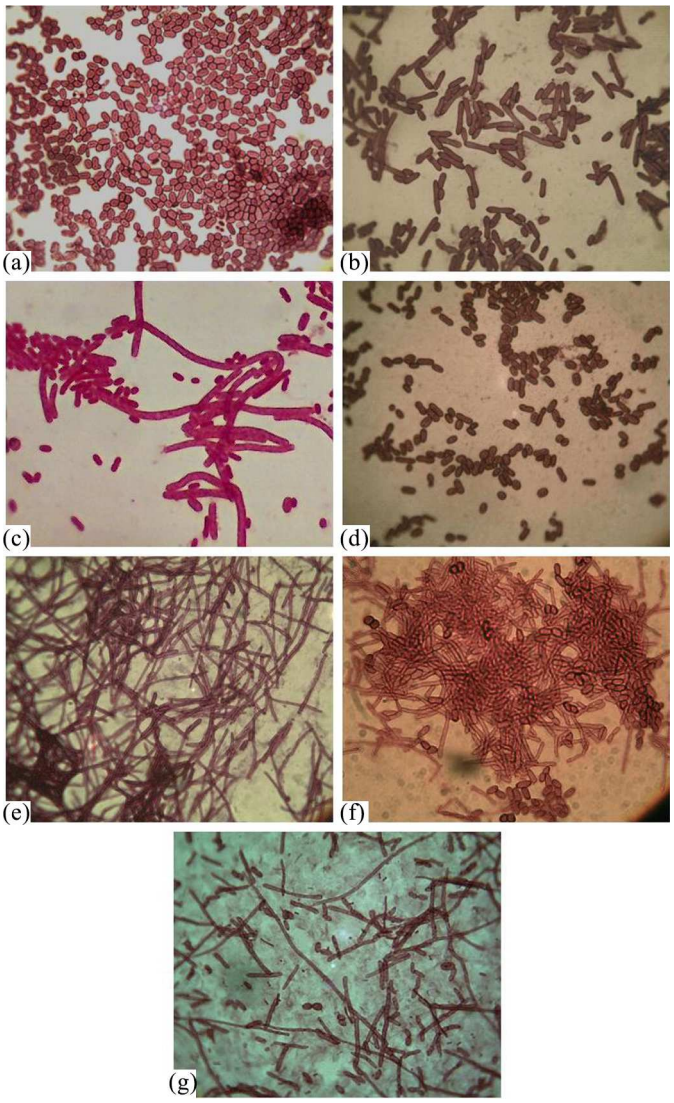

Figure 9. Effect of carboxylic acids and PEG addition in the growth medium on the bacterial cell morphology of strain producer *A. chroococcum* (LM, ×900): (a) S + 5 mM VA (12 h addition) 72 h; (b) S + 35 mM VA (12 h addition) 72 h; (c) S + 20 mM OA (12 h addition) 72 h; (d) S + 20 mM 4MVA (12 h addition), 72 h; (e) S + 150 mM PEG 300 (0 h addition), 72 h; (f) S + 150 mM PEG 300 (12 h addition), 72 h; (g) S + 20 mM VA (12 h addition) + 150 mM PEG 300 (18 h addition), 72 h. *Note*: PEG, poly(ethylene glycol); VA, valeric (pentanoic) acid; OA, octanoic acid; 4MVA, 4-methylvaleric acid.

Conclusion

Therefore, the introduction of carboxylic acids and EG *derivatives* into *A. chroococcum* 7B culture could be considered as one approach for the production of a series of PHA copolymers. We demonstrated that *A. chroococcum* 7B was able to synthesize not only PHB homopolymer and its main copolymer PHB–HV but also alternative PHB copolymers: P(3HB-3H4 MV), PHB–PEG, and PHB–HV–PEG, when its precursors (valeric acid, 4MVA, DEG, TEG, PEG 300, PEG 400) are used as additional carbon sources for copolymers biosynthesis. It was shown also that use of the additional carbon sources caused inhibition of cell growth, decrease in polymer yields, fall in polymer molecular weight, and decrease in 3HV content in produced PHB–HV–PEG copolymer that were depended on the nature of the additive (carboxylic acids or EG *derivatives*) and the timing of its addition to the growth medium. The observed relations could be coupled with the different roles that the carboxylic acids and EG *derivatives* play in the metabolism of bacterial cell. Currently, we are working to adjust the materials by tailoring the compositions achieve a balance between biocompatibility, physicochemical properties, processing ability, and device fabrication.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by Russian Science Foundation, project # 15-15-10014. The authors thank S.G. Alekseeva (G.S. Petrov Institute of Plastics) for excellent technical assistance and valuable discussions. The equipment of User Facilities Center of Moscow State University (including in framework of Development Program of MSU to 2020) and A.N. Bach Institute of Biochemistry RAS was used in the work.

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