ISSN 0026-2617, Microbiology, 2007, Vol. 76, No. 6, pp. 704–711. © Pleiades Publishing, Ltd., 2007.
Original Russian Text © T.G. Volova, G.S. Kalacheva, I.V. Kozhevnikov, A. Steinbüchel, 2007, published in Mikrobiologiya, 2007, Vol. 76, No. 6, pp. 797–804.

EXPERIMENTAL ARTICLES

Biosynthesis of Multicomponent Polyhydroxyalkanoates by *Wautersia eutropha*

T. G. Volova[a], G. S. Kalacheva[b,1], I. V. Kozhevnikov[b], and A. Steinbüchel[c]

[a] *Institute of Biophysics, Siberian Branch, Russian Academy of Sciences, Krasnoyarsk, Russia*
[b] *Krasnoyarsk State University, Krasnoyarsk, Russia*
[c] *Institute of Molecular Biology and Biotechnology, Munster, Germany*

Received June 26, 2006

Abstract—The effect of carbon supply on polyhydroxyalkanoate (PHA) synthesis by bacteria *Wautersia eutropha* was studied. Synthesis of multicomponent PHA composed of short- and long-chain monomers (C_4–C_8) by two natural strains (H16 and B5786) under mixotrophic conditions (CO_2 + alkanoic acids as cosubstrates) was demonstrated for the first time. The PHA composition was shown to be dependent on the cosubstrate type. In the presence of odd fatty acids, four- and five-component polymers were synthesized; hydroxybutyrate, hydroxyvalerate, and hydroxyheptanoate were the major monomers, while hydroxyhexanoate and hydroxyoctanoate were minor. In the presence of even fatty acids, PHA contained not only the corresponding molecules (hydroxyhexanoate and hydroxyoctanoate), but also hydroxyvalerate; synthesis of four-component PHA which contain mainly hydroxybutyrate and hydroxyhexanoate (up to 18 mol %) is therefore possible. A series of four- and five-component PHA was synthesized and their physicochemical characteristics were determined.

Key words: Wautersia eutropha, autotrophic and mixotrophic growth, multicomponent polyhydroxyalkanoates.

DOI: 10.1134/S0026261707060082

Analysis of the literature confirms active research in the field of synthesis and structure of polymers based on fatty acid derivatives. Biodegradable polyesters, apart from polylactides and polyglycolides, include polyhydroxyalkanoates (PHA), polymers of microbial origin. Their most valuable feature is the possibility to synthesize polymers of different composition and therefore with different physicochemical characteristics. To achieve this goal, however, fundamental knowledge is required concerning the mechanisms of synthesis of PHA with a given structure and its correlation with the physicochemical characteristics of the polymers. Known PHA include polyesters of different chemical structure, from highly crystalline thermoplastics to thermolabile rubber-like elastomers. PHA are subdivided into three groups, short-chain (PHA_{SC}), medium-chain (PHA_{MC}), and long-chain (PHA_{LC}). PHA_{SC} consist of the monomers containing three to five atoms in their carbon chain (C_3–C_5); PHA_{MC}, from C_6 to C_{14}; and PHA_{LC}, over C_{17} and C_{18}. Among these polymers, PHA_{SC} including hydroxybutyrate (PHB) and copolymers of hydroxybutyrate (HB) and hydroxyvalerate (HV) (PHB/PHV) are the best known. Active research on PHA_{MC} and PHA_{LC} began recently; information is still scarce concerning the producers capable of synthesis of new types of multicomponent PHA containing medium- and short-chain monomers (PHA_{SC+MC}).

Although numerous producers of PHA have been characterized (over 300 species), only a small number of them are capable of synthesizing PHA_{SC+MC}. The known natural strains include: *Pseudomonas* sp. A33 (synthesizes copolymers of HB and nine monomers with chain lengths fromC_{12} to C_{16}) [1]; *Pseudomonas putida* Gpo-1 (synthesizes PHA_{MC} of varying composition) [2]; *Thiococcus pfennigii, Aeromonas hydrophila, Aeromonas caviae,* and *Ectothiorhodospira shaposhnikovii* (synthesize copolymers of HB and hydroxyhexanoate, HH) [3–8]; and *Aeromonas punctata* and *Wautersia eutropha* (formerly *Ralstonia eutropha* [9]), which synthesize PHA containing C_4–C_8 monomers [3, 10–12]. Genetically modified producers are also use for PHA synthesis. Among the known strains are *Pseudomonas* strains with the cloned *Ralstonia* PHA synthesis genes [13]; *R. eutropha* with the *Pseudomonas* synthase gene; *E. coli* with the PHA synthase genes from *Aeromonas* and *Ralstonia* (synthesize HB and HH copolymers) [6, 14]; *R. eutropha* with the *Pseudomonas fluorescens* GK-13 genes (synthesizes PHA from C_4–C_{12} monomers) [15]; and *W. eutropha* with the *Pseudomonas* sp. 61-3 cloned genes (synthesizes HB copolymers with hydroxyoctanoate, HO) [16].

[1] Corresponding author; e-mail: kalach@ibp.ru

In order to obtain multicomponent PHA, specific conditions of carbon nutrition are required. To synthesize medium- and long-chain PHA, complex carbon sources are usually applied, which include the major carbon source supplemented with the salts of fatty acids with different lengths of a carbon chain (C_6–C_9 and more) as cosubstrates.

Since *Wautersia eutropha* synthesize polymers of diverse composition (predominately PHA_{SC}) with high yields (up to 80–90%) and utilize a variety of substrates (hydrogen–CO_2 mixtures, sugars, organic acids, alcohols, and industrial or agricultural waste), these bacteria are among the most promising PHA producers [13, 17–20]. HB is the dominant monomer in *Wautersia* PHA; the ratio of HV in specialized modes can reach 80–90 mol % [21], while HH and HO levels do not exceed 1–2 mol % [10, 22–24].

The goal of the present work was to carry out comparative research on the formation, accumulation, and chemical structure of $PHA_{SC + MC}$ by *Wautersia eutropha* strains H16 and B5786 under different conditions of carbon nutrition.

MATERIALS AND METHODS

Two strains of *Wautersia eutropha* were investigated, H16 and B5786. Strain H16 have been isolated by H. G. Schlegel and has been thoroughly studied in a number of European laboratories. Strain B5786 was isolated in Russia; it is a fast-growing variant of strain *W. eutropha* Z1 [25] which has been isolated by the group of G.A. Zavarzin [26]. Bacteria were grown on a Schlegel mineral salt medium [27] in batch culture under sterile conditions in 1-l flasks (at 0.5 mark-to-space ratio) on a shaker at 30°C. The mixture of H_2 and CO_2 was used as a source of carbon and energy in the autotrophic mode and fructose in the heterotrophic mode. The initial ratio of CO_2, O_2, and H_2 in the gas mixture was 1 : 2 : 6 (vol/vol). Fructose concentration under heterotrophic growth conditions was maintained at 10 g/l. In order to obtain maximum synthesis and accumulation of PHA, bacteria were grown under nitrogen limitation during the first stage of cultivation and in nitrogen-free medium during the second stage; pH was maintained at 7.0 and 30°C [28]. The composition of the gas mixture was monitored with serial gas analyzers and an LKhM-80 chromatograph (katharometer detector, argon as carrier gas). Fructose concentration in the medium was determined with the resorcinol method. Salts of fatty acids (valerate, hexanoate, heptanoate, and octanoate) (Sigma) were introduced into the medium as cosubstrates for the synthesis of multicomponent polymers (0.5–2.0 g/l). The concentrations of fatty acids were determined by gas chromatography after hexane extraction from acidified samples of the medium. The relevant alkane acids were used as internal standards.

Biomass accumulation in the culture was monitored by dry weight and optical density. The total polymer content in the biomass and the monomer composition were determined by chromatography of the fatty acid methyl ethers after methanolysis of dry biomass on a GSD plus chromato–mass spectrometer (Hewlett Packard, United States) [12]. The polymer and lipids were extracted from dry biomass with chloroform–ethanol (2 : 1 vol/vol); the polymer was then separated from the lipids by hexane precipitation. The chemical structure of the polymer was determined by chromato–mass spectrometry after repeated precipitation. Retention times and mass spectra of the monomers were used for their identification.

To determine the thermal characteristics and crystallinity of PHA samples, films obtained from solutions were used. The thermal characteristics were determined by derivatography on an MOM device (Hungary) with simultaneous registration of the timelines of differential thermal analysis (DTA), thermogravimetry (TG), and derivative thermogravimetry (DTG). Samples of 0.1-mm thick PHA films of different composition were placed in platinum crucibles; analysis was conducted under an inert gas within the 20–300°C temperature range, and the heating rate was 5°C/min. The temperatures of fusion and decomposition were determined as the temperatures of the maximum heat absorbance for the corresponding endothermic effects. The accuracy of temperature measurements by derivatograms was ±1°C. X-ray structure analysis was carried out on a D8 ADVANCE X-ray spectrometer (Bruker, Germany) with a graphite monochromator on a reflected beam. In order to determine the degree of crystallinity C_x, scan-step spectra were obtained with a step of 0.04° and 2-s exposures to determine the intensity at a given point (the 40 kV × 40 μA mode).

RESULTS AND DISCUSSION

Under autotrophic growth conditions under nitrogen limitation and a C_1 substrate (CO_2), the biomass yield and the polymer yield of both strains were practically identical. After 48-h cultivation of strains H16 and B5786, the biomass yield was 5.8 and 6.1 g/l; the final polymer concentrations were 63.0 and 61.4%, respectively. The polymer synthesized by both strains consisted mostly of HB (96.6–99.2 mol %); HV (0.6–2.8 mol %) and HH (0.2–0.7 mol %) were detected as minor components (Table 1).

Synthesis of multicomponent PHA is a rather complicated technical problem. A number of factors should be taken into account. High PHA yields are impossible in rapidly growing cultures (with the exception of *Alcaligenes latus*); special growth modes are therefore required to obtain high polymer yields together with the overall high biomass productivity. Moreover, the rates of monomer incorporation into the polymer are different for the monomers with different lengths of the carbon chain. The PHA monomer ratio is therefore unsta-

Table 1. Dynamics of the composition of the polymers synthesized by two *Wautersia eutropha* strains under autotrophic, mixotrophic, and heterotrophic growth conditions and supplemented with odd and even fatty acids (mol %)

Carbon substrate	Cultivation time, h	B5786					H16				
		HB	HV	HH	HHep	HO	HB	HV	HH	HHep	HO
CO_2	12	98.80	1.00	0.20	ND	ND	99.20	0.60	0.20	ND	ND
	24	97.80	1.50	0.70	ND	ND	97.00	1.70	1.30	ND	ND
	36	97.30	2.20	0.50	ND	ND	97.90	1.60	0.50	ND	ND
	48	96.50	3.00	0.50	ND	ND	98.20	1.20	0.60	ND	ND
	48 PHA*	96.60	2.80	0.60	ND	ND	97.60	1.70	0.70	ND	ND
CO_2 + C5	12	96.05	2.04	1.91	ND	ND	94.24	4.00	1.76	ND	ND
	24	32.80	67.05	0.16	ND	ND	37.84	61.71	0.14	0.31	ND
	36	31.02	68.40	0.06	0.20	0.01	34.08	65.62	0.10	0.20	tr.
	36 PHA*	31.22	68.40	0.13	0.20	0.04	36.01	63.64	0.12	0.21	0.02
	48	57.56	42.32	0.05	0.07	tr.	58.62	41.29	tr.	0.09	tr.
	48 PHA	56.65	41.71	0.05	0.04	ND	58.15	41.71	0.08	0.06	tr.
CO_2 + C7	12	97.54	0.89	1.57	ND	ND	99.51	0.49	ND	ND	ND
	24	52.67	44.60	0.70	1.87	0.16	39.01	60.62	tr.	0.37	ND
	36	41.18	57.25	0.22	1.30	0.05	30.67	68.93	ND	0.40	ND
	36 PHA	41.94	56.30	0.71	1.01	0.05	36.10	63.52	0.07	0.28	0.03
	48	53.51	46.04	0.03	0.42	ND	59.64	40.20	ND	0.16	ND
	48 PHA	52.25	47.33	0.09	0.33	ND	59.36	40.48	0.06	0.10	ND
CO_2 + C6	12	98.87	1.04	0.09	ND	ND	99.41	0.59	ND	ND	ND
	24	86.49	2.71	9.79	ND	1.01	89.50	2.81	6.88	ND	0.81
	36	80.38	2.31	16.67	ND	0.64	88.24	1.85	9.48	ND	0.43
	36 PHA	81.81	3.69	13.76	ND	0.75	81.86	3.74	13.68	ND	0.72
	48	86.83	1.37	10.29	ND	1.51	89.77	1.72	8.01	ND	0.50
	48 PHA	85.89	2.05	11.44	ND	0.62	85.60	2.76	10.84	ND	0.84
CO_2 + C8	12	96.58	0.37	2.05	ND	ND	94.57	3.61	3.02	ND	ND
	24	91.25	3.40	3.02	ND	2.33	89.37	3.48	3.77	ND	3.38
	36	93.30	2.32	2.33	ND	2.05	91.62	2.57	3.60	ND	2.21
	36 PHA	72.14	24.80	1.11	0.23	0.72	86.07	9.32	2.59	0.04	1.99
	48	94.50	1.23	3.05	ND	1.22	94.09	1.66	2.94	ND	1.31
	48 PHA	91.70	4.59	2.55	ND	1.17	93.31	3.34	2.38	ND	0.97
Fructose	12	97.62	1.73	0.65	ND	ND	97.9	1.57	0.53	ND	ND
	24	99.55	0.30	0.15	ND	ND	99.42	0.36	0.22	ND	ND
	36	99.48	0.37	0.15	ND	ND	99.51	0.42	0.07	ND	ND
	48	99.59	0.28	0.13	ND	ND	99.65	0.32	0.03	ND	ND
	48 PHA	99.58	0.27	0.15	ND	ND	99.52	0.29	0.19	ND	ND
Fructose + C5	12	99.55	0.45	tr.	ND	ND	99.46	0.54	tr.	ND	ND
	24	99.67	0.23	0.10	ND	tr.	99.74	0.24	0.01	ND	0.01
	36	71.59	28.37	0.04	ND	ND	62.04	37.96	tr.	ND	ND
	36 PHA	67.45	32.40	0.09	0.05	ND	61.81	38.00	0.11	0.04	ND
	48	74.48	25.47	0.05	ND	ND	66.58	33.35	0.07	ND	ND
	48 PHA	78.16	21.03	0.81	ND	ND	73.17	26.74	0.09	ND	ND
Fructose + C6	12	99.92	0.08	tr.	ND	ND	99.46	0.54	tr.	ND	ND
	24	99.69	0.15	0.16	ND	ND	99.60	0.24	0.16	ND	ND
	36	97.35	0.58	1.99	ND	0.08	97.88	0.80	1.28	ND	0.04
	36 PHA	95.70	1.22	3.00	ND	0.08	96.97	1.72	1.30	ND.	0.01
	48	97.71	0.95	1.30	ND	0.04	98.01	0.78	1.19	ND	0.02
	48 PHA	97.06	1.54	1.29	ND	0.11	97.55	1.25	1.16	ND	0.04
Fructose + C8	12	99.42	0.58	tr.	ND	ND	99.64	0.36	tr.	ND	ND
	24	99.72	0.21	0.07	ND	ND	99.70	0.29	0.01	ND	ND
	36	99.04	0.28	0.23	ND	0.45	98.76	0.45	0.40	ND	0.39
	36 PHA	98.43	1.00	0.39	ND	0.18	98.47	0.68	0.54	ND	0.31
	48	97.67	1.99	0.20	ND	0.14	97.05	0.89	1.31	ND	0.75
	48 PHA	98.72	0.77	0.32	ND	0.19	96.97	1.17	1.20	ND	0.66

Notes: 36 PHA and 48 PHA, isolated and purified polymer; ND, not detected; tr., trace.

ble during the cultivation period. Finally, alkane fatty acids which are applied as cosubstrates are toxic to most of microbial cultures; their maximum permissible concentrations should therefore be determined for each acid and for each producer. Thus, the synthesis in dense cultures of high levels (over 50–60%) of multicomponent PHA with medium- and long-chain monomers as major components is highly problematic.

We have previously developed a cultivation mode [28] which accounted both for the different rates of incorporation into PHA for monomers with different lengths of the carbon chain and for toxicity of the salts of fatty acids added as cosubstrates. Fig. 1 presents the results of cultivation and PHA synthesis on mixed carbon substrates (CO_2, valerate, hexanoate, etc.) by strains H16 and B5786. The data presented demonstrate that the dynamics of PHA synthesis and composition by these strains was different depending on the co-substrate added (length of the carbon chain) (Fig. 1, Table 1).

Introduction of valerate into 12-h cultures (biomass, 2.7–2.8 g/l; polymer content, 22%) resulted in an increased biomass yield (to 4.1–4.8 g/l) and polymer yield (to 48.9–63.8%) for both strains (Fig. 1a). The polymer content in the cells by the end of the experiment exceeded 90%. Both strains synthesized four- and five-component polymers. HB and HV were identified as the predominant monomers; HH, hydroxyheptanoate (HHep), and HO were the minor components. However, the inclusions of HH and HO were irregular. The ratios of the PHA fractions depended on the duration of cultivation after the cosubstrate introduction (Table 1). Since the composition of the purified PHA samples obtained from 36- and 48-h cultures was practically identical to that of the corresponding biomass samples after methanolysis (Table 1), the pool of free monomers not incorporated into the polymer was absent. By varying the amount of valerate introduced into the culture and of the subsequent cultivation duration, the polymers were obtained with HV ratios of up to 85 mol % for both strains (Table 2).

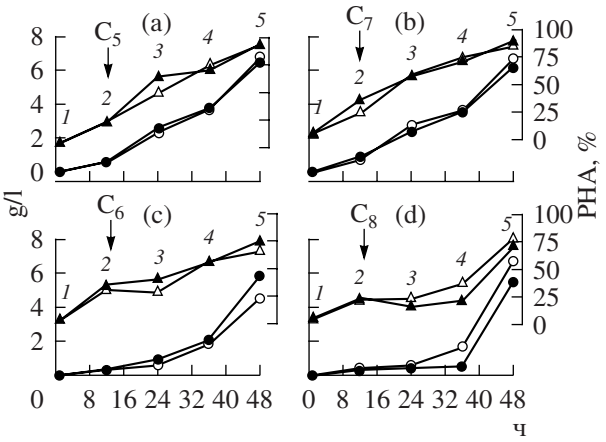

Fig. 1. Dynamics of PHA synthesis in autotrophically grown *W. eutropha* strains B5786 and H16 supplemented with valerate (a), heptanoate (b), hexanoate (c), and octanoate (d).▲, B 5786 PHA, g/l; △, H16 PHA, g/l; ●, B5786 PHA, % dry weight; ○, H16 PHA, % dry weight; *1, 2, 3, 4, 5,* sampling points.

When grown on mixtures of CO_2 and enanthic acid (Fig. 1b, Table 1), bacteria synthesized polymers containing mostly HV and HB. The introduction of HHep was insignificant (less than 2 mol %), and that of HH and HO was low and irregular (Table 1). The composition of the polymer samples did not differ from the composition of the corresponding biomass samples after methanolysis (Table 1); it varied with the duration of bacterial cultivation. The polymer yield was comparable to that in the above experiment with valerate (over 90%).

In the course of growth on the mixtures of CO_2 and salts of even fatty acids, multicomponent PHA of a different composition were formed.

Table 2. Physicochemical characteristics of the multicomponent polymers synthesized by *W. eutropha* under autotrophic growth conditions

Polymer sample no.	PHA composition, mol %					PHA characteristics		
	HB	HV	HH	HHep	HO	C_x, %	T_{fus}, °C	T_{dec}, °C
1	11.22	88.46	0.13	0.16	0.04	49	146	210
2	36.01	63.64	0.12	0.21	0.02	51	150	213
3	41.94	56.30	0.71	0.98	0.06	48	153	230
4	52.25	47.33	0.09	0.33	ND	46	158	234
5	79.61	1.50	18.03	ND	0.85	53	155	253
6	81.81	3.69	13.76	ND	0.74	60	156	253
7	83.38	4.47	11.63	ND	0.52	62	157	257
8	90.63	2.29	6.52	ND	0.56	65	159	256
9	91.70	4.59	2.55	ND	1.17	74	163	265
10	99.76	0.24	tr.	ND	ND	74	168	268

For example, when hexanoate was introduced into the medium, both strains synthesized PHA with HB and long-chain HH as the major components (Fig. 1c, Table 1). The dynamics of the polymer accumulation was typical of the autotrophic culture; the maximum of HH inclusion into the polymer (up to 13 mol %) occurred 24 h after the fatty acid introduction. The polymer content in the cells was by then over 50%. Apart from HH, HO (0.6–0.8 mol %) and HV (2.05–3.74 mol %) were detected in the polymer. By the end of the experiment, the polymer content in the cells increased to 70%; the ratios of HH and HV decreased to 11 and 2.0–2.8 mol %, respectively, while HO content remained the same (0.6–0.8 mol %). The composition of the polymer isolated and purified from two strains was practically identical; it did not differ from the results obtained by methanolysis of the biomass samples (Table 1). The observed increase in the HO fraction may indicate formation of the monomer precursors not only by β-oxidation of fatty acids, but by elongation of their acyl chains.

Octanoate is more toxic to the strains used; at 0.5 g/l it inhibited both cell growth and polymer accumulation (Fig. 1d). Growth and polymer synthesis resumed at lower octanoate concentrations. By the end of the experiment, PHA concentration in the cells was 70–77% and the biomass yield was 7.8–8.7 g/l. In this experiment, the composition of the polymers obtained 12 h after the introduction of octanoate was substantially different from the monomer composition of the samples; HV content was significantly higher (24.8 and 9.32 mol % for B5786 and H16, respectively), HHep was present (0.04–0.23 mol %), and HO incorporation was decreased (2.05–2.21 in the biomass polymer and 0.72–1.99 mol % in the isolated polymer). By the end of the experiment, the monomer composition in the isolated polymers and in the biomass samples was virtually identical (HB, 91.7 and 93.3; HV, 4.6 and 3.3; HH, 2.55 and 3.34; and HO, 1.0 and 1.2 mol % for strains B5786 and H16, respectively) (Table 1.).

Thus, the possibility to obtain multicomponent PHA containing short- and medium-chain monomers was demonstrated for the first time for two *W. eutropha* strains grown under mixotrophic conditions with a complex carbon source (CO_2 + fatty acid). Apart from the predominant hydroxybutyrate and hydroxyvalerate, PHA samples containing hydroxyhexanoate as a major component (up to 18 mol %) were obtained.

Under organotrophic growth conditions on fructose, the polymer yield was higher than in the autotrophic mode (over 90% of the dry biomass for both strains). Addition of valerate and hexanoate had no effect on this value; by the end of the experiment, the polymer content was 88 and 92% for strains B5786 and H16, respectively. Octanoate inhibited polymer biosynthesis, and PHA content in the cells did not exceed 60%. When grown heterotrophically on a mixed substrate (fructose + fatty acid), both strains synthesized a three-component PHA containing HB (over 97%) with minor inclusions of HV and HH (Table 1). The mode with valerate addition was exceptional in that a three-component PHA was synthesized with up to 26% content of hydroxyvalerate; HH was detected as a minor component.

Fig. 2 demonstrates two typical chromatograms of PHA isolated from *W. eutropha* H16 supplemented with heptanoate and *W. eutropha* B5786 supplemented with octanoate. Mass spectra of all five monomers confirm their identification.

Varying the amounts of supplements (odd and even fatty acids) and the fermentation duration in the autotrophic mode enabled us to obtain a family of PHA_{SC} and PHA_{MC}; its physicochemical characteristics are presented in Table 2. Our previous finding concerning the effect of HV on redistribution of the regular and amorphous phases of the polymer (leading to its decreased crystallinity) was thus confirmed. This effect, however, was revealed only when HV content did not exceed 25–30 mol %; HV content above 50 mol % did not result inC_x changes. Increased HV content in the polymer affected its thermal characteristics within the whole range investigated; it was most pronounced at high HV levels exceeding 35–40 mol %. Introduction of minor amounts of HHep and HO (0.98 and 1.2 mol %, respectively) had no effect on the crystallinity of the polymer.

The experimental PHA samples containing, apart from HB, also hydroxyhexanoate as a macro component (from 2 to 18 mol %) are of special interest. Analysis of X-ray spectra revealed a regular dependence of the degree of crystallinity (C_x) on the ratio of HH (Table 2). The degree of PHA crystallinity decreased uniformly with increasing HH ratio. Thus, similar to HV, HH incorporation into the polymer makes it less crystalline and more suitable for manufacturing purposes. PHA thermal characteristics also changed with increasing HH ratio. Within the investigated range (from several to 18 mol %), both T_{fus} and T_{dec} decreased when HH content increased; however, no breach was detected between the temperatures of fusion and decomposition.

The results obtained demonstrate that the direction of PHA synthesis by *W. eutropha* strains H16 and B5786 is identical. According to our present knowledge [29], the PHA composition is determined by the substrate specificity of PHA synthase (polymerase), one of the key enzymes of PHA synthesis. Comparison of amino acid sequences of synthases from *W. eutropha* strains H16 and B5786 revealed very high homology (99%); the insignificant differences in their primary structure are localized in a short C-terminal nonidentical fragment between the amino acid residues 561 and 572 [30].

According to some publications, *W. eutropha* is a promising producer not only of PHB, but of the poly(HB-co-HV) copolymer [21, 31]. Incorporation of monomers with more than five atoms in the carbon

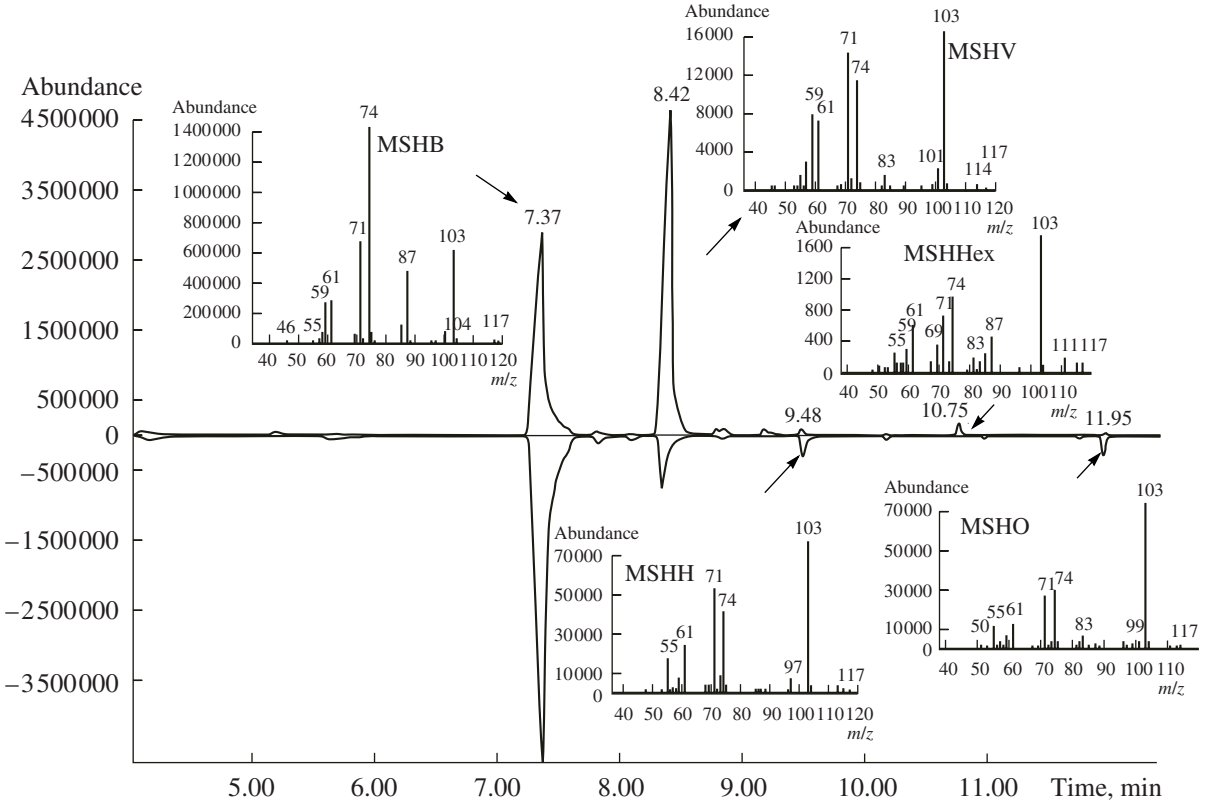

Fig. 2. Ion chromatograms of the polymers obtained from autotrophically grown *W. eutropha* H16 supplemented with heptanoate (top), *W. eutropha* B5786 supplemented with octanoate (bottom, mirrored), and mass spectra of the corresponding monomers: MSHB, hydroxybutyrate with retention time 7.37; MSHV, hydroxyvalerate, 8.42; MSHH, hydroxyhexanoate, 9.48; MSHHep, hydroxyheptanoate, 10.75; and MSHO, hydroxyoctanoate, 11.95.

chain was revealed only in specially engineered strains [22, 23] or when β-oxidation of fatty acids was inhibited [24]. Our results demonstrate that under autotrophic conditions without additional substrates (fatty acids), known strains synthesize a three-component PHA with minor inclusions of HV and HH. Under mixotrophic conditions (CO_2 + fatty acid), both strains synthesize four- or five-component polymers with predomination of HB, HV, of HH. The high level of HV incorporation into the polymer, which was observed when odd fatty acids were applied, is understandable and agrees with the published data [21, 31]. High levels of HH incorporation into the polymer by *Wautersia* strains, however, have not been previously reported, in spite of the interest in this PHA type. The producer of a HB/HH copolymer, *Aeromonas hydrophyla* has been recently isolated. The wild strain accumulates up to 45% of poly(HB/HH); the HH fraction is 10–13% [6, 7, 14]. Our results demonstrate that under mixotrophic growth conditions both *W. eutropha* strains have higher polymer content than *A. hydrophyla* the quality of these polymers is comparable.

It can be concluded that in *W. eutropha* strains H16 and B5786 grown under mixotrophic conditions, odd fatty acids (valerate and heptanoate) promote the synthesis of four- and five-component PHA with hydroxybutyrate and hydroxyvalerate as the major components, and hydroxyhexanoate and hydroxyoctanoate as the minor ones. Even fatty acids (hexanoate and octanoate) are involved in the synthesis of the corresponding monomers (hydroxyhexanoate and, to a lesser degree, hydroxyoctanoate) and promote inclusion of small amounts of hydroxyvalerate into the polymer.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research (project no. 05-04-08024ofi-a), the Russian Federation Ministry of Education and Science, and the U.S. Civilian Research & Development Foundation for the independent countries of the former Soviet Union (CRDF and MES RF REC) (project BP1MO2), and the Interdisciplinary Projects Program of Siberian Division, Russian Academy of Sciences (project no. 24).

REFERENCES

1. Lee, E.L., Jendrossek, D., Schirmer, A., Choi, C.V., and Steinbüchel, A., Biosynthesis of Copolymers Consisting of 3-Hydroxybutyric Acid and Medium-Chain-Length 3-Hydroxyalkanoic Acids from 1,3-Butandiol or from

3-Hydroxybutyrate by *Pseudomonas* sp. A33, *Appl. Microbiol. Biotechnol.,* 1995, vol. 42, pp. 901–909.

2. Hartmann, R. Hany, R., Geiger, T., Egli, T., Witholt, B., and Zinn, M. Tailored Biosynthesis of Olefinic Medium-Chain-Length Poly[(*R*)-3-Hydroxyalkanoates] in *Pseudomonas putida* GPol with Improved Thermal Properties, *Macromol,*, 2004, vol. 70, pp. 6780–6785.
3. Liebergesell, M., Mayer, F., and Steinbüchel, A., Analysis of Polyhydroxyalkanoic Acid-Biosynthetic Genes of Anoxygenic Phototrophic Bacteria Reveals Synthesis of a Polyester Exhibiting An Unusual Composition, *Appl. Microbiol. Biotechnol.*, 1993, vol. 40, pp. 292–300.
4. Doi, Y., Kitamure, S., and Abe, H., Microbial Synthesis and Characterization of Poly(3-Hydroxybutyrate-co-3-Hydroxyhexanoate), *Macromol.*, 1995, vol. 28, pp. 4822–4828.
5. Chen, G.Q., Zhang, G., and Lee, S.Y., Industrial Production of Poly(Hydroxybutyrate-co-Hydroxyhexanoate), *Appl. Microbiol. Biotechnol.*, 2001, vol. 57, pp. 50–55.
6. Qiu, Y.-Z., Ouyang, S.-P., Shen, Z., Wu, Z., and Chen, G.-Q., Metabolic Engineering for the Production of Copolyesters Consisting of 3-Hydrohybutyrate and 3-Hydrohyhexanoate by *Aeromonas hydrophila, Macromol. Bioscience*, 2004, vol. 4, pp. 255–261.
7. Han, J., Qiu, Y.-Z., Liu, D.-C., and Chen, G.-Q., Engineered *Aeromonas hydrophila* for Enhanced Production of Poly(3-Hydroxybutyrate-Co-3-Hydroxyhexanoate) with Alterable Monomers Composition, *FEMS Microbiol Lett*, 2005, vol. 239, pp. 195–201.
8. Zhang, S., Kolvek, S., Goodwin, S., and Lenz, R.W., Poly(Hydroxyalkanoic Acid) Biosynthesis in *Ectothiorhodospira shaposhnikovii*: Characterization and Reactivity of a Type III PHA Synthase, *Biomacromol.*, 2004, vol. 5, pp. 40–48.
9. Vaneechoutte, M., Kämpfer, P., De Baere, T., Falsen, E., and Verschraegen, G., *Wautersia* gen. nov., a Novel Genus Accommodating the Phylogenetic Lineage Including *Ralstonia eutropha* and Related Species, and Proposal of *Ralstonia* [*Pseudomonas*] *syzygii* (Roberts et al. 1990) comb. nov, *Int. J. Syst. Evol. Microbiol.,* 2004, vol. 54, pp. 317–327.
10. Liebergesell, M., Hustede, E., Timm, A., Steinbüchel, A., Fuller, R.C., Lenz, R.W., and Schlegel, H.G., Formation of Poly(3-Hydroxyalkanoates) by Phototrophic and Chemolithotrophic Bacteria, *Arch. Microbiol.*, 1991, vol. 155, pp. 416–421.
11. Volova, T.G., Belyaev, O.G., Gitel'zon, I.I., Kalacheva, G.S., Lukovenko, S.G., and Plotnikov, V.F., Microbial Heteropolymeric Polyhydroxyalkanoates: Synthesis and Properties, *Dokl. Akad. Nauk*, 1996, vol. 347, pp. 256–258 [*Dokl. Biophys.* (Engl. Transl.), vols. 346–348, pp. 22–26].
12. Volova, T.G., Kalacheva, T.S, and Plotnikov, V.F., Biosynthesis of Heteropolymeric Polyhydroxyalkanoates by Chemolithoautotrophic Bacteria, *Mikrobiologiya*, 1998, vol. 67, no. 4, pp. 512–517 [*Microbiology* (Engl. Transl.), vol. 67, no. 4, pp. 420–424].
13. Fukui, T. and Doi, Y. Efficient Production of Polyhydroxyalkanoates from Plant Oils by *Alcaligenes eutrophus* and its Recombinant Strain, *Appl. Microbiol. Biotechnol.,* 1998, vol. 49., pp. 333–336.
14. Qiu, Y.-Z., Han, J., and Chen, G.-Q., Metabolic Engineering of *Aeromonas hydrophila* for the Enhanced Production of Poly(3-Hydroxybutyrate-Co-3-Hydroxyhexanoate), *Appl. Microbiol. Biotechnol.*, 2006, vol. 69, pp. 537–542.
15. Noda, I., Green, P.R., Satkowski, M.M., and Schechtman, L.A., Preparation and Properties of a Novel Class of Polyhydroxyalkanoate Copolymers, *Biomacromol.*, 2005, vol. 6, pp. 580–586.
16. Delamarre, S.C.. and Batt, C.A., Comparative Study of Promoters for the Production of Polyhydroxyalkanoates in Recombinant Strains of *Wautersia eutropha, Appl. Microbiol. Biotechnol.*, 2006, vol. 71, no. 5, pp. 668–679.
17. Tanaka, K., Ishizaki, A., Kanamaru, T., and Kawano, T., Production of Poly(D-3-Hydroxybutyrate) from CO_2, H_2 and O_2 by High Cell Density Autotrophic Cultivation of *Alcaligenes eutrophus, Biotethnol. Bioeng.*, 1995, vol. 45, pp. 268–275.
18. Bormann, E.J. and Roth, M., The Production of Polyhydroxybutyrate by *Methylobacterium rhodesianum* and *Ralstonia eutropha* in Media Containing Glycerol and Casein Hydrolysates, *Biothechnol. Lett.*, 1999, vol. 21, pp. 1059–1063.
19. Ishizaki, A., Tanaka, K., and Taga, N., Microbial Production of Poly-D-3-Hydroxybutyrate from CO_2, *Appl. Microbiol. Biotechnol.*, 2001, vol. 57, pp. 6–12.
20. Volova, T.G., Kalacheva, G.S., and Altuhova, O.V., Autotrophic Synthesis of PHAs by *Ralstonia eutropha* in the Presence of Carbon Monoxide, *Appl. Microbiol. Biotechnol.*, 2002, vol. 58, pp. 675–678.
21. Doi, Y., Tamaki, A., Kunioka, M., and Soga, K. Production of Copolyesters of 3-Hydroxybutyrate and 3-Hydroxyvalerate by *Alcaligenes eutrophus* from Butyric and Pentanoic Acids, *Appl. Microbiol. Biotechnol.*, 1998, vol. 28, pp. 330–334.
22. Dennis, D., McCoy, M., Stangl, A., Valentin, H.E., and Wu, Z., Formation of Poly(3-Hydroxybutyrate-Co-3-Hydroxyhexanoate) by PHA Synthase from *Ralstonia eutropha, J. Biotechnol.*, 1998, vol. 64, pp. 177–186.
23. Antonio, R.V., Steinbüchel, A., and Rehm, B.H.A., Analysis of the *in vivo* Substrate Specificity of the PHA Synthase from *Ralstonia eutropha*: Formation of Novel Copolyesters in Recombinant *Escherichia coli,* FEMS *Microbiol. Lett.*, 2000, vol. 182, pp. 111–117.
24. Green, P.R., Kemper, J., Schechtman, L., Guo, L., Satkowski, M., and Fiedler, S., Steinbüchel, A., and Rehm, B.H.A., Formation of Short Chain Length/Medium Length Polyhydroxyalkanoate Copolymers by Fatty Acid β-Oxidation Inhibited *Ralstonia eutropha, Biomacromol.*, 2002, vol. 3, pp. 208–213.
25. Stasishina, G.N. and Volova, T.G., RF Patent No. 2053292 (1992), *Bull. Izobret.*, 1996, no. 1.
26. Zavarzin, G.A., *Litotrofnye Mikroorganizmy*, (Lithotrophic Microorganisms), Moscow: Nauka, 1972.
27. Schlegel, H.G., Kaltwasser, H., and Gottschalk, G., Ein Submersverfahren zur Kultur wasserstoffoxydierender Bakterien: wachstumphysiologische Untersuchungen, *Arch. Mikrobiol.*, 1961, vol. 38, pp. 209–222.
28. Volova, T.G., Kalacheva, G.S., Konstantinova, V.M., and Puzyr', A.P., Effect of Growth Conditions on Polyhydroxybutyrate Accumulation by Hydrogen-Oxidizing

Bacteria, *Prikl. Biokhim. Mikrobiol.*, 1992, vol. 28, pp. 221–229.

29. Wei, Y., Jia, Y. Tian, J., Snell, K.D., Müh, U., Sinskey, A.J., Lambalot, R.H., Walsh, C.T., and Stubbe, J.A., Class I and III Polyhydroxyakanoate Sythases from *Ralstonia eutropha* and *Allochromatium vinosum*: Characterization and Substrate Specificity Studies, *Arch. Biochem. Biophys.*, 2001, vol. 394, pp. 87–98.
30. Kozhevnikov, I.V., Volova, T.G., Tran Hai, Steinbüchel, A., and Volodina, E.V., Clining and Molecular Organization of *Ralstonia eutropha* polyhydroxyalkanoate synthase (*phaC*) gene, *Vestn. Krasnoyarsk. Gos. Univ.*, 2005, no. 5, pp. 214–223.
31. Longan Shang, Seong Chun Yim, Hyun Gyu Park, and Ho Nam Chang, Sequential Feeding of Glucose and Valerate in a Feed-Batch Culture of *Ralstonia eutropha* for Production of Poly(Hydroxybutyrate-Co-Hydroxyvalerate) with High 3-Hydroxyvalerate Fraction, *Biotechnol. Prog.*, 2004, vol. 20, pp. 140–144.