 DOI: 10.1002/masy.200850901

Biosynthesis of Multi-Component Polyhydroxyalkanoates by the Bacterium *Wautersia eutropha*

Tatiana G. Volova,[1,2] *Galina S. Kalacheva,*[*1] *Alexander Steinbüchel*[3]

Summary: The study addresses the effect of different conditions of carbon nutrition on synthesis of polyhydroxyalkanoates by the bacterium *Wautersia eutropha*. In experiments with two wild type strains (H16 and B5786), it has been first found that under mixotrophic growth conditions – CO_2 + co-substrate (alkanoic acids) – bacteria can synthesize multi-component PHAs, consisting of short- and medium-chain-length monomers with carbon chains containing 4 to 8 atoms. It has been shown that PHA composition is determined by the type of the co-substrate. Fatty acids with odd number of carbons induce bacteria to synthesize four- and five-component PHAs with hydroxybutyrate, hydroxyvalerate as major monomers and hydroxyhexanoate, hydroxyheptanoate and hydroxyoctanoate as minor, occasionally occurring, ones. Fatty acids with even number of carbons induce synthesis of not only their respective monomers (hydroxyhexanoate and hydroxyoctanoate) but also hydroxyvalerate, making possible synthesis of four-component PHAs, containing hydroxybutyrate and hydroxyhexanoate as major components (up to 18 mol%). A family of short- and medium-chain-length four- and five-component PHAs were synthesized and their physicochemical properties examined.

Keywords: autotrophic and mixotrophic growth; multi-component polyhydroxyalkanoates; *Wautersia eutropha*

Introduction

Much consideration has been given recently to synthesis and investigation of polymers based on derivatives of carbonic acids. Among biodegradable polyesters a special place is occupied by polyhydroxyalkanoates (PHAs) – polyesters of microbial origin. The main advantage of PHAs is feasibility of synthesizing variously composed polymers, i.e. polymers with different physicochemical properties.

Over 300 various PHA producers have been described, but just a few have been found to be able to synthesize $PHA_{SCL+MCL}$. These are such wild-type strains as *Pseudomonas sp.* A33, which synthesizes hydroxybutyrate (HB) copolymers with various monomers, from C_{12} to C_{16};[1] *Pseudomonas putida* GPo-1, which synthesizes medium-chain-length PHAs of various compositions;[2] *Thiococcus pfennigii, Aeromonas hydrophila, Aeromonas caviae*, and *Ectothiorhodospira shaposhnikovii*, which synthesize HB – hydroxyhexanoate (HHx) copolymers;[3–6] *Aeromonas punctata* and *Wautersia eutropha*, which synthesize PHAs with carbon chains containing 4 to 8 carbon atoms.[3,7–9]

Synthesis of multi-component PHAs requires specific conditions of carbon nutrition. As a rule, medium- and long-chain-length PHAs are synthesized using complex carbon substrates that contain the main carbon substrate and hydrocarbon

[1] Institute of Biophysics SB RAS, 660036 Krasnoyarsk, Russia
E-mail: kalach@ibp.ru
[2] Siberian Federal University, Krasnoyarsk, Russia
[3] Institute of Molecular Microbiology and Biotechnology, Münster, Germany

acid salts with carbon chains of various lengths (6 to 9 atoms and longer) as co-substrates.

The bacterium *W. eutropha* (formerly known as *Ralstonia eutropha*) is one of the most promising PHA producing organisms: it can synthesize polymers of various chemical structures, mainly PHA_{SCL}, and give high polymer yields (up to 80–90%), utilizing a wide range of substrates – hydrogen – carbon dioxide mixtures, sugars, organic acids, alcohols, industrial and agricultural wastes.[10–14] The predominant monomer in the PHAs of *W. eutropha* is HB; another monomer, hydroxyvalerate (HV), can amount to 80–90 mol% under specified conditions;[15] however, reported levels of HHx and hydroxyoctanoate (HO) do not exceed 1–2 mol%.[7,16–18]

The purpose of the study was to investigate and compare accumulation of $PHA_{SCL+MCL}$ by *W. eutropha* strains H16 and B5786 under different conditions of carbon nutrition and analyze their chemical structure.

Materials and Methods

The objects of the study were two strains of *Wautersia eutropha* – H16 and B5786. Strain H16, isolated by Professor H. Schlegel, is the most heavily investigated strain of *W. eutropha* in EC states and strain B5786, a fast-growing variant of *W. eutropha Z*1, isolated by Academician G.A. Zavarzin's research team – in Russia. Bacteria were batch-cultured under strictly aseptic conditions using Schlegel's method[19] in 1-l flasks filled to 50% of their volume, on a thermostatic shaker; the procedure promoting PHA synthesis, which was described elsewhere was used.[9] Under autotrophic conditions, the carbon source was CO_2 and under heterotrophic conditions – fructose. Bacteria were cultured in standard Schlegel mineral salts medium[19] deficient in NH_4Cl. To induce synthesis of multi-component polymers, the culture medium was supplemented with such co-substrates as salts of alkanoic acids (valerate, hexanoate, heptanoate, and octanoate) (Sigma Chem. Co.) at concentrations of 0.5–2.0 g/L.

Accumulation of the biomass in the culture was monitored by measuring the dry matter weight and optical density of the culture. Dry biomass samples were subjected to methanolysis and the total polymer content of the biomass and monomer compositions were determined by the chromatography of methyl esters of fatty acids on a GCD-Plus gas chromatograph-mass spectrometer (Hewlett Packard, USA).[14] Polymer and lipids were extracted from biomass with a chloroform-ethanol mixture (2:1 v/v) and then the polymer was separated from lipids by precipitation with hexane. The chemical structure of the extracted and twice precipitated polymer was analyzed in a similar manner, using the chromatograph-mass spectrometer. Monomers were identified by their retention time and mass spectra. PHA films prepared by the solution casting technique were used to investigate temperature characteristics and crystallinity of PHAs. Temperature characteristics of the polymers were measured with a derivatograph (MOM, Hungary), which simultaneously registered curves of differential thermal analysis (DTA) and performed thermogravimetry (TG) and derivative thermogravimetry (DTG). 0.1 mm thick PHA samples of various compositions were placed into platinum crucibles. The polymers were analyzed in the inert gas medium; the heating was conducted at a rate of 5 °C/min, from 20 to 300 °C. Melting points and temperatures for the onset of decomposition were determined as temperatures of heat absorption peaks of the corresponding endothermic effects. The error of derivatogram-based determination of endothermic effect temperatures was ± 1 °C. The X-ray structure analysis was conducted using a D8 ADANCE X-ray spectrometer (Bruker, Germany) (graphite monochromator on a reflected beam). To determine the degree of crystallinity, C_x, spectra were taken in a scan-step mode, with step 0.04^0, exposure time 2', to measure intensity at point. The operating mode of the instrument was $40\ kV \times 40\ \mu A$.

Results and Discussion

In the autotrophic culture, when bacteria were grown on a sole carbon substrate (CO_2) and their growth was limited by nitrogen deficiency, polymer yields produced by both the strains were almost identical. After 48 h of fermentation, strains H16 and B5786 yielded 5.8 and 6.1 g/L of biomass, respectively, the final polymer concentration being 63.0 and 61.4%. In the polymer synthesized by both strains, the predominant fraction was that of HB (96.6–99.2 mol%); minor components were HV (0.6–2.8 mol%) and HHx (0.2–0.7 mol%) (Table 1).

Data on bacterial growth and PHA synthesis on mixed carbon substrates (CO_2 + valerate, hexanoate, etc.) in H16 and B5786 cultures are shown in Fig. 1. After the 12 h culture was supplemented with valerate (the biomass yields 2.7–2.8 g/l and polymer content 22%) (Fig. 1, A), the biomass yields of both cultures increased to 4.1–4.8 g/L and polymer yields to 48.9–63.8%. At the end of the experiment, intracellular polymer content exceeded 90%. Both strains synthesized 4- and 5-component polymers. The major monomers were HB and HV and the minor ones – HHx, hydroxyheptanoate (HHp), and HO. However, HHx and HO were not regularly registered. Percentages of PHA fractions varied depending on how much time had passed after addition of a co-substrate (Fig. 1, A). The composition of recovered and purified PHAs (36 h and 48 h cultures) was the same as that of biomass samples (Table 1). By varying amounts of added valerate and time of the subsequent culture, induction of both strains to synthesize PHAs containing up to 85 mol% HV was possible (Table 2).

Bacteria grown on the CO_2 – heptanoic acid mixed substrate (Table 1) synthesized polymers that mostly consisted of HV and HB. HHp fractions were insignificant (below 2 mol%) and HHx and HO inclusions were small and unstable (Table 1). Qualitative composition of polymer samples was the same as that of biomass samples and varied depending on culture duration. Polymer yield was similar to that in the above-described experiment with valerate (over 90%) (Fig. 1, B).

When the strains were grown on mixtures of CO_2 and salts of fatty acids with even number of carbons, they synthesized multi-component PHAs of other compositions (Table 1).

With hexanoate as a co-substrate, both strains synthesized PHAs with HB and the medium-chain-length HHx as the major fractions (Table 1). Polymer accumulation pattern was characteristic of autotrophic culture, and the polymer had the maximal HHx content (up to 13 mol%) in 24 h after the addition of the fatty acid. By that time, intracellular polymer content had exceeded 50%. In addition to HHx, the polymers also contained HO (0.6–0.8 mol%) and HV (2.05–3.74 mol%). At the end of the experiment, intracellular polymer concentration reached 70% (Fig. 1, C); the HHx fraction decreased to 11 mol%, the HV fraction to 2.0–2.8 mol%, while the HO content remained unchanged – 0.6–0.8 mol%. The compositions of the recovered and purified polymers synthesized by the two strains were identical and did not differ from those of biomass samples (Table 1). The increase in HO may indicate that monomer precursors can be formed in the cell not only due to fatty acid oxidation but also in the course of acyl chain elongation.

Addition of octanoate, which is more toxic for the strains, inhibited culture growth and polymer synthesis even at concentration 0.5 g/l (Fig. 1, D). When octanoate concentration in the culture medium decreased, biomass growth and polymer synthesis were resumed. At the end of the experiment intracellular PHA concentration was 70–77% and biomass yield – 7.8–8.7 g/L. In this experiment, qualitative composition of the polymers recovered in 12 h after addition of octanoic acid was significantly different from that of biomass samples: the polymers contained enhanced HV percentages (24.8 mol% for B5786 and 9.32 mol% for H16), an HHp fraction was present, and the HO fraction was smaller (2.05–2.21 mol% in biomass

Table 1.
Composition of polyesters recovered from the two strains B5786 and H16 of *Wautersia eutropha* grown in autotrophic, mixotrophic, and heterotrophic cultures, which were supplemented with fatty acids with even (C_6 or C_8) and odd (C_5 or C_7) number of carbons. The compositions of the obtained PHAs are provided in mol%.

Supplement	Culture Duration (h)	B5786					H16				
		HB	HV	HHx	HHp	HO	HB	HV	HHx	HHp	HO
CO_2	12	98.80	1.00	0.20	N.d.	N.d.	99.20	0.60	0.20	N.d.	N.d.
	24	97.80	1.50	0.70	N.d.	N.d.	97.00	1.70	1.30	N.d.	N.d.
	36	97.30	2.20	0.50	N.d.	N.d.	97.90	1.60	0.50	N.d.	N.d.
	48	96.50	3.00	0.50	N.d.	N.d.	98.20	1.20	0.60	N.d.	N.d.
	48 PHA[a)]	96.60	2.80	0.60	N.d.	N.d.	97.60	1.70	0.70	N.d.	N.d.
CO_2+C_5	12	96.05	2.04	1.91	N.d.	N.d.	94.24	4.00	1.76	N.d.	N.d.
	24	32.80	67.05	0.16	N.d.	N.d.	37.84	61.71	0.14	0.31	N.d.
	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	N.d.	58.15	41.71	0.08	0.06	Tr.
CO_2+C_7	12	97.54	0.89	1.57	N.d.	N.d.	99.51	0.49	N.d.	N.d.	N.d.
	24	52.67	44.60	0.70	1.87	0.16	39.01	60.62	Tr.	0.37	N.d.
	36	41.18	57.25	0.22	1.30	0.05	30.67	68.93	N.d.	0.40	N.d.
	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	N.d.	59.64	40.20	N.d.	0.16	N.d.
	48 PHA	52.25	47.33	0.09	0.33	N.d.	59.36	40.48	0.06	0.10	N.d.
CO_2+C_6	12	98.87	1.04	0.09	N.d.	N.d.	99.41	0.59	N.d.	N.d.	N.d.
	24	86.49	2.71	9.79	N.d.	1.01	89.50	2.81	6.88	N.d.	0.81
	36	80.38	2.31	16.67	N.d.	0.64	88.24	1.85	9.48	N.d.	0.43
	36 PHA	81.81	3.69	13.76	N.d.	0.75	81.86	3.74	13.68	N.d.	0.72
	48	86.83	1.37	10.29	N.d.	1.51	89.77	1.72	8.01	N.d.	0.50
	48 PHA	85.89	2.05	11.44	N.d.	0.62	85.60	2.76	10.84	N.d.	0.84
CO_2+C_8	12	96.58	0.37	2.05	N.d.	N.d.	94.57	3.61	3.02	N.d.	H.o.
	24	91.25	3.40	3.02	N.d.	2.33	89.37	3.48	3.77	N.d.	3.38
	36	93.30	2.32	2.33	N.d.	2.05	91.62	2.57	3.60	N.d.	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	N.d.	1.22	94.09	1.66	2.94	N.d.	1.31
	48 PHA	91.70	4.59	2.55	N.d.	1.17	93.31	3.34	2.38	N.d.	0.97
Fructose	12	97.62	1.73	0.65	N.d.	N.d.	97.90	1.57	0.53	N.d.	H.o.
	24	99.55	0.30	0.15	N.d.	N.d.	99.42	0.36	0.22	N.d.	H.o.
	36	99.48	0.37	0.15	N.d.	N.d.	99.51	0.42	0.07	N.d.	H.o.
	48	99.59	0.28	0.13	N.d.	N.d.	99.65	0.32	0.03	N.d.	H.o.
	48 PHA	99.58	0.27	0.15	N.d.	N.d.	99.52	0.29	0.19	N.d.	H.o.
Fructose +C_5	12	99.55	0.45	Tr.	N.d.	N.d.	99.46	0.54	Tr.	N.d.	H.o.
	24	99.67	0.23	0.10	N.d.	Tr.	99.74	0.24	0.01	N.d.	0.01
	36	71.59	28.37	0.04	N.d.	N.d.	62.04	37.96	Tr.	N.d.	H.o.
	36 PHA	67.45	32.40	0.09	0.05	N.d.	61.81	38.00	0.11	0.04	H.o.
	48	74.48	25.47	0.05	N.d.	N.d.	66.58	33.35	0.07	N.d.	H.o.
	48 PHA	78.16	21.03	0.81	N.d.	N.d.	73.17	26.74	0.09	N.d.	H.o.
Fructose +C_6	12	99.92	0.08	Tr.	N.d.	N.d.	99.46	0.54	Tr.	N.d.	H.o.
	24	99.69	0.15	0.16	N.d.	N.d.	99.60	0.24	0.16	N.d.	H.o.
	36	97.35	0.58	1.99	N.d.	0.08	97.88	0.80	1.28	N.d.	0.04
	36 PHA	95.70	1.22	3.00	N.d.	0.08	96.97	1.72	1.30	N.d.	0.01
	48	97.71	0.95	1.30	N.d.	0.04	98.01	0.78	1.19	N.d.	0.02
	48 PHA	97.06	1.54	1.29	N.d.	0.11	97.55	1.25	1.16	N.d.	0.04
Fructose +C_8	12	99.42	0.58	Tr.	N.d.	N.d.	99.64	0.36	Tr.	N.d.	H.o.
	24	99.72	0.21	0.07	N.d.	H.o.	99.70	0.29	0.01	N.d.	N.d..
	36	99.04	0.28	0.23	N.d.	0.45	98.76	0.45	0.40	N.d.	0.39
	36 PHA	98.43	1.00	0.39	N.d.	0.18	98.47	0.68	0.54	N.d.	0.31
	48	97.67	1.99	0.20	N.d.	0.14	97.05	0.89	1.31	N.d.	0.75
	48 PHA	98.72	0.77	0.32	N.d.	0.19	96.97	1.17	1.20	N.d.	0.66

[a)] 36 or 48 PHA - isolated and purified polyesters; C_5, C_6, C_7, C_8- pentanoic, hexanoic, heptanoic, octanoic acids; HB– hydroxybutyrate; HV- hydroxyvalerate; HHx- hydroxyhexanoate; HHp– hydroxyheptanoate; HO– hydroxyoctanoate; N.d.– not detected; Tr.– trace amounts.

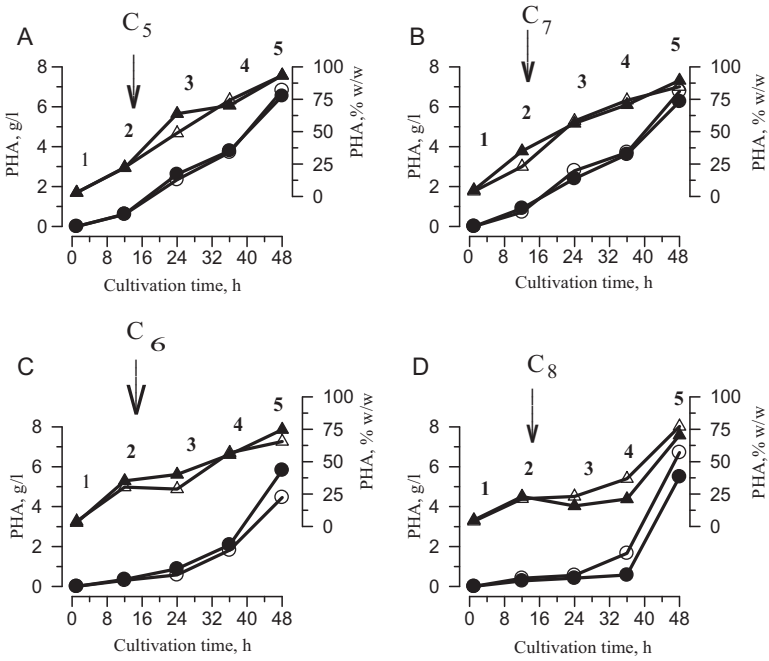

Figure 1.
Dynamics of PHA synthesis for two strains of *Wautersia eutropha*, B5786 and H16, grown under specific conditions. ●- B5786 PHA, g/l; ○- H16 PHA, g/l; ▲ - PHA, % B5786 dry matter; △- PHA, % H16 dry matter. The numbers (1, 2, 3, 4, 5) indicate the times when samples were withdrawn for further analysis. The bacterial growth on CO_2 + valerate (C_5) -**A**; CO_2 + heptanoate (C_7) - **B**; CO_2 + hexanoate (C_6) - **C**; CO_2 + octanoate (C_8) - **D**.

polymer vs. 0.72–1.99 mol% in the recovered polymer). At the end of the experiment, compositions of the recovered polymers and biomass samples were similar: HB – 91.7–93.3; HV – 4.6–3.3; HHx – 2.55–3.34; and HO – 1.0–1.2 mol%, for B5786 and H16, respectively (Table 1).

When the same culture procedure was used to grow bacteria on heterotrophic substrate (fructose + a fatty acid), both

Table 2.
Chemical structure and properties of multi-component polymers of *Wautersia eutropha*.

Sample No.	Strain	PHA composition, mol%					PHA properties		
		HB	HV	HHx	HHp	HO	C_x, %	T_m, °C	T_d, °C
1	B5786	11.22	88.46	0.13	0.16	0.04	49	146	210
2	H16[a)]	36.01	63.64	0.12	0.21	0.02	51	150	213
3	B578[a)]	41.94	56.30	0.71	0.98	0.06	48	153	230
4	B578[a)]	52.25	47.33	0.09	0.33	N.d.	46	158	234
5	B5786	79.61	1.50	18.03	N.d.	0.85	53	155	253
6	H16[a)]	81.81	3.69	13.76	N.d.	0.74	60	156	253
7	H16[a)]	83.38	4.47	11.63	N.d.	0.52	62	157	257
8	B5786	90.63	2.29	6.52	N.d.	0.56	65	159	256
9	B578[a)]	91.70	4.59	2.55	N.d.	1.17	74	163	265
10	B5786	99.76	0.24	Tr.	N.d.	N.d.	74	168	268

[a)] PHA samples obtained in the above described experiments; HB– hydroxybutyrate; HV- hydroxyvalerate; HHx- hydroxyhexanoate; HHp– hydroxyheptanoate; HO– hydroxyoctanoate; N.d.– not detected; Tr.– trace amounts; C_x- the degree of crystallinity; T_m– melting point; T_d– decomposition point.

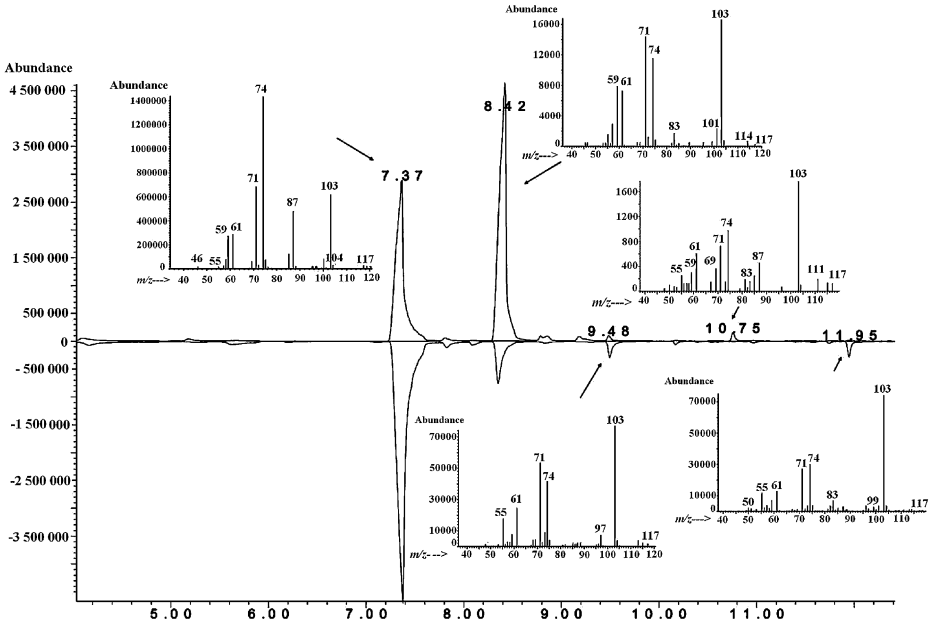

Figure 2.
Ion chromatograms of polymers extracted from *Wautersia eutropha* H16 biomass supplemented with heptanoate (above) and *Wautersia eutropha* B5786 supplemented with octanoate (mirror below) and mass spectra of respective monomers – HB with retention time 7.37, HV– 8.42, HHx– 9.48, HHp– 10.75, HO– 11.95 min.

strains synthesized three-component PHAs that contained more than 97 mol% HB and minor fractions of HV and HHx, no matter what co-substrate was used (Table 1). One exception was the experiment with valerate as a co-substrate: a three-component PHA with HV amounting to 26 mol% and HHx as a minor component was synthesized.

To illustrate, Fig. 2 shows two chromatograms of PHAs recovered from H16 culture with heptanoate and B5786 culture with octanoate and mass spectra of all the 5 monomers, which confirm their identity.

By culturing bacteria autotrophically and varying amounts of added acids with even and odd number of carbons and fermentation duration, we managed to obtain a family of short-and medium-chain-length PHAs, whose physicochemical properties are presented in Table 2. These results confirm our earlier evidence that the presence of the HV fractions is a significant factor in redistribution of the amorphous and crystalline phases in the polymer, reducing the degree of crystallinity. However, these changers are only caused by HV fractions from a few mol% to 25–30 mol%, the presence of HV over 50 mol% does not cause a change in C_x. Larger HV fractions influenced temperature characteristics of the polymer, and the most significant influence was observed when the HV fraction exceeded 35–40 mol%. Minor fractions – HHp (0.98 mol%) and HO (1.2 mol%) – did not affect the degree of crystallinity of the polymer. It was particularly interesting for us to investigate our first samples of PHAs that contained as major fractions not only HB but also HHx (2 to 18 mol%). Analysis of X-ray spectra revealed a clear relationship between the degree of crystallinity of a PHA and its HHx percentage (Table 2). As the HHx fraction grew, the degree of crystallinity of the PHA steadily decreased. Hence, similarly to HV, presence of HHx in the polymer decreases its crystallinity and improves its processability. Temperature characteristics of the PHA also changed as the HHx percentage increased. As the HHx fraction increased (within the studied range, from a

few mol% to 18 mol%), both T_m and T_d of the PHA decreased, but no difference between the melting point and the temperature for the onset of decomposition was registered.

Analysis of the results obtained first of all shows identical direction of PHA synthesis by the H16 and B5786 strains of *W. eutropha*. The accepted notion is that qualitative composition of a PHA is determined by substrate specificity of its PHA synthase (polymerase), a key enzyme of the PHA cycle. Analysis of amino acid sequences of the *W. eutropha* B5786 synthase and comparison with the primary structure of the *W. eutropha* H16 synthase showed a very high identity (99%) and revealed quite insignificant differences in the primary structures of the synthases of these two strains, which are located in the short non-identical fragment of the C-terminal domain, between the 561 and 572 amino acid sequences.[20]

It has been reported that *W. eutropha* can successfully produce not only PHB but also a copolymer – poly(HB-co-HV).[15] Incorporation of monomers of carbon numbers over five was observed only under inhibition of β-oxidation of fatty acids[18] or in specially constructed strains.[16,17] The results indicate that under autotrophic conditions, the study strains can synthesize a three-component PHA with minor HV and HHx fractions, even without added acids. Cultured mixotrophically ($CO2 + a$ fatty acid), both strains synthesize four- and five-component polymers, with HB, HV or HHx as major monomers. The high HV percentage that was registered in experiments with fatty acids with odd number of carbons is a natural result, consistent with the literature data.[15] However, large fractions of HHx in the polymer synthesized by *W. eutropha* have not been reported in the literature, although this PHA type is of great interest to researchers. A producer of the HB/HHx copolymers – *Aeromonas hydrophyla* – has been isolated recently. The wild-type strain accumulates up to 45% poly(HB/HHx), with the HHx fraction 10–13 mol%.[4–6] The results show that both of the study strains, when cultured mixotrophically, produce a polymer of a composition, which is not inferior in quality to the polymer of *A. hydrophyla*.

Acknowledgements: This study was financially supported by grant of Russian Foundation for Basic Research (RFBR) No07-03-0012a, and the Program for Interdisciplinary Projects of the Siberian Branch of the RAS (Project No. 24).

[1] E. L. Lee, D. Jendrossek, A. Schirmer, C. V. Choi, A. Steinbüchel, *Appl. Microbiol. Biotechnol.* **1995**, *42*, 901.
[2] R. Hartmann, R. Hany, T. Geiger, T. Egli, B. Witholt, M. Zinn, *Macromol.* **2004**, *70*, 6780.
[3] M. Liebergesell, F. Mayer, A. Steinbüchel, *Appl. Microbiol. Biotechnol.* **1993**, *40*, 292.
[4] Y. Z. Qiu, S. P. Ouyang, Z. Shen, Z. Wu, G. Q. Chen, *Bioscience.* **2004**, *4*, 255.
[5] J. Han, Y. Z. Qiu, D. C. Liu, G. Q. Chen, *FEMS Microbiol Lett.* **2005**, *239*, 195.
[6] Y. Z. Qiu, J. Han, G. Q. Chen, *Appl. Microbiol. Biotechnol.* **2006**, *69*, 537.
[7] M. Liebergesell, E. Hustede, A. Timm, A. Steinbüchel, R. C. Fuller, R. W. Lenz, H. G. Schlegel, *Arch. Microbiol.* **1991**, *155*, 416.
[8] T. G. Volova, O. G. Belyaeva, G. S. Kalacheva, V. F. Plotnikov, *Doklady RAN (Articles of the Russian Academy of Sciences).* **1996**, *346*, 558 (in Russian).
[9] T. G. Volova, G. S. Kalacheva, V. F. Plotnikov, *Mikrobiologiya (Microbiology)* **1998**, *67*, 512 (in Russian).
[10] K. Tanaka, A. Ishizaki, T. Kanamaru, T. Kawano, *Biotethnol. Bioeng.* **1995**, *45*, 268.
[11] T. Fukui, Y. Doi, *Appl. Microbiol. Biotechnol.* **1998**, *49*, 333.
[12] E. J. Bormann, M. Roth, *Biothechnol. Lett.* **1999**, *21*, 1059.
[13] A. Ishizaki, K. Tanaka, N. Taga, *Appl. Microbiol. Biotechnol.* **2001**, *57*, 6.
[14] T. G. Volova, G. S. Kalacheva, O. V. Altuhova, *Appl. Microbiol. Biotechnol.* **2002**, *58*, 675.
[15] Y. Doi, A. Tamaki, M. Kunioka, K. Soga, *Appl. Microbiol. Biotechnol.* **1998**, *28*, 330.
[16] D. Dennis, M. McCoy, A. Stangl, H. E. Valentin, Z. Wu, *J. Biotechnol.* **1998**, *64*, 177.
[17] R. V. Antonio, A. Steinbüchel, B. H. A. Rehm, *FEMS Microbiol. Lett.* **2000**, *182*, 111.
[18] P. R. Green, J. Kemper, L. Schechtman, L. Guo, M. Satkowski, S. Fiedler, A. Steinbüchel, B. H. A. Rehm, *Biomacromol.* **2002**, *3*, 208.
[19] H. G. Schlegel, H. Kaltwasser, G. Gottschalk, *Arch Mikrobiol.* **1961**, *38*, 209.
[20] I. V. Kozhevnikov, T. G. Volova, Tran Hai, A. Steinbüchel, E. V. Volodina, *Vestnik Krasnoyarskogo gosuniversiteta (Bulletin of the Krasnoyarsk State University).* **2005**, *5*, 214 (in Russian).

