Microbiology, Vol. 74, No. 1, 2005, pp. 54–59. Translated from Mikrobiologiya, Vol. 74, No. 1, 2005, pp. 63–69.

EXPERIMENTAL ARTICLES

The Synthesis of Hydroxybutyrate and Hydroxyvalerate Copolymers by the Bacterium *Ralstonia eutropha*

T. G. Volova and G. S. Kalacheva

Institute of Biophysics, Siberian Division, Russian Academy of Sciences, Krasnoyarsk, 660036 Russia

Received December 8, 2003; in final form, April 12, 2004

Abstract—The paper deals with the study of the synthesis of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) copolymers by the bacterium *Ralstonia eutropha* B-5786 grown under different carbon nutrition conditions (growth on carbon dioxide, fructose, and CO_2–valerate and fructose–valerate mixtures). The parameters to be analyzed included the yield of biomass; the yield, synthesis rate, and composition of copolymers; the activity of the key enzymes of polyhydroxyalkanoate (PHA) synthesis (β-ketothiolase, acetoacetyl-CoA reductase, and PHA synthase); the maximum tolerable concentration of valerate to the bacterium; and the conditions that govern the incorporation of hydroxyvalerate into copolymers. This allowed the relationship between cultivation conditions and the proportion of monomers in the copolymers to be deduced. We were able to synthesize a range of 3HB/3HV copolymers and found that the thermal characteristics and the degree of crystallinity of such copolymers depend on the molar fraction of 3HV.

Key words: poly(hydroxybutyrate-*co*-hydroxyvalerate), controlled synthesis, β-ketothiolase, *Ralstonia eutropha.*

There is increasing research activity directed toward the study of polyhydroxyalkanoate (PHA) synthesis by microorganisms with the aim of obtaining PHAs with specified properties [1]. Of great interest in this respect is the bacterium *Ralstonia eutropha* (formerly, *Alcaligenes eutrophus*), which is able to synthesize polyhydroxybutyrate, copolymers of hydroxybutyrate and hydroxyvalerate, and hydroxyalkanoate terpolymers [2, 3]. The proportion between components in these polymers may vary, depending on the physiological and biochemical characteristics of the producing strains and their cultivation conditions.

Microorganisms produce 3-hydroxyvalerate (3HV) through the condensation of propionyl-CoA and acetyl-CoA with the involvement of β-ketothiolase. The molar fraction of 3HV in the PHAs synthesized by bacteria from the genus *Ralstonia* depends on the proportion between acetate and propionate in the growth medium and may reach 50% of the dry weight of cells [4]. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), abbreviated poly(3HB-*co*-3HV), is synthesized with the involvement of the enzymes β-ketothiolase, acetoacetyl-CoA reductase, and PHA synthase (PHA polymerase).

Experiments carried out by Doi *et al.* showed that, when valerate was the only carbon source in the growth medium of *R. eutropha* NCIMB 11599, the fraction of 3HV in the PHA synthesized by this strain reached 85 mol %. In a cultivation medium with a mixture of 5-chlorovalerate and valerate as the carbon source, this strain synthesized a terpolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-5-hydroxyvalerate) in an amount of 46% of the dry biomass, the molar fraction of 5HV in the terpolymer reaching 52% [5, 6]. When the *R. eutropha* strains NCIMB 11599 and H16 were grown on a mixture of butyric and pentanoic acids, the molar fraction of 3HV in the PHAs synthesized by these strains reached 90 and 75 mol %, respectively. Butyrate and valerate may be incorporated into poly(3HB-*co*-3HV) without forming acetyl-CoA and propionyl-CoA by β-ketothiolase [7]. When grown on the fatty acids C_2 through C_{22}, *Alcaligenes* sp. AK201 was able to synthesize poly(3HB-*co*-3HV) in an amount of 50% of the dry biomass. In this case, the molecular mass of the copolymers was found to be dependent on the length of the carbon chain of the fatty acid used for the growth of the strain [8].

The obtaining of PHA copolymers with a high molar fraction of hydroxyvalerate is a difficult task since the short-chain propionic and valeric fatty acids are typically toxic to the producing strains. This problem can be solved by using microorganisms tolerant to short-chain fatty acids and by optimizing their cultivation conditions. For instance, Kobayashi *et al.* [9] optimized the cultivation conditions of *A. eutrophus* grown on propionate in a fed-batch mode in pH-stat. By varying the proportion of carbon and nitrogen in the nutrient medium, the authors showed that the incorporation of 3HV into PHA increases with the feeding and growth rates of the bacterium. Squio *et al.* [10] employed a phosphate-feeding strategy to study the relationship between the yields of biomass and copolymers with dif-

0026-2617/05/7401-0054

ferent molar fractions of 3HV. Choi *et al.* [11] showed the possibility of using threonine-overproducing mutants of *Alcaligenes* sp. to obtain poly(3HB-*co*-3HV) with a high molar fraction of 3HV.

The above short review of literature data shows that poly(3HB-*co*-3HV) copolymers are commonly manufactured by using nutrient media with propionic and valeric acids or their salts, which are quite expensive substrates. In searching for a low-cost alternative, Marangoni *et al.* [12] studied the effect of oleic acid and found that the addition of this substance as a cosubstrate to the nutrient medium of *R. eutropha* considerably augmented the yield of poly(3HB-*co*-3HV) without influencing the biomass yield or the molar fraction of 3HV in the copolymer. Of great interest in this regard is the ability of the bacterium *R. eutropha* to synthesize PHA under autotrophic conditions, that is, from the low-cost inorganic substrates H_2 and CO_2 [13–16].

The aim of this work was to investigate the synthesis of hydroxybutyrate and hydroxyvalerate copolymers by the bacterium *R. eutropha* grown under different carbon nutrition conditions.

MATERIALS AND METHODS

Experiments were carried out with the bacterial strain *Ralstonia eutropha* B-5786 [17].

The strain was cultivated in a batch mode in a 10-l fermentor containing 3 l of a mineral medium. The fermentor was equipped with an uncovered stirring turbine (1000 rpm). During cultivation under autotrophic conditions, a gas mixture (CO_2, O_2, and H_2 in a volume ratio of 1 : 2 : 6) was continuously bubbled through the growing culture at a flow rate of 8–12 l/min. During cultivation under heterotrophic conditions, the growing culture was continuously fed with a fructose solution so that the current concentration of fructose in the medium did not exceed 10 g/l. The highest rate of PHA synthesis was obtained by using a two-stage batch fermentation, when bacterial growth was limited by a nitrogen deficiency at the first stage and cultivation was continued in the nitrogen-free medium at pH 7.0 and temperature 30°C at the second stage [18].

The composition of the gas mixture was analyzed with an LKhM-80 gas chromatograph equipped with a katharometer. The carrier gas was argon. The concentration of fructose in the medium was determined with resorcinol [19]. Bacterial growth was evaluated by the mass of dry cells. The composition of PHA and its content in the biomass were determined by analyzing the methyl esters of fatty acids after the methanolysis of dry biomass samples. This analysis was carried out with a GSD Plus chromatograph–mass spectrometer (Hewlett Packard, United States) [20]. The calculated parameters were the biomass yield X (g/l) and the total (V, g/l) and specific (μ, h^{-1}) rates of copolymer synthesis.

Enzymes were assayed using cell-free extracts prepared by the sonication of cell suspensions at 4°C for a total of 4 min in 1-min bursts, followed by centrifugation at 14000 rpm for 20 min. The activity of the key enzymes of PHA synthesis was determined by the known methods with the use of a UVICON 943 two-beam recording spectrophotometer (Italy). The activity of β-ketothiolase was determined by the thiolysis of acetoacetyl-CoA using the extinction coefficient 1.726×10^4 mol^{-1} cm^{-1} at 303 nm [21]. Acetoacetyl-CoA reductase was assayed from the oxidation rate of NADPH, which was monitored at 340 nm. The activity of PHA synthase was determined from the rate of reduction of 5,5'-dithio-bis-2-nitrobenzoic acid by the SH groups of CoA, which was released in the reaction of monomer condensation. The reaction product was measured at 412 nm using an extinction coefficient equal to 13600 mol^{-1} cm^{-1}.

Protein concentration in the cell-free extracts was measured by the method of Lowry *et al.*

PHA was extracted from the biomass with chloroform and purified by precipitation with ethanol. The molecular mass of poly(3HB-*co*-3HV) was evaluated with a capillary viscosimeter (30°C; capillary diameter 0.34 mm). The degree of crystallinity of poly(3HB-*co*-3HV) was determined with a D8 ADVANCE X-ray spectrometer (Bruker, Germany) equipped with a graphite monochromator on a reflected beam. The operation parameters were as follows: scan steps 0.04°; 2-s exposure; 40 kV; 40 μA). The thermal characteristics of poly(3HB-*co*-3HV), namely, the melting temperature T_m and the degradation temperature T_d, were determined by differential scanning calorimetry in an inert atmosphere. Polymer samples were heated from room temperature to 500°C at a rate of 5°C/min.

The results obtained were statistically processed by routine methods [23] with the aid of the Microsoft Excel software.

RESULTS AND DISCUSSION

The highest yields of PHA were observed when the strain *R. eutropha* B-5786 was grown in a two-stage batch mode. At the first stage, the strain was grown at a limiting concentration of the source of nitrogen (50% of the physiological demand). At the second stage, the cultivation of the strain was continued in the nitrogen-free medium. At the first stage, the bacterium accumulated 5–12 g/l biomass (depending on the cultivation time) and 40–50% PHA with respect to the cell mass. This allowed the duration time of the second stage to be reduced, so that the total time of fermentation was 60–70 h [18].

Figure 1a shows a typical dynamics of the major culture parameters of *R. eutropha* B-5786 synthesizing PHA under autotrophic conditions. At the first stage (30–35 h of cultivation under nitrogen limitation), the biomass reached 10 g/l, whereas the specific growth rate decreased from 0.15–0.17 to 0.08–0.10 h^{-1}. At the beginning of the first stage, the absolute and specific

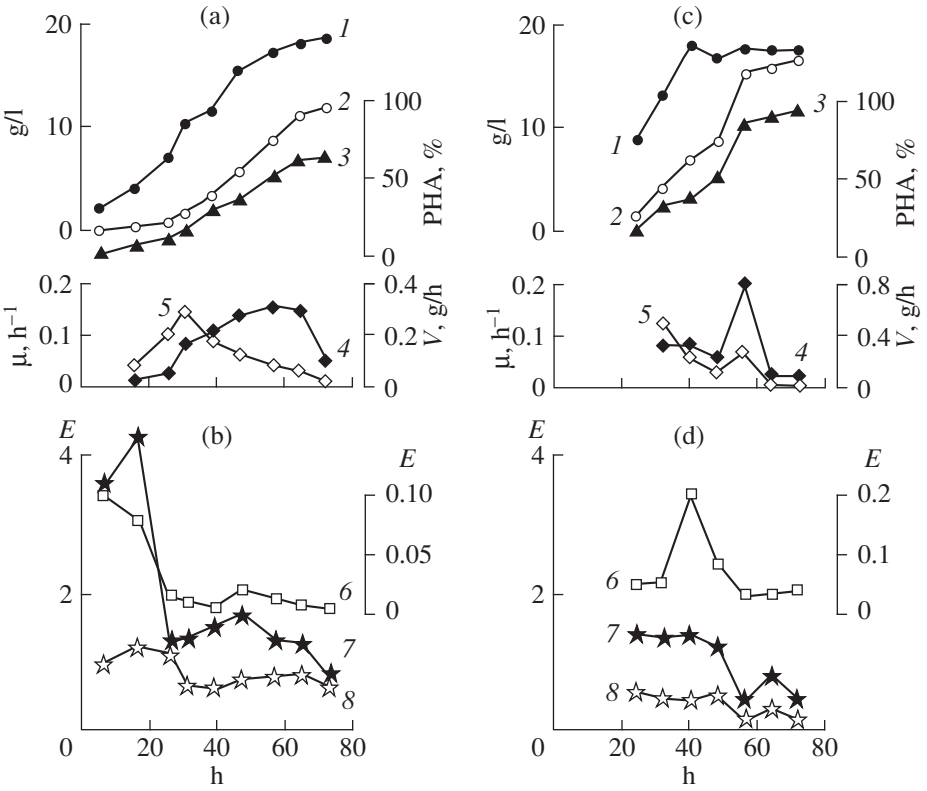

Fig. 1. The dynamics of growth parameters and the activities of enzymes involved in the PHA synthesis in the bacterium *R. eutropha* B-5786 grown (a, b) autotrophically and (c, d) heterotrophically: (*1*) biomass, g/l; (*2*) PHA, g/l; (*3*) PHA, %; (*4*) absolute rate of PHA synthesis, *V* (g/h); (*5*) specific rate of PHA synthesis, μ, h^{-1}; (*6*) PHA synthase; (*7*) β-ketothiolase; (*8*) acetoacetyl-CoA reductase. Enzyme activities are given in U (μmol/(min mg protein)).

rates of PHA synthesis were low (0.013 g/h and 0.08 h^{-1}, respectively). By the end of the first stage, the cellular content of PHA reached 50%, whereas the absolute and specific rates of PHA synthesis increased to 0.31 g/h and 0.146 h^{-1}, respectively. At the second cultivation stage, the bacterium retained its high rates of PHA synthesis. By the end of the experiment (72 h of cultivation), the content of PHA in the cells and culture liquid reached 63% and 18 g/l, respectively, whereas the PHA synthesis rates decreased to the original values (in some experiments, even to zero).

During the first hours of cultivation (15–20 h), when the concentration of PHA in the cells was low and the specific growth rate was relatively high, the activity of the enzymes involved in PHA synthesis was high (Fig. 1b). Namely, the activities of β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase, and PHA synthase were 3.57–4.26, 0.98–1.23, and 0.08–0.10 U, respectively (here, U = μmol/(min mg protein)). By the end of the first cultivation stage, the activities of the three enzymes decreased to 1.29–1.34, 0.66–1.13, and 0.009–0.015 U, respectively. At the second stage, the activities of acetoacetyl-CoA reductase and PHA synthase virtually did not change, whereas that of β-ketothiolase (this enzyme catalyzes the first step of PHA synthesis and the last step of its endogenous degradation) showed a further decline during the last 20–25 h of cultivation.

The strain *R. eutropha* B-5786 grew faster (by 25–30%) when cultivated heterotrophically on fructose than when cultivated autotrophically and accumulated up to 95% PHA (Fig. 1c). As soon as after 56 h of cultivation, the cellular content of PHA reached 80%, the total rate of PHA synthesis being 0.79 g/h. The specific rate of PHA synthesis reached a maximum (0.126 h^{-1}) by the 32nd hour of cultivation. The dynamics of the enzymes of PHA synthesis during heterotrophic growth was similar to that during autotrophic growth (Fig. 1d). The statistical analysis of enzyme activities in terms of Student's *t*-test statistics showed that the mean values of β-ketothiolase activity during the autotrophic and heterotrophic growth of *R. eutropha* B-5786 are comparable, the activity of acetoacetyl-CoA reductase is lower during the heterotrophic growth, and the difference in the activities of PHA synthase in these two cases is statistically insignificant.

The mass spectroscopic analysis of the PHA synthesized by *R. eutropha* B-5786 grown on monosubstrates (either CO_2 or fructose) showed that it contained 99.6 ± 0.3 mol % 3-hydroxybutyrate (3HB) and 0.1–0.7 mol % 3-hydroxyvalerate (3HV) (Figs. 2c, 2f). The presence of 3HV in the PHA synthesized by *R. eutropha* B-5786

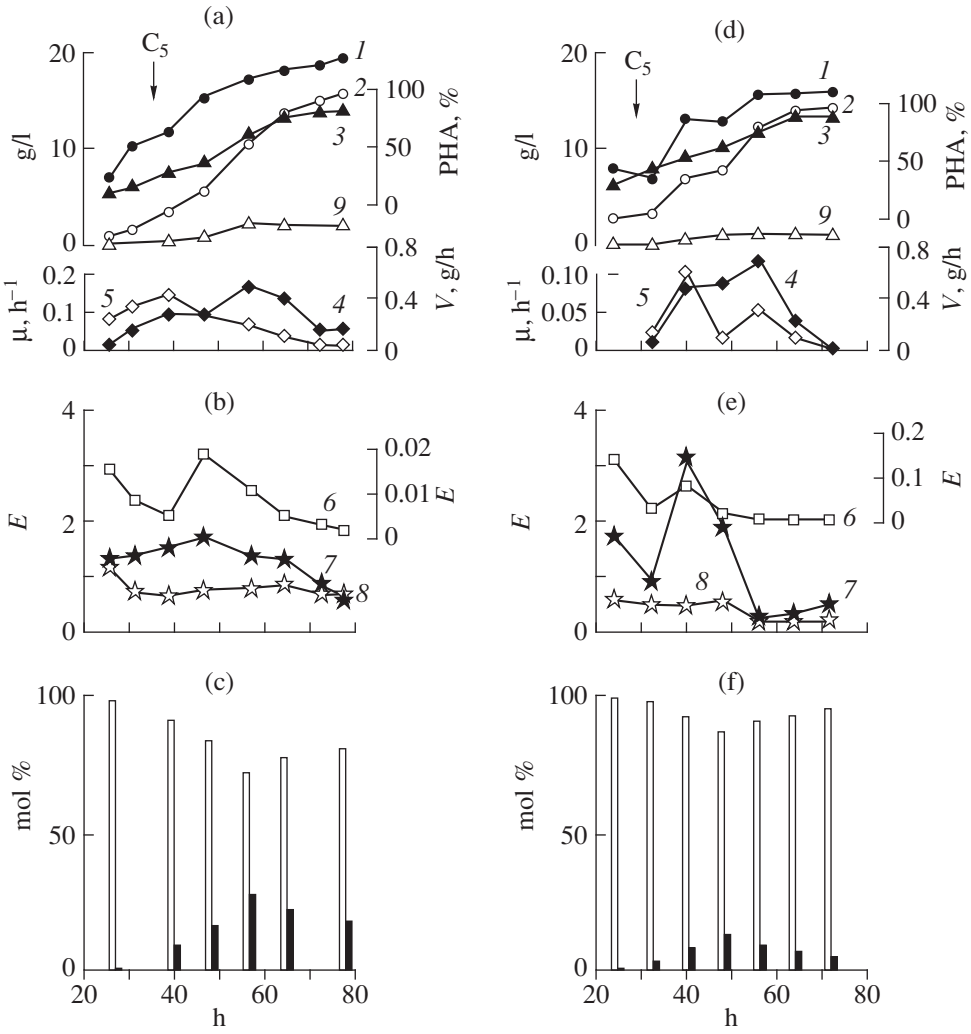

Fig. 2. The dynamics of growth parameters, the composition of PHA, and the activities of enzymes involved in the PHA synthesis by the bacterium *R. eutropha* B-5786 grown on (a–c) CO_2 + valerate and (d–f) fructose + valerate. Curves *1–8* correspond to those in Fig. 1; curve *9* shows the concentration of β-hydroxyvalerate in g/l. In panels c and f, the open and dark bars represent the molar fractions of, respectively, 3HB and 3HV in the PHA.

in the medium that contained no propionate or valerate suggests that this bacterium can utilize β-hydroxyacyl derivatives or their precursors produced biosynthetically, for instance, in the β-oxidation pathway. This suggestion is in agreement with the high level of ketothiolase activity in the early cultivation period (Fig. 1b). Indeed, bacteria of the genus *Ralstonia* have at least three ketothiolases, one of which is involved in the β-oxidation pathway and can provide substrates for PHA synthesis [1].

Experiments with mixed growth substrates (Fig. 2) showed that, as soon as 30 min after the addition of valerate to the cultivation medium, the hydroxy derivative of this fatty acid appeared in the PHA synthesized by *R. eutropha* B-5786, irrespective of the major carbon substrate used (CO_2 or fructose). The molar fraction of 3HV tended to increase in the course of cultivation (Figs. 2c, 2f), reaching a maximum 10–15 h after the addition of valerate to the medium. In general, the dynamics of biomass, copolymer yield, and enzyme activities during cultivation on the mixed substrates (Figs. 2a, 2b, 2d, 2e) were almost the same as in the case of cultivation on the monosubstrates.

The calculation of carbon balance from the data presented in Fig. 2 showed that virtually all the valerate added to the medium (2 g/l in the case of autotrophic growth and 1 g/l in the case of heterotrophic growth) was incorporated into the PHA as soon as after 10–15 h, when the concentration of poly(3HB-*co*-3HV) in the culture reached 8–10 g/l (60% of the biomass), and the molar fraction of 3HV in the poly(3HB-*co*-3HV) was 28 and 13 mol % (in the cases of autotrophic and heterotrophic growth, respectively). The high degree of valerate incorporation into poly(3HB-*co*-3HV) (almost 100%) suggests that valerate is hydroxylated directly

The effect of cultivation conditions on the composition and physicochemical properties of the poly(3HB-*co*-3HV) synthesized by *R. eutropha* B-5786

Carbon source	Number of valerate additions	PHA, %	Molar fraction (mol %) of		PHA properties		
			3HB	3HV	M_r, kDa	C_x, %	T_m, °C
CO_2	–	78	99.8	0.2	250	74	168
Fructose	–	86	99.9	0.1	320	72	170
CO_2 + valerate	1	45	85.7	14.3	400	64	162
CO_2 + valerate	2	60	70.1	29.9	280	51	160
CO_2 + valerate	3	68	57.0	43.0	370	54	159
CO_2 + valerate	4	76	37.0	63.0	290	52	152
Fructose + valerate	1	70	83.0	17.0	180	59	158
Fructose + valerate	2	82	65.4	34.6	380	56	160

and not in the β-oxidation pathway. This suggestion is in agreement with the experimental results of Doi *et al.* [7].

In the course of further cultivation, the molar fraction of 3HV in poly(3HB-*co*-3HV) tended to decrease to, respectively, 7 and 19 mol % after 25–35 h of cultivation under heterotrophic and autotrophic conditions (Figs. 2c, 2f). In this case, the absolute content of 3HV in the culture (in g/l) remained nearly unchanged, indicating that the observed decrease in the molar fraction of 3HV was due to the continuing synthesis of 3HB and the termination of 3HV synthesis (because of the exhaustion of valerate in the medium) rather than to the degradation of poly(3HB-*co*-3HV).

It should be noted that valerate concentrations higher than 2 g/l are toxic to the strain *R. eutropha* B-5786. For this reason, we attempted to augment the molar fraction of 3HV in the poly(3HB-*co*-3HV) by adding valerate to the cultivation medium of *R. eutropha* B-5786 in portions. The data presented in the table show how the proportion between the molar fractions of 3HB and 3HV in the poly(3HB-*co*-3HV) synthesized by *R. eutropha* B-5786 can be varied from 9 : 1 to 1 : 9 by varying the cultivation conditions. The yield of poly(3HB-*co*-3HV) can reach 70–75% of the cell mass. As the molar fraction of 3HV in poly(3HB-*co*-3HV) increased, the degree of polymer crystallinity, as well as the melting and degradation temperatures, decreased. The decrease in the polymer crystallinity (C_x) was particularly noticeable when the molar fraction of 3HV in poly(3HB-*co*-3HV) increased to 30–35 mol % (table). At the same time, the molar fraction of 3HV showed no statistically significant effect on the molecular mass of poly(3HB-*co*-3HV).

Thus, the proportion between the molar fractions of 3HB and 3HV in the poly(3HB-*co*-3HV) synthesized by *R. eutropha* B-5786 can be varied within wide limits by varying the cultivation conditions of this bacterium with due consideration for the toxicity of valerate.

ACKNOWLEDGMENTS

This work was supported by grant no. 02-04-49674 from the Russian Foundation for Basic Research and by grant no. 2218 from the International Science and Technology Center.

REFERENCES

1. Slater, S., Houmiel, K.L., Tran, M., Mitsky, T.A., Taylor, N.B., Padgette, S.R., and Gruys, K.J., Multiple β-Ketothiolases Mediate Poly(β-Hydroxyalkanoate) Copolymer Synthesis in *Ralstonia eutropha, J. Bacteriol.*, 1998, vol. 180, no. 8, pp. 1979–1987.
2. Volova, T.G., Kalacheva, G.S., and Plotnikov, V.F., Biosynthesis of Heteropolymeric Polyhydroxyalkanoates by Chemolithoautotrophic Bacteria, *Mikrobiologiya*, 1998, vol. 67, pp. 512–517.
3. Kemper, J., Schechtman, L., Guo, L., Satkowski, M., Fiedler, S., Steinbuchel, A., and Rehm, H.A., Formation of Short-Chain/Medium-Chain-Length Polyhydroxyalkanoate Copolymers by Fatty Acid β-Oxidation–Inhibited *Ralstonia eutropha, Biomacromolecules*, 2002, vol. 3, pp. 208–213.
4. Doi, Y., *Microbial Polyesters*, New York: VCH, 1990.
5. Doi, Y., Segawa, A., Kawaguchi, Y., and Kunioka, M., Cyclic Nature of Poly(3-Hydroxyalkanoate) Metabolism in *Alcaligenes eutrophus, FEMS Microbiol. Lett.*, 1990, vol. 67, pp. 165–170.
6. Doi, Y., Kitamura, S., and Abe, H., Microbial Synthesis and Characterization of Poly(3-Hydroxybutyrate-*co*-3-Hydroxyhexanoate), *Macromolecules*, 1995, vol. 28, pp. 4822–4828.
7. 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.
8. Akiyama, M., Taima, Y., and Doi, Y., Production of Poly(3-Hydroxyalkanoates) by a Bacteria of the Genus *Alcaligenes* Utilizing Long-Chain Fatty Acid, *Appl. Microbiol. Biotechnol.*, 1992, vol. 37, pp. 698–701.
9. Kobayashi, G., Tanaka, K., Iton, H., Tsuge, T., Sonomoto, K., and Ishizaki, A., Fermentative Production of

P(3HB-*co*-3HV) from Propionic Acid by *Alcaligenes eutrophus* in Fed-Batch Culture with the pH-Stat Continuous Substrate Feeding Method, *Biotechnol. Lett.*, 2000, vol. 22, pp. 1067–1069.

10. Squio, C.R., Marangoni, C., De Vecchi, C.S., and Aragao, G.M.F., Phosphate Feeding Strategy during Production Phase Improves Poly(3-Hydroxybutyrate-*co*-3-Hydroxyvalerate) Storage by *Ralstonia eutropha, Appl. Microbiol. Biotechnol.*, 2003, vol. 61, pp. 257–260.
11. Choi, G.G., Kim, M.W., Kim, J.-Y., and Rhee, Y.H., Production of Poly(3-Hydroxybutyrate-*co*-3-Hydroxyvalerate) with High Molar Fraction of 3-Hydroxyvalerate by a Threonine-Overproducing Mutant of *Alcaligenes* sp. SH-69, *Biotechnol. Lett.*, 2003, vol. 25, pp. 665–670.
12. Marangoni, C. and Furigo, Jr., Falcao de Aragao, G.M., Oleic Acid Improves Poly(3-Hydroxyvalerate) Production by *Ralstonia eutropha* in Inverted Sugar and Propionic Acid, *Biotechnol. Lett.*, 2000, vol. 22, pp. 1635–1638.
13. Tanaka, K., Ishizaki, A., Kanamaru, T., and Kawano, T., Production of Poly(D-3-Hydroxybutyrate) from CO_2, H_2, and O_2 by the High Cell Density Autotrophic Cultivation of *Alcaligenes eutrophus, Biotechnol. Bioeng.*, 1995, vol. 45, pp. 268–275.
14. 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.
15. 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, no. 5, pp. 675–678.
16. Volova, T.G. and Voinov, N.A., The Technological Parameters of the Hydrogen Bacterium *Ralstonia eutropha* Synthesizing Polyhydroxyalkanoates, *Prikl. Biokhim. Mikrobiol.*, 2003, vol. 39, no. 2, pp. 166–170.
17. RF Patent No. 2053292, Byull. Izobret., No. 1 (1966).
18. Volova, T.G., Kalacheva, G.S., Konstantinova, V.M., and Puzyr', A.P., The Effect of Growth Conditions on the Accumulation of Polyhydroxybutyrate by Hydrogen Bacteria, *Prikl. Biokhim. Mikrobiol.*, 1992, vol. 28, pp. 221–22.
19. Ermakov, A.I., Arasimovich, V.V., Smirnova-Ikonnikova, M.I., Yarosh, N.P., and Lukovnikova, G.A., *Metody biokhimicheskogo issledovaniya rastenii* (Methods for the Biochemical Study of Plants), Leningrad: Kolos, 1972.
20. Brandl, H., Gross, R.A., Lenz, R.W., and Fuller, C.W., *Pseudomonas oleovorans* As a Source of Poly(β-Hydroxyalkanoates) for Potential Application As Biodegradable Polyesters, *Appl. Environ. Microbiol.*, 1988, vol. 54, pp. 1977–1982.
21. Senior, P.J. and Dawes, E.A., The Regulation of Poly-β-Hydroxybutyrate Metabolism in *Azotobacter beijerinckii, Biochem. J.*, 1973, vol. 134, pp. 225–238.
22. Valentin, H.E. and Steinbüchel, A., Application of Enzymatically Synthesized Short-Chain-Length Hydroxy Fatty Acid Coenzyme A Thioesters for Assay of Polyhydroxyalkanoic Acid Synthases, *Appl. Microbiol. Biotechnol.*, 1994, vol. 40, pp. 699–709.
23. Plokhinskii, N.A., *Algoritmy biometrii* (Biometric Algorithms), Moscow: Mosk. Gos. Univ., 1980.