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EXPERIMENTAL ARTICLES

The Autotrophic Synthesis of Polyhydroxyalkanoate by *Alcaligenes eutrophus* in the Presence of Carbon Monoxide

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Abstract—The CO-resistant strain B5786 of the hydrogen-oxidizing bacterium *Alcaligenes eutrophus* was found to be able to synthesize polyhydroxyalkanoates (PHAs) under the conditions of growth limitation by nitrogen deficiency (the factor that promotes PHA synthesis) and growth inhibition by carbon monoxide. The gas mixtures that contained from 5 to 20 vol % CO did not inhibit the key enzymes of PHA synthesis–β-ketothiolase, acetoacetyl-CoA reductase, hydroxybutyrate dehydrogenase, and PHA synthase. In the presence of CO, cells accumulated up to 70–75 wt % PHA (with respect to the dry biomass) without any noticeable increase in the consumption of the gas substrate. Chromatographic–mass spectrometric analysis showed that the PHA synthesized by *A. eutrophus* is a copolymer containing more than 99 mol % β-hydroxybutyrate and trace amounts of β-hydroxyvalerate. The PHA synthesized under the conditions described did not differ from that synthesized by *A. eutrophus* cells from electrolytic hydrogen.

Key words: hydrogen-oxidizing bacteria, polyhydroxyalkanoates (PHAs), synthesis, CO inhibition.

Polyhydroxyalkanoates (PHAs), which are a group of diverse polymers synthesized by various microorganisms as reserve substances, are extensively studied due to their potential application as biodegradable polymeric materials. The production cost of PHAs is mainly determined by the cost of raw materials. This calls for the search of low-cost substrates necessary for the growth of producing microorganisms and new natural and transgenic PHA-producing strains [1].

PHAs can be produced from different substrates, such as carbon dioxide + hydrogen, sugars, alcohols, organic acids, and waste products of the alcohol, sugar, oil, and other industries, including some toxic compounds [2]. For instance, it was shown that PHAs can be synthesized by microorganisms growing on toxic octane and octanoate [3], methacrylic acid [4], sodium benzoate, and phenol [5].

In recent years, there has been increasing interest in the use of low-rank coals, whose reserves are huge and whose cost is relatively low, as substrates for the microbial production of PHAs [6]. Fuchtenbuch and Steinbuchel showed that the bacteria *Pseudomonas oleovorans* and *Rhodobacter ruber* growing on humic acids (water-soluble coal liquefaction products) synthesize the terpolymer of 3-hydroxyhexanoate, 3-hydroxydecanoate, and 3-hydroxydodecanoate and the copolymer of hydroxybutyrate and hydroxyvalerate, respectively [7].

Some hydrogen-oxidizing bacteria, such as *Alcaligenes eutrophus* Z1 [8] and its fast-growing variant *A. eutrophus* B5786 [9], are resistant to carbon monoxide. These strains were shown to be able to grow on the converter gas and the coal and lignin gasification products, which contain hydrogen and CO [10, 11].

The aim of the present work was to investigate the synthesis of polyhydroxyalkanoates by hydrogen-oxidizing bacteria growing autotrophically under the conditions of growth inhibition by CO.

MATERIALS AND METHODS

Experiments were carried out with the CO-resistant strain B5786 of the hydrogen-oxidizing bacterium *Alcaligenes eutrophus*, which is characterized by a high rate of PHA synthesis under autotrophic conditions.

The strain was cultivated autotrophically in a batch mode in a 10-l fermentor containing 3 l of a mineral medium with the use of a gas mixture containing carbon dioxide and hydrogen as the sources of carbon and energy. The control gas mixture contained CO_2, O_2, and H_2 in a volume ratio of 1 : 2 : 6. The highest rate of PHA synthesis was obtained using a two-stage batch-mode fermentation with the growth limitation by nitrogen deficiency at the first stage and with cultivation in a nitrogen-free medium at the second stage [12].

The effect of CO on PHA synthesis was studied by varying its concentration in the gas mixture from 5 to 25 vol % at a constant volume proportion of the other gas components. The composition of the gas mixture was analyzed using an LKhM-80 gas chromatograph equipped with a katharometer. The carrier gas was argon.

The parameters recorded were the cell biomass, which was evaluated from the optical culture density

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and by the mass of dry cells, the PHA content of the biomass, and the consumption of the components of the gas mixture. Using these parameters, we calculated the biomass yield X (g/l), the gas mixture expenditure Q (l/g biomass), and the biomass yield with respect to the hydrogen consumed, Y_{H_2} (g biomass/g).

Enzymes were assayed using cell-free extracts prepared by sonicating cell suspensions at 4°C for 4 min in 1-min bursts. Debris was removed by centrifugation, and the supernatant was assayed for the activity of the key enzymes of PHA synthesis (β-ketothiolase, acetoacetyl-CoA reductase, hydroxybutyrate dehydrogenase, and PHB synthase) [13]. 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. Acetoacetyl-CoA reductase and hydroxybutyrate dehydrogenase were assayed by the oxidation of NADPH and the reduction of NAD^+, respectively, using the extinction coefficient of NADH and NADPH = 6.22×10^{-3} μmol^{-1} cm^{-1} at 340 nm. PHB synthase was assayed by the reaction in which CoA releasing from 3-hydroxybutyryl-CoA interacted with 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). The reaction product was measured at 412 nm (the extinction coefficient = 13600 mol^{-1} cm^{-1}) using an UVIKON 943 two-beam recording spectrophotometer.

Protein concentration was measured by the method of Lowry *et al.*

The CO-oxidizing activity of cell-free extracts was measured with methylene blue (the extinction coefficient = 37.1 mol^{-1} cm^{-1}) using a Shimadzu UV-300 spectrophotometer [14]. The activity of membrane hydrogenase was measured with methyl viologen using the extinction coefficient = 13 mol^{-1} cm^{-1} at 600 nm [15]. The concentration of cytochromes was measured as described by Probst and Schlegel [16].

The intracellular content and composition of PHA were determined by analyzing the methyl esters of fatty acids after the methanolysis of 4-mg biomass samples. The analysis was carried out using a Hewlett Packard GCD plus chromatograph–mass spectrograph (United States). Lipids were extracted from freshly collected cells with an ethanol–chloroform (1 : 2, v/v) mixture. After the hydrolysis of the defatted biomass, the fatty acids (FAs) of the firmly bound lipids were extracted with hexane. Fatty acids were analyzed as described previously [17].

The molecular mass of PHB was evaluated viscosimetrically; its temperature characteristics were determined by differential scanning calorimetry (DSC), and the degree of crystallinity was determined by x-ray diffraction analysis on a DRON-2.0 autodiffractometer equipped with a graphite monochromator (2.0 (Cu$K\alpha$-radiation).

RESULTS AND DISCUSSION

Carbon monoxide is a strong respiratory poison that inactivates the iron-containing electron carriers of the respiratory chain. Investigations showed that increasing concentrations of CO inhibited the growth of the hydrogen-oxidizing bacterium *A. eutrophus* Z1 and the carboxydobacterium *S. carboxydohydrogena* Z1062 cultivated in a complete nutrient medium in a turbidostat mode [8, 18]. In this case, the activity of hydrogenase, which is the key enzyme of hydrogen metabolism, and the concentration of cytochromes increased and the membrane apparatus of cells became more developed. The consumption of the growth substrate (hydrogen) per unit biomass increased by 1.5–2 times against the background of the high protein-synthesizing activity of ribosomes.

High PHA yields in the bacterium *A. eutrophus* can be obtained under the conditions of unbalanced growth, when protein synthesis is limited while carbon and energy sources are in excess. For instance, under nitrogen deficiency, the content of PHA may reach 80% of the dry mass of cells. However, under nitrogen deficiency (or other conditions unfavorable for growth), the efficiency of hydrogen utilization by the hydrogen-oxidizing bacterium is low, so that the consumption of the gas substrate rises [8, 13].

The effect of two unfavorable factors, nitrogen deficiency and the presence of carbon monoxide, on the growth and PHA synthesis in microorganisms was not previously studied. For this reason, in the experiment on the synthesis of PHA by the CO-inhibited *A. eutrophus* culture, we evaluated not only the PHA yield and the activity of the key enzymes of PHA synthesis but also the consumption and the efficiency of utilization of hydrogen, as well as the hydrogenase activity and the chemical composition of cells.

Taking into account the dependence μ/S_{CO} that was revealed in our earlier study with *A. eutrophus* [18], this bacterium was grown on gas mixtures containing from 5 to 25 vol % CO. In the presence of such concentrations of CO, cells accumulated up to 70–75 wt % PHA (with respect to the mass of dry cells) whereas the biomass yield slightly decreased only when the concentration of CO in the gas mixture was above 20 vol % (Fig. 1).

CO presumably did not inhibit the enzymatic system responsible for PHA synthesis, since the activity of the key enzymes of PHA synthesis (β-ketothiolase, acetoacetyl-CoA reductase, hydroxybutyrate dehydrogenase, and PHB synthase) were detected in all growth phases of the *A. eutrophus* B5786 culture irrespective of the presence of CO (Fig. 2).

The gas metabolism of *A. eutrophus* weakly depended on the CO concentration in the gas phase (Table 1). The stoichiometry of gas consumption from the $CO_2 : O_2 : H_2$ mixture, which is a measure of the efficiency of utilization of the energy substrate, also did not considerably change in the presence of CO as com-

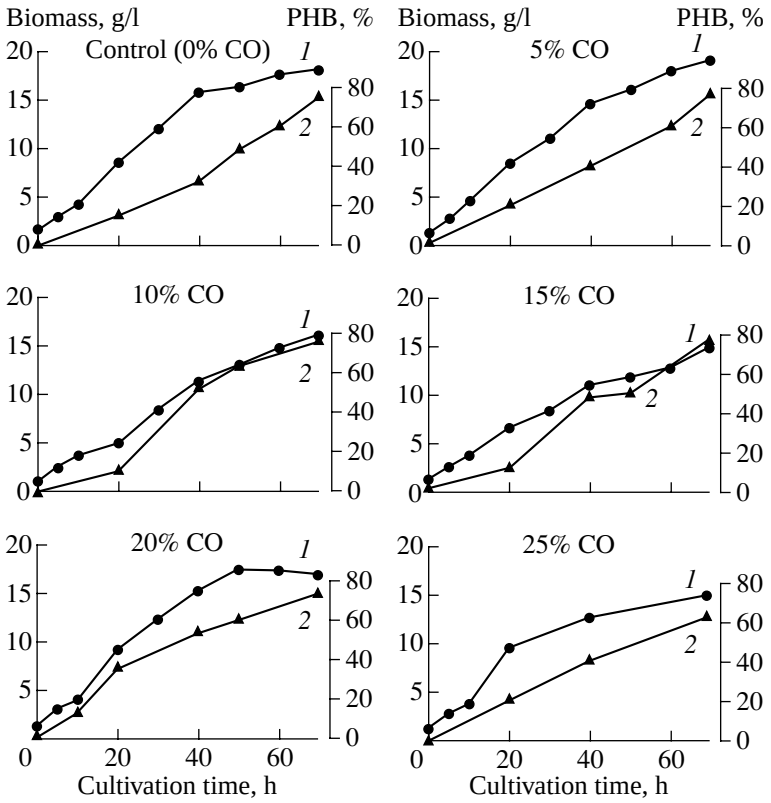

Fig. 1. The accumulation of (*1*) biomass and (*2*) PHA in the *A. eutrophus* B5786 cultures grown in the presence of different concentrations of CO (vol %).

pared with the stoichiometric values observed for the nitrogen-limited batch culture accumulating PHA.

To understand why CO did not exert a noticeable effect on the ability of *A. eutrophus* to synthesize PHA, we analyzed the effects of two unfavorable factors, nitrogen deficiency and CO, on the physiological and biochemical characteristics of the PHA-synthesizing bacterium cultivated in batchwise and continuous modes (Tables 1–3).

The hydrogenase activity of cells synthesizing PHA in the batch culture in the presence of CO was 1.3–1.5 times higher than that of cells cultivated continuously under optimum conditions (Table 2). In the presence of 20 vol % CO and the absence of nitrogen deficiency, the hydrogenase activity of cells increased even more (almost twofold). In this case, there was an increase in the gas consumption and a decrease in the biomass yield with respect to the hydrogen consumed. On the other hand, 50% nitrogen deficiency did not affect the activity of hydrogenase in the chemostat culture but lowered it about twofold in the batch culture, in spite of a rise in the consumption of the gas substrate.

A similar effect was exerted by CO on the concentration of cytochromes (Table 2). Namely, the concentration of all cytochromes increased in the presence of CO used either separately or in combination with a nitrogen deficiency. At the same time, the nitrogen deficiency itself did not affect the content of cytochromes in cells grown either batchwise or continuously.

It should be noted that cell-free extracts of *A. eutrophus* B5786 were able to oxidize CO at a rate of 30–50 μmol CO/(min g protein). At the same time, the CO oxidation rate by intact cells was low. Therefore, CO should be considered as an inhibitor, rather than a substrate for *A. eutrophus* cells.

The defense response of cells to CO may be related to changes in the permeabilities of their cell wall and cytoplasmic membrane, which are mainly determined by the fatty acid composition of the membrane lipids. Previous studies showed that the composition of the *A. eutrophus* lipids and fatty acids is typical of gram-negative bacteria and that the fatty acids of the cytoplasmic membrane of this bacterium are represented by saturated, monounsaturated (monoenoic), and cyclopropane acids. In the bacterial cells grown under optimum conditions, membrane lipids were dominated by the monoenoic $C_{16:1}$ and $C_{18:1}$ FAs (about 55% of the total FAs) and saturated palmitic acid (up to 36% of the total FAs). The content of cyclopropane acids was low (no more than 0.4%) [19]. Under unfavorable growth conditions, the content of unsaturated fatty acids in *A. eutrophus* cells decreased and that of saturated FAs and cyclopropane acids increased.

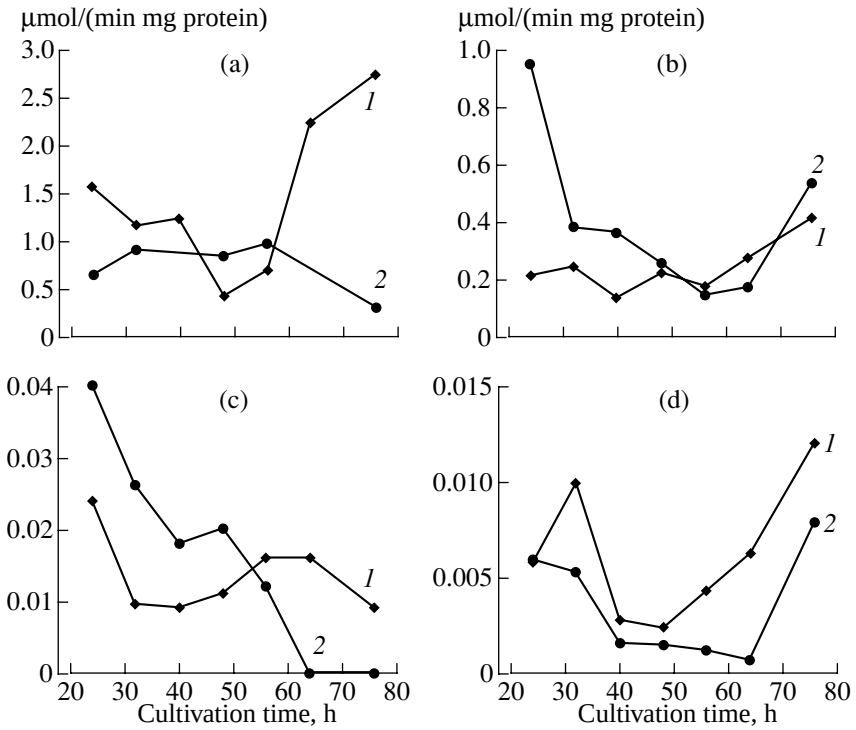

Fig. 2. Dynamics of (a) β-ketothiolase, (b) acetoacetyl-CoA reductase, (c) hydroxybutyrate dehydrogenase, and (d) PHA synthase activities in the *A. eutrophus* B5786 cells synthesizing PHA in the (*2*) presence and (*1*) absence of CO.

The presence of CO in the gas phase of the *A. eutrophus* cultures accumulating PHAs did not considerably affect the total content of membrane lipids and the fatty acid composition of the cell-wall lipopolysaccharides. In the course of the cultivation of *A. eutrophus* cells under nitrogen deficiency in the absence of CO, the relative content of unsaturated palmitoleic acid decreased from 27.8 to 10.5% and that of *cyclo*-C_{17} acid increased from 0.9 to 21.6%. The degree of the *cis*-vaccenic acid cyclization somewhat increased. The relative content of *cyclo*-C_{19} acid increased from a trace amount to 2.8%. At the same time, the content of the major saturated fatty acid, palmitic acid, in bacterial lipids changed insignificantly. The relative content of the straight-chain C_{14}, C_{16}, and C_{18} fatty acids decreased from 1.9 to 0.7%, from 42 to 37%, and from 1.2 to 0.7%, respectively, whereas the relative content of the branched-chain *anteiso*-C_{15}, *iso*-C_{15}, and *iso*-C_{16} fatty acids increased from 0.15 to 1.8%, from 0.24 to 0.6%, and from 0.16 to 0.74%, respectively. The saturation degree

Table 1. Some parameters characterizing the gas metabolism of *A. eutrophus* B5786 under different growth conditions

Growth conditions	PHA content of cells, %	Gas mixture consumption (Q), l/g biomass	Volume stoichiometry of gas consumption ($CO_2 : O_2 : H_2$)	Biomass yield on hydrogen (Y_{H_2}), g biomass/g H_2
Control (continuous culture)	Traces	9.6 ± 0.7	1 : 2.0 : 6.0	1.56 ± 0.10
Batch culture under nitrogen deficiency + CO (vol %):				
10	76.2	14.4	1 : 3.6 : 11.0	0.90 ± 0.07
15	76.8	15.5	1 : 3.3 : 10.9	0.92 ± 0.06
20	72.8	16.6	1 : 2.8 : 9.0	0.89 ± 0.07
Nitrogen deficiency:				
continuous culture	28	13.7	1 : 3.6 : 8.2	0.82 ± 0.07
batch culture	76.4	15.4	1 : 3.0 : 9.1	0.92 ± 0.06
Continuous culture in the presence of 20 vol % CO	Traces	16.0	1 : 3.8.2 : 9.7	0.84 ± 0.07

Table 2. Cytochrome concentrations and hydrogenase activity in cell-free extracts of *Alcaligenes eutrophus* B5786 grown under different conditions

Growth conditions	PHA content of cells, %	Cytochromes, nmol/mg protein			Hydrogenase, μmol H_2/(min mg protein)
		c	*b*	*a*	
Control (continuous culture)	Traces	0.56 ± 0.17	0.35 ± 0.11	0.07 ± 0.02	540 ± 40
Batch culture under nitrogen deficiency + CO (vol %):					
10	76.2	0.66 ± 0.17	0.39 ± 0.11	0.05 ± 0.01	560 ± 30
15	76.8	0.60 ± 0.16	0.45 ± 0.14	0.06 ± 0.02	640 ± 20
20	72.8	0.89 ± 0.23	0.51 ± 0.12	0.09 ± 0.02	760 ± 40
Nitrogen deficiency:					
continuous culture	28.4	0.51 ± 0.19	0.39 ± 0.13	0.06 ± 0.02	590 ± 28
batch culture	76.4	0.49 ± 0.16	0.30 ± 0.12	0.05 ± 0.01	296 ± 18
Continuous culture in the presence of 20 vol % CO	Traces	1.45 ± 0.30	0.96 ± 0.20	0.16 ± 0.03	1050 ± 38

Table 3. The fatty acid composition of the membrane lipids of *A. eutrophus* B5786 cells grown under different conditions

Fatty acid	Nitrogen deficiency + CO						Nitrogen deficiency without CO						CO inhibition	Nitrogen deficiency
	12 h	24 h	36 h	40 h	60 h	70 h	12 h	24 h	36 h	48 h	60 h	70 h		
10 : 0	0.32	0.04	0.04	0.08	0.04	0.05	0.18	0.03	0.04	Traces	0.03	0.01	Traces	Traces
12 : 0	0.19	0.08	0.03	0.09	0.14	0.16	0.15	0.04	0.05	0.03	0.04	0.01	Traces	0.10
13 : 0	0.18	0.03	0.06	0.09	0.04	0.10	0.04	0.03	0.02	Traces	0.02	Traces	Traces	2.30
iso-14 : 0	0.07	0.04	0.06	0.09	0.09	0.14	0.04	0.05	0.09	0.07	0.10	0.07	Traces	Traces
14 : 1	0.10	0.04	0.03	0	0	0	0.06	0.02	0.02	0.01	0	0	0.40	0.20
14 : 0	2.71	1.28	1.04	1.79	2.28	2.24	1.89	0.91	0.95	0.80	1.02	0.71	0.07	0.20
anteiso-15 : 0	0.16	0.24	0.28	0.47	0.50	0.69	0.15	0.63	1.05	0.94	2.34	1.82	Traces	Traces
iso-15 : 0	0.21	0.11	0.14	0.10	0.15	0.13	0.24	0.31	0.39	0.31	0.57	0.39	Traces	Traces
15 : 0	0.20	0.09	0.09	0.14	0.17	0.21	0.12	0.05	0.07	0.07	0.07	0.06	1.00	0.10
iso-16 : 0	0.16	0	0.15	0.26	0.19	0.28	0.16	0.28	0.38	0.41	0.77	0.74	Traces	Traces
16 : 1Δ7	29.03	28.31	20.50	12.81	9.48	7.50	27.77	27.50	27.49	23.43	17.42	10.49		
16 : 1Δ9	1.31	1.13	2.32	0.54	0.88	0.67	1.0	0.62	0.92	0.73	0.22	0.57	24.10	19.70
16 : 0	46.69	46.14	47.68	58.45	58.54	61.32	42.04	38.72	37.79	40.17	40.01	37.37	38.10	46.10
17 : 1Δ8	0.29	0.20	0.28	0.45	0.48	0.61							Traces	Traces
17 : 1Δ11	0.87	0.83	0.46	1.81	0	2.20	1.45	1.06	0.90	1.17	0.35	0.24	Traces	Traces
cyclo-17 : 0	1.29	1.73	5.50	2.81	10.00	4.27	0.89	0.87	1.64	1.03	13.02	21.63	4.90	15.90
17 : 0	0.10	0.06	0.04	0.10	0.07	0.06	0.09	0.08	0.12	0.08	0.06	0.09	Traces	0.10
18 : 1Δ11	14.86	18.70	20.28	19.07	15.38	18.22	22.56	27.70	27.19	28.74	22.19	22.25	26.20	13.80
18 : 0	1.16	0.84	0.65	0.64	0.60	0.69	1.17	0.92	0.72	0.92	0.55	0.73	5.20	0.50
cyclo-19 : 0	0.06	0.13	0.38	0.21	0.79	0.45	0	0.18	0.18	1.09	1.22	2.83	Traces	1.0
Σ_{sat}	53.54	50.79	56.13	65.32	73.78	70.8	47.18	43.10	44.48	47.09	59.82	66.45	49.47	66.3
Σ_{unsat}	46.46	49.21	43.87	34.68	26.22	29.2	52.84	56.90	56.52	52.91	40.18	33.55	50.0	33.7
Sat. degree	1.15	1.03	1.28	1.88	2.81	2.42	0.89	0.76	0.79	0.89	1.49	1.98	0.97	1.97

* Cultivation time, h.

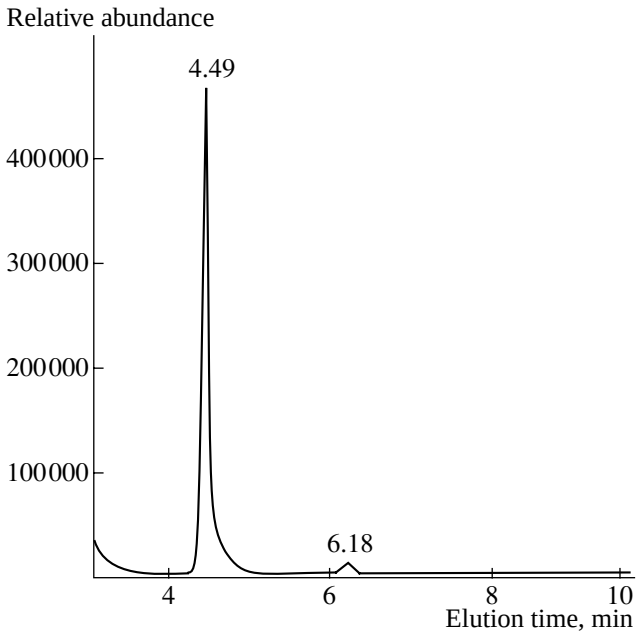

Fig. 3. Elution profile of the hydrolysis products of the PHA synthesized by *A. eutrophus* B5786 cells in the presence of 20 vol % CO. Peaks with elution volumes of 4.49 and 6.18 correspond to the methyl esters of β-hydroxybutyrate and β-hydroxyvalerate, respectively.

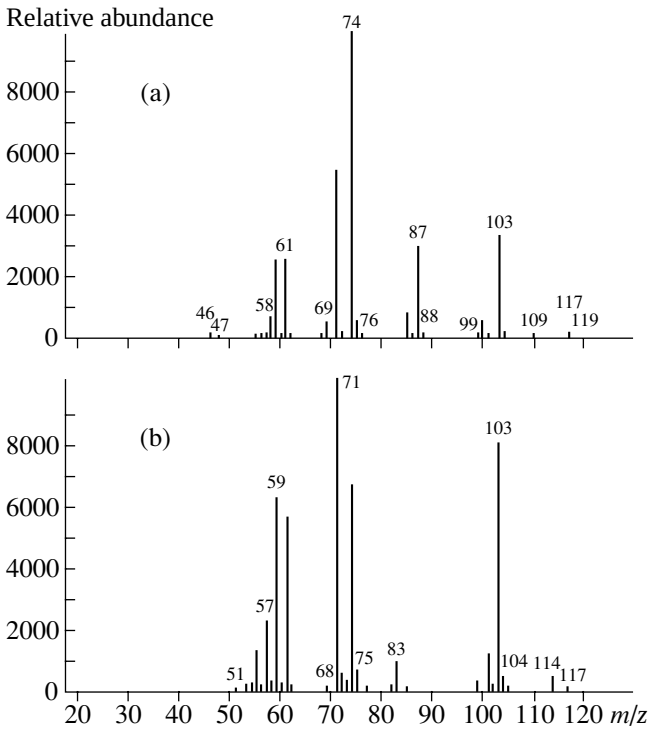

Fig. 4. The mass spectra of the methyl esters of (a) β-hydroxybutyrate and (b) β-hydroxyvalerate, which constitute, respectively, 99.6 and 0.4 mol % of the PHA synthesized by *A. eutrophus* B5786 cells in the presence of 20 vol % CO.

of membrane lipids increased more than twofold, primarily due to the cyclization of monoenoic acids. It is known that, under unfavorable growth conditions, monoenoic acids convert into cyclopropane acids with the involvement of cyclopropane synthase, which uses unsaturated fatty acids to acylate the phospholipids of the cytoplasmic membranes of gram-negative bacteria [20]. A similar enhancement of the cyclization of monoenoic fatty acids under nitrogen deficiency was observed for the continuous *A. eutrophus* culture (Table 1). The elevated content of cyclopropane fatty acids was, however, associated with a rise in the content of saturated fatty acids, mainly palmitic acid.

In the presence of CO in the gas phase of the nitrogen-deficient *A. eutrophus* culture, the accumulation of PHA was accompanied by about a twofold rise in the degree of lipid saturation. In this case, the relative content of saturated $C_{16:0}$ fatty acid increased from 46.7 to 61.3%, the content of cyclopropane acids increased from 1.3 to 10% (after 60 h of cultivation), and that of monoenoic $C_{16:1}$ acid decreased from 29 to 7.5%. The content of *cis*-vaccenic acid in the presence of CO was considerably lower than under nitrogen deficiency alone and changed insignificantly in the course of cultivation. In the continuous *A. eutrophus* culture, the effect of CO was similar. Namely, CO augmented the saturation degree of membrane lipids due to the increased content of $C_{16:0}$ fatty acid and, to a lesser degree, of cyclopropane acids (Table 3).

Thus, the enhanced saturation of the FAs of membrane lipids under the action of CO makes the cytoplas-

Table 4. Some physicochemical parameters of the PHAs synthesized by *A. eutrophus* B5786 cells grown on gas mixtures containing different amounts of CO

CO concentration, vol %	Molecular mass of PHA, kDa	Crystallinity index, %	Temperature characteristics, °C		
			T_{soft}	T_{melt}	T_{degr}
0	$(200–800) \times 10^3$	68–72	110	162	198
10	432×10^3	65	112	168	200
15	640×10^3	71	109	165	197
20	420×10^3	68	105	169	200
25	720×10^3	70	110	167	196

mic membrane more rigid and less permeable. These changes in the membrane properties may protect cellular structures from the detrimental effect of CO and explain why it has almost no effect on the physiological and biochemical characteristics of *A. eutrophus* cultures.

The chromatographic analysis of the chemical composition of the PHA synthesized by *A. eutrophus* in the presence of CO showed that it is a polymer of β-hydroxybutyric acid (the relative content is more than 99 mol %) with a small amount of β-hydroxyvaleric acid (0.24–0.48 mol %) (Fig. 3). The chromatographic data were confirmed by the mass spectrometry of the methyl esters of hydroxybutyrate and hydroxyvalerate (Figs. 4a and 4b, respectively).

The PHA synthesized in the presence of CO showed no difference from the PHA synthesized from pure electrolytic hydrogen in molecular mass, temperature characteristics, and crystallinity index (Table 4).

Thus, the results obtained show the absence of any significant effect of CO on the physiological and biochemical characteristics of the *A. eutrophus* B5786 culture grown under nitrogen deficiency and provide evidence that PHA can be synthesized with high yields in the presence of CO.

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